



**PROCEEDINGS OF ABSTRACTS
III. INTERNATIONAL
BIOLOGICAL & LIFE SCIENCES
CONGRESS
BIOLIC 2025**

16-19 NOVEMBER, 2025

Megasaray Westbeach Hotel, Antalya, Turkey



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**Organized by
Trakya University
International Researchers Association**

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WELCOME NOTES

You are welcome to our III. International Biological and Life Sciences Congress (BIOLIC) which is organized by Trakya University and the International Researchers Association. The congress will be held in Megasaray Westbeach Hotel, Antalya, Turkey, on 16-19 November, 2025 will be normal as well as with online participation.

Our meeting is a premier international science, technology and business forum focusing on Agriculture, Biology and Life Sciences. The program will include oral talks by invited prominent scientists and oral and e poster presentations by participants in selected topics. The Congress is intended that the subjects to be kept broad in order to provide opportunity to the science and research community to present their works as oral or poster presentations in a friendly environment of Antalya, Turkey to share their knowledge and experience and benefit from each other.

The first meeting has been organized in Lviv, Ukraine in 2019 by Trakya University, with part of more than 200 participants from all over the world with 376 scientific papers. In the 2nd congress, there were 68 orals and 64 poster presentation in the congress both joining and presenting normal and online with 101 participants from 22 different countries from the world. The 3rd congress will gather scientists from around the world, and present their recent achievements. The attendees will have ample opportunities for learning, reconnecting, engaging and networking with colleagues from academia and industry as well as meeting with various exhibitors.

As there have been many different scientific meetings around the world, we aimed to bring three different communities together, namely science, research and private investment groups considering practical information sharing that is of value for researchers and scientists from around the world, in a friendly environment of Antalya, Turkey to share their knowledge and experience and benefit from each other as well as prospects to overcome the limitation for sustainable crop production to feed the world.

There are record participation in our 3rd BIOLIC Congress with 600 papers contributed by about 1400 authors from 41 different countries from the world. 233 oral and 205 poster presentations existed in the congress program both joining and presenting normal and online presentations by 188 normal and 144 online as total by 322 participants.

With care for our nature and environment, we aim the green congress, meaning that as little as possible papers will be used. Abstract book is published in electronic book and is distributed to the participants by e mail for online participants. All the e-posters are prepared in electronic form and then submit to via the congress e mail and exhibited in electronical poster boards as well as in online e poster hall in our web page during the congress.

Congress Topics:

Agriculture, Forestry, Life Sciences, Agricultural Engineering, Aquaculture and Biosystems, Animal Science, Biomedical science, Biochemistry and Molecular Biology, Biology, Bioengineering, Biomaterials, Biomechanics, Biophysics, Bioscience, Biotechnology, Botany, Chemistry, Chemical Engineering, Earth Sciences, Environmental Science, Food Science, Genetics and Human Genetics, Medical Science, Machinery, Pharmaceutical Sciences, Physics, Soil Science.

We would like to thank all of you for joining this congress and we would like to give also special thanks to our sponsors and collaborators for giving us a big support to organize this event.

Prof Dr Yalcin KAYA
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ECONOMICAL WHEAT PRODUCTION IN SERBIA AND THE WORLD

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ABSTRACT

Wheat (*Triticum* sp.) is the most important grain that is grown all over the world. In world grain production, according to the quantity produced, it ranks second, right after corn. Geopolitical changes in the world directly affect international trade and the markets of agricultural and food products, as well as the state of the international grain market. The area under wheat varied during the observed period. The highest average yield was recorded in 2022 in the world (3704.4) and Europe (4514.4), and the lowest average yield was recorded in 2000. Wheat production in Serbia varied in the period 2020-2024, while the yield recorded continuous growth (except for 2022), in 2024 it reached 5.8t ha⁻¹.

Keywords: Wheat, sown areas, yield.

INTRODUCTION

Wheat (*Triticum* sp.) is the most important grain used for human consumption. Wheat is the dominant crop in temperate countries. It originates from Asia and southern Europe. According to Zhukovski (1957), it was cultivated before 6500 BC in Iraq and 5-6000 years ago. BC in Egypt and Asia Minor. Early Neolithic farmers domesticated wild grasses and, through centuries of cultivation, they gave origin to the bread wheat we know today (Highfield 2025; Popović et al., 2010; 2020; 2025). Modern wheat, a hexaploid species, evolved through the introgression of numerous previous ploidies (Tilak 2024), with different qualitative and quantitative traits. Advances in genetics and breeding led to the creation of highly productive wheat varieties, which enabled a significant increase in yield per unit area (Ikanović et al., 2010; Malešević et al., 2012; Lakić et al., 2018; Dražić et al., 2021). Wheat accounts for one fifth of the total calories and protein consumed by the world's population, ensuring security of food, worldwide (Erenstein 2022; Popović et al., 2025).

Varieties of different origins are grown under different soil and climate conditions and show great variation in traits (Curtis, 2002). Wheat is a long-day annual plant, but early-maturing varieties thrive in all day lengths - from 12h (Andes in Ecuador) to 20h (Rampart, Alaska). Wheat is grown in all parts of the world except low-lying areas in the tropics - in Britain up to 150m, Switzerland 1200m, tropics 2400-3000m (Marić, 1987).

Jaćimović et al. (2018) point out that in further efforts to increase wheat production, man has two factors at his disposal: variety as a biological tool and production technology. The correct choice of variety according to the results of numerous researches has a very significant role in achieving stable production that enables high yields of good quality seeds (Popović, 2008). The

world is facing climate changes and they affect the production of wheat by reducing its yield, increasing it in the form of increased costs and a necessary deviation from traditional cultivation (Regović et al. 2023). Wheat contributes to human nutrition with dietary fiber components, essential amino acids and phytochemicals and vitamins (B1, B2, E, K, PP). The grain contains 64-69% carbohydrates, 10-14% water, 2-3% cellulose, 1.5-2% fat and 10-21% protein depending on the climate. 13.5% content of protein in grain is taken as the international wheat standard. In Serbia, it is 13-15% (Jevtić et al. 1989; Popović, 2010). However, wheat products, in some people, are responsible for a number of adverse reactions, including intolerances (especially celiac disease) and allergies (respiratory and food) (Shewry 2009). The specific requirements of producers of health-safe and high-quality food require that alternative grains (the brawny types of wheat, durum wheat, durum wheat, and einkorn wheat) are increasingly grown (Ugrenović et al. 2012), millet, buckwheat, and Ph.D. (Popović et al., 2025). In addition to being used in human and animal nutrition, wheat is also used in the form of products: flour, bran, semolina, sprouts, feed flour. Straw is used for the production of paper, cellulose, building boards, hats, baskets, etc. (Đorović 1992). The expansion of the EU and the globalization of trade as well as the high level of competition in other countries around the world (Voicilas 2020) affect the cereal market in the region. The largest part of wheat turnover goes through world exchanges.

The factors that will affect the decrease in wheat production are: the decrease in the amount of fresh water and its increasing contamination; soil degradation and in terms of chemistry (increase in acidity and salinity); climatic diseases and the emergence of new diseases (Serafimov et al, 2025) and pests (a new race of stem rust Ug99) that can reduce yields by 50% (Brown et al. 2008). One of the extremely negative phenomena of global warming will be heat shocks, which in the grain pouring phase can have negative consequences for both yield and quality. Due to the great importance of wheat, the goal of this study was to examine the production of wheat in the world and in our country.

MATERIAL AND METHOD

Yields and areas are the main determinants of wheat production. Areas depend on geographical and economic factors and yields depend on variety, cultivation technology and climatic conditions. In the work, the areas under wheat and its production were monitored. Data on wheat production in the world and in Serbia were used (FAO 2025; FAOSTAT_data_en_10-26-2025; and Statistical Office of Serbia).

RESULTS AND DISCUSSION

International trade and agri-food markets are directly affected by geopolitical changes. Consequently, they also affected the state of the international grain market. Table 1 shows data on harvested areas under wheat in the world and in Europe. In the observed period, the harvested areas under wheat in the world were the largest in 2021 and 2023. And in Europe in 2021 and 2022. The highest average yield was in 2022 in the world (3704.4 kg ha⁻¹) and in Europe (4514.4 kg ha⁻¹). The lowest average yield was recorded in the world and in Europe in 2000.

Table 1. Areas and yield of wheat in the world and Europe (2019-2023)

Parameter	World		Europe	
Year	Harvested area (ha)	Average yield (tha ⁻¹)	Harvested area (ha)	Average yield (tha ⁻¹)
2019	214000000	3,575	62387726	4,2657
2020	217000000	3,4996	61529895	4,141
2021	220000000	3,5229	62881169	4,2834
2022	219000000	3,7044	62743856	4,5144
2023	220000000	3,625	61481984	4,3795

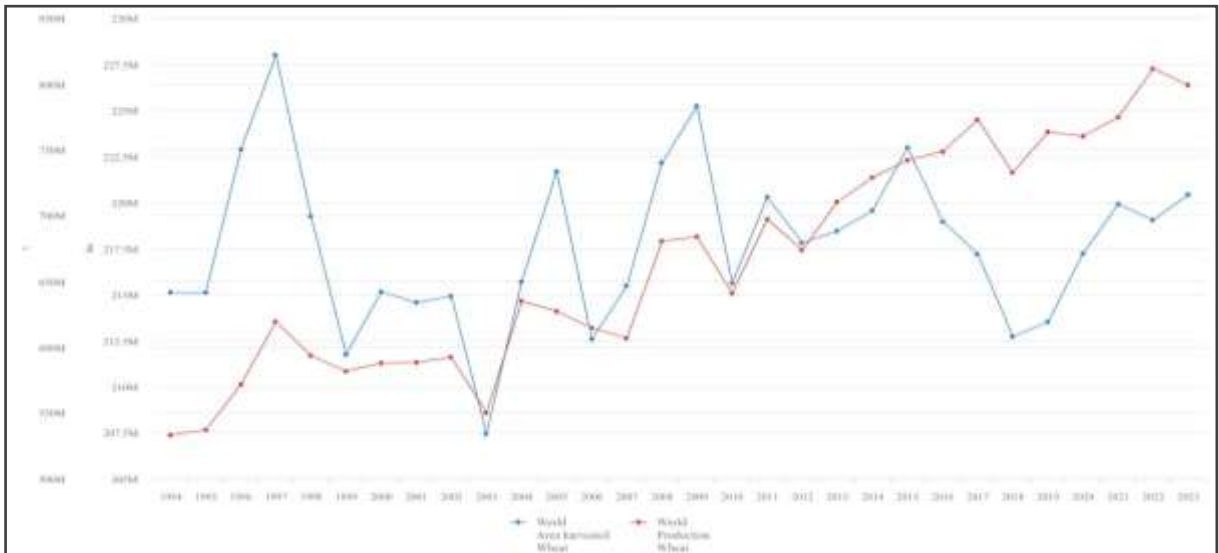


Figure 1. Areas and production of wheat in the world from 1994 to 2023

Figure 1 shows the trend of production and areas under wheat in the world over 30 years. It is noted that there are several sudden increases and decreases in both production and harvested areas. The highest harvested area was in 1996, while the highest production was recorded in 2022. The lowest harvested area was in 2003, when the third lowest yield was achieved in the observed period, after those from 1994 and 1995. In 2019 and 2020, high wheat production was achieved despite the reduced harvested area.

Figure 2 shows the production of wheat as a crop, and it ranks fourth, after the production of sugar cane, maize and rice. As for wheat production by country, in the observed period the largest production was achieved by China, followed by India and the United States, Figure 3. Among the 10 largest producers are Russia, France, Canada, Australia, Germany, Pakistan and Ukraine.

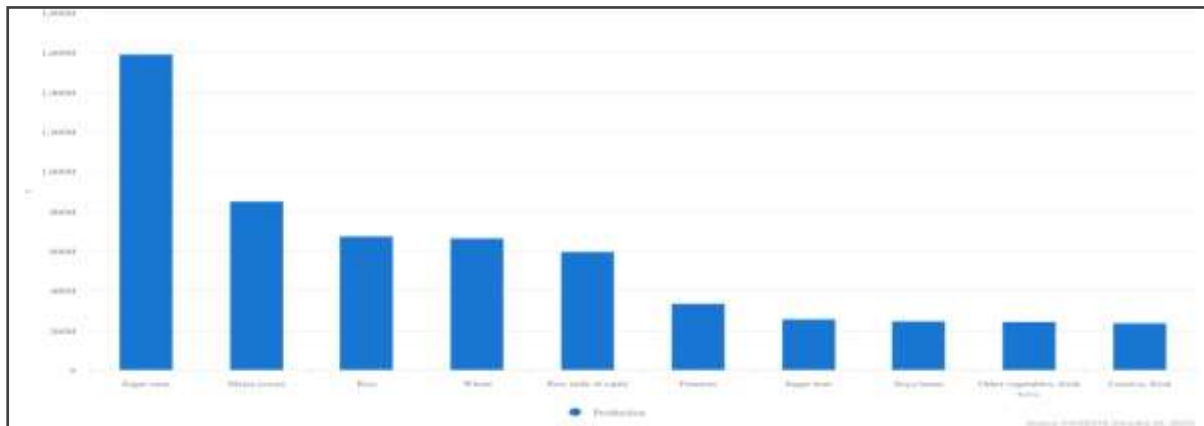


Figure 2. The most produced crops in the world (1994-2023)

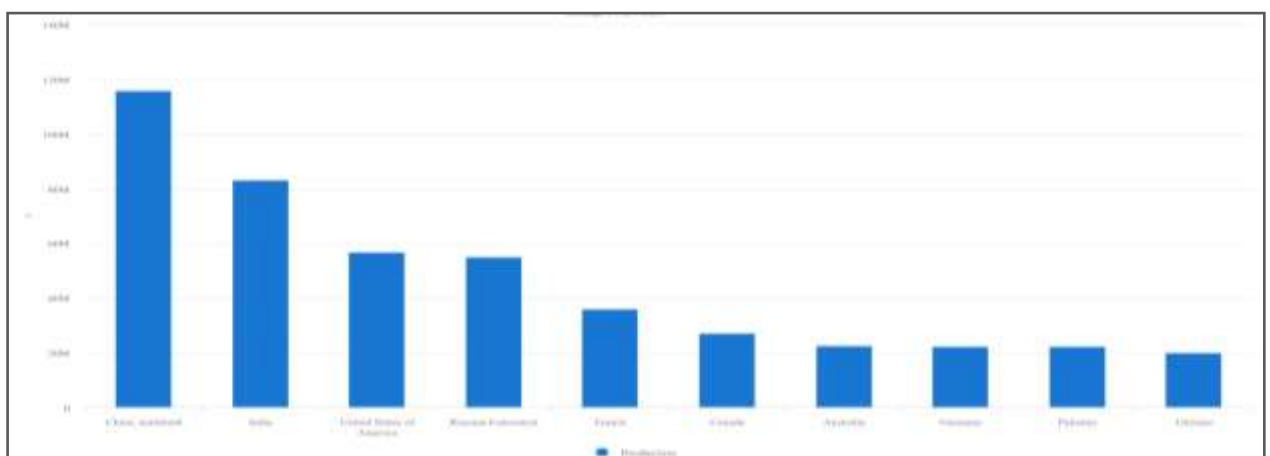


Figure 3. Wheat production in the world (1994-2023)

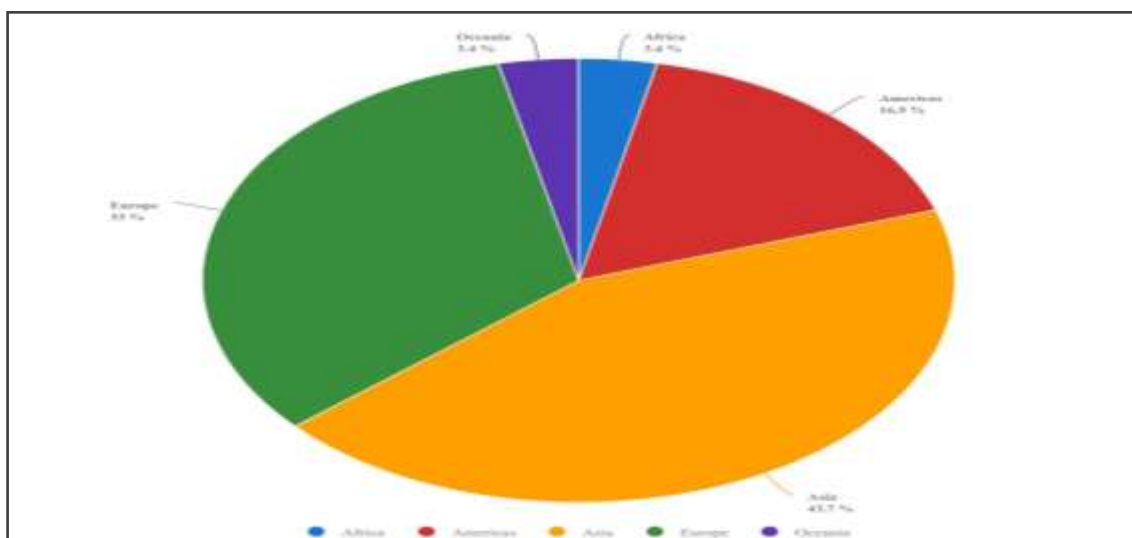


Figure 4. Wheat production in the world by region (1994-2023)

Figure 4 shows wheat production by region. Asia has the largest share in world wheat production (43.7%), while the smallest belongs to Africa and Oceania (3.4%).

Table 2 shows the state of production of agricultural goods in Serbia, which shows a trend of growth in 2022 and 2023, while in 2024 there was a decline in the production of all agricultural goods, including grain.

The production varied in the observed period, while the yield recorded continuous growth (except in 2022) and in 2024 it was 5.8t ha⁻¹. In 2024, there were the least areas under wheat due to the observed period, so the recorded production value was the lowest despite the highest yields.

Table 2. Production of agricultural goods, 2020-2024

Grain production	Year				
	2020	2021	2022	2023	2024
Description	Million RSD				
Production of agricultural goods and services	632360.9	821507.6	707213.2	752671,7	712127.8
Plant production	454422.6	544202.2	590920.8	521272,1	477347.2
Cereals (including seeds)	170698.6	216992.9	225652.3	187950.2	149220.6

Source: Publikacije.stat.gov.rs

Table 3 shows the state of wheat production in Serbia. The area sown with cereals grew from 2020 to 2022, while in 2023 it decreased by 10%.

Table 3. Areas, yield and production of wheat, 2020-2024

Years	Grain	Wheat		
		Area, ha	Yield, t ha ⁻¹	Production, t
2020	1740456	344922	4.9	2873503
2021	1770188	360403	5.7	3442308
2022	1724728	375104	5.3	3109827
2023	1766058	409500	5.7	3448700
2024	1678906	356919	5.8	2900536

Source Publikacije.stat.gov.rs

Data on the expected production of wheat shown according to the situation on May 23, 2025. Based on the state of the crops on the day of the assessment, the expected production of wheat is 3605 thousand tons, which is 24.3% more compared to the production achieved last year. The yield per hectare is estimated based on the appearance of the plants, stage of development, sowing density, applied agro-technical measures and weather conditions (<https://www.stat.gov.rs/sr-latn/vesti/statisticalrelease/?p=17028>).

Wheat is grown on all continents from the north of Norway to the south of South Africa and from Japan to the extreme west of Canada. Seen from the aspect of proper use of land, as an important agricultural resource, the small presence of wheat in our country is very unfavorable because wheat and other small grains represent a factor of soil stabilization and "rest" after plant species that require intensive work on plots. Wheat is mostly used in food (about 53%) of the total production in developed countries and about 85% in developing countries (Pena, 2007). It is also used as animal feed and somewhat less for industrial purposes. In the last few years,

wheat has also been used to obtain bio-ethanol, but in small quantities. Regarding nutrition, there are a large number of final products made from wheat flour, but globally, wheat flour is used to make fermented bread (Europe, America and Australia), unfermented, unleavened bread (India, Mexico, parts of Africa) and as cooked dough, i.e. noodles (China and Japan). Of the total wheat production, about 10% is durum wheat, which is used to make pasta, i.e. spaghetti, macaroni and other pasta. In our country, wheat is used to make bread and other similar products almost exclusively of the fermented type, although recently unleavened breads have also appeared, but also as animal feed. On average, about 10-15% of the total wheat production is used for animal feed, while the amount used for other purposes is practically statistically negligible. The production of wheat in the world records a constant trend of growth due to the increased human population and the share of that population in which the diet is dominated by bread (Denčić et al., 2009).

Wheat represents a fundamental food culture that has played a key role in human civilization for millennia (Velimirović 2021). Increased awareness of changes in the environment and the health benefits of a plant-based diet (Robles-Zazueta 2023) has led, in the world, to a direction towards greater production of technologically and nutritionally quality wheat. Ensuring global food security in the future means applying a multidisciplinary approach to wheat cultivation and production technology, in accordance with changing diets, consumer preferences and climate change (Vouchko et al. 2025).

CONCLUSION

- Wheat represents a very important fundamental food crop, which is why in the world there has been a direction towards the greatest possible production of technologically and nutritionally quality wheat.
- To ensure global food security in the future, a multidisciplinary approach to wheat cultivation technology will be applied, in accordance with consumer preferences and climate change.
- According to prediction, the production of wheat will move on an upward trajectory due to the increase in the number of the population and thus the need for an increasing amount of food.

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THE EVALUATION OF NODULATING ABILITY OF ALFALFA IN MIXTURES

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ABSTRACT

A field experiment was conducted to study the nodulation ability of two alfalfa varieties, alone and in mixtures with grass crops. The alfalfa varieties Pleven 6 and Dara were used, and the cocksfoot variety Dabrava was used for the mixtures with grass crops. The ratio of legumes to grass crops in the mixtures was 50:50% and 80:20%, respectively. Soil monoliths were taken to determine the number of nodules formed on the alfalfa plants and the level of nodule formation. It was found that the number of nodules formed (16 nodules/plant) on the Dara alfalfa plants was 33.3% higher than that on the Pleven 6 plants (12 nodules/plant). In mixtures of alfalfa and cocksfoot in a 50:50% ratio, the number of nodes was found higher than that formed only by alfalfa in both varieties, with the Dara variety having 26.8% more. In mixtures of alfalfa and cocksfoot in a ratio of 80:20%, the number of nodes formed was the highest due to the larger proportion of the legume component. The number of nodes formed in the alfalfa variety Dara was 26.7% higher than that in the variety Pleven 6, and 15.8% higher in the mixture in a ratio of alfalfa Dara: cocksfoot 80:20% compared to alfalfa alone. In terms of nodule formation, the plants were ranked as follows: Dara alfalfa in mixtures with cocksfoot in a ratio of 80:20% (45.5), Pleven 6 alfalfa in mixtures with cocksfoot in a ratio of 80:20% (43.0), alfalfa variety Dara – single crop (42.0), alfalfa variety Pleven 6 – single crop (40.5) and alfalfa variety Pleven 6 in mixtures with – single crop in a ratio of 50:50% (40.5). The higher potential for alfalfa nodule formation in mixtures with cocksfoot in a ratio of 80:20% was associated with more efficient use of nitrogen for nodule formation.

Keywords: Alfalfa, Nodule formation, Mixtures

INTRODUCTION

Biological nitrogen fixation (BNF) is a major natural source of nitrogen and plays a crucial ecological and agronomic role in the structure of sustainable agricultural systems (Soumare et al., 2020; Yu and Zhuang, 2020). It represents the most efficient way to supply nitrogen through leguminous crops, contributing to high yields and improved protein content. Utilizing the nitrogen-fixing potential of legumes, including alfalfa, significantly reduces the need for synthetic mineral fertilizers (Uçar, 2023). Increasing fertilizer application often leads to diminishing yield returns and adverse environmental impacts, without improving resource-use efficiency. Therefore, optimizing agricultural practices remains a key strategy for sustainable production. Better integration between crop and livestock systems can also enhance

nitrogen-use efficiency at both local and global scales (Soussana and Lemaire, 2014; Anas et al., 2020).

Large-scale data (Lassaletta et al., 2014) indicate that promoting symbiotic nitrogen fixation as a natural nitrogen source enhances nitrogen-use efficiency. Thus, incorporating more legumes into crop rotations is of great importance. The ecological relevance of nodulation and nitrogen fixation has been convincingly demonstrated in previous studies (Vasileva and Ilieva, 2012; Vasileva, 2017; Vasileva and Tariq, 2018; Goyal et al., 2021; Ladha et al., 2022; Vasileva et al., 2024).

Nitrogen derived from BNF is a major contributor to the soil nitrogen pool, directly available to plants and less susceptible to denitrification and leaching losses (Kot's, 2001; Graham, 2008). Annual nitrogen fixation can range between 50 and 450 kg ha⁻¹, with approximately 30–80% of plant nitrogen derived from fixed nitrogen (Wang et al., 2024).

Alfalfa (*Medicago sativa* L.), as one of the most important forage legumes, is particularly effective in nitrogen fixation. The proportion of nitrogen derived from fixation (%Ndfa) varies widely depending on variety, soil, and management conditions, typically ranging from 50 to 80% of total plant nitrogen (Issah et al., 2020). Under optimal conditions, alfalfa can fix up to 200–450 kg N ha⁻¹ year⁻¹ (Undersander et al., 2011), significantly reducing fertilizer needs and promoting sustainable soil and resource management (Navarro et al., 2025).

Because of their nitrogen-fixing capacity, legumes particularly forage legumes are valuable components in grass-legume mixtures. Nitrogen fixed by the legume is partly transferred to the soil, where it becomes available to the companion grass species. Mixtures containing legumes are typically more productive and have higher protein content, resulting in more balanced chemical composition and improved nutritional value of the forage biomass. Numerous studies have confirmed that legumes are preferred components in mixed crops (Pukhtaievych et al., 2019; Vasileva and Kaya, 2024; Ma et al., 2025). According to Vasileva and Ilieva (2016), yields in mixtures are more stable, while Vasileva and Vasilev (2012, 2017) found that nitrogen-use efficiency is higher in mixed than in monoculture systems.

Research on the nodulation potential of legume-nonlegume mixtures, involving nitrogen-fixing and nitrogen-assimilating species, is a priority in sustainable agriculture. It was hypothesized that increasing the legume proportion in the mixtures enhances nodulation due to improved nitrogen use efficiency. Therefore, this study aimed to determine the nodulation ability of two alfalfa varieties grown alone and in mixtures with cocksfoot (*Dactylis glomerata* L.) at ratios of 50:50% and 80:20%.

MATERIAL AND METHOD

The field experiment was conducted to assess the nodulation ability of two alfalfa (*Medicago sativa* L.) varieties grown as sole crops and in mixtures with cocksfoot (*Dactylis glomerata* L.). The experimental design followed a randomized complete block layout with four replications. Each experimental plot measured 5.60 m². The experimental variants were as follows:

1. Alfalfa variety Pleven 6 (100%);
2. Alfalfa variety Dara (100%);
3. Alfalfa variety Pleven 6 + cocksfoot (50:50%);
4. Alfalfa variety Dara + cocksfoot (50:50%);
5. Alfalfa variety Pleven 6 + cocksfoot (80:20%);
6. Alfalfa variety Dara + cocksfoot (80:20%).

The alfalfa varieties Pleven 6 and Dara were developed at the Institute of Forage Crops, Pleven, Bulgaria (Kertikov and Kertikova, 2016), while the cocksfoot variety Dabrava was also bred at the same institute (Katova and Naydenova, 2017). The seeding rate for both, alfalfa and cocksfoot was 25 kg ha⁻¹, with a row spacing of 12.5 cm. Sowing was done manually. All agronomic practices during the growing season followed standard recommendations.

To determine nodulation parameters, soil monoliths measuring 20 x 30 x 40 cm were collected from the first growth stage of each replicate in all variants (Beck et al., 1993). The root mass of alfalfa plants was gently washed under running tap water, and the following indicators were measured: root length (cm) within the root-inhabited soil layer; specific root length (root length per gram of root mass); fresh root mass (g/plant); and dry root mass (g/plant), after drying at 60°C.

Nodule formation was evaluated by counting the number of nodules on both main and lateral roots, expressed as the average number of nodules per plant. The nodulation rate were calculated. The nodulation rate was classified according to the location of nodules: (1) on the main root; (2) on lateral roots near the main root; (3) scattered along the root system; and (4) plants without nodules.

Experimental data were analyzed statistically using SPSS software (Version 24.0, IBM Corp., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to evaluate the effects of variety and mixture ratio on root and nodulation traits. When significant differences were detected, mean comparisons were conducted using the Least Significant Difference (LSD) test at a significance level of $p < 0.05$. Results are presented as mean values $\pm$ standard deviation (SD). The assumptions of normality and homogeneity of variance were verified before analysis.

RESULTS AND DISCUSSION

Based on plant morphometry root mass and nodulation-related indicators several biometric parameters of nodule formation were evaluated. The length and dry weight of the root mass were found to be highest in plants of the alfalfa variety Dara grown as a sole crop (Table 1).

Regarding the number of nodules per plant, which is generally a varietal trait, it was found that the Dara variety produced 16 nodules per plant - 33.3% more than Pleven 6, which formed 12 nodules per plant. In mixtures of alfalfa and cocksfoot at a 50:50% ratio, the number of nodules was higher than in pure alfalfa stands for both varieties, with a 26.8% increase observed for Dara.

For mixtures at an 80:20% ratio, the number of nodules was the highest, corresponding to the greater proportion of the legume component. In the Dara variety, nodulation was 26.7% higher than in Pleven 6, and 15.8% higher in the Dara + cocksfoot (80:20%) mixture compared to Dara grown alone.

Table 1. Root mass of alfalfa in mixtures with cocksfoot

Variants	LRM	SLRM	FWRM	DWRM	Nodules
	cm	cm/g	g/plant	g/plant	number/plant
Alfalfa Pleven 6 (100%)	40.0	1.86	21.4	0.32	12.0
Alfalfa Dara (100%)	48.5	2.62	18.5	0.47	16.0
Alfalfa Pleven 6+cocksfoot (50:50%)	39.5	4.28	9.2	0.24	14.0
Alfalfa Dara+cocksfoot (50:50%)	29.0	2.55	11.4	0.19	17.8
Alfalfa Pleven 6+cocksfoot (80:20%)	34.5	1.88	18.3	0.24	15.0
Alfalfa Dara+cocksfoot (80:20%)	34.0	1.82	18.7	0.25	19.0
Average	48.5	4.28	21.4	0.47	17.8
STDEV	6.7	0.94	4.8	0.10	2.5
Max	48.5	4.28	21.4	0.47	19.0
Min	29.0	1.82	9.2	0.19	12.0

LRM, Length of root mass; SLRM, specific length of root mass; FWRM, fresh weight of root mass; DWRM, dry weight of root mass.

Statistical evaluation

The analysis of variance revealed a significant effect of the experimental variant on the number of nodules formed per plant ($F = 6.19$, $p = 0.0017$). According to the LSD test ($p < 0.05$), the Dara alfalfa variety, especially when grown in mixture with cocksfoot at a 50:50% ratio, produced significantly more nodules compared to Pleven 6 and its respective mixtures. This confirms the strong influence of varietal characteristics and legume proportion on nodule formation.

Root and nodulation characteristics

The mean values of root and nodulation parameters are presented in Table 1, and the statistical differences between treatments are shown in Table 2. The Dara variety + cocksfoot (50:50%) mixture exhibited the highest mean number of nodules (17.8 per plant), significantly exceeding the values for Pleven 6 plants (12.0) and its mixtures. The Dara + cocksfoot (80:20%) and Dara (100%) treatments also maintained high nodulation levels.

Table 2. Statistical differences between treatments related to nodules

Variants	Mean $\pm$ SD	Groups (LSD 0.05)
Alfalfa Pleven 6 (100%)	12.0 $\pm$ 2.0	c
Alfalfa Dara (100%)	16.0 $\pm$ 1.8	ab
Alfalfa Pleven 6+cocksfoot (50:50%)	14.0 $\pm$ 0.8	bc
Alfalfa Dara+cocksfoot (50:50%)	17.8 $\pm$ 2.3	a
Alfalfa Pleven 6+cocksfoot (80:20%)	15.0 $\pm$ 1.2	bc
Alfalfa Dara+cocksfoot (80:20%)	15.0 $\pm$ 2.3	ab

Different letters indicate statistically significant differences between means at $p < 0.05$ (LSD test).

The ranking of plants based on nodulation rate was as follows: Dara + cocksfoot (80:20%) (45.5), Pleven 6 + cocksfoot (80:20%) (43.0), Dara (100%) (42.0), Pleven 6 (100%) (40.5), and Pleven 6 + cocksfoot (50:50%) (40.5) (Figure 1). The enhanced nodule formation in the 80:20% mixtures was attributed to the larger legume proportion and the more efficient utilization of nitrogen for nodule development.

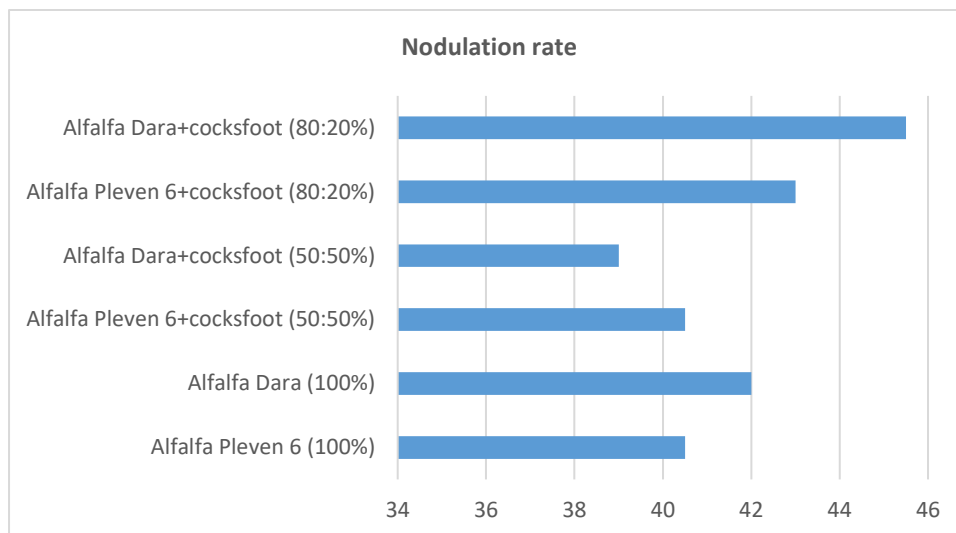


Figure 1. Level of clod formation in alfalfa mixtures with cocksfoot

These findings align with the conclusions of Vasileva and Tariq (2018) and Pukhtaievych et al. (2019), who also reported greater nodulation and nitrogen efficiency in mixed cropping systems containing a higher legume proportion. Similarly, Vasileva and Vasilev (2012, 2017) demonstrated that nitrogen-use efficiency is higher in legume-grass mixtures than in monocultures.

The present results reaffirm that increasing the legume component in mixtures promotes not only improved nitrogen fixation but also enhances the symbiotic potential and root development of alfalfa. Consequently, the combination of Dara alfalfa with cocksfoot at ratios favoring the legume component (particularly 80:20%) proved most effective for achieving higher nodule formation and potentially greater nitrogen accumulation.

CONCLUSIONS

The study demonstrated that the nodulation ability of alfalfa (*Medicago sativa* L.) varied significantly between varieties and depending on their proportion in mixtures with cocksfoot (*Dactylis glomerata* L.). The Dara variety formed 33.3% more nodules per plant (16 nodules) than Pleven 6 (12 nodules). In mixtures of alfalfa and cocksfoot at a 50:50% ratio, the number of nodules was higher than in pure alfalfa stands for both varieties, with a 26.8% increase recorded for Dara. The mixtures with an 80:20% ratio showed the highest nodulation levels due to the larger proportion of the legume component. The Dara variety exhibited 26.7% higher nodulation than Pleven 6 under the same conditions, and 15.8% more nodules in the Dara + cocksfoot (80:20%) mixture compared to alfalfa grown alone. Based on nodulation rate, the ranking was as follows: Dara + cocksfoot (80:20%) > Pleven 6 + cocksfoot (80:20%) > Dara (100%) > Pleven 6 (100%) > Pleven 6 + cocksfoot (50:50%). The higher potential for nodule formation in the 80:20% mixtures was associated with more efficient nitrogen utilization for symbiotic development.

These results contribute to the optimization of mixed crops or the selection of varieties with high nodulation activity. They also highlight the essential role of leguminous forage crops and legume-grass mixtures in building sustainable and environmentally friendly agricultural systems.

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STRATIGRAPHIC THICKNESS DETERMINATION OF MURAL PAINTINGS FROM THE JANI AND VASILI ATELIER USING EDXRF

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ABSTRACT

The study of post-Byzantine artistic techniques gives us information about the evolution of materials and methods used by historical painters. This work presents our research on 18th-century murals from the atelier of the Albanian painters Jani and Vasili. The investigation covered frescoes from several key sites, including the Monastery of Saint Michael in Nivan (1779), the Church of Saint Nikolla in Dhrovjan (1796), and the Monastery of Saint Mary of Athali in Himara (1795). The aim of this study was to apply and test a method for determining the thickness of pictorial layers, using a minimally invasive approach on micro-samples analyzed ex-situ at the Institute of Applied Nuclear Physics.

To fully characterize the samples' palette and stratigraphy, we used a combination of analytical techniques. Energy Dispersive X-Ray Fluorescence (EDXRF) revealed the elemental makeup of the layers, allowing us to detect iron (Fe) in pigments like red and yellow ochres and Prussian blue, as well as lead (Pb) in lead white. For molecular identification, we turned to Raman Spectroscopy, which was crucial for identifying carbon-based black pigments. This dual-technique approach was essential for confirming the composition of the uppermost layer, a necessary first step to correctly interpret the EDXRF data for thickness calculations. The thickness itself was calculated using the principles of the Lambert-Beer law. We created theoretical calibration curves with Monte Carlo (MC) simulations, which modeled how the thickness of a pigment layer affects the attenuation of X-ray signals, such as the change in the Pb $L\alpha/L\beta$ intensity ratio from a lead white ground layer.

The calculated thicknesses were validated against direct physical measurements from paint cross-sections, which were prepared from the micro-samples and analyzed with optical microscopy. We measured the stratigraphic thicknesses on these cross-sections using ImageJ software. Our findings show that the proposed EDXRF and simulation method gives good results for simple structures of one or two layers, with the calculated values showing strong agreement with the physical measurements. For more complex structures of three or more layers, however, the determination error becomes significantly over 30%—which highlights the limitations of this technique when dealing with highly complex stratigraphies.

Keywords: EDXRF, Raman Spectroscopy, Layer Thickness, Monte Carlo Simulation, Post-Byzantine Painting

INTRODUCTION

The post-Byzantine period (1453-1830 AD) holds significant historical and cultural importance as Orthodox religious painting, particularly in the form of portable icons and murals, continued traditional practices in the Byzantine style, ensuring the persistence of Christian Orthodox identity despite the Ottoman rule [1]. This tradition was inherently conservative,

consciously resisting the prevailing Western European shifts toward oil painting on canvas by persistently employing egg yolk tempera on gessoed wooden panels [2]. The application of paint followed a well-defined, multi-layer manner, where superimposed, rather than intermixed, layers were used to build up forms [3]. For monumental art, a mixed technique (fresco-secco) was generally employed, recently documented as a mixed fresco-lime painting method often incorporating proteinaceous binders [3]. Key artistic materials included high-purity, extremely thin gold leaves for backgrounds and haloes, reflecting the preciousness and spiritual significance of the divine glory being represented [4], and traditional pigments like cinnabar, yellow ochres, indigo, and green earths [4]. The analytical investigations reveal an evolution in material use, including the growing employment of metal powders for highlights from the mid-18th century onward- a shift perhaps indicating a minor move toward more physiocratic painting-and a tendency among late post-Byzantine painters to rapidly adopt new synthetic pigments like Prussian blue and lead chromate, demonstrating an openness to handy new materials despite the conservative nature of the art form [5].

One of the aspects of studying multilayered materials, such as cultural heritage objects or coatings is the determination of painting layer thicknesses using sophisticated quantitative methods [6]. The measurement of layer thickness is generally achieved through methods categorized broadly as transmission/absorption based, emission-based using calibration, or fundamental parameter models, often enhanced by specialized instrumentation or computational techniques. The Fundamental Parameter approach to quantitative X-ray Fluorescence analysis relies on the FP method, which describes the relationship between net-line intensities and elemental concentrations, often assuming homogeneous composition [7]. However, due to the inherent complexity of analytical expressions, especially for secondary and tertiary excitations, iterative computer methods like Monte Carlo (MC) simulations are often utilized to model the spectrum by adjusting layer structures until calculated results match the experimental data [8]. Simpler, empirical approaches include the Absorption Mode, which relies on measuring the attenuation of the substrate signal by the overlying layer, often following the Lambert-Beer law [9], or the Emission Mode, which correlates the measured fluorescence intensity of the layer material itself directly against external calibration standards of known thickness [9].

A highly effective non-destructive approach for determining stratigraphy and layer thickness in multilayered samples relies on measuring X-ray intensity ratios derived from characteristic lines of the same element, such as K_a/K_b or L_a/L_b [10]. This method exploits the principle of differential attenuation: the characteristic lines K_a , K_b to be absorbed more strongly as it travels through the overlaying material or the layer itself [11]. This differential attenuation causes the measured ratio to deviate from its expected value, the ratio for an infinitely thin sample [10]. This measured ratio is functionally related to the thickness (d) and the linear attenuation coefficients (μ) of the material causing the attenuation [10]. This technique is applied in two main contexts: quantifying the thickness of the layer itself (for example, the self-attenuation, such as using the Au- L_a/L_b ratio for gold leaf) [10], or determining the thickness of a superimposed coating layer by measuring the attenuation of the characteristic ratio of an element located in the underlying substrate (for example using the Cu- K_a/K_b ratio of a copper base to calculate the thickness of an overlying gold layer) [6].

MATERIAL AND METHODS

As a first step, we performed a visual examination using the Raman Video Micro-Sampling System BAC 151C, which is a compact Raman microscope belonging to the B&W Tek portable Raman system. For molecular analysis, a B&W Tek i-Raman Ex Raman spectrometer was used, operating with a wavelength of 1064 nm and a laser power of 4.3 mW.

Microsamples from black areas of the frescoes were also analyzed by EDXRF spectrometry with an Artax 800 spectrometer produced by Bruker. This device is equipped with a Rhodium (Rh) anode, operates in direct excitation mode, and uses a Silicon Drift Detector. During the measurement, the generator was operated at 30 kV and 200 μ A, and the samples were measured for 50s with a dead time of 6%. Finally, the stratigraphic thicknesses were determined by using the open-source software ImageJ.

While the results of this paper are based on the direct analytical model, the theoretical calibration curves are generated using the Monte Carlo (MC) method. This simulation is used to build a virtual sample of the stratigraphy. As illustrated in Figure 1, the simulation models the X-ray behavior for a substrate (e.g., Pb White) covered by varying, known thicknesses of an overlying layer. This generates a calibration curve that plots the Pb $L\alpha/L\beta$ ratio against the layer thickness, which can then be used to determine the thickness of an unknown sample from its measured ratio.

As we have mentioned one the physical principles used to mathematically describe the attenuation suffered by a beam of X-ray photons as it passes through a material is the Lambert-Beer law. The law describes an exponential decay of X-ray intensity on the material properties and the distance traveled by the photons [12]. The relationship is generally expressed as: $I = I_0 \exp [-\mu \rho t]$, where I_0 is the initial intensity of the X-ray beam, I is the transmitted intensity after crossing the absorbing medium, μ is the mass attenuation coefficient in $\frac{cm^2}{g}$, which quantifies the interaction probability per unit mass of the absorbing material [12]. This coefficient is calculated as weighted sum of the mass attenuation coefficients of all constituent elements in the medium $\mu = \sum w_i \mu_i$ [12]. ρ is the density of the material in g/cm^3 , $\mu \rho$ is the linear attenuation coefficient in cm^{-1} , representing the interaction probability per unit length [12], and t (or d or l) is the path length or the physical thickness of the medium traversed by the X-ray beam [12]. This exponential decrease in intensity occurs because, as the beam crosses matter, photons are removed from the beam primarily through photoelectric absorption and, to a lesser extent, scattering Compton and Rayleigh [13]. The energy of the unabsorbed photons remains generally unchanged [13].

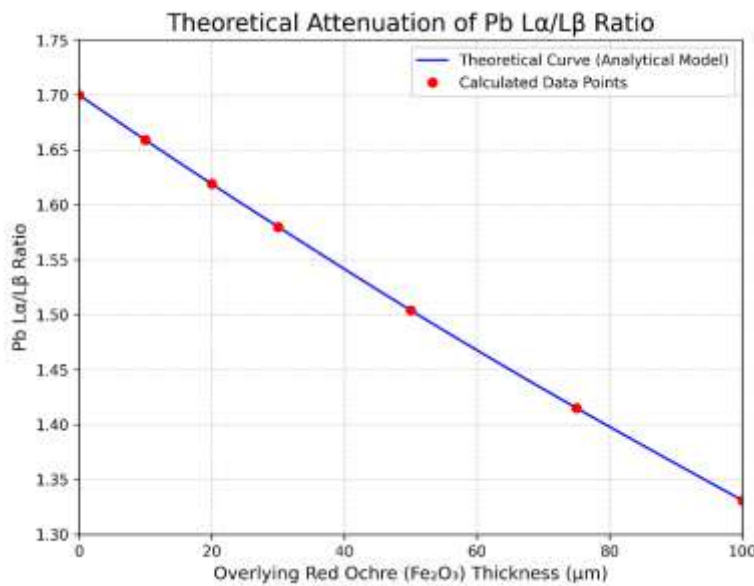


Figure. 1. Illustrative calibration curve for the differential attenuation method. This analytical model, which replicates the results of a Monte Carlo simulation, plots the calculated Pb $L\alpha/L\beta$ ratio against the known thickness

of an overlying red ochre (Fe_2O_3) layer. This curve provides a graphical method to determine an unknown sample's layer thickness from its experimentally measured La/Lb ratio.

For a sample that is not infinitely thin, the intensity of the measured characteristic X-rays is a complicated function of the thickness, composition, and XRF geometry [11]. The distance traveled by the photons (absorption path length) depends on the angle of the incident primary beam (ϕ_1) or the exit angle of the fluorescent beam ϕ_2 relative to the surface [11]. For a monochromatic beam traversing a thickness t in a specific layer, the effective path length is $t/\sin\phi$ [11]. The Lambert-Beer law provides the theoretical foundation for how the layer thickness t directly impacts the detected X-ray intensity I .

For a known, single element layer of increasing thickness t , the measured fluorescence intensity of that element increases but eventually levels off at a “saturation thickness” because the X-rays generated far beneath the surface are completely attenuated before reaching the detector [9]. In quantitative XRF models such in modifications of the Sherman equation, used for a layer of finite thickness T through a complex attenuation factor that contains the core exponential decay term [12]. This absorption term accounts for both primary and fluorescent attenuation along the path, relating I_i and I_0 using the sample's thickness T and density ρ [12]:

$$\text{Attenuation Factor} \propto 1 - \exp \left[-\rho T \left(\frac{\mu(E_0)}{\sin\phi_1} + \frac{\mu(E_i)}{\sin\phi_2} \right) \right] \quad [12]$$

If the sample is layered, the attenuation factor must calculate the total X-ray removal along the entire path length, which involves summing the exponential attenuation contributions from every overlying layer (k) with thickness t_k and specific mass attenuation coefficient μ_k [14]. This means that the layer thickness t is directly related to the observed intensity I via its exponential influence on photon survival [14]. In practical analysis, thickness can be determined in the Absorption Mode by measuring the reduction in the X-ray signal I emitted by the substrate (or underlying infinite layer) as the overlying layer thickness d absorbs the signal according to the relationship $I = I_0 e^{-md}$ [13].

Another physical principle that uses internal characteristic X-ray intensity ratios, such as the case of Pb La/Lb ratio to determine the thickness of an overlaying layer is the differential attenuation [6]. The core physical principle relies on the energy difference between the two characteristic X-ray lines emitted by the same element. For example, the La and Lb lines of lead (Pb) are produced by electron transitions within the lead atom, resulting in photons with slightly different energies [15]. For lead, the La_{11} line energy is 10.55 keV and Lb_{11} line energy is 12.61 keV [15]. When the lead substrate is covered by an overlying layer (the attenuating material), the emitted La and Lb photons must pass through this layer before reaching the detector [6]. The attenuation of X-rays in matter is strongly dependent on the photon energy [6]. Since the La line has lower energy than the Lb line, it experiences stronger absorption by the overlying material [6].

This differential attenuation causes the measured, Pb (La/Lb) to shift significantly away from the value typically observed for an infinitely thin sample [6]. The amount of this change in the ratio is directly and exponentially related to the thickness and the attenuation coefficients of the covering layer [6]. This relationship allows the calculation of the thickness (d) of the covering layer, assuming the lead substrate is infinitely thick [16]. The intensity ratio method is effective for quantification in layered systems due to several intrinsic advantages, particularly in comparison to measuring absolute intensities. It directly reflects the thickness of the attenuating material, it removes the geometric influences and identify both the element's position and the layer thickness [10].

RESULTS AND DISCUSSIONS

To apply the differential attenuation method [6], we have chosen to analyze four representative samples consisting of a two-layer stratigraphy being an ochre paint layer on top

of a calcium carbonate CaCO_3 ground layer. Firstly, we measured the thickness using ImageJ software from the cross-section images referred to in Table 1, calibrating the software using a millimetric sheet. The XRF spectra for these samples, such as those shown in Figure 4 and Figure 5, were used to extract the experimental $\text{Ca } K\alpha/K\beta$ ratio labeled as R_{measured} . This value, along with the reference ratio for the ground layer only CaCO_3 was measured to be around 7, was used as an input in the theoretical model. We also used the standard densities for hematite Fe_2O_3 , 5.26 g/cm^3 , and goethite FeOOH , 4.28 g/cm^3 , for the top painted layer. The uppermost painted layer was identified using Raman Spectrometry and both yellow and red ochre characteristic wavenumbers were found. We are presenting both Raman measurements for yellow and red ochre in the figure below.

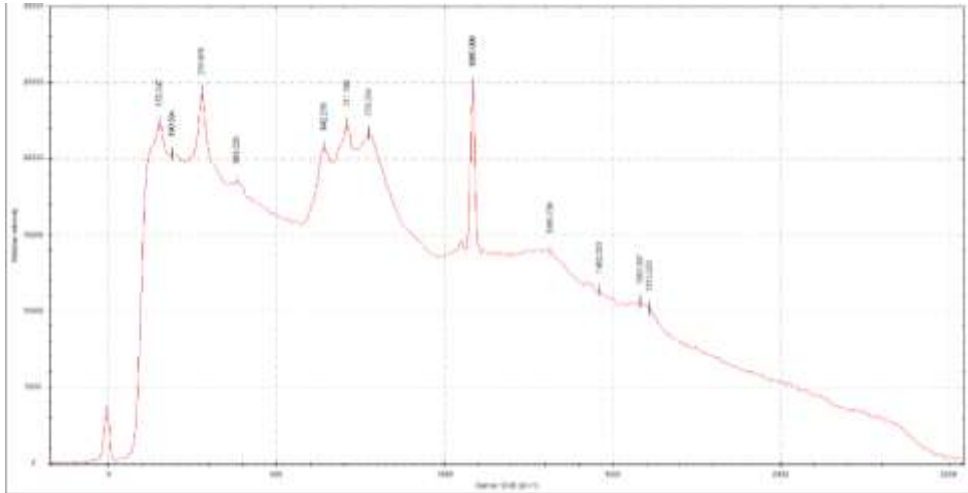


Fig.3. Red ochre Raman spectra representation. Monastery of Saint Michael, Nivan, 1064 nm.

Again we confirm the presence of $CaCO_3$ from peaks at 1085 cm^{-1} , indicating a symmetric mode in C-O bond stretching, the peak at 711 cm^{-1} indicating in-plane bending and low energy peaks at wavenumbers, $279, 153\text{ cm}^{-1}$ [17]. The red ochre pigment in this sample is confirmed by the presence of Hematite Fe_2O_3 at wavenumbers 279 and 617 cm^{-1} and Goethite at wavenumber 380 cm^{-1} [17].

The final thickness ($t_{calculated}$) is presented in Table 2. The calculated results are very close to those obtained using the ImageJ software. All four samples have low determination errors, ranging from 3.6% to 11.2%. This is a good agreement that validates both methods as reliable tools for quantifying the thickness of simple two layered structures.

Table 1. Microscopic picture of samples, front view and cross-section painted layer.

Sample	Picture	Cross-section
1. Red Ochre, Monastery of Saint Michael, Nivan		
2. Yellow Ochre, Monastery of Saint Michael, Nivan		
3. Ochre North Side, Saint Mary of Athali		
4. Red Ochre Saint Koll, Dhrovjan		

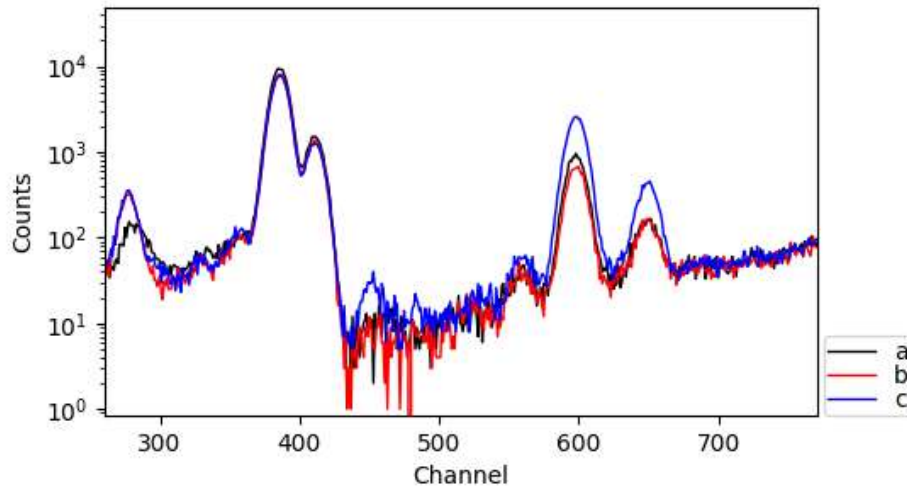


Fig.4. Nivan Red and Yellow Ochre spectra superimposed on the ground layer spectra. Red ochre in black (a), Yellow Ochre in blue (c) and ground layer in red (b).

The plot in Fig.4. shows the attenuation of the Ca $K\alpha$ (channel 380) and Ca $K\beta$ (channel 410) peaks from the ground layer (b) as they pass through the overlying ochre layers (a, c). This visible reduction, and the resulting change in the $K\alpha/K\beta$ ratio, forms the basis of the thickness calculation.

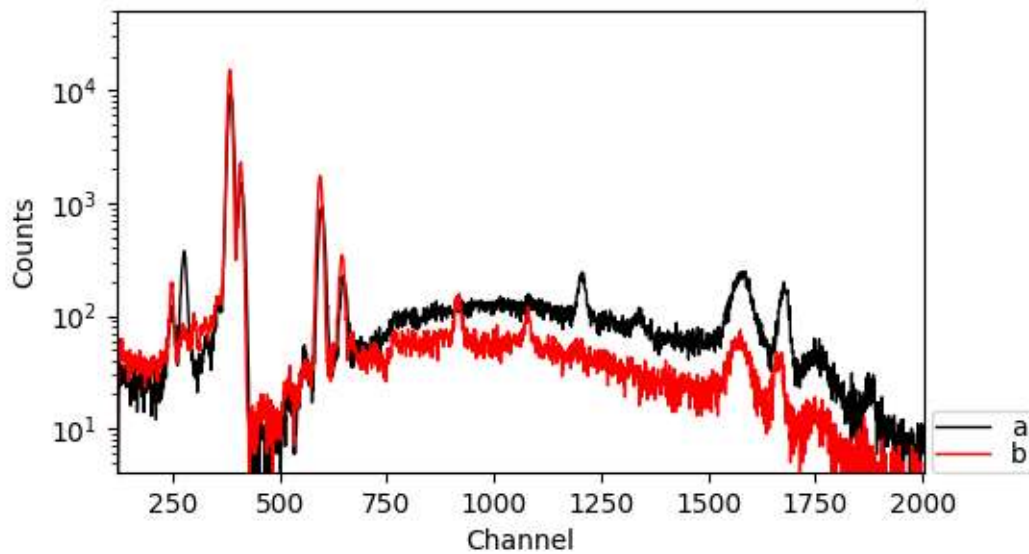


Fig.5. Yellow Ochre b (red spectra) superimposed on the ground layer a (black spectra).

In Figure 5. We have an illustration of a two-layer stratigraphy, the ground layer being defined by strong Ca $K\alpha/K\beta$ peaks (channels 380 to 410), while top layer is identified by the presence of strong Fe $K\alpha/K\beta$ peaks (channels 640-705) which are characteristic peaks in the ochre pigment. The attenuation of the Ca signal by this overlying iron-based layer is clearly visible and the intensity ratios are used in this study, to calculate the layer thickness, presented in table 2.

Table.2. Summary of calculations of painted layer thickness

Sample ID	Stratigraphy	$R_{measured}$	T imageJ (μm)	T calculated	Error (%)
Red Ochre, Monastery of Saint Michael, Nivan	Red Ochre (Fe_2O_3) on $CaCO_3$	6.10	25.0	27.8	11.2
Yellow Ochre, Monastery of Saint Michael, Nivan	Yellow Ochre ($FeO(OH)$) on $CaCO_3$	6.40	20.0	18.5	7.5
Ochre North Side, Saint Mary of Athali	Yellow Ochre ($FeO(OH)$)	6.00	28.0	29.0	3.6
Red Ochre Saint Koll, Dhrovjan	Red Ochre (Fe_2O_3) on $CaCO_3$	5.90	35.0	31.2	-10.8

The minor experimental errors observed are expected and can be attributed to the density approximation and the sample heterogeneity. The model uses the density of pure, crystalline minerals. The actual paint layer, which includes an organic binder and has natural porosity, possesses a lower "effective" density. This is a primary source of systematic error in the calculation. As seen in the cross-section images in Table 1, the layers are not perfectly uniform. Variations in layer flatness or pigment particle size can lead to slight discrepancies between the large spot size of the XRF and the specific path measured in the cross-section.

Crucially, this validation applies *only* to these simple 2-layer structures. The calculation, which only accounts for one attenuating layer, cannot solve the two unknown thicknesses and produces errors well over 30%. This confirms that for complex stratigraphies, a more advanced model (such as a 3-layer iterative calculation or a full Monte Carlo simulation) is required.

CONCLUSIONS

In this study we applied a differential attenuation method, based on the Ca $K\alpha/K\beta$ ratio, for determining pigment layer thickness on 18th-century post-Byzantine murals. The primary finding is that for simple 2-layer systems (ochre on a $CaCO_3$ substrate), the calculated thicknesses show good agreement with ImageJ measurements from cross-sections. All tested samples showed low experimental errors, between 3.6% and 11.2%. This validation confirms the method as a reliable, minimally invasive tool for quantifying simple pictorial layers.

We should mention that the method has some boundaries in application. The validation applies only for simple 2-layer structures. As noted in the discussion section, the 3-layer model produces significant errors (over 30%) when applied to complex, multi-layer stratigraphies because it cannot solve for multiple unknown attenuating layers. This confirms that while the differential attenuation method is a powerful tool, it must be applied with a clear understanding of the sample's stratigraphy. For complex cases, more advanced models like multi-layer iterative calculations or Monte Carlo simulations are required.

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OPTIMIZATION OF EXPERIMENTAL CONDITIONS FOR THE SKYRAY 6000B XRF SPECTROMETER IN CULTURAL HERITAGE PIGMENT ANALYSIS

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ABSTRACT

The study of pigments from cultural heritage objects was conducted using energy dispersive X-ray fluorescence (EDXRF) to establish optimal analytical methodologies. This work centers on the SkyRay 6000B spectrometer, a new instrument at the Institute of Applied Nuclear Physics acquired through the ALB1009 project in collaboration with the International Atomic Energy Agency (IAEA). The purpose of our study is to outline the optimization of measurement conditions necessary for performing high-resolution elemental analysis of artistic and archaeological materials.

Key experimental parameters were refined, including the determination of the system's geometry and the implementation of various radiation filters. This allows for the creation of specialized conditions for analyzing the full range of elements, from light elements (low Z) to medium and heavy elements (high Z). To integrate the instrument into existing laboratory workflows, a spectral converter was developed using Python, enabling data from the SkyRay 6000B to be processed with established software packages such as AXIL, PYMCA, and Bruker's ARTAX.

To validate the system's analytical performance, data from pigment samples taken from cultural objects and archaeological sites were measured with both the SkyRay 6000B and a Bruker ARTAX 800A spectrometer. This paper will present a comparison of the results. Furthermore, a quantitative analysis of a certified tin standard is shown, providing a direct comparison of the systems' quantification algorithms, geometry, and detection limits.

Keywords: X-ray fluorescence, Pigment analysis, Quantitative analysis, Cultural heritage, Spectrometer optimization

1. Introduction

The study of cultural heritage objects, such as paintings, statues, and medieval manuscripts, is considered of utmost importance because these artefacts hold significant artistic, historical, and archaeological value [1]. Investigating these unique items allows scholars to gain new insights into aspects crucial to art history, such as establishing the origin and age of the objects [2], documenting the evolution of materials and techniques used over centuries [2], aiding in the authentication of pieces or the distinction between original work and later restorations [2]. Material analysis is therefore crucial to understand the composition, the selection of raw materials, and the identification of materials within the artwork's layers reveals the creative process of masters and workshops [3]. Critically, precise knowledge of the constituent materials, particularly the identification of pigments and the reconstruction of their layered structures (stratigraphy) [4], is mandatory for conservators and restorers to design effective, correct and long-lasting preservation treatments [2].

Since artworks are often unique samples of high historical value, for which subsampling or movement is generally prohibited [2], the ideal analytical approach must be nondestructive, versatile and fast [1]. Energy Dispersive X-ray Fluorescence is widely used in this field because it meets these requirements, allowing for the rapid and non-invasive elemental characterization of materials in air, which is fundamental to addressing questions of provenance, manufacturing procedure, and conservation status [1].

Energy Dispersive X-ray Fluorescence (EDXRF) is widely recognized as a foundational and powerful analytical tool for spectrochemical determination of nearly all elements present in a sample, making it widely employed in the field of cultural heritage [5]. Its suitability stems from its core features: the technique is nondestructive, multi-elemental and rapid [5]. EDXRF allows for the qualitative and quantitative analysis of elements from Na ($Z=11$) to U ($Z=92$) [5]. Crucially, for analysing fragile artefacts, EDXRF is non-invasive and permits analysis without requiring any sample preparation, ensuring the integrity of the object [6]. The ability to penetrate deeply into matter is a defining feature, which means EDXRF can gather information across multiple pictorial layers, including the preparation ground and underlying pigment layers, although interpreting complex, mixed signal required careful analysis [7].

The field employs a variety of instrumentation, distinguishable by scale and capability, ranging from handheld, battery-operated field units to high-power laboratory units requiring significant space [5]. Portable XRF systems are essential because they allow for in situ analysis, enabling the investigation of masterpieces that cannot be moved or sampled, such as large paintings or frescoes on high walls [7]. These portable devices are compact and often use radioisotope sources or miniature low-power X-ray tubes, offering the critical advantages of fieldwork mobility [5]. In contrast, stationary benchtop or laboratory XRF spectrometers typically use high-power X-ray tubes and often include vacuum chambers or inert gas flushing [5]. This ability to operate under vacuum drastically improves the detection and quantification of light elements ($Z < 10$ keV), which are otherwise strongly absorbed by air [5]. Benchtop systems generally deliver superior energy resolution and higher detection limits, making them a good choice when high accuracy is necessary for complex analysis [6].

This paper will present a comparison of the results. Furthermore, a quantitative analysis of a certified tin standard is shown, providing a direct comparison of the systems' quantification algorithms, geometry, and detection limits. The purpose of our study is to outline the optimization of measurement conditions necessary for performing high-resolution elemental analysis of artistic and archaeological materials.

2. Materials and Methods

The instrumentation for this study involved two EDXRF systems to serve for primary analysis and cross-validation. The first instrument is the Skyray 6000B, a high-end, stationary EDXRF spectrometer. This system is equipped with an electro-cooled Ultra-High-Resolution Detector (UHRD) with specified energy resolution of 125 ± 5 eV. Its X-ray tube operates a variable voltage from 5kV to 50kV. One distinctive feature for this work is the instrument integrated vacuum pump system, which is essential for eliminating air absorption and enabling the accurate detection of low-content light elements, such as Sodium (Na), Magnesium (Mg) and Silicon (Si). The other instrument used for comparative validation is Bruker Artax 800A. The Artax 800A is a portable micro-XRF (μ -XRF) spectrometer featuring an air-cooled Rhodium (Rh) fine-focus X-ray tube and a Peltier-cooled, nitrogen-free XFlash Silicon Drift Detector (SDD). This detector provides a high energy resolution of < 150 eV. In contrast to the

benchtop's vacuum, the ARTAX800A is designed for in-situ work and employs a Helium (He) purging system to reduce photon absorption for light element analysis (Na to Ar).

The reliable identification of pigments demands analytical techniques capable of detecting elements spanning a vast atomic range, from light elements such as Sodium (Na), Aluminium (Al) and Silicon (Si), constituents of historical pigments like lapis lazuli and many binders [4], to heavy elements like lead (Pb) and mercury (Hg) [6]. The accurate measurement of these low atomic number elements using EDXRF presents a significant technical challenge [8]. Based on the physical principles of XRF, this difficulty stems primarily from two complementary effects. First, light elements exhibit a fundamentally low fluorescence yield (ω_k) [5]. Fluorescence yield describes the probability that an inner-shell electron vacancy, created by the primary X-ray beam, is filled by a radiative transition (emission of a characteristic photon), rather than a non-radiative transition that ejects an Auger electron [5]. For light elements (generally $Z < 21$), the competing Auger process is predominantly favored, resulting in a substantially lower output of characteristic X-rays (with ω_k values often < 0.2) compared to medium or heavy elements [5]. Second, the low energy X-rays characteristic of light elements are subject to strong absorption by the atmosphere [9]. The mass attenuation coefficient for X-rays increases significantly at low energies, causing substantial absorption losses in the air gap between the sample and the detector [1]. Consequently, conventional EDXRF measurements performed in air are often limited to detecting photons greater than approximately 2 keV [1]. To achieve this sensitivity and improve the detection limits for low-Z elements, the air path must either be eliminated by using a vacuum chamber or substituted with a low-Z inert gas, typically Helium (He) flushing [1]. This severe absorption also creates a restricted escape path for characteristic fluorescence radiation of low-Z elements, meaning that the critical penetration depths is often only a fraction of a micrometer, thus limiting compositional information largely to the immediate surface layers of the artefact [5].

2.1 Analytical samples

The samples analyzed for this study are: "Ochre Arch first north side", which appears with a bright yellow paint layer on a white base and with a crushed surface. The dimensions of the sample are 1cm X 1cm. And the second sample, "Ochre above Koli" is a small ochre sample with a complex red-brown paint layer, measuring 0.5cm X 0.5 cm. The samples analyzed in this study are presented in figures.1-2.



Fig 1. Ochre Arch 1 North Side. Dimensional representation and microscopic view of the painting's surface.



Fig 2. Ochre above Koli. Dimensional representation and microscopic view of the painting's surface.

For quantitative validation, a certified reference material (CRM) was used, as specified in the abstract. The standard was a Bruker Standard Reference (SUS, approx. RC 36/16), a certified high alloy with copper (Cu) base. Its elemental composition is respectively Cu (76.8%), Pb (12.0%), Sn (8.0%), Ni (1.5%), Zn (1.1%), Sb (0.5%), S (0.03%), Fe (0.02%), As (0.02%), P (0.01%), and Bi (0.01%). This standard was used to directly compare the quantification algorithms of the two spectrometers, particularly for assessing the accuracy of measuring major elements Pb, Sn and the sensitivity for trace elements As, Bi within the alloy matrix.

2.2 Optimization of experimental conditions

To overcome the analytical hurdles explained above in the paper, and to ensure optimal sensitivity across the elemental range required for comprehensive pigment analysis, the experimental conditions, namely the X-ray tube voltage (kV), current (mA), and primary beam filtration, must be carefully chosen for selective excitation [1]. The tube voltage is paramount, as the incident X-ray energy must exceed the characteristic absorption edge energy of the analyte for fluorescence to occur [1]. Low kV settings, eg. 4kV-8kV are generally used, typically with no filter, to maximize the flux of low-energy bremsstrahlung continuum necessary to excite light elements like Na, Al and Si [1]. Conversely, for medium to high Z elements, the kV is optimally set to 1.5 to 2 times the absorption edge energy of the elements of interest [1]. The tube current (mA) regulates the X-ray output intensity, which must be carefully adjusted to manage the detector's limited total counting capacity (typically 1-50 kcps) and prevent spectral degradation from high dead time or pulse pile-up [1].

Primary beam filters modify the polychromatic tube spectrum to maximize the peak to background (P/B) ratio for specified elemental group [1]. For instance, a 0.2 mm Aluminum (Al) filter serves as an absorption filter that selectively reduces the low energy side of the bremsstrahlung spectrum and removes tube L-lines, resulting in high P/B ratios for transition metal elements [1]. Similarly, metal filters such as those incorporating Nickel (Ni) or other transition metals (ex. Copper or Titanium) are typically used in conjunction with optimized kV settings (25-50 kV), to selectively suppress interfering lines or tailor the excitation band, thereby improving detection limits for targeted medium or heavy elements [1]. Effective pigment characterization therefore relies on acquiring a sequence of spectra using varied filter/kV combinations to ensure optimal excitations across the entire periodic table range [1].

3. Results and Discussions

An important methodological step is the creation of a “spectral converter” using Python programming language, as outlined in the abstract. This custom utility was developed to solve data interoperability challenges that arise from different data formats. The script was designed to read the native output files from both the Skyray EDX6000B and the Bruker ARTAX 800A, which store spectral data as text-based energy (x-axis) and intensity (y-axis) pairs. The program automatically parses these files and reformats the data into standard. axil spectrum format, a very important step for integrating the new instrumentation into our established, analytical workflow. For all subsequent spectral processing, two primary software packages were employed: PyMca, a state of the art, open-source Python toolkit developed at the ESRF [10],

and QXAS (Quantitative X-ray Analysis System), an IAEA distributed package that integrates the foundational AXIL algorithm [11]. This software combination was used to perform robust qualitative analysis on pigment samples and comprehensive quantitative analysis on metallic artifacts.

Experiments have been carried out using different measurement conditions related to the use of different combinations of Voltage and Current for the X-ray tube. As well as the use of different filters for the primary beam of radiation to reduce as much as possible the effect of Bremsstrahlung braking radiation that occurs during the interaction of X-ray radiation with matter.

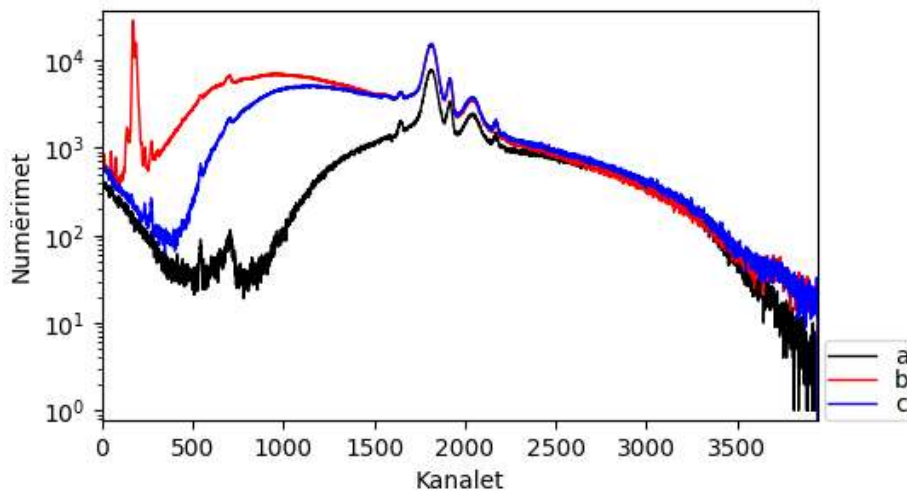


Fig.3. Evaluation of the contribution of the use of primary X-ray filters on the sample. a) NiAl filter, b) No filter, c) 0.2mm Al filter. (Acquisition parameters: 40kV, 100uA, 60s)

It is noted that the use of the Ni-Al alloy filter significantly improves the quality of the spectrum by maximally reducing the contribution of the L lines of the Rh anode on the left side of the spectrum and filtering a significant part of the Bremsstrahlung radiation on the right side of the spectrum. Regarding the use of different voltage and current conditions during spectrum acquisition, it should be mentioned that the voltage change is related to the number of chemical elements that we want to excite in our sample. While the current change is related to the amount of Counts max that we want to obtain. Both conditions are determined by agreement during the development of the experiment. In the following presentation, four spectra of boric acid H_3BO_3 have been obtained with a fixed current condition of $60\mu A$ and voltages that start with 20kV and continue with 30kV, 40kV and 50kV.

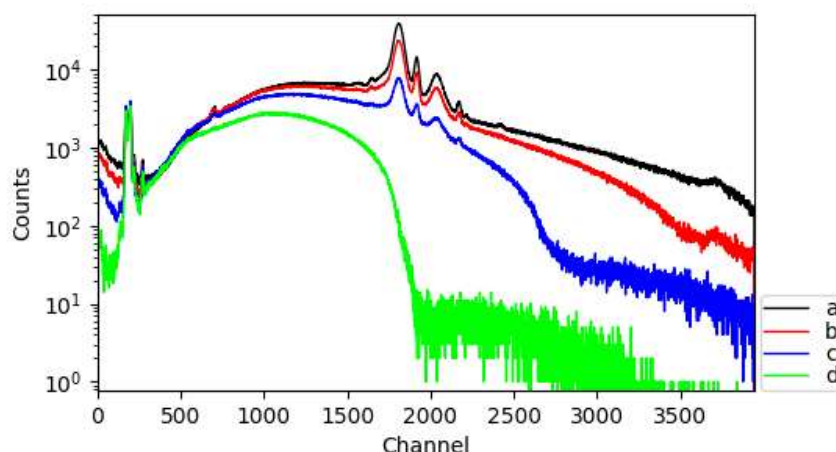


Figure 4. Presentation of boric acid spectra measured with a current of 60 μ A and with different voltages: a) 50kV, b) 40kV, c) 30kV and d) 20kV.

The main idea lies in the fact that since the anode material of the X-ray tube is Rh, it is necessary that the voltage of the X-ray tube exceeds the energy level of Rh itself to excite it into X-ray fluorescence with its K_a and K_b lines. In this way we obtain information on the Rayleigh and Compton peaks of the anode which help us during our daily work by analyzing pigment composite materials. From Figure 4, it is noted that for the voltage value of 20kV the K_a and K_b lines do not appear in the spectrum in the Rayleigh and Compton excitation of Rh because Rh needs at least 22keV of energy to be excited in XRF.

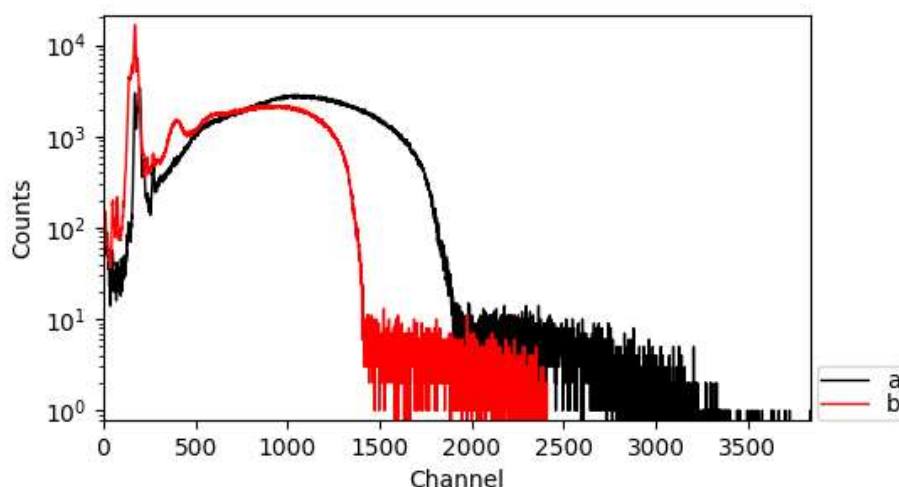


Figure 5. a) Boric Acid measured at 20kV and 60 μ A. b) Boric Acid measured at 15kV and 100 μ A.

From Figure 5, it is understood that increasing the voltage increases the information we expect from elements with higher atomic numbers Z, while increasing the current increases the height of the peak intensities and this is observed in the L lines of Rh on the left side of the spectrum.

In this section we will study the application of the different measurement conditions with the instrument Skyray EDX6000B and Bruker ARTAX 800A for the same samples, respectively “Ochre Arch first north side” and “Ochre above Koli”, both belong to the church of Saint Mary of Athali, Himara, Albania, a cultural heritage object painted by master Vasili in 1795.

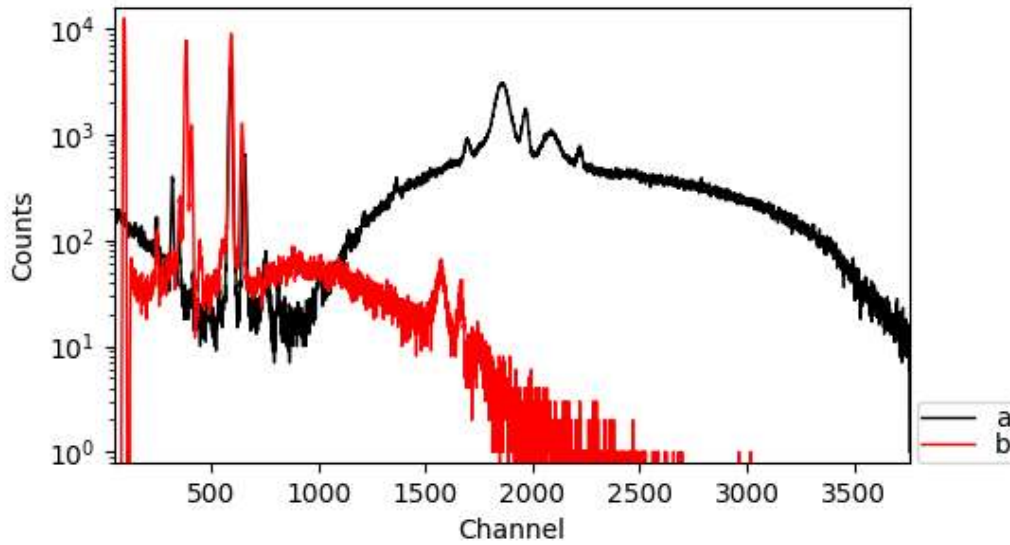


Fig. 6. Representation of XRF measurement for “Ochre Arch first north side”, a) SKYRAY EDX6000B, b) Bruker ARTAX 800.

The measurement conditions differ from one measurement to the other. The measurement with SKYRAY 6000B were performed under vacuum for 120s, 40kV and 60 μ A with a Ni-Al thin filter. While the measurements with Bruker ARTAX 800A were performed without vacuum or helium flush for 100s, 40kV and 300 μ A. As we can clearly see from Figure 6, the spectra are the same only for the middle Z elements, on identifying nearly the same, the presence of Fe, while they differ a lot on low energy part of the spectrum, with the Skyray EDX 6000B performing better, excluding the argon (Ar) peaks and providing visibility for magnesium (Mg) peaks. Ni-Al filter helps in discriminating and having better peak to background ratio and this is visible in the regions from channel 600 to channel 1700. Elements like Ni and Cu are almost non-existent in the ARTAX 800A measurement.

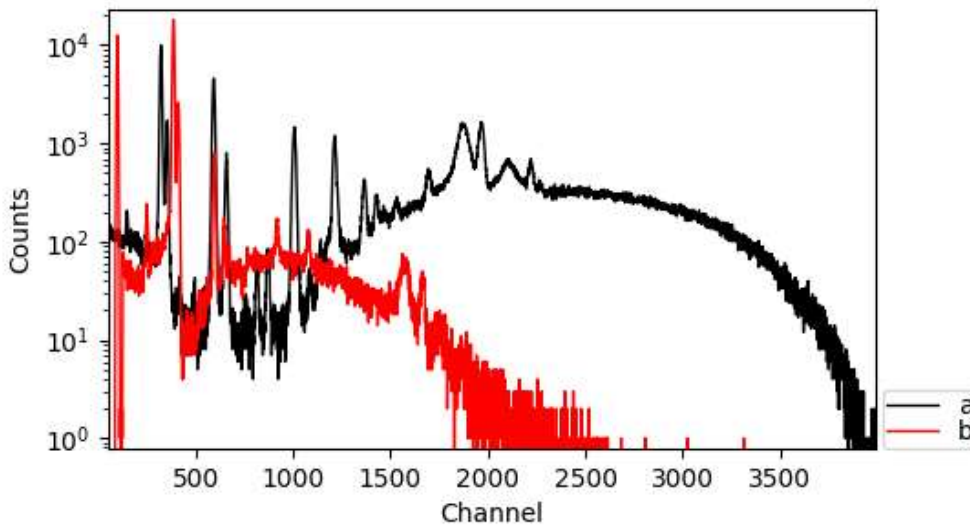


Fig. 7. Representation of XRF measurement for “Ochre above Koli”, a) SKYRAY EDX6000B, b) Bruker ARTAX 800.

The spectra overlay presented in Figure 7 represents a considerable difference between the two measurement conditions, as described in the paragraph above. In this sample, we are dealing with a mixed compound pigment. The EDX6000B (black), clearly reveals the sample's composite nature showing strong characteristic peaks for Iron (Fe K-lines) that come from the red ochre, along with high intensity Mercury (Hg L-lines) and Sulfur (S K-Lines) peaks, which together confirm the presence of *cinnabar HgS*. The differences can clearly be seen in the low energy part of the spectrum where EDX6000B has huge counting rate for the detection of sulfur K line. On the other hand, the ARTAX800A (red), acquired in air atmosphere, shows nearly a signal cutoff in this region, due to atmospheric absorption. Another thing to consider is that despite using five times lower current (60 μ A) than the ARTAX (300 μ A), the EDX6000B registers peak intensities that are orders of magnitude higher, noting the fact that spectra are represented in logarithmic scale in the vertical axis (intensities).

Quantitative X-ray Fluorescence analysis employs several mathematical methods for converting measured fluorescent intensities into concentrations, broadly categorized into the Fundamental Parameter (FP) Method and the Influence Coefficient methods (often referred to as Empirical Coefficient methods) [5]. The Fundamental Parameter method is distinct because it relies on the physical theory of X-ray production, leveraging fundamental constants such as mass-attenuation coefficients and fluorescence yields, rather than purely empirical relationships derived from standards [5]. This theoretical approach results in a computationally complex process involving the iterative solution of a set of equations that relate the observed intensity of one element to the concentrations of all elements present in the sample [5].

A key advantage of the FP method is its utility for semi-quantitative analysis of completely unknown samples, often requiring few or no standards [5]. In contrast, Empirical Coefficients methods operate based on empirical relationships describing the observed X-ray intensity ratio and concentration, using coefficients derived from measured specimens via regression analysis [1]. While influence coefficient algorithms possess a simpler mathematical structure, they generally necessitate the use of more standards compared to FP methods to ensure accurate calibration, although if a large suite of well-characterized standards like the unknown sample is used, they can yield very high analytical accuracy [1].

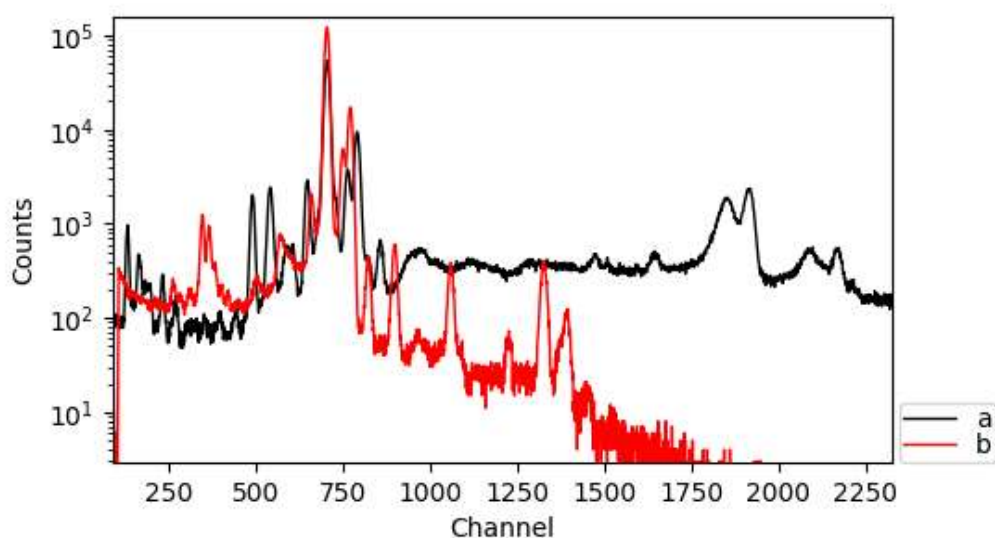


Fig. 8. Bruker Standard Reference (SUS, approx. RC 36/16), measured with EDX600B and ARTAX800A with respective conditions.

A direct comparison of the quantitative capabilities of the two spectrometers is complex and dependent on the chosen analytical conditions. The spectral overlay presented in Fig. 8 demonstrates a difficult challenge. The Skyray 6000B spectrum in black (a), run at 40kV with a NiAl filter but no vacuum, exhibits a high, complex background continuum (the broad hump from channel 1000 to 2250).

This elevated background would compromise the accuracy of any quantification algorithm, such as those in PyMca or QXAS, by complicating the deconvolution of the complex alloy peaks (Cu-Pb-Sn-Zn). In this specific scenario where the optical path between x-ray tube, sample and detector is filled with air in both instruments, the ARTAX 800A spectrum in red (b), run at 30kV, provides a cleaner signal with a superior peak to background ratio for these major elements.

CONCLUSIONS

This comparison only serves to underscore the central thesis of this paper, the necessity of optimization. As proven in the analysis of the red ochre/cinnabar pigment (Fig 7), the EDX6000B primary advantage is its vacuum capability. When the vacuum is engaged (Fig 7, spectrum a) the detection limits are fundamentally improved, enabling the detection of critical light elements like Sulfur (S), Aluminum (Al) and Silicon (Si). These elements are completely absent in all air path measurements from both instruments, providing their loss is due to atmospheric absorption, not detector limitations. Furthermore, the optimized geometry of the Skyray 6000B in vacuum is demonstrably superior, delivering orders of magnitude higher signal intensity at a fraction of the tube current.

Building the python based spectral converter made it possible to process data from two different instruments in the same environment software using PyMca and QXAS. The final remark of this work is that optimization is task dependent. The spectrometrist aims to adapt the measurement conditions on his purposes and make sure to know his instruments as much as possible. Both ARTAX 800A and EDX6000B gave good results depending on the sample being studied and the measurement conditions chosen to perform the analysis. The optimization parameters for the analysis of cultural heritage materials were established. The systematic testing of filters and X-ray tube settings (Figs. 3-5) demonstrated that spectral quality is highly dependent on these choices, with the NiAl filter providing a superior peak-to-background ratio for general analysis.

Finally, the comparative analysis of the certified standard (Fig. 8) underscored the central thesis: **optimization is paramount**. It showed that an un-optimized, air-path measurement on the SkyRay 6000B can be inferior to a properly configured measurement on the ARTAX 800A. This confirms that the SkyRay 6000B is a high-precision laboratory instrument whose superior performance is only unlocked through the systematic application of its full capabilities, especially its vacuum path. This work establishes a dual-instrument (laboratory and *in-situ*) methodology, providing a new standard of practice for future cultural heritage research in our lab.

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MORPHOLOGICAL ANALYSIS OF *PRIMULA VERIS* LEAVES FROM THREE DIFFERENT LOCATIONS IN ALBANIA

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ABSTRACT

Albania's diverse climate and rich natural landscapes provide favorable conditions for the growth of wild medicinal and aromatic plants. *Primula veris*, commonly known as cowslip, is one of the most widespread medicinal species in Europe and Albania. This herbaceous plant from the Primulaceae family typically grows in mountain meadows and forest edges.

In this study, we analyzed *P. veris* populations collected from three regions in Albania—Tropojë (601 m a.s.l.), Librazhd (1146 m a.s.l.), and Qafë-Shtamë (1114.8 m a.s.l.)—to evaluate potential phenotypic differences related to environmental conditions. Morphological traits including leaf length, leaf width, number of flowers per plant, and root length were compared across the three sites. To better understand the influence of habitat characteristics, soil temperature, pH, moisture, and fertility were also measured at each location.

The number of flowers per plant differed significantly among populations, with Qafë-Shtamë and Tropojë plants producing more flowers than those from Librazhd. The longest roots were recorded in Tropojë (mean = 19.2 cm), although root differences were not statistically significant. Leaf size varied notably, with Qafë-Shtamë specimens exhibiting the longest and widest leaves, followed by Librazhd, while Tropojë plants had the narrowest leaves.

Although Qafë-Shtamë and Librazhd are located at similar elevations, they showed clear differences in both leaf morphology and flower production, likely due to contrasting local environmental conditions. Qafë-Shtamë supported both larger leaves and more flowers, potentially linked to higher soil fertility (319 $\mu\text{S}/\text{cm}$) and greater moisture availability. Tropojë, at a lower elevation, exhibited high flower output and longer roots but narrower leaves. These findings suggest that phenotypic variation in *P. veris* is influenced not solely by altitude, but by the interaction between elevation and site-specific ecological factors, underscoring the species adaptability across diverse habitats.

Key words: *Primula veris*, photosynthetic pigments, chlorophyll meter CCM-200

INTRODUCTION

The genus *Primula*, belonging to the family Primulaceae, is widely distributed across the world. It is native to North America, Europe, Algeria, Morocco, much of Asia, and also Albania. These plants are mainly found in temperate climatic zones, ranging from the tropics to polar regions. This wide distribution of the genus *Primula* indicates its remarkable adaptability and ability to survive under diverse environmental conditions (“Plants of the World Online” 2025).

The genus name *Primula* is derived from the Latin word *primulus*, meaning “first” or “foremost” (Ghazanfar & Edmondson, 2014), reflecting the fact that this plant is among the first to appear in spring.

Primula veris is traditionally used for its expectorant, anti-inflammatory, and antioxidant properties, but recent studies also highlight its broader pharmacological potential. Prokofiev *et al.* (2023) showed that *P. veris* extract exhibits cardioprotective effects by reducing oxidative damage and improving myocardial function under chronic alcohol-induced stress. The leaves and flowers of *Primula veris* are particularly valuable due to their high content of bioactive compounds. Ethanolic extracts from these plant parts have been shown to contain significant amounts of phenolic compounds, including kaempferol, quercetin, and gallic acid, which contribute to strong antioxidant activity (Lupitu *et al.*, 2018). Flavonoids, one of the major bioactive groups found in *Primula veris*—have been shown in a recent systematic review to be effective and generally safe in treating a wide range of viral acute respiratory tract infections, including the common cold and influenza (Yao *et al.*, 2022).

The flowers and leaves of *Primula veris* are particularly valuable both medicinally and pharmacologically. According to the European Medicines Agency (EMA, 2012), the flowers and roots are traditionally used as expectorants for the treatment of coughs and are incorporated into herbal preparations. In addition, ethanolic extracts from the leaves and flowers have been shown to contain significant amounts of bioactive phenolic compounds, including kaempferol, quercetin, and gallic acid, which contribute to strong antioxidant activity (Prokofiev *et al.*, 2023).

Primula veris is a plant that typically grows in well-lit or partially shaded areas and is commonly found on dry to moderately moist soils. The soils where this species occurs are generally low in fertility and nutrient content. Additionally, *P. veris* is absent from saline environments due to its minimal tolerance to soil salinity (Hill *et al.*, 2004). *P. veris* thrives better in habitats with higher altitude where the insolation is higher, humidity is lower and generally is drier habitat (Lluga-Rizani *et al.*, 2022).

Primula veris L., a valuable medicinal and ornamental species, exhibits growth and reproductive traits that are strongly influenced by environmental conditions and developmental stage. Leaf expansion, flower production, and secondary metabolite accumulation can be affected by light availability, soil fertility, and moisture. Recent studies have demonstrated that tailored LED lighting, particularly with red light spectra, can enhance both vegetative growth and the production of bioactive compounds in *P. veris* (Tsivelika *et al.*, 2025). Understanding how environmental factors interact with developmental processes is therefore essential for optimizing the cultivation and conservation of this species.

This study focuses on plants from three areas in Albania: Qafë-Shtamë, Tropojë and Librazhd which are recognized for spontaneous flora, and share similar climatic conditions situated in the Northern and Central zones of Albania. The study was conducted to assess phenotypic variation in *Primula veris* populations by analyzing leaf morphology (length and width), the number of flowers per plant, and root length, in order to evaluate potential differences related to local environmental conditions.

MATERIAL AND METHOD

This study focuses on plants from three areas in Albania: Qafë-Shtamë, Tropojë and Librazhd which are recognized for spontaneous flora, and share similar climatic conditions situated in the Northern and Central zones of Albania (Kopali *et al.*, 2013). The study was conducted across three to evaluate the development of *Primula veris*. The specific locations and their respective altitudes are as follows:

Coordinate zones:

- **Qafë-Shtamë** N: 41°31'15.1"
(1114.8 m a.s.l.) E: 19°53'38.0"
- **Tropojë** N: 42°15'29.8"
(601 m a.s.l.) E: 20°13'05.1"
- **Librazhd** N: 41°19'01.6"
(1146 m a.s.l.) E: 20°23'09.1"



Figure 1. The natural habitat of *Primula veris* in the Qafë-Shtamë area in Albania

Plant materials were collected from these three areas during the period of April to May 2025 to capture the plants at their peak developmental stage. To maintain consistency and accuracy, plants were selected according to uniform criteria, including age and health and were collected at the same stage of blossoming

In Qafë-Shtamë, *Primula veris* was found in a well-drained area with moist soil (Figure 1). The atmosphere was humid with abundant mist and the plants grew in semi-shaded conditions under pined trees. Soil temperature at the time of collection ranged from 7-9.4 °C at 10:00 a.m. Most plants were at peak flowering, although some individuals had not yet flowered and showed limited stem development. In Qafë-Shtamë, soil and environmental parameters were measured at the time of plant collection. Soil fertility was 319 µS/cm, moisture content was 52%, and pH was 6.5. Soil temperature was 9.4°C, while sunlight exposure level was 5559



lux. Atmospheric humidity in the area was 55%. These measured parameters indicate that the plants were growing under medium shaded conditions with moderate humidity and moderately fertile soil, providing favorable conditions for *Primula veris* growth.

Figure 2. The natural habitat of *Primula veris* in the Tropojë area in Albania



Figure 3. The natural habitat of *Primula veris* in the Librazhd area in Albania

In Tropojë, *Primula veris* was found in a partially shaded area (Figure 2). *Primula veris* was observed in its natural habitat, a forested area with scattered, thin oak trees. Soil temperature measured during plant collection ranged from 9 to 10°C at 9:17 a.m. The plants grew on humus-rich soil formed from decomposed fallen leaves.

Primula veris in Librazhd was found in an area with moist soil. Soil temperature measured during plant collection ranged from 5.4 to 6°C at 11:00 a.m. The species grew in its natural habitat, a forested area with sparse oak trees and abundant fallen oak leaves (Figure 3). Flowers were located near the bases of the oak trunks, and Northern moss was also observed. The humus layer in this area was moister and looser than in Tropojë, with fewer rocks. Environmental measurements indicated moderate soil fertility (48 $\mu\text{S}/\text{cm}$), soil moisture at 48%, pH 6.5, and soil temperature 8.0°C. Light intensity was 12,419 lux, and atmospheric humidity was 32%. It suggests the plants were in a **well-lit area, possibly under light canopy or in open spots**, receiving diffuse or filtered sunlight.

The plants brought from the 3 selected areas were transported to a laboratory for further morphometric analysis, including leaf number, length, and width, Figure X.



Figure 4. *Primula veris* in the natural habitat in pots after collection and in laboratory

Primula veris plants were selected in a large natural area. All the leaves of each plant were measured, the measurements of the leaves for the length were made at its longest point and for the width at the widest point. Measurements were made for all plants to provide representative data for each habitat.

RESULTS AND DISCUSSION

Measurements of leaf number, leaf length and width, flower number and root length were conducted to assess morphological differences among *Primula veris* plants from the three study areas in Albania.

Based on the study's results, *Primula veris* plants in the Qafë-Shtamë area show superior development compared to those from the other study sites. The largest leaf dimensions, both in length and width, were recorded in plants from this area, as presented in Table 1.

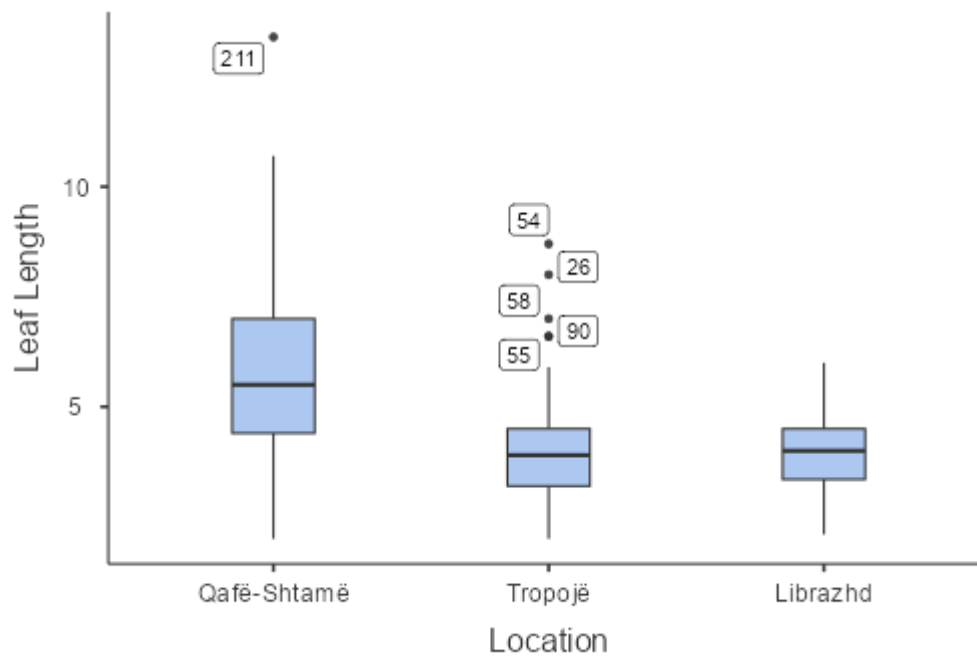


Figure 5. Boxplot of leaf length across the three study locations

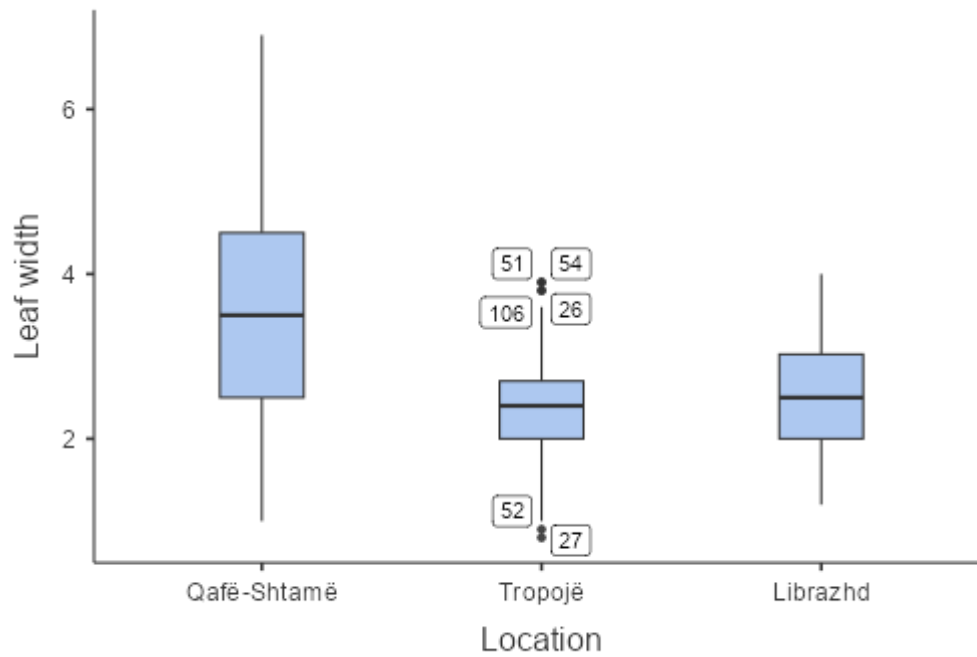


Figure 6. Boxplot of leaf width across the three study locations

Primula veris plants in Qafë-Shtamë exhibited the largest leaf length and width, significantly exceeding those observed in Tropojë and Librazhd (Figure 5 and 6). The similarity in leaf dimensions between Tropojë and Librazhd may reflect comparable environmental conditions at these sites, whereas the enhanced vegetative development at Qafë-Shtamë likely results from local factors promoting growth.

The plants grew in well-drained, moist soil under semi-shaded conditions beneath pine trees (Figure 1). Unlike the other sites, the ground at Qafë-Shtamë was free of fallen leaves, allowing more sunlight to reach the base of the plants, whereas Tropojë and Librazhd had leaf litter from oak trees covering the soil, which may have partially reduced light availability (Figure 2 and 3). This greater exposure to sunlight at the ground level likely stimulated leaf expansion, contributing to the larger leaf dimensions observed.

Additionally, high soil fertility (319 $\mu\text{S}/\text{cm}$) at Qafë-Shtamë would have provided abundant nutrients to support this enhanced leaf growth. Experimental studies also suggest that red light, absorbed by phytochromes, promotes leaf expansion in *P. veris*, even without a proportional increase in leaf biomass (Tsivelika et al., 2025). Together, the combination of better light availability and fertile soil at Qafë-Shtamë likely underlies the superior vegetative development of this population compared to Tropojë and Librazhd.

The number of flowers per plant varied among the three study sites. In Tropojë, individual stems generally produced the highest number of flowers, with an average of 8 open flowers per stem. In Qafë-Shtamë, stems had a slightly lower flower count, averaging 6 flowers, while in Librazhd, flower numbers were the lowest, averaging 4 flowers per stem. In contrast, Librazhd plants, located at higher altitude with colder temperatures and occasional late snow, likely experienced environmental stress that limited reproductive output, resulting in fewer flowers per stem.

All plants were in full bloom, indicating that differences in flower number reflect site-specific growth conditions rather than differences in phenological stage.



Figure 7. Measurement of *Primula veris* roots and observation of root morphology

The root lengths of *Primula veris* did not differ significantly among the three study sites. On average, plants from Qafë-Shtamë had roots measuring 16.6 cm, those from Tropojë 19.2 cm, and plants from Librazhd 15.9 cm. Although Tropojë plants showed slightly longer roots on average, these differences were not statistically significant, suggesting that root growth in this species is relatively consistent across the environmental conditions of the three study areas.

Table 1. Data on the length and width of *Primula veris* leaves from Qafë-Shtamë area

Leaf no.	1	2	3	4	5	6	7	8	9	10
Length	9	3.6	5	3	10.6	9.2	7	5	6	3
Width	5.7	2.3	3.2	1.2	6	5.9	2.8	3	3.5	2

Leaf no.	11	12	13	14	15	16	17	18	19	20
Length	6.4	6.6	6	3.2	2	7.2	10.7	8.6	7	6
Width	3.4	4.4	3.5	2.3	1.5	4.6	6.7	5.5	3.3	3.5

Leaf no.	21	22	23	24	25	26	27	28	29	30
Length	5.2	4.5	6.5	5.3	5	4	3	6.3	5	6
Width	3.5	2.2	4.1	3.5	2.9	2.9	2	4.5	4	4.5

Leaf no.	31	32	33	34	35	36	37	38	39	40
Length	4.3	3	4.5	5.5	6.5	4	3	4.5	3	4.5
Width	2.5	1.5	3	3.7	4.6	2	1.5	3.2	2	3

Leaf no.	41	42	43	44	45	46	47	48	49	50
Length	8.5	3	8	6	7.8	5	5	2.5	3	5
Width	5	2.3	4.4	4	5	2	3.3	1.5	2	3.2

Leaf no.	51	52	53	54	55	56	57	58	59	60

Length	6.5	3.9	8.5	7.6	7	4.5	3	3	2.5	5.1
Width	4.3	2	5.5	4.7	4.5	3.5	2.5	2.4	2	4.2

Leaf no.	61	62	63	64	65	66	67	68	69	70
Length	3.4	4.7	4.9	5.5	6.3	3.5	5.2	5.3	5.5	3
Width	2.5	3.7	3	4.3	5	1.5	3.5	3.6	4	2

Leaf no.	71	72	73	74	75	76	77	78	79	80
Length	9	7.7	6.5	5.5	5.6	7.7	5.8	8	4.6	3.3
Width	4.5	4.5	3.9	3.2	3.5	5	4.3	5.3	3	1

Leaf no.	81	82	83	84	85	86	87	88	89	90
Length	9.5	6	6.7	3	13.4	10.5	8.3	5.5	5.5	6.5
Width	5.3	3	4	2.4	6.2	6.9	4.3	4	4.3	5.1

Leaf no.	91	92	93	94
Length	7.6	7.6	6	6
Width	5	4.9	3.9	4

Table 2. Data on the length and width of *Primula veris* leaves from Librazhd area

Leaf no.	1	2	3	4	5	6	7	8	9	10
Length	3.6	4.2	2.1	4.4	4	4.5	5.5	4.5	3	5.1
Width	2	2.6	1.5	3.1	2	2.4	2.9	3.2	2.1	3.1

Leaf no.	11	12	13	14	15	16	17	18	19	20
Length	6	4.2	3.2	4	3.2	4	4.9	3.6	2.3	3.8
Width	4	2.2	1.5	2.5	2.3	1.5	3.1	2.5	2.1	2.5

Leaf no.	21	22	23	24	25	26	27	28	29	30
Length	5.6	5	3.2	4.7	5.2	5.3	5	2.5	3.5	4
Width	3.9	2.5	2.9	3.4	3.3	3.2	3	1.5	2.5	2

Leaf no.	31	32	33	34	35	36	37	38	39	40
Length	4	3.9	3.5	4.5	3.4	2.6	3.5	4.5	4	3.5
Width	3.3	3.2	1.2	3	2.3	1.5	2	3	2.5	1.9

Leaf no.	41	42	43	44
Length	2.5	3	4.2	3.1
Width	1.9	1.9	2.6	2.2

Table 3. Data on the length and width of *Primula veris* leaves from Tropojë area

Leaf no.	1	2	3	4	5	6	7	8	9	10
Length	3.4	3.1	5.2	3.9	3	4.5	4.2	3.6	3	2
Width	2	2.1	2.3	2.4	2.2	2.5	2.2	2.3	2	2.5

Leaf no.	11	12	13	14	15	16	17	18	19	20
Length	3.6	4.5	3.1	3.6	3.5	2	4	3.5	5	3.3
Width	1.8	2.5	1.7	2.2	1.5	1.1	2.2	2	2.6	2

Leaf no.	21	22	23	24	25	26	27	28	29	30
Length	4	2.5	3	3.5	2.5	8	2.5	5.3	3.6	3.4
Width	2.9	1.9	1.8	2.2	1.1	3.8	0.8	3	1	2

Leaf no.	31	32	33	34	35	36	37	38	39	40
Length	3.2	3	2	3	3	3.5	5	3.3	2.1	5.7
Width	2.5	2.2	1.5	2	2.2	2.1	3.2	2.5	1.6	3.6

Leaf no.	41	42	43	44	45	46	47	48	49	50
Length	3.6	3.5	5.5	5.5	5.1	3	3.5	3.6	4	4.5
Width	2.5	2.5	2.9	3.2	2.7	2	1.5	2.3	2.6	3.1

Leaf no.	51	52	53	54	55	56	57	58	59	60
Length	5.9	3	2.5	8.7	6.6	4.1	4.1	7	4.2	3.5
Width	3.9	0.9	1.6	3.9	3.2	2.5	2	3.4	2.4	2.5

Leaf no.	61	62	63	64	65	66	67	68	69	70
Length	4	5.5	3.5	4.5	4.1	4.3	5.2	3.9	3.6	3.3
Width	2.2	3	3.5	3	2.2	2.7	3.1	2.5	2.2	2.1

Leaf no.	71	72	73	74	75	76	77	78	79	80
Length	2.9	2.3	5.3	4.1	3.6	6.6	4	3.6	3.9	4.3
Width	2.3	5.1	3.1	2.4	2.1	3.4	2.3	2.5	2.3	3.2

Leaf no.	81	82	83	84	85	86	87	88	89	90
Length	2.5	3.5	4.1	2	2.9	5	3.5	4	4.3	4
Width	2.1	2.7	3.1	1.5	2.2	3.1	2.5	2.6	3.1	2.5

Leaf no.	91	92	93	94	95	96	97	98	99	100
Length	3	5.2	3.3	3.6	4.3	3	2.6	4.5	4.5	4.8
Width	1.5	3.8	2.1	2.8	2.8	2.3	2	2.2	2.7	3.5

Leaf no.	101	102	103	104	105	106	107	108	109	200
Length	4.5	5.4	4	5.2	2.2	4.5	5.1	4.6	3	4

Width	2.5	2.7	2.5	2.5	1.6	2.5	3.1	2.7	1.7	2.2

Leaf no.	101
Length	4.5
Width	2.2

CONCLUSION

The study demonstrates that environmental conditions significantly influence the morphological traits of *Primula veris* in Albania. Plants from Qafë-Shtamë exhibited superior vegetative development, with the largest leaves in both length and width, likely resulting from a combination of higher soil fertility, well-drained moist soil and greater sunlight exposure due to the absence of leaf litter and the semi-shaded canopy of pine trees.

Tropojë and Librazhd populations showed smaller leaf dimensions, with Tropojë plants producing the highest number of flowers per stem, while Librazhd plants, growing at higher altitude with colder temperatures and occasional late snow, had the fewest flowers, reflecting environmental constraints on reproductive output. These findings highlight the importance of soil and light conditions in shaping the growth and reproductive characteristics of *P. veris*, suggesting that both vegetative and reproductive traits are closely linked to site-specific ecological factors.

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VARIATION IN CHLOROPHYLL CONTENT AND STOMATAL INDEX IN *PRIMULA VERIS* ACROSS THREE LOCATIONS IN ALBANIA

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ABSTRACT

Understanding the variation of chlorophyll content and stomatal traits within a species across different environments offers valuable insights into plant adaptive strategies. This study investigates chlorophyll content and stomatal index in *Primula veris* populations from three ecologically distinct regions in Albania: Tropojë (601 m a.s.l.), Kukës (561 m a.s.l.) and Librazhd (1146.2 m a.s.l.). Chlorophyll content, an indicator of photosynthetic potential, was measured using the CCM-200 chlorophyll content meter. Stomatal index on both adaxial (upper) and abaxial (lower) leaf surfaces was assessed using the EVOS XL Core Imaging System, following epidermal membrane removal to expose the stomatal layer for microscopic observation.

Statistical analysis revealed significant interpopulation differences. Kukës specimens exhibited the highest median chlorophyll content (17.0), while Tropojë recorded the highest individual value (30.3). Librazhd plants showed the highest stomatal index on the adaxial surface (16.4), indicating a potential adaptive response to microclimatic or altitudinal stress.

The observed variation is likely determined by elevation-related environmental factors. Librazhd, at the highest altitude, exhibited increased stomatal index, likely indicating physiological adjustment to reduced atmospheric CO₂ availability and increased stress variability. In contrast, the mid-elevation site of Kukës supported higher chlorophyll accumulation, suggesting more favorable conditions for photosynthetic activity. These findings highlight how differences in elevation can influence physiological strategies in *P. veris*, with chlorophyll content and stomatal traits acting as useful indicators of the species ability to adapt to different environmental conditions.

Key words: *Primula veris*; chlorophyll content; stomatal index; chlorophyll meter CCM 200

INTRODUCTION

The roots of *Primula veris* are particularly rich in bioactive compounds primverin and primulaverin (Bączek et al., 2017). The species is known for a wide range of pharmacological activities, including expectorant, anti-inflammatory, diuretic, antifungal, secretolytic, sedative, antioxidant and antimicrobial effects (Papadhopuli, 1976; Bogenschutz-Godwin et al., 2002; EMA, 2012;

Primula veris is traditionally valued for its therapeutic potential in treating skin disorders, exhibiting anti-bleeding and hemostatic effects, as well as antifungal, antibacterial, and

antiseptic properties useful against mycoses, dermatophyte infections, and ulcer-related infections (Tsioutsiou et al., 2022).

Nowdays, Albania is ranked among the leading Mediterranean countries for the export of several medicinal and aromatic plants, among which *Primula veris* is included (Pazari 2014). Albania's long isolation on the 20th-century has preserved its rich diversity and aromatic plants (Pieroni, 2012).

A 2021 BBC report highlighted the economic importance on Albania's wild harvested medicinal and aromatic plants such as *Primula veris*. The industry provides significant opportunities and independence for local plant collector women in Kukes while supporting traditional knowledge.

Although previous studies have documented the traditional uses of *P. veris* and some of its ecological distribution there is a limited knowledge about its physiological variability, particularly chlorophyll content and stomatal traits, across different habitats in Albania.

The study encompasses three areas in Albania: Tropojë, Kukës and Librazhd. The territory of Albania is divided into four agro-ecological zones with distinct ecological characteristics (Shundi, 2003; Kopali et al., 2007). Our three study areas are all located on Northern and central mountainous zone that is exposed to high quantities of snow, number of frost days and share similar climatic conditions.

The present study aims to investigate differences in chlorophyll content and stomatal index of *Primula veris* plants from three locations providing insights into their physiological adaptation.

Chlorophyll content index (CCI) provides a rapid and reliable indicator of plant health and photosynthetic efficiency (Pavlovic et al., 2014).

Stomata are crucial for balancing water loss and photosynthetic performance, and their patterning can change in response to environmental cues such as light and CO₂, though the underlying mechanisms remain incompletely understood (Casson & Gray, 2008).

MATERIAL AND METHOD

The study was conducted across three regions located in Albania (Tropojë, Kukës and Librazhd) to evaluate the development of *Primula veris*. The specific locations are as follows:

- **Tropojë:** N: 42°15'29.8"
E: 20°13'05.1"
- **Kukës:** N: 42°02'16"
E: 20°20'17"
- **Librazhd:** N: 41°19'01.6"
E: 20°23'09.1"

The selected sites include both mountainous and lowland regions of Albania and are selected due to their favorable conditions for medicinal and aromatic plant growth, and the fact that they are known as natural habitats of *Primula veris*.

Plant sampling took place between April and May 2025, corresponding to the peak flowering and full vegetative development of the species. In each area, individuals of similar age, size, and overall health were selected to ensure consistency across sites. Only plants at the same flowering stage were included in the study. Individuals were collected at sufficient distances from each other, and only fully expanded, mature, undamaged leaves were taken for analysis.

Chlorophyll content was measured on site in all leaves using a CCM 200 chlorophyll meter. Clean leaves were placed between the meter's sensors, and three readings were taken

per leaf, with the average value used for analysis. After field measurements, the plants were transported to the laboratory for further analysis on stomatal traits (Figure 1).

The plants were transported to the laboratory under shaded and cool conditions, where they were maintained in a stable environment until analyses were performed. From each plant, fully expanded, mature, and undamaged leaves were selected and used immediately for stomatal analysis. For each leaf, at least five epidermal peels were prepared, and from each peel, stomatal and epidermal cells were counted in five randomly selected microscopic fields. This approach provided enough repeated measurements to obtain clear and reliable values of the stomatal index for both the upper and lower epidermis.



Figure 1. *Primula veris* in its natural habitat during field measurements and in the laboratory

The stomatal index was evaluated on both the adaxial (upper) and abaxial (lower) surfaces of the leaves. For this analysis, the epidermal membrane was carefully removed to expose the stomatal layer, allowing clear microscopic observation. The measurements were performed using the EVOS XL Core Imaging System, shown in Figure 2.

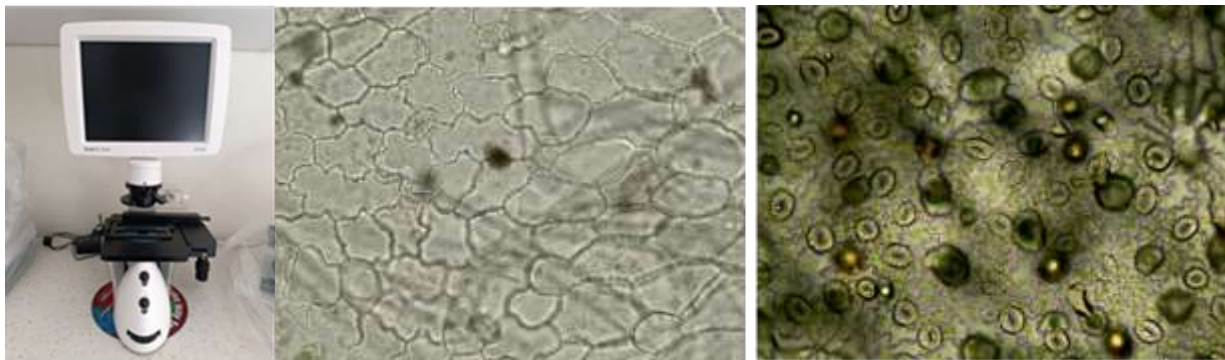


Figure 2. EVOS XL Core microscope (left) and images of stomatal cells on the adaxial and abaxial leaf surfaces of *Primula veris* (right)

Using the EVOS XL Core Imaging System, the number of stomata and stomatal apparatus on each surface was counted, and the stomatal index was calculated based on these counts to quantify stomatal density and distribution.

The formula used for the calculation of stomatal index:

$$SI = \frac{\text{the total number of stomata}}{\text{the total number of stomata} + \text{total number of epidermal cells}} \times 100 \text{ (Poole \& Kürschner, 2020).}$$

RESULTS AND DISCUSSION

The measurement of chlorophyll content and the calculation of stomatal index in each leaf surface were made with the purpose to show the differences between individual plants in three different areas in Albania.

Measurements of chlorophyll content differed significantly among the three *Primula veris* populations. The Kukës population showed the highest median chlorophyll content (17.0), while the highest individual value was recorded in Tropojë (30.3). Librazhd specimens exhibited the lowest overall chlorophyll values. Statistical tests (a non-parametric Kruskal–Wallis test) confirmed that plants from Kukës differs significantly from both Tropojë and Librazhd plants related to chlorophyll content.

Based on the results, *Primula veris* plants from Kukës show the highest chlorophyll content index (CCI) and Tropojë exhibits the widest range and greatest variability in CCI values. (Figure 3).

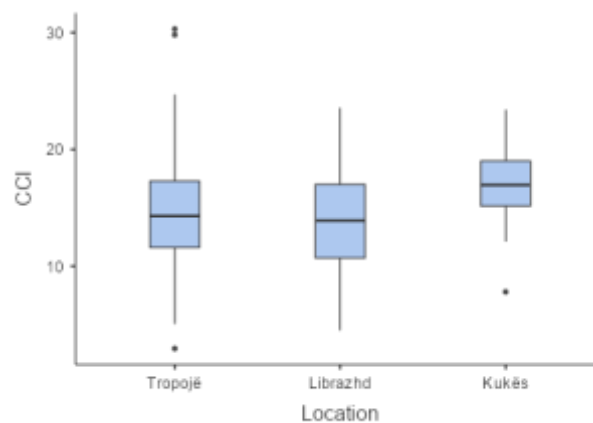


Figure 3. Boxplot of chlorophyll content index (CCI) across the three study locations

The chlorophyll pigment in *Primula veris* plants from the Tropojë area (Table 1) and the Librazhd area (Table 3) show approximately similar values.

The *Primula veris* plants in Tropoja area in Figure 4 were developed at a height 601 meters above sea level.



Figure 4. The natural habitat of *Primula veris* in Tropoja area

Primula veris plants in Kukës area, located at an elevation of 561 meters above sea level, were observed to be full in flowers, as illustrated in Figure 5.



Figure 5. The natural habitat of *Primula veris* in Kukës area (left) and in Librazhd area (right)

Primula veris plants were identified at an altitude of 1146.2 meters above sea level in Librazhd area, where they were found to be rarely distributed.

Table 1. Chlorophyll content in leaf samples from *Primula veris* plants in Tropoja area

Plant number	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Plant 1	9.43	9.66	14	12.86	8.86	10.8	11.13	11.5
Plant 2	7	10.8	19.6	13.2	15.75			
Plant 3	14.15	8.4	15.76					
Plant 4	12.2	13.1	10.6					

Plant 5	15.05	16.5	16.5	15.4	9			
Plant 6	10.6	6.9	8.8	11.8				
Plant 7	14.5	15.7	6.3	11.9				
Plant 8	15.6	10.4	16.2					
Plant 9	17.13	12.4	9.83	17.93	11.7	14.85		
Plant 10	14.2	15.23	14.16	10.2	12.8			
Plant 11	8.8	7.5	13.93	13.96				
Plant 12	18.6	13	13.7	10	20.96			
Plant 13	19.3	30.3	20	19.8	18			
Plant 14	29.8	19	24.7	17.8				

As shown in Table 1, *Primula veris* plants from the Tropojë area exhibited the highest recorded CCI value of 30.3.

Table 2. Chlorophyll content in leaf samples from *Primula veris* plants in Kukës area

Plant Number	Leaf 1	Leaf 2	Leaf 3	Leaf 4
Plant 1	15.95	22.93	17.95	15.1
Plant 2	23	16	21.1	18.7
Plant 3	19.1	13.7	18.4	15.3
Plant 4	15	17.3	7.8	
Plant 5	22.8	23.4	16.6	

As shown in Table 2 most of the *Primula veris* plants studied in the Kukës area had 4 leaves per plant, with an average chlorophyll content of 17.

Table 3. Chlorophyll content in leaf samples from *Primula veris* plants in Librazhd area

Plant number	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
Plant 1	19.8	13.4	22.8	21.2		
Plant 2	17	15.5	13.4	15.5	15.8	
Plant 3	16.4	15.4	8.9	16.9	18.4	16.7
Plant 4	15.1	12.2	19.1	11.6	21.1	19.9
Plant 5	11.2	14.2	22.6	14.7	22.6	
Plant 6	14.7	19	12.2	10.7	12.3	
Plant 7	21.1	15.9	17.3	14.9	12.4	
Plant 8	13.7	20.1	13.7	18.2	19.6	
Plant 9	10.9	11.4	15.7	15.8		

As shown in Table 3 most of the *Primula veris* plants studied in the Librazhd area had 5 leaves per plant, with an average chlorophyll content of 13.7.

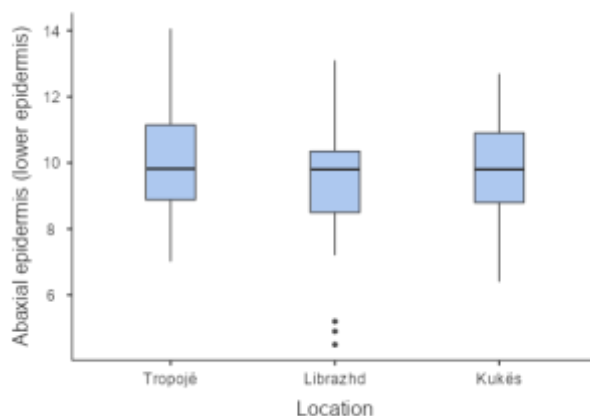


Figure 6. The stomatal index of the abaxial (lower) epidermis across three study locations

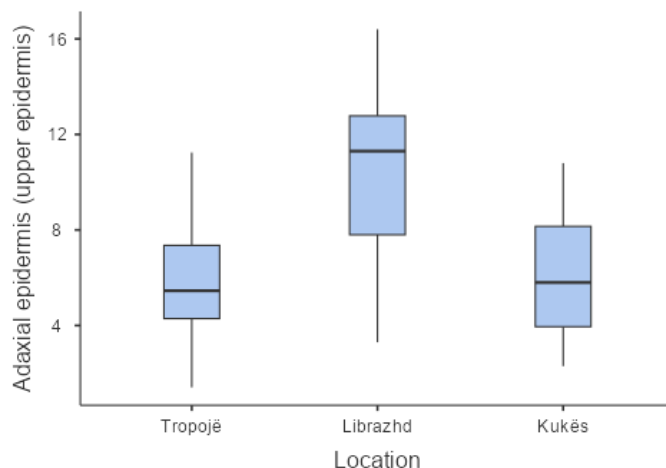


Figure 7. The stomatal index of the adaxial (upper) epidermis across three study locations

Stomatal observations also revealed clear differences between the adaxial and abaxial surfaces of *Primula veris* leaves. In *Primula veris* plants from the areas taken in study the epidermal cells on the upper (adaxial) surface are larger than those on the lower (abaxial) surface (Figure 8). Similar observations have been reported in other *Primula* species, such as *P. auriculata*, where anomocytic stomata were found on both leaf surfaces (Akçin et al., 2023). Stomata were generally more numerous on the abaxial surface, which is typical for many mesophytic plants, while fewer stomata were observed on the adaxial surface.

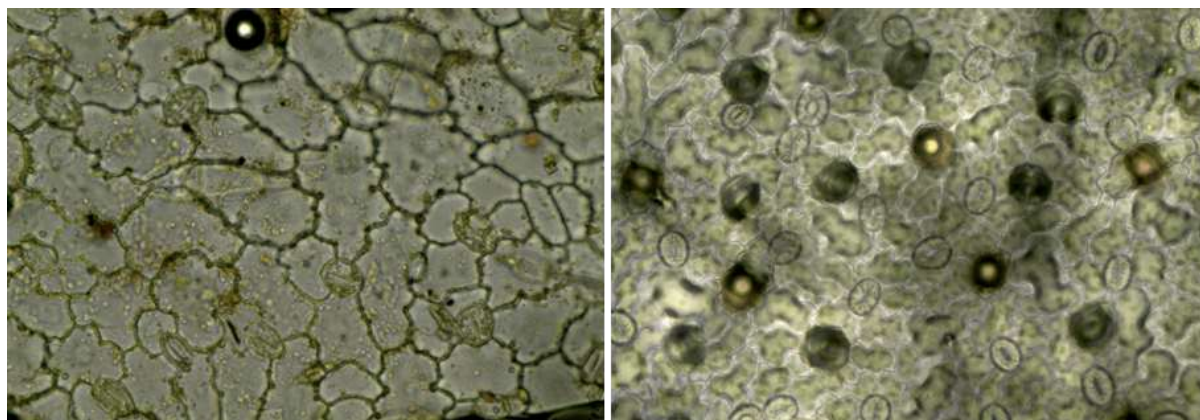


Figure 7. Epidermal cells of *Primula veris* on the adaxial (upper, left) and abaxial (lower, right) leaf surfaces at the same magnification.

Table 4. Stomatal index values of the adaxial (upper) and abaxial (lower) leaf surfaces of *Primula veris*

		Tropojë	Kukës	Librazhd
Adaxial Surface	Leaf	5.85%	5.5%	10.5%
Abaxial Surface	Leaf	10.13%	9.7%	9.4%

In the Librazhd area, the stomatal index on the adaxial (upper) leaf surface of *Primula veris* was higher than in the other studied locations, which is unusual since this species typically has more stomata on the abaxial (lower) surface. This atypical pattern may be influenced by

both local and elevation-related environmental factors. Notably, snowfall had occurred just a few days before sampling, which is uncommon for April and May and may have imposed additional stress on the plants. Such conditions, low temperatures and occasional late snow may have triggered adaptive responses, including an increased stomatal index on the upper epidermis, as a physiological adjustment to maintain gas exchange and photosynthetic efficiency under stress.

Although *Primula veris* plants from these study areas belong to the same agro-ecological zone according to the classification of Shundi (2003) and Kopali et al. (2007), they exhibit notable variations in chlorophyll content and stomatal index.

CONCLUSION

This study demonstrated significant variation in both chlorophyll content and stomatal traits among *Primula veris* populations from Tropojë, Kukës, and Librazhd.

This study revealed clear variation in chlorophyll content and stomatal traits among *Primula veris* populations from Tropojë, Kukës, and Librazhd. Plants from Kukës showed the highest chlorophyll content and visually appeared healthy and fully flowering. Tropojë and Kukës populations, growing in drier soils, exhibited similar stomatal indices, suggesting comparable anatomical adaptations for water use. In contrast, Librazhd plants at higher altitude showed an increased adaxial stomatal index, likely as an adaptive response to higher soil moisture and environmental variability, including occasional late snow. These results highlight the plasticity of *P. veris* in response to local conditions and provide important baseline data for its physiology, adaptation, and conservation in Albania. These findings highlight that *Primula veris*, even within the same agro-ecological zone, exhibits plasticity in physiological and anatomical traits in response to local environmental conditions.

The results provide valuable baseline data for further studies on the adaptation, physiology, and conservation of this medicinal and aromatic plant species in Albania.

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FOOD SAFETY AWARENESS AND BEHAVIOR: TOWARD INTEGRATED APPROACHES FOR SUSTAINABLE PUBLIC HEALTH

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ABSTRACT

Introduction: Food safety represents a vital dimension of sustainable public health, closely linked to nutrition, environmental hygiene, and consumer behaviour. Despite improvements in food systems, limited awareness and inconsistent practices among consumers continue to contribute to foodborne diseases. An integrated approach combining education, regulation, and behavioural insight is essential to promote safe food practices and public health resilience. **Aim:** This study explores consumer awareness, attitudes, and behaviours toward food safety, emphasizing the need for integrated and preventive health strategies. **Methodology:** A cross-sectional mixed-method design was applied, combining a structured survey of 500 consumers with focus group discussions in Tirana. The data explored knowledge, hygiene practices, risk perception, and socio-demographic influences. Quantitative analyses identified behavioural predictors, while qualitative findings provided contextual understanding. **Results and Discussion:** Results reveal high awareness but gaps between knowledge and practice. Education level, access to food safety information, and post-pandemic behavioural changes emerged as key determinants of safe practices. Findings support the need for community-based and interdisciplinary interventions that integrate food safety into broader health and sustainability agendas. **Conclusion:** Enhancing food safety awareness through integrated health education and behavioural interventions can foster safer consumer practices and sustainable public health outcomes.

Keywords: food safety, consumer behaviour, integrated health, sustainability, public health

1. INTRODUCTION

Food safety is a fundamental component of sustainable public health, influencing disease prevention, food system resilience, and overall well-being. Globally, foodborne illnesses affect an estimated 600 million people annually, resulting in significant morbidity, mortality, and economic burden (WHO, 2022). In many developing regions, including the Western Balkans, food safety challenges are exacerbated by informal markets, inconsistent regulatory enforcement, and varying levels of consumer awareness.

Despite advancements in food control systems, studies emphasize that consumer behaviour remains a critical determinant of food safety outcomes (Charlesworth & Mullan, 2023). Unsafe practices such as inadequate handwashing, cross-contamination, improper storage temperatures, and inadequate cooking contribute to household-level risk. The COVID-

19 pandemic further reshaped public perception and behaviours regarding hygiene, yet its lasting influence remains uneven.

This paper expands on the importance of an integrated approach that merges public health education, behavioural insights, and sustainability principles. Understanding consumer behaviours and underlying determinants is essential for designing effective interventions that support safer food environments and healthier communities.

The purpose of this study is to evaluate consumer knowledge, attitudes, and behaviours related to food safety, and to identify key determinants influencing safe practices. The study aims to:

1. Assess the level of awareness and understanding of food safety principles among consumers.
2. Examine the behavioural practices related to food purchasing, handling, storage, and preparation.
3. Analyze socio-demographic and contextual factors associated with safer food behaviours.
4. Provide evidence-informed recommendations for integrated, community-based food safety interventions.

Literature Review

Food safety awareness is shaped by knowledge, socio-economic conditions, and behavioural tendencies. Recent studies indicate strong awareness but inconsistent translation of knowledge into safe practices (Meixner et al., 2024). Behavioural theories such as the Health Belief Model and Theory of Planned Behavior provide a framework to understand consumers' risk perception and decision-making processes.

1.1 Knowledge-Practice Gap

Consumers may recognize food safety risks but fail to apply safe practices consistently due to convenience, lack of motivation, or misunderstanding (Erol et al., 2023).

1.2 Socio-Demographic Influences

Education level, income, gender, and household composition significantly influence risk perception and hygiene behaviours. Women traditionally participate more in food preparation and often demonstrate higher safety awareness.

1.3 Post-Pandemic Behavioural Shifts

Public health messages during COVID-19 increased attention to hygiene; however, long-term maintenance remains uncertain.

1.4 Integrated Public Health Approaches

Successful interventions incorporate education, behavioural nudges, regulatory enforcement, and community engagement (Charlesworth & Mullan, 2023).

MATERIAL AND METHOD

Study Design: A cross-sectional mixed-methods design was employed combining: Quantitative survey of 500 consumers in Tirana and Qualitative focus groups (3 groups, 8–10 participants each). **Sample Selection:** Participants were selected using stratified random sampling by: Age, Gender, Education, Residential area (urban/suburban). Survey tool included: Knowledge assessment (15 items), Self-reported hygiene behaviours (20 items), Risk perception scale, Socio-demographic questionnaire. Focus groups explored beliefs, barriers, post-pandemic changes, and sources of information. **Data Analysis** were Quantitative: Descriptive statistics, chi-square tests, logistic regression and Qualitative: Thematic analysis.

RESULTS AND DISCUSSIONS

Participant Characteristics

Table 1. Socio-demographic profile of participants (N = 500)

Characteristic	Percentage (%)
Female	58%
Male	42%
University degree	47%
Secondary education	38%
Primary education	15%
Urban residence	72%
Suburban/peripheral	28%

Awareness Levels

High awareness observed in:

- Hand hygiene (92%)
- Food expiration labels (87%)
- Safe cooking temperatures (66%)

Table 2. Food Safety Awareness Scores

Knowledge Item	Correct Response (%)
Cross-contamination risks	78%
Refrigerator storage zones	54%
Reheating guidelines	63%

Behavioural Practices

Despite awareness, gaps in practice persisted:

- Only 49% follow recommended refrigeration temperatures
- 38% report occasional cross-contamination
- 41% reheat food incorrectly

Qualitative findings highlight convenience, time limitations, and "underestimation of risk" as major barriers.

Behavioral Predictors

Regression analysis identified significant predictors:

Table 3. Predictors of Safe Food Practices

Predictor	Significance ($p < 0.05$)	Influence
Education level	Yes	Positive
Access to information	Yes	Positive
Post-pandemic hygiene habits	Yes	Moderately positive
Age	No	Neutral

Table 4. Correlation Between Education and Food Safety Behavior

Behavior Domain	r	p -value
Hygiene practices	.42	$< .001$
Storage practices	.37	$< .001$
Cross-contamination prevention	.29	$< .01$

Table 4 presents the correlation coefficients examining the relationship between education level and three key domains of food safety behavior: hygiene practices, storage practices, and cross-contamination prevention. All three correlations are positive and statistically significant, indicating that higher levels of education are consistently associated with safer food handling practices.

The strongest correlation was observed between **education and hygiene practices** ($r = .42, p < .001$), suggesting that individuals with higher education are more likely to adopt proper handwashing, cleaning, and sanitation behaviors in daily food preparation. This moderate-to-strong association highlights the influence of formal education on risk awareness and compliance with basic safety guidelines.

Similarly, **storage practices** demonstrated a significant positive correlation with education ($r = .37, p < .001$). Participants with more advanced education showed better adherence to recommended refrigeration practices, food separation, and expiration date monitoring. This finding implies that education contributes to greater knowledge of food spoilage risks and proper household storage management.

The correlation between education and **cross-contamination prevention** was somewhat weaker but still statistically significant ($r = .29, p < .01$). Although the association is modest, it still indicates that individuals with higher education levels are more likely to follow practices such as separating raw and cooked foods and properly sanitizing cutting surfaces. The weaker correlation may reflect the fact that cross-contamination behaviors are less intuitive and may require more targeted education or practical demonstrations than other safety domains.

Overall, the data show that **education is a significant predictor of safer food handling behavior across multiple domains**. These results support the broader literature indicating that education enhances both knowledge and risk perception, thereby contributing to improved food safety practices in household environments.

Table 5. Predictors of Safe Food Handling Behavior

Predictor	β	p-value
Education level	0.41	< .001
Access to credible information	0.33	< .01
Post-pandemic hygiene habits	0.27	< .05

Table 5 summarizes the results of the regression analysis assessing the strongest predictors of safe food handling behavior. Three variables emerged as statistically significant predictors: education level, access to credible information, and post-pandemic hygiene habits, each contributing uniquely to explaining variations in consumer practices.

Education level was the strongest predictor ($\beta = 0.41, p < .001$), indicating that individuals with higher educational attainment are substantially more likely to engage in safe food handling behaviors. This reinforces previous findings showing that education enhances health literacy, risk recognition, and the adoption of evidence-based household safety practices.

Access to credible information also showed a significant positive effect ($\beta = 0.33, p < .01$). Consumers who regularly accessed reliable food safety sources—such as public health agencies, nutrition experts, or verified digital platforms—demonstrated better compliance with recommended behaviors. This finding highlights the importance of information quality, suggesting that increasing the availability and visibility of trustworthy sources could meaningfully improve public safety outcomes.

Post-pandemic hygiene habits emerged as a significant but comparatively weaker predictor ($\beta = 0.27, p < .05$). The COVID-19 pandemic led to widespread changes in personal and household hygiene practices, many of which appear to have persisted and now contribute indirectly to safer food handling. These habits include more frequent handwashing, increased surface sanitation, and heightened awareness of contamination risks. Although the effect size is smaller, its significance indicates that sustained behavioral shifts resulting from public health crises can play a valuable role in long-term food safety improvements.

Taken together, the results show that safe food handling behavior is shaped by a combination of structural factors (education), informational access, and habitual practices, demonstrating the value of integrated, multi-level public health interventions.

DISCUSSIONS

Results support global findings: awareness is high, but behaviours lag behind. Education level correlates with safer practices, consistent with previous research (Meixner et al., 2024). Post-pandemic behavioural improvements show potential but require reinforcement. Integrated interventions that combine public health messaging with behavioural insights may help overcome habitual and motivational barriers.

The findings of this study align closely with global research trends indicating that, although consumer awareness of food safety principles is relatively high, a significant discrepancy persists between knowledge and actual behaviour. This knowledge–practice gap continues to be one of the most persistent challenges in public health and risk communication. The results reinforce earlier studies (Charlesworth & Mullan, 2023; Meixner et al., 2024), demonstrating that awareness alone is insufficient to guarantee safe food handling practices. Instead, behaviour is shaped by multiple interconnected factors—educational background, perceived vulnerability, social norms, time constraints, and post-pandemic behavioural changes.

One of the strongest predictors in this study was the education level, which showed a positive and statistically significant association with safe food handling behaviours. Individuals with higher education tend to seek out more information, interpret risk-related messages more accurately, and demonstrate higher adherence to hygiene recommendations. This finding is consistent with Erol et al. (2023), who reported that consumers with university-level education are more likely to engage in preventive behaviours such as proper storage, temperature control, and cross-contamination avoidance. However, education alone does not fully eliminate unsafe habits, indicating that behavioural interventions must also target motivational and contextual drivers of behaviour.

Another important determinant emerging from the data was post-pandemic changes, which appear to have had a temporary positive impact on hygiene behaviours—particularly handwashing frequency, surface sanitization, and cautiousness during food purchasing. However, focus group discussions revealed that many of these behaviours have weakened since the acute phase of the pandemic ended. This suggests that once perceived risk diminishes, individuals tend to revert to pre-pandemic habits, a pattern also identified by global studies on behavioural fatigue. Thus, maintaining momentum in hygiene practices requires ongoing reinforcement, reminder systems, and community-level engagement strategies.

The study also highlights the influence of access to accurate food safety information, which was significantly associated with safer behaviours. Consumers who relied on credible sources—public health institutions, trained professionals, and verified digital platforms—demonstrated considerably higher adherence to safe handling practices compared to those who obtained information informally or through social media. This underscores the role of quality risk communication in shaping public behaviour and mitigating misinformation, which has become especially prevalent in the digital age.

While awareness was high in areas such as hand hygiene and expiration date checking, considerable gaps were observed in behaviours related to temperature control, proper storage, reheating, and cross-contamination avoidance. These areas represent critical control points in household food safety. The persistence of such unsafe practices suggests the presence of *behavioural barriers*, including lack of time, misconceptions about risk severity, overconfidence, limited kitchen space, and perceived inconvenience. These findings complement the growing body of behavioural science research showing that consumer decisions are often impulsive, habitual, and influenced by environmental cues rather than deliberate knowledge-based reasoning.

Overall, the results highlight the necessity of integrating food safety into broader sustainability, health promotion, and behaviour-change initiatives. Conventional educational approaches alone are insufficient; instead, multidimensional interventions are needed, combining clear communication, environmental restructuring, social influence, regulatory frameworks, and nudges that support safe routines in daily life. Such an integrated public health model aligns with the One Health concept and with contemporary frameworks for sustainable community health.

CONCLUSION

Food safety continues to represent a central pillar of sustainable public health, influencing not only individual well-being but also broader societal outcomes such as healthcare costs, food security, economic resilience, and trust in food systems. Despite encouraging levels of knowledge among consumers, the findings of this study reveal persistent behavioural inconsistencies that heighten the risk of foodborne diseases at the household level. As long as improper storage, cross-contamination, and unsafe cooking practices remain common, the potential for outbreaks and preventable illnesses remains substantial.

This study underscores the need for an integrated and system-wide approach that addresses food safety not merely as a technical or regulatory issue but as a behavioural, educational, and social challenge. The evidence demonstrates that sustainable improvements require:

- **Robust public health education**, designed to translate knowledge into practical skills and everyday habits.
- **Behavioural science applications**, including nudges, reminders, habit-formation techniques, and choice architecture strategies that make safe practices easier and more intuitive.
- **Community engagement**, particularly through schools, municipalities, and local health centres, to enhance collective awareness and shared norms.
- **Strengthened regulatory frameworks**, ensuring that food safety guidelines are accessible, enforceable, and aligned with contemporary scientific standards.

Ultimately, meaningful progress will depend on long-term, interdisciplinary initiatives that bring together public health authorities, educators, behavioural scientists, food industry stakeholders, and consumers. Creating a culture of food safety requires continuous reinforcement, culturally adapted educational materials, and digital tools that support real-time decision-making. By integrating these components, societies can empower individuals, reduce preventable illness, and advance sustainable public health outcomes.

7. Recommendations

Building on the findings of the study, the following recommendations are proposed to strengthen food safety awareness and behaviour at both individual and systemic levels:

1. Develop Community-Based Food Safety Training Programs

Local health institutions, NGOs, and educational centres should organize regular workshops, cooking demonstrations, and community awareness campaigns that focus on practical, hands-on skills rather than solely theoretical knowledge. These programs should be culturally adapted and accessible to diverse demographic groups.

2. Integrate Food Safety Topics into School Curricula

Introducing food safety concepts at an early age can foster lifelong healthy habits. Schools should incorporate modules on hygiene, nutrition, environmental sustainability, and safe cooking practices across multiple age groups. Youth engagement is critical for creating future communities that value safety and sustainability.

3. Strengthen Behaviour-Focused Interventions (e.g., nudges, reminders, labels)

Food safety messaging should be improved through visual cues, refrigerator stickers, color-coded storage guides, simplified temperature charts, and digital reminders delivered through mobile applications. Such tools make safe choices more intuitive and help consumers maintain healthy habits over time.

4. Expand Digital Tools and Public Health Communication Platforms

Developing mobile apps, interactive websites, and social media campaigns can provide real-time advice on food storage, expiration dates, contamination risks, and safe preparation techniques. Digital platforms are essential for combating misinformation and reaching younger demographics.

5. Promote Cross-Sector Collaboration

Strengthening cooperation among public health agencies, educational institutions, consumer protection authorities, and the food industry is essential. Partnerships can facilitate consistent messaging, improved regulatory enforcement, and coordinated community outreach.

6. Enhance Monitoring and Evaluation Systems

Regular surveys, audits, and behavioural assessments should be conducted to track progress, identify emerging risks, and ensure that interventions remain effective and relevant.

7. Support Vulnerable Populations

Targeted interventions should be developed for elderly individuals, low-income households, and populations with limited access to safe food storage facilities, ensuring that no group remains disproportionately at risk.

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TRACE ELEMENT DISTRIBUTION IN RESERVOIR SEDIMENTS:A COMPARATIVE STUDY OF ATIKHISAR AND ALIBEY DAM LAKES

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ABSTRACT

Reservoirs are of great importance for drinking water supply, irrigation, energy production, and flood control in the Marmara Region, where population density and anthropogenic pressures are high. Among these, Atikhisar Dam Lake, the main drinking water source of Çanakkale, and Alibey Dam Lake, serving Istanbul, are two strategically important reservoirs. Understanding the sediment quality of such water bodies is essential, since sediments act both as sinks and secondary sources of pollutants, influencing ecological health and water quality. In this study, selected trace elements (Li, Mn, Ni, Ba) were measured in surface sediments collected from both reservoirs during dry and wet seasons. The results demonstrated spatial and temporal variations, with Atikhisar Dam generally showing lower lithium and manganese concentrations compared to Alibey Dam, where markedly higher levels were detected, especially for manganese. Nickel values were comparable between the reservoirs, while barium concentrations were higher in Atikhisar. These findings indicate that local geology, catchment characteristics, and possible human activities contribute to the observed differences between the two lakes. Overall, the study emphasizes the necessity of regular monitoring of reservoir sediments to safeguard water resources and ecosystem health. By providing baseline information on trace metal levels, this research contributes to sustainable water management strategies for the Marmara Region, where the preservation of dam lakes is critical for meeting the growing demand for clean water and ensuring long-term ecological balance.

Keywords: Marmara Region Reservoirs, Trace elements, Seasonal variation

INTRODUCTION

Sediments play a crucial role in aquatic ecosystems as both sinks and potential secondary sources of contaminants (Muhammad et al., 2024; Yüksel et al., 2024; Tokatlı et al., 2025). Among these, trace elements are of particular concern due to their persistence, bioaccumulation potential, and possible ecological and human health impacts (Haq et al., 2023; Haque et al., 2023; Muhammad et al., 2025). The assessment of trace element levels in sediments is therefore widely recognized as an essential approach for understanding pollution dynamics, evaluating water quality, and developing effective management strategies for freshwater resources (Varol et al., 2022; Köse et al., 2023; Ustaoglu et al., 2022; 2025).

Reservoirs are of strategic importance in densely populated regions, providing drinking water, irrigation, energy generation, and flood regulation. In Türkiye's Marmara Region, increasing urbanization and anthropogenic pressures heighten the need for careful monitoring of such critical water resources. Atikhisar Dam Lake, the main drinking water source for

Çanakkale, and Alibey Dam Lake, serving the megacity of Istanbul, are two major reservoirs that play vital roles in meeting regional water demand. The ecological integrity and water quality of these reservoirs directly influence public health, socio-economic stability, and sustainable development (Anonymous, 2021a; Anonymous, 2021b; Tokatlı et al., 2023; 2024; Varol and Tokatlı, 2024).

The present study aims to investigate the concentrations of selected trace elements (Li, Mn, Ni, Ba) in the surface sediments of Atikhisar and Alibey Dam Lakes under dry and wet seasonal conditions. By comparing spatial and temporal variations between these two reservoirs, the study provides insights into the factors influencing sediment quality and highlights the importance of regular monitoring. The findings are expected to contribute to a better understanding of reservoir ecosystem health and support sustainable water management policies in the Marmara Region.

MATERIALS AND METHOD

Research Area and Sample Collection

Study area, selected locations on the reservoirs and the coordinated information about the sampling stations are given in Figure 1. Surface sediment samples were collected during the dry (late summer) and wet (late winter) seasons of 2022–2023 using a Hydrobios branded Ekman Grab (EPA, 2001a).

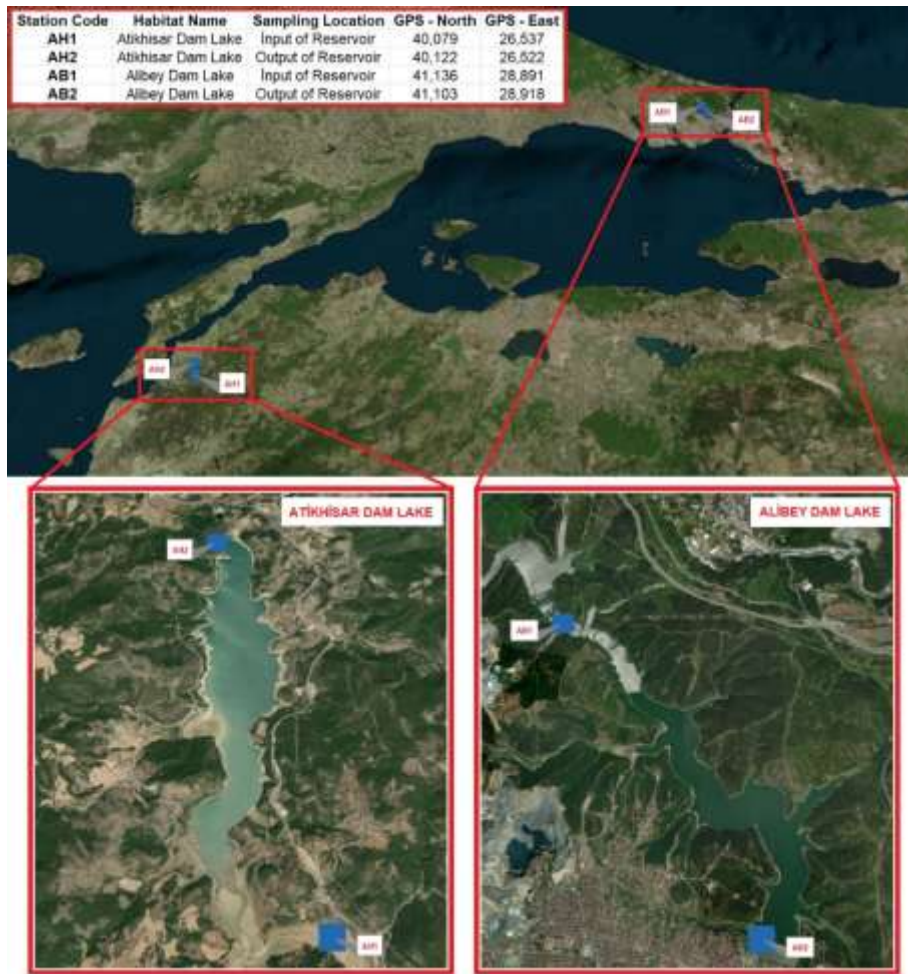


Figure 1. Research area and selected sampling stations

Element Analysis

Sediment samples were oven-dried at 105 °C for 3 hours, and 0.25 g aliquots were digested in a CEM Mars Xpress microwave system using $\text{HClO}_4\text{--HNO}_3$ (1:3). The digestion was carried out at 200 °C for 30 minutes, after which the solutions were filtered (0.45 µm), diluted to 100 ml with ultrapure water, and analyzed for Li, Mn, Ni, and Ba using an Agilent 7700 ICP-MS. All analyses were performed in triplicate at the accredited Central Laboratory of Trakya University (TS EN / ISO IEC 17025) following standard protocols (EPA, 1998; 2001b).

RESULTS AND DISCUSSION

The concentrations of lithium (Li), manganese (Mn), nickel (Ni), and barium (Ba) in the surface sediments of Atikhisar and Alibey Dam Lakes exhibited clear spatial and seasonal differences (Figure 2).

In Atikhisar Dam Lake, Li concentrations were relatively low (mean 0.93–1.50 ppm), while in Alibey Dam Lake they were markedly higher (3.85–5.58 ppm), indicating possible lithological influence or catchment-specific anthropogenic inputs.

Manganese levels showed the highest variability, ranging from 199.5 ppm (AH2, wet season) to 1936.4 ppm (AB2, wet season). The exceptionally elevated Mn concentrations in Alibey suggest either strong geogenic enrichment or potential human-induced contributions, particularly considering the dam's proximity to densely populated and industrialized areas of Istanbul.

Nickel concentrations were generally moderate, with Atikhisar ranging between 7.5 and 15.5 ppm and Alibey between 8.3 and 20.4 ppm. Although these values are below international sediment quality guideline thresholds for ecological risk, the higher Ni content in Alibey sediments warrants attention.

Barium concentrations presented a contrasting pattern: Atikhisar exhibited consistently higher levels (81.7–93.9 ppm) compared to Alibey (19.4–75.9 ppm). These variations can be attributed to differences in geological background, sedimentation processes, and land use in the respective catchments.

Overall, the results suggest that Alibey Dam Lake is more enriched in Li and Mn, while Atikhisar has higher Ba concentrations. Such findings highlight the importance of regular sediment monitoring, as dams supplying drinking water to large populations are vulnerable to both natural and anthropogenic influences. Identifying spatial and seasonal trends in trace element accumulation not only improves our understanding of reservoir ecosystem dynamics but also provides essential data for effective water resource management in the Marmara Region.

The geological composition of a watershed fundamentally influences aquatic sediment chemistry, often elevating essential and toxic elements beyond natural background levels (Çiçek et al., 2013, 2014; Tokatlı and Helvacıoğlu, 2020; Tokatlı and İslam, 2022; Mia et al., 2023; Mutlu et al., 2023). Elevated macro and micro element concentrations are key indicators of anthropogenic impact, with potential deleterious effects on ecosystems (Köse et al., 2023; Tokatlı et al., 2023; Din et al., 2023). Thus, high sediment element levels at specific sites likely stem from combined geological and anthropogenic sources.

CONCLUSIONS

This study revealed distinct spatial and seasonal variations in the sediment concentrations of Li, Mn, Ni, and Ba in the Atikhisar and Alibey Dam Lakes, underscoring a complex interplay

of geogenic and anthropogenic factors. The significant enrichment of Li and Mn in Alibey Dam Lake sediments is likely attributable to the combined influence of the catchment's lithology and anthropogenic pressures from the adjacent urban and industrial zones of Istanbul. Conversely, the consistently higher Ba levels in Atikhisar Dam Lake point to a stronger control by the local geological background. Although the observed Ni concentrations remained below critical ecological risk thresholds, their spatial distribution further supports the differential impact of human activities between the two reservoirs. Ultimately, these findings highlight the vulnerability of critical drinking water supplies to trace metal accumulation and emphasize the necessity of implementing continuous sediment quality monitoring programs. Such proactive measures are indispensable for safeguarding public health and ensuring the sustainable management of water resources in the Marmara Region.

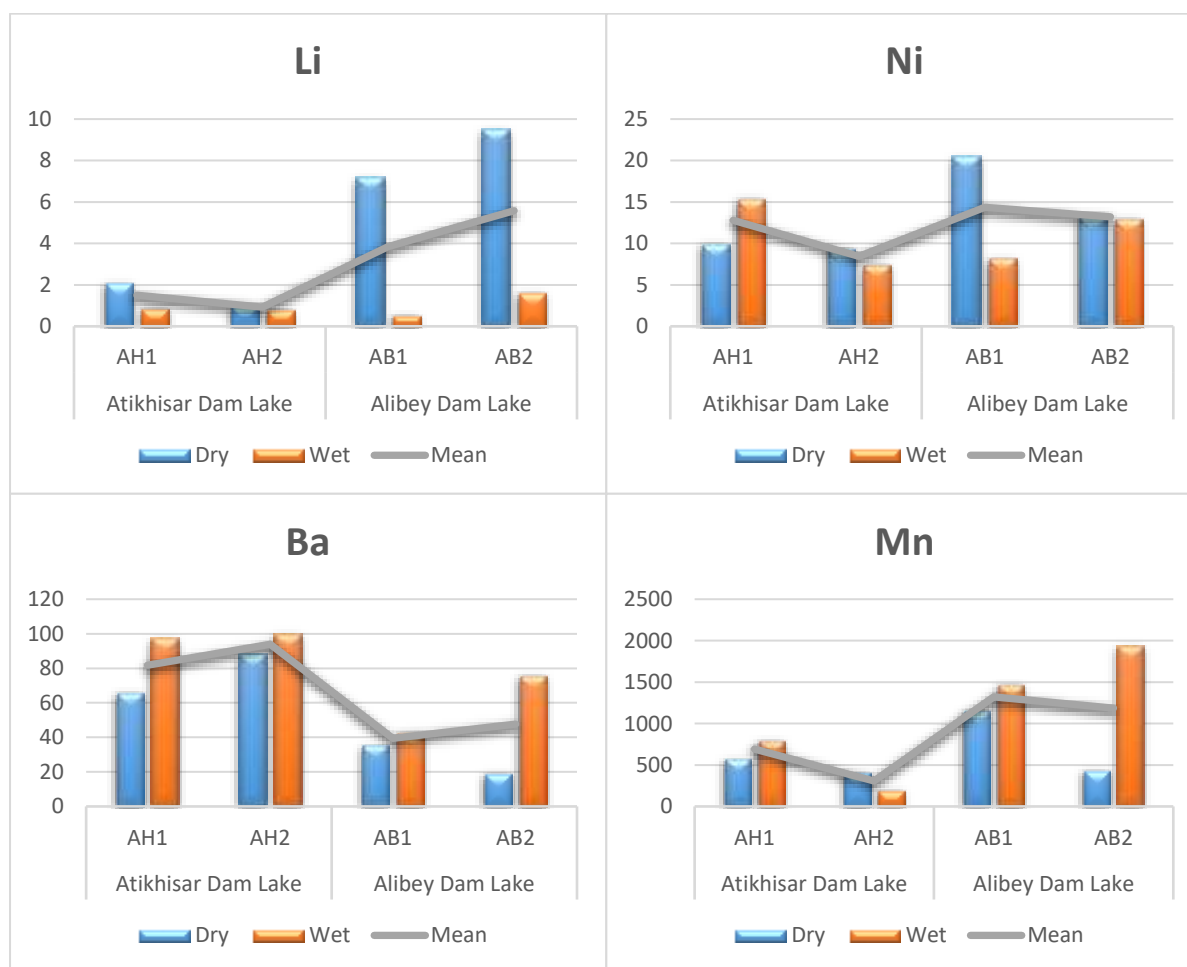


Figure 2. Detected element levels reservoir sediments

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INVESTIGATING SEDIMENT CONTAMINATION USING GEOACCUMULATION AND POLLUTION LOAD INDICES: A CASE STUDY OF ATIKHISAR AND ALIBEY DAM LAKES

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ABSTRACT

Atikhisar and Alibey Dam Lakes are crucial water resources in the Çanakkale and Istanbul regions of Türkiye, respectively, serving purposes from drinking water to irrigation, thus making their sediment quality a direct indicator of ecosystem health and human exposure risk. This study evaluates the extent of heavy metal pollution (Li, Mn, Ni, Ba) in the sediments of these reservoirs by employing two fundamental environmental indices: the Geoaccumulation Index (I_{geo}), which assesses metal enrichment by comparing current concentrations to background levels, and the Pollution Load Index (PLI), providing a simple, comparative measure of overall site contamination. Sediment samples were collected from two specific sites (inlets and outlets of reservoirs – AH1, AH2; AB1, AB2) in each lake during dry and wet seasons, with mean I_{geo} and PLI values calculated from the analysed metal concentrations. The results from Atikhisar Dam Lake (mean I_{geo} : -2.218 to -2.759; mean PLI: 0.099 to 0.143) and Alibey Dam Lake (mean I_{geo} : -1.969 to -2.094; mean PLI: 0.168 to 0.172) consistently yielded negative I_{geo} values and PLI values significantly below 1. This uniform classification across all sampling points indicates that the sediments are unpolluted with respect to the analysed elements Li, Mn, Ni, and Ba. The findings conclusively demonstrate that there is no significant ecological risk or pollution burden from these specific metals in the sediments of either dam lake. This study provides a critical baseline for future monitoring efforts and underscores the current effectiveness of watershed management practices in preventing heavy metal contamination in these vital water bodies.

Keywords: Atikhisar and Alibey dam lakes, Geoaccumulation Index, Pollution Load Index

INTRODUCTION

The assessment of trace element levels in sediments is widely recognized as an essential approach for understanding pollution dynamics, evaluating water quality, and developing effective management strategies for freshwater resources (Haq et al., 2023; Muhammad et al., 2025). To accurately evaluate sediment quality and potential ecological risks, various indices have been developed and applied worldwide (Yüksel et al., 2024; Tokatlı et al., 2025). Among these, the Geoaccumulation Index (I_{geo}) is particularly valuable for assessing the extent of metal pollution by comparing current concentrations to geological background values, while the Pollution Load Index (PLI) provides a simple, comparative measure of overall contamination at a sampling site by integrating multiple metal concentrations. These indices have proven to be effective tools for monitoring and managing sediment quality in freshwater systems (Varol et al., 2022; Ustaoglu et al., 2025).

Providing drinking water, irrigation, energy generation, and flood regulation, reservoirs are of vital strategic importance in densely populated areas. In Türkiye's Marmara Region, the combined effect of increasing urbanization and human pressures intensifies the need for rigorous monitoring of these essential water sources. As the main drinking water source for Çanakkale, Atikhisar Dam Lake, and a critical supplier for the metropolis of Istanbul, Alibey Dam Lake, both play indispensable roles in meeting the region's water needs. The overall health and quality of these reservoirs directly determine public health, economic stability, and sustainable development. (Anonymous, 2021a; Anonymous, 2021b; Tokatlı et al., 2023; 2024; 2025; Varol and Tokatlı, 2024).

The objective of this research was to quantify the ecological risk posed by heavy metal contamination (Li, Mn, Ni, and Ba) in the sediments of Atikhisar and Alibey Dam Lakes, two indispensable water resources in Türkiye by using two key environmental indices, the Geoaccumulation Index (I_{geo}) and the Pollution Load Index (PLI). Ultimately, this study was designed to provide robust data on the current state of these reservoirs, thereby supporting ongoing efforts to ensure the long-term sustainability and safety of these critical water supplies.

MATERIALS AND METHODS

Research Area and Sample Collection

Study area, selected locations on the reservoirs and the coordinated information about the sampling stations are given in Figure 1. Surface sediment samples were collected during the dry (late summer) and wet (late winter) seasons of 2022–2023 using a Hydrobios branded Ekman Grab (EPA, 2001a).

Element Analysis

Sediment samples were oven-dried at 105 °C for 3 hours, and 0.25 g aliquots were digested in a CEM Mars Xpress microwave system using $\text{HClO}_4\text{--HNO}_3$ (1:3). The digestion was carried out at 200 °C for 30 minutes, after which the solutions were filtered (0.45 µm), diluted to 100 ml with ultrapure water, and analyzed for Li, Mn, Ni, and Ba using an Agilent 7700 ICP-MS. All analyses were performed in triplicate at the accredited Central Laboratory of Trakya University (TS EN / ISO IEC 17025) following standard protocols (EPA, 1998; 2001b).

Calculation of Indices

According to Muller (1969), the Geo – Accumulation Index (I_{geo}) is calculated as given below. Where, “Cn” is the concentration of the investigated elements, and “Bn” is the geochemical background score of the investigated elements. In order to balance the probable variations of the reference values resulting from the lithogenic effects, the coefficient of 1.5 was used. I_{geo} has 7 classes as described in Table 1.

$$I_{geo} = \log_2 \left(\frac{C_n}{1.5 \times B_n} \right)$$

According to Hakanson (1980), the Contamination Factor (CF) is calculated as given below. Where, “Cn” is the concentration of the investigated elements, and “Bn” is the background value of the investigated elements. CF has 4 classes as described in Table 1.

$$CF = \frac{C_n}{B_n}$$

The following formula can be used in order to calculate the Pollution Load Index (PLI) (Tomlinson et al., 1980):

$$PLI = (CF1 \times CF2 \times CF3 \times \dots \times CFn)^{(1/n)}$$

Table 1. Quality classes for I_{geo} , CF and PLI

I_{geo}	Risk Grades	CF	Risk Grades
$I_{geo} < 0$	Practically uncontaminated	$CF < 1$	Low contamination
$0 < I_{geo} < 1$	Uncontaminated to moderately contaminated	$1 < CF < 3$	Moderately contamination
$1 < I_{geo} < 2$	Moderately contaminated	$3 < CF < 6$	Considerably contamination
$2 < I_{geo} < 3$	Moderately to heavily contaminated	$6 < CF$	Very high contamination
$3 < I_{geo} < 4$	Heavily contaminated	PLI	Value
$4 < I_{geo} < 5$	Heavily to extremely contaminated	$PLI < 1$	Heavy metal pollution
$5 < I_{geo}$	Extremely contaminated	$1 < PLI$	No heavy metal pollution

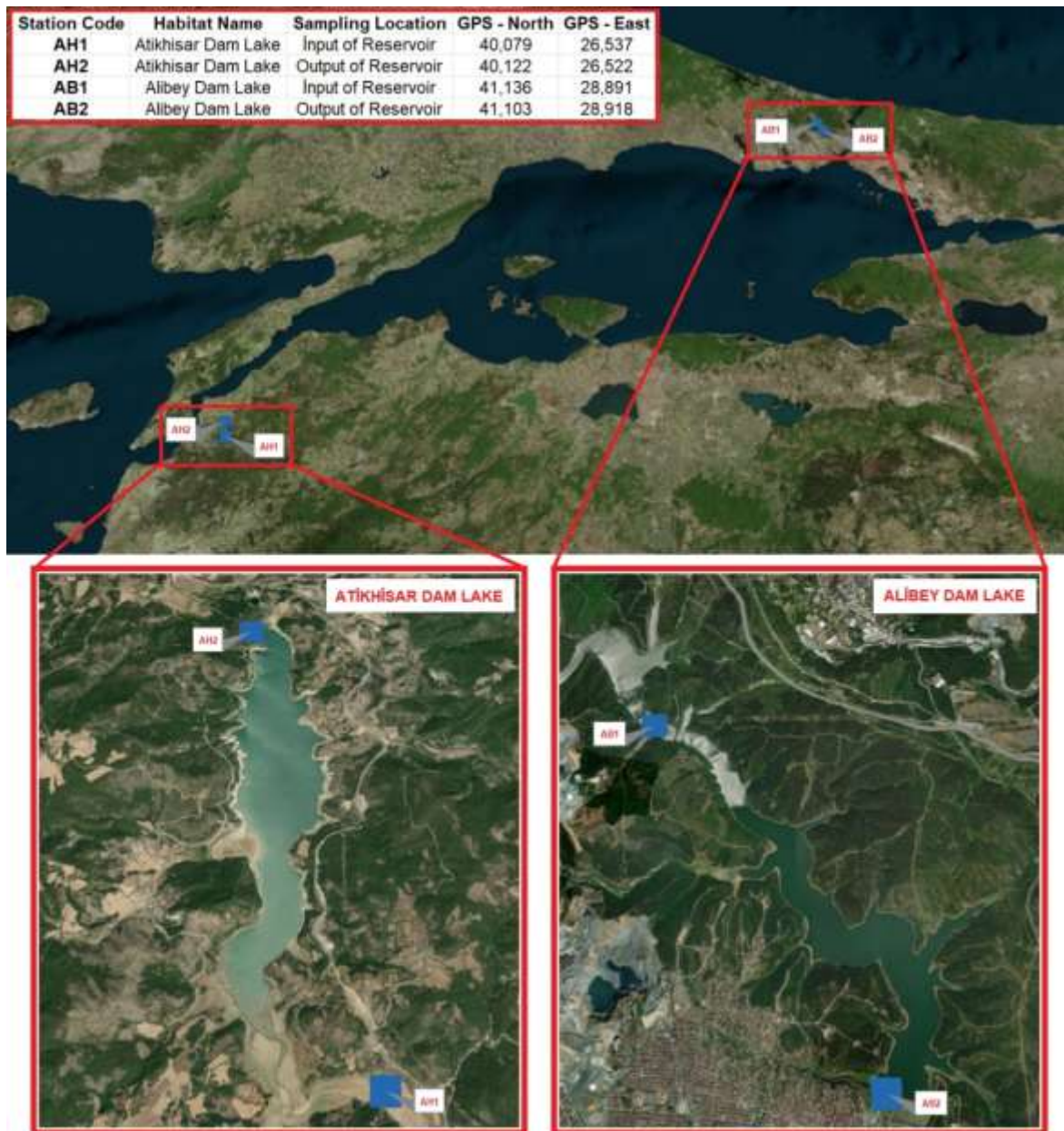


Figure 1. Research area and selected sampling stations

RESULTS AND DISCUSSION

The data from our analysis of the Geoaccumulation Index (I_{geo}) and the Pollution Load Index (PLI) for the sediments of Atikhisar and Alibey Dam Lakes reveal that both reservoirs are currently free from significant heavy metal pollution associated with lithium (Li), manganese (Mn), nickel (Ni), and barium (Ba) (Figure 2 and 3). All calculated mean I_{geo} values were negative, ranging from -2.218 to -2.759 for Atikhisar Dam Lake and -2.094 to -1.969 for Alibey Dam Lake. According to the classification system, negative I_{geo} values indicate that the sediments are unpolluted with respect to the heavy metals Li, Mn, Ni, and Ba. This consistent finding across all sampling points and seasons shows that the concentrations of these metals in the sediments do not exceed their background levels. Furthermore, the PLI values consistently remained below 1, with mean values ranging from 0.099 to 0.143 for Atikhisar and 0.168 to 0.172 for Alibey. A PLI value of less than one is a clear indicator that there is no pollution burden on the studied sites.

The geological makeup of a watershed and anthropogenic activities collectively influence aquatic sediment chemistry (Mia et al., 2023; Mutlu et al., 2023). While some elements may be naturally elevated, high concentrations often indicate human-induced pollution with potential negative effects on ecosystems (Tokatlı et al., 2023; Din et al., 2023). While our findings conclusively demonstrate that the sediments are unpolluted with respect to the analysed elements (Li, Mn, Ni, and Ba), this does not preclude the possibility of contamination by other toxic metals or pollutants. Numerous studies conducted in the region, particularly within Istanbul's freshwater ecosystems, have consistently highlighted significant anthropogenic pressures (Tokatlı et al., 2023; 2024; 2025). Therefore, the continuous monitoring and ecotoxicological evaluation of these vital dam lakes are essential for safeguarding both the ecosystem and human health.

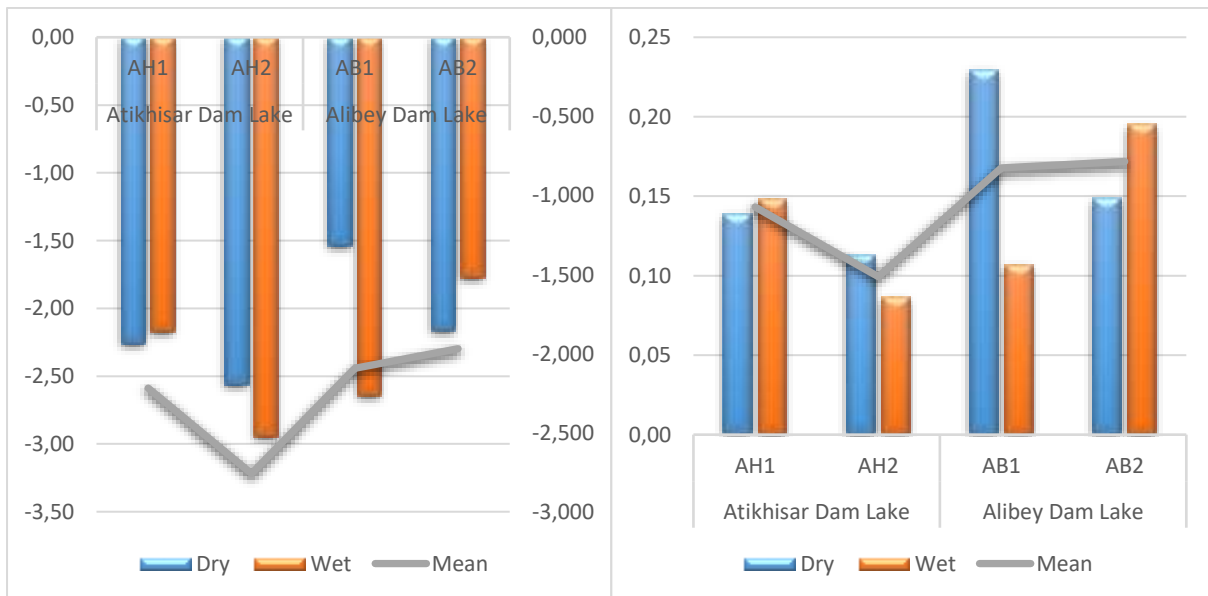


Figure 2. Results of multinomial ecologic indices (I_{geo} left; PLI right)

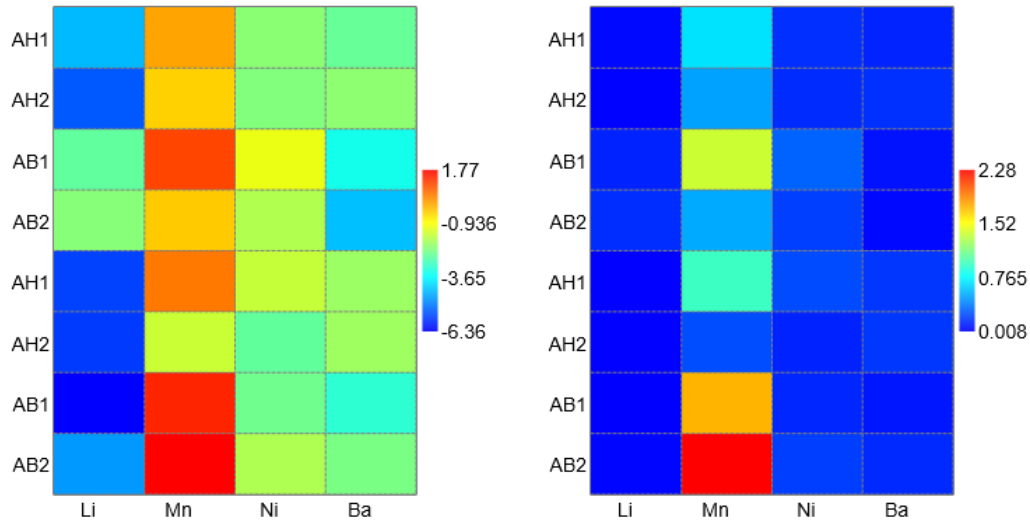


Figure 3. Results of monomial ecologic indices (I_{geo} left; CF right)

CONCLUSIONS

This study assessed the heavy metal pollution status of Atikhisar and Alibey Dam Lakes by analyzing the Geoaccumulation Index (I_{geo}) and Pollution Load Index (PLI). The consistently negative I_{geo} values and PLI values below 1 for the analysed elements (Li, Mn, Ni, and Ba) indicate that the sediments of both reservoirs are currently unpolluted. These results provide a critical baseline for future environmental assessments and reflect a favorable status for these water resources. Nevertheless, it is crucial to recognize that this finding does not exclude the presence of other pollutants. Therefore, ongoing monitoring and ecotoxicological evaluations remain a necessity to protect these vital ecosystems and to ensure public health in the face of persistent anthropogenic pressures in the region.

ACKNOWLEDGEMENTS

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SILICA NANOPARTICLES AND THEIR CURRENT BIOMEDICAL APPLICATIONS

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ABSTRACT

Silica, or silicon dioxide (SiO_2), is among the most extensively researched substances in science and engineering. It is commonly found as quartz or sand, although it exists in numerous crystalline and amorphous forms. Crystalline silica has been utilized for millennia in the manufacture of glass and concrete, while contemporary applications encompass high-temperature ceramics and refractory materials. In crystalline silica, silicon is coordinated by four oxygen atoms, with each oxygen atom bonding to two silicon atoms. This constitutes a cohesive network of tetrahedra featuring core silicon atoms and bridging oxygen atoms. The $\text{Si}-\text{O}-\text{Si}$ bond denotes the siloxane functional group.

Various processes for silica nanoparticle preparation are utilized, including plasma synthesis, chemical vapor deposition, microemulsion processing, combustion synthesis, sol-gel processing, and hydrothermal procedures. Recent endeavors in silica nanoparticle synthesis concentrate on regulating size, shape, and surface reactivity. The sol-gel method is extensively utilized and preferred for nanoparticle synthesis due to its advantages, including the ability to conduct synthesis at low temperatures, achieve desired pH levels for high purity, and control reaction kinetics by adjusting the composition of the reaction mixture.

Silica-based nanoparticles (SNPs) have garnered significant interest in biomedical applications over the past decades owing to their exceptional biocompatibility and controlled architectures. They have been utilized in various biomedical domains, including diagnostics, biosensing, and medication delivery. The medical applications of SNP in anti-cancer, anti-microbial, and theranostic contexts are particularly notable due to their remarkable efficacy in delivering various small compounds and, more recently, biologics to specified locations. The physical and chemical characteristics of SNP, including a substantial specific surface area, adjustable particle size and porosity, as well as exceptional biodegradability and biocompatibility, render them an optimal platform for drug delivery and diagnostics. In this study, brief information about silica nanoparticles and recent research examples will be given. Keywords: Silica Nanoparticles, Nanotechnology, Biomedical Applications

INTRODUCTION

Silica (SiO_2) nanoparticles have garnered significant interest in nanomedicine during the past twenty years. Their popularity arises from (i) precisely controllable particle size (ranging from a few nanometers to hundreds of nanometers), (ii) adjustable internal architecture (solid versus mesoporous, varying pore sizes and volumes), (iii) extensive surface area (notably in MSNs) facilitating substantial drug loading, (iv) uncomplicated and varied surface chemistry (silane coupling agents permit the attachment of polymers, peptides, antibodies, and imaging agents), and (v) generally favorable biocompatibility in comparison to numerous inorganic

alternatives. These attributes enable silica platforms to serve as transporters, imaging scaffolds, adjuvants, and therapeutic "platforms" for combination therapies(Xu et al., 2023).

1. Types of Silica Nanoparticles

Solid Silica Nanoparticles (solid SiNPs): Solid silica nanoparticles (SiNPs) denote predominantly non-porous silica spheres or particles, generally produced without a templated pore structure. The canonical method is the Stöber process, which involves base-catalyzed hydrolysis and condensation of a silica alkoxide precursor, often tetraethyl orthosilicate (TEOS), in an alcoholic solution including water and ammonia(Liberman et al., 2014).

The Stöber method involves the hydrolysis of TEOS (or another alkoxy silane) under basic circumstances using an ammonia catalyst, resulting in silanol species that condense into siloxane (Si–O–Si) networks, ultimately producing silica particles(Saha et al., 2024). The particle size and dispersity can be precisely adjusted by modifying reaction parameters, including TEOS concentration, water/TEOS ratio, ammonia concentration, alcohol/water solvent ratio, TEOS addition rate, temperature, and reaction duration. For instance, elevating ammonia concentration or diminishing TEOS concentration often results in a reduction of particle size(Liberman et al., 2014). Recent mechanistic investigations indicate that the Stöber process can be delineated by two pathways: initial nucleation from hydrolyzed TEOS monomers, succeeded by growth through silanol condensation onto seeds. The chronological distinction of these phases facilitates enhanced size regulation(Han et al., 2017).

These solid SiNPs feature a compact silica matrix, negligible or absent mesopore structure, and a surface abundant in silanol (Si–OH) groups, facilitating surface functionalization using silane coupling agents. Due to their relatively uncomplicated structure, they are frequently employed as inert cores or scaffolds: for instance, as carriers for dyes or imaging agents; as platforms for additional functionalization (polymer grafting, targeting ligands); as biosensing labels; or as components for hybrid systems (coated with metals, polymers, biomolecules). Benefits: excellent colloidal stability (contingent upon surface modification), relatively simple synthesis, regulated size, monodispersity, and ease of functionalization. Limitations: the insufficient interior pore volume restricts the loading of substantial payloads (such as high-mass medication molecules or macromolecular therapies) in comparison to porous alternatives; also, the dense matrix may impede release or restrict the diffusion of guests(Li et al., 2019; Liberman et al., 2014).

Mesoporous silica nanoparticles (MSNs): Mesoporous silica nanoparticles (MSNs) are silica nanoparticles designed with structured mesopores (typically 2–50 nm in diameter) and substantial interior surface areas and pore volumes, rendering them ideal for biomedical delivery and controlled release applications(Paris & Vallet-Regí, 2020).

MSNs are generally produced using sol-gel reactions involving TEOS (or other silanes), accompanied by a structure-directing surfactant (such as cetyltrimethylammonium bromide, CTAB) that generates micelles and molds the mesopore architecture. Following the condensation of silica around the micelles, the surfactants are eliminated (by calcination or solvent extraction) to produce the pore network. The particle size, pore diameter, pore volume, pore morphology, and overall structure can be modified by varying the surfactant type and concentration, swelling agents, co-solvents, silica precursor concentration, reaction duration, and calcination/etching procedures. Larger holes can be attained through the utilization of swelling agents, such as trimethylbenzene(Narayan et al., 2018; Vallet-Regí et al., 2018).

The extensive internal pore network facilitates substantial loading of therapeutic substances (small compounds, macromolecules, nucleic acids) through adsorption, encapsulation, or covalent anchoring within the mesopores (Benkő et al., 2025). The mesoporous architecture safeguards sensitive cargoes (e.g., enzymes, siRNA) from premature degradation and facilitates regulated release kinetics by diffusion via pores, triggered "gate" processes, or external stimuli (Paris & Vallet-Regí, 2020). MSNs can be modified on their external surface using silane chemistry to incorporate targeting ligands (peptides, antibodies, aptamers), "gatekeepers" (pH-responsive polymers, redox-cleavable linkers), and imaging moieties, enhancing their efficacy for targeted and stimuli-responsive distribution (Zhou et al., 2018). They have been thoroughly examined in cancer drug delivery, gene therapy, combination therapy (chemotherapy and phototherapy), and other high-payload interventions (Benkő et al., 2025). MSNs are generally desirable for biomedical application and their structure effect functionality of process. For example; The performance, encompassing loading capacity and release kinetics, is significantly affected by physical characteristics such as particle size, pore size, pore volume, surface charge, morphology, and surface functionalization. A study indicated that mesoporous silica nanoparticles (MSNs) with a smaller size (about 64 nm) and greater pore volume (0.89 cc/g) exhibited enhanced encapsulation performance (92.2%) relative to bigger MSNs (approximately 174 nm) with a reduced pore volume (0.356 cc/g) in doxorubicin loading (Fathy et al., 2023).

2. Synthesize Methods

The traditional synthetic methodologies for silica nanoparticles (SiNPs) are based on the sol-gel chemistry of alkoxy silanes and encompass the renowned Stöber method for producing monodisperse solid SiNPs, the surfactant-directed or "soft-templating" technique for mesoporous silica nanoparticles (MSNs), and sol-gel co-condensation (or modification) strategies for integrating functional silanes during synthesis (Benkő et al., 2025).

The Stöber method, developed by Stöber, Fink, and Bohn in 1968, involves the base-catalyzed hydrolysis of tetraethyl orthosilicate (TEOS) in an alcoholic medium, typically ethanol, combined with water and ammonia. This reaction produces silanol intermediates that condense into siloxane (Si-O-Si) networks, forming spherical silica colloids. Size regulation of these colloids is influenced by varying factors such as concentrations of TEOS and ammonia, the water/ethanol ratio, ionic strength, temperature, and stirring. Increased amounts of ammonia or water lead to faster hydrolysis and condensation, resulting in larger particles and greater polydispersity. Recent studies have focused on the kinetics of the process, particularly how the balance between hydrolysis and condensation impacts size and monodispersity. The Stöber method remains a widely used technique for producing silica spheres in applications like optical labels, core-shell structures, and as inert carriers, thanks to its simplicity and reproducibility (dos Santos da Silva & dos Santos, 2023; Florensa et al., 2022; Han et al., 2017).

The soft-templating or surfactant-directed synthesis method is utilized to create mesoporous silica nanoparticles (MSNs) using amphiphilic surfactants like cetyltrimethylammonium bromide (CTAB). These surfactants form micelles in solvent systems, serving as templates for silica condensation via sol-gel chemistry of TEOS and other silanes. After removing the surfactant, an ordered mesoporous silica framework is formed, with pore sizes typically between 2 to 50 nm. The characteristics of MSNs, such as shape, pore size, and surface area, can be modified by varying surfactant type, concentration, and other synthesis parameters, resulting in a versatile carrier for medicinal agents (Gai et al., 2016; Mehmood et al., 2017; Pal & Bhaumik, 2013).

Sol-gel modifications, especially co-condensation methods, facilitate the incorporation of functional organosilanes into the silica matrix during particle synthesis. This technique integrates silica precursors (e.g., TEOS) and organoalkoxysilanes (e.g., APTES) under templating conditions, resulting in silica nanoparticles or mesoporous silica nanoparticles (MSNs) with evenly dispersed functional groups. The co-condensation of TEOS and APTES augments amine functionalization and boosts loading/release properties relative to surface grafting alone (Gai et al., 2016; Mahmoodi et al., 2017).

3. Biomedical Applications

Pharmaceutical Delivery and Regulated Release: Mesoporous Silica Nanoparticles (MSNs) are extensively utilized for the delivery of small-molecule medicines, biologics (including peptides and siRNA), and in combination therapy. Their benefits encompass substantial loading capacity by adsorption into the pores, safeguarding of sensitive cargos (e.g., siRNA or enzymes) from degradation, and the implementation of modular "gatekeeper" systems (such as pH-, redox-, enzyme-, or light-responsive caps) to facilitate release at the target location. These attributes enhance efficacy and diminish negative effects (Benkő et al., 2025; Vallet-Regí et al., 2022; Watermann & Brieger, 2017).

Imaging and Theranostics: Silica nanoparticles function as superior scaffolds for multimodal imaging and theranostic applications. Fluorophores and radionuclides may be integrated into the silica matrix or affixed to the surface; the inclusion of magnetic particles or gold shells facilitates MRI/CT/PA contrast enhancement. Ultrasmall silica nanoparticles (USNs) have advanced in fluorescence-guided surgery owing to their advantageous clearance and luminescence (F. Chen et al., 2018).

Photothermal and Photodynamic Therapies (PTT/PDT): MSNs infused with photosensitizers or plasmonic elements (e.g., gold shells) facilitate light-induced tumor ablation through photothermal or photodynamic processes. The silica framework inhibits the aggregation of photosensitizers, enhances tumor accumulation and selectivity, and can be integrated with chemotherapy or immunotherapy for synergistic effects (Walia & Acharya, 2015).

Applications of Antimicrobials and Antivirals: Silica nanoparticles have been investigated as antimicrobial carriers (e.g., for transporting antibiotics, antiseptics, and metal ions) and for direct antibacterial or antiviral functions when modified with cationic polymers or metal species. Recent study suggests the potential antiviral application of silica-based particles as delivery systems for antiviral medicines and as direct antiviral nanostructures (Tng & Low, 2023).

Biosensing and Diagnostics: Silica nanoparticles, owing to their extensive surface area and customizable surfaces, are optimal for use as labels in immunoassays, lateral flow tests, and fluorescence-based detection systems. MSNs also facilitate stimuli-responsive sensors, wherein an analyte initiates cargo release that generates a measurable signal (Mladenović et al., 2024).

Tissue engineering, hemostasis, and wound healing: Silica, including mesoporous silica microparticles, is integrated into composite scaffolds for bone regeneration, as bioactive silica fragments can promote osteogenesis. Likewise, silica particles are utilized in hemostatic medicines and wound healing, where functionalized surfaces enhance cell adhesion or facilitate regulated local administration of growth factors (L. Chen et al., 2019).

4.Examples

MSNs for combinatorial chemotherapy and phototherapy: Multifunctional MSNs containing chemotherapeutics alongside photosensitizers or plasmonic cores have demonstrated improved tumor regression in animal models owing to synergistic effects(L. Chen et al., 2019). Ultrasmall silica nanoparticles in surgical imaging: Ultrasmall, dye-doped silica nanoparticles exhibiting renal clearance characteristics have advanced significantly toward clinical evaluation as fluorescence-guided probes(Singh et al., 2023). Stimuli-responsive sentinel pH- and redox-responsive caps have been developed for MSNs to selectively release cargo in tumor microenvironments, enhancing therapeutic indices in animal experiments(Benkő et al., 2025).

CONCLUSION

Silica nanoparticles, particularly mesoporous and ultrasmall types, offer a highly adaptable platform for several biomedical applications, including controlled drug delivery, multimodal imaging, theranostics, biosensing, and regenerative medicine. Recent advancements in surface functionalization, stimuli-responsive release mechanisms, and hybridization with other nanomaterials have produced promising preclinical outcomes. Nonetheless, the implementation of these improvements in clinical settings necessitates meticulous consideration of issues including biodegradation, long-term safety—incorporating genetic and epigenetic elements—scalable production, and adherence to regulatory standards. Current interdisciplinary research focused on addressing these problems suggests that silica nanoplatforms are poised to significantly influence the future of precision medicine.

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A PHOTOCATALYTIC STUDY OF A CATIONIC DYE DEGRADATION VIA COBALT MONOXIDE

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ABSTRACT

Currently, water contamination represents a significant ecological hazard for numerous countries worldwide due to population growth and ongoing industrial expansion. There is a significant focus on creating sustainable, environmentally friendly technologies to adhere to stringent environmental standards. The utilization of standard approaches has achieved little success in addressing the obstinate characteristics of industrial wastewater from dye-based effluents. Advanced oxidation processes (AOPs) generate reactive radical species like hydroxyl radicals and superoxide anions, which effectively oxidize organic materials. Photodegradation of organic pollutants offers advantages like complete mineralization, low concentration of pollutants, and no by-products. This process is proposed as a new technology in wastewater treatment due to its advantages. AOPs can be classified into many varieties based on their generating principles and the oxidizing agents utilized, including Fenton, ozonation, ultrasonic, electrochemical oxidation, H_2O_2/UV , and photocatalysis. Photocatalysis is an efficient and environmentally sustainable therapy within AOPs, generating highly reactive hydroxyl radicals upon exposure to light while maintaining low operational costs.

Cobalt monoxide (CoO) is a notable semiconductor with an energy band gap ranging from 2.4 eV to 2.7 eV . It crystallizes in two forms: the cubic rock salt structure and the hexagonal wurtzite phase. Despite the thermodynamic stability of the cubic close-packed structure, the hexagonal form of CoO exhibits intriguing electrical, optical, magnetic, and electrochemical properties that are advantageous for various potential applications, including catalysis, gas sensors, magnetic data storage devices, and lithium-ion batteries.

This study has designed the structural characterization of CoO particles utilizing FT-IR and Raman Spectroscopy. Methylene blue (MB) was chosen as a dye employed in the textile sector, namely for cotton dyeing, and is classified as a wastewater pollutant. The photocatalytic degradation of MB has been assessed under solar-simulated light irradiation. Additionally, the photocatalytic test conditions, such as catalyst dose (0.25 g/L, 0.50 g/L, 0.75 g/L, and 1.0 g/L) and initial MB concentration (10 mg/L, 20 mg/L, and 30 mg/L), were also investigated. The photocatalytic test results indicated that the maximum decolorization efficiency of MB was observed with the decreased initial dye concentration.

Keywords: Photocatalysis, Methylene Blue, Cobalt Oxide, Photocatalytic Degradation

INTRODUCTION

The industrial expansion, especially the swift development of the textile manufacturing sector, has emerged as a major contributor to global water pollution. The textile dyeing process is chemically demanding and produces significant quantities of effluent, including various persistent organic contaminants. During the dyeing process, it is estimated that about 85–90% of the applied dyes properly bond to the fabric fibers, while approximately 10–15% remain unbound and are discharged into wastewater with salts, surfactants, dispersion agents, and other auxiliary chemicals utilized during processing (Al-Tohamy et al., 2022a; Dutta, 2022; Yaseen & Scholz, 2019). These waste streams are typically distinguished by elevated color intensity, chemical oxygen demand, biological oxygen demand, and total dissolved solids, rendering them some of the most intricate industrial effluents to process. The durability of synthetic dyes, predominantly sourced from coal-tar or petroleum-derived aromatic chemicals, exacerbates their elimination due to their resilience against light, heat, and microbial destruction (Liu et al., 2024).

Dye compounds, even at minimal quantities (below 1 mg L⁻¹), can substantially impact the aquatic ecosystem by obstructing sunlight penetration and disrupting photosynthesis in aquatic flora and phytoplankton. The decline in photosynthetic activity causes oxygen depletion, leading to hypoxic or anoxic conditions that endanger aquatic species. Dyes and their breakdown products may be poisonous, carcinogenic, or mutagenic, presenting hazards to aquatic organisms and human populations exposed to contaminated water sources (Dutta, 2022; Yaseen & Scholz, 2019).

In light of these considerations, it is universally acknowledged that efficient wastewater treatment is essential prior to the discharge of textile effluents into natural water bodies. Attaining compliance with environmental regulations, such as the EU Water Framework Directive or WHO guidelines, necessitates sophisticated and integrated treatment systems that can effectively manage the chemical complexity and stability of dye-contaminated wastewaters. Consequently, the use of multi-stage treatment systems that integrate physical, chemical, and biological processes is essential for reducing environmental impacts and facilitating safe water reuse in the textile sector (Al-Tohamy et al., 2022a; Liu et al., 2024).

Conventional wastewater treatment systems are extensively employed for treating industrial effluents and enabling reuse; yet, the treatment methodology must be customized according to the effluent's composition, characteristics, and concentration. It is increasingly acknowledged that no singular treatment unit is adequate for the comprehensive elimination of dye-laden effluent from textile processes. Although biological systems have cost advantages, their kinetics are sluggish, and their degrading efficacy for synthetic dyes is often insufficient. Conversely, basic physical and chemical techniques like flocculation, filtration, and sedimentation frequently yield inadequate treatment and may result in secondary pollution (e.g., sludge production or residual coloration) when employed independently (Al-Tohamy et al., 2022b).

Due to the intrinsic limitations of traditional biological and physicochemical treatment systems, advanced oxidation processes (AOPs) have arisen as highly efficient and versatile chemical oxidation technologies for the degradation of a broad range of recalcitrant organic pollutants, such as textile dyes, phenols, and surfactants. AOPs depend on the in-situ production of extremely reactive oxygen species (ROS), primarily hydroxyl radicals ($\cdot\text{OH}$) and superoxide radicals ($\text{O}_2^{\cdot-}$), which possess elevated oxidation potentials and can non-selectively mineralize

persistent organic compounds into CO₂, H₂O, and inorganic ions (Mirza et al., 2020; Zhang et al., 2021). These procedures include several approaches such as Fenton and photo-Fenton treatments, ozonation, ultrasonic oxidation, electrochemical oxidation, H₂O₂/UV systems, and heterogeneous photocatalysis. Each approach differs in its mechanism of radical generation, operational parameters, and appropriateness for specific pollutants. Fenton-type systems rely on Fe²⁺/H₂O₂ reactions but face challenges like iron sludge formation and a limited optimal pH range, while ozonation is efficient for decolorization but may encounter high operational costs and partial mineralization (Khan et al., 2024).

Photocatalysis has garnered significant interest as one of the most sustainable and energy-efficient AOPs. In photocatalytic processes, light-activated semiconductor materials (such as TiO₂, ZnO, Fe₂O₃, Sb₂O₃, and g-C₃N₄) absorb photons with energies at or above their band gap, thereby promoting electrons (e⁻) from the valence band to the conduction band and producing positively charged holes (h⁺). The charge carriers subsequently interact with surface-adsorbed species to produce ·OH and O₂·⁻ radicals, which oxidize complex dye molecules into smaller, less damaging intermediates, ultimately resulting in mineralized end-products (Khan et al., 2024; Kumar et al., 2025).

Furthermore, photocatalysis is regarded as environmentally friendly, necessitating no supplementary chemical reagents and able to harness sun irradiation, thus presenting a viable green alternative for extensive wastewater treatment. Recent studies have concentrated on strategies including band-gap engineering, heterojunction formation, and metal/non-metal doping to augment light absorption, enhance charge separation, and inhibit electron-hole recombination, thus improving degradation rates and quantum efficiencies (Kumar et al., 2025).

Cobalt-oxide semiconductors, specifically cobalt(II) monoxide (CoO) and cobalt(III) oxide (Co₃O₄), are increasingly recognized as efficient photocatalysts for wastewater treatment owing to their advantageous band structures and chemical stability. Co₃O₄ has been employed in photoelectrocatalytic processes to dissolve recalcitrant organic compounds in water, markedly increasing the production of ROS, such as ·OH and O₂·⁻ that break down persistent pollutants (Zhao & Ma, 2023). In heterogeneous photocatalysis, metal-oxide nanocomposites augment photocatalytic effectiveness by enhancing light absorption and diminishing electron-hole recombination. Cobalt-oxide photocatalysts efficiently decompose wastewater contaminated with textile dyes, reducing chemical oxygen demand and toxicity. Their magnetic and semiconducting characteristics enable catalyst recovery, offering a more environmentally friendly option for wastewater treatment. Challenges persist in optimizing synthesis for elevated surface area, adjusting bandgap for visible light activation, and maintaining catalyst stability in corrosive aqueous conditions (Chowdhury et al., 2020; Moridon et al., 2019; Zhao & Ma, 2023).

MATERIAL AND METHOD

CoO and MB were obtained from Sigma Aldrich and used without further treatment. All aqueous solutions were prepared with distilled water. The chemical structure of the MB dye is given in Figure 1.

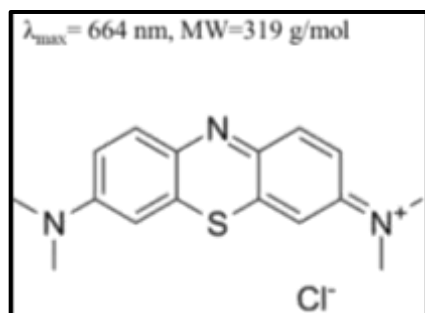


Figure 1. The chemical structures of MB.

FT-IR spectroscopy was employed using a Thermo Scientific Nicolet 6700 spectrometer equipped with an attenuated total reflection accessory. FT-IR measurements were performed in 32 scans at a resolution of 4 cm^{-1} in the range of $1600\text{--}374 \text{ cm}^{-1}$. Raman spectroscopy measurements were carried out using a Jobin Yvon LabRam confocal microscope Raman spectrometer equipped with a charge-coupled device detector and a holographic notch filter. It was outfitted with an 1800 grooves/mm grating, and all measurements were performed using a $200 \mu\text{m}$ entrance slit. The Raman excitation was supplied by 632.8 nm photons from a He-Ne laser with a total power of 20 mW .

The photocatalytic activity tests of the CoO specimen were assessed by degrading the MB solution (10 mg/L). CoO (0.25 g/L) in 50 mL MB solution was placed in a Pyrex reactor container, which was illuminated from the top with an OSRAM Ultra-Vitalux lamp, as a solar simulation (300 W). The resulting mixture was rapidly filtered using $0.22 \mu\text{m}$ cellulose acetate filters.

RESULTS AND DISCUSSION

According to the FT-IR spectrum Figure 2 (a), the cubic CoO exhibits two distinct peaks at 558 and 681 cm^{-1} , as recorded in the literature, which are closely linked to Co-O bending vibrations (Elfadl et al., 2023). The Raman spectrum of CoO across the range of $550\text{--}1000 \text{ cm}^{-1}$ has one distinct peaks at 670 cm^{-1} (Figure 2 (b)). The pronounced narrow bands at 670 cm^{-1} . A distinct peak at 670 cm^{-1} for octahedrally faceted CoO nanocrystals, approximately 60 nm in size (Shinagawa et al., 2022).

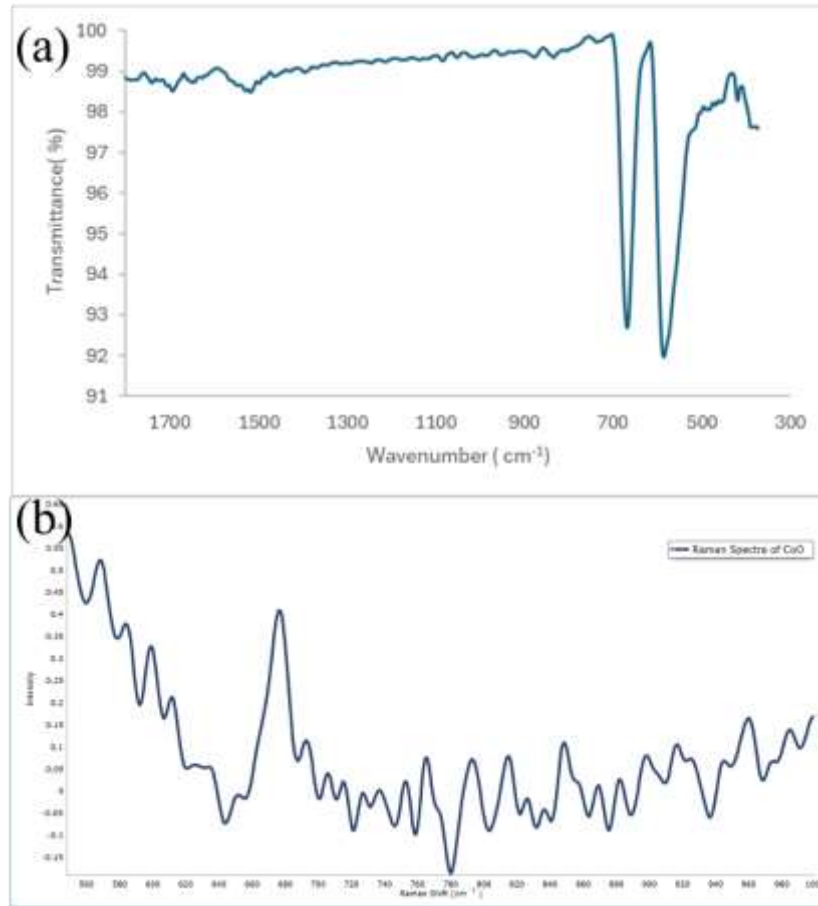


Figure 2. (a) FT-IR and (b) Raman spectrum of CoO specimen.

The photocatalytic removal efficiency of MB cationic dye was calculated by equation (1) at $\lambda_{\max} = 664 \text{ nm}$:

$$\text{Removal efficiency, \%} = \left(\frac{A_0 - A_t}{A_0} \right) \times 100 \quad (1)$$

where,

A_0 : absorbance of MB at initial time t ,

A_t : absorbances of MB at time t ,

A series of photocatalytic MB degradation tests was performed using CoO amount (0.25 g/L) in a 50 mL MB solution (10 mg/L) over a period of irradiation time, and the results were shown in Figure 3 (a).

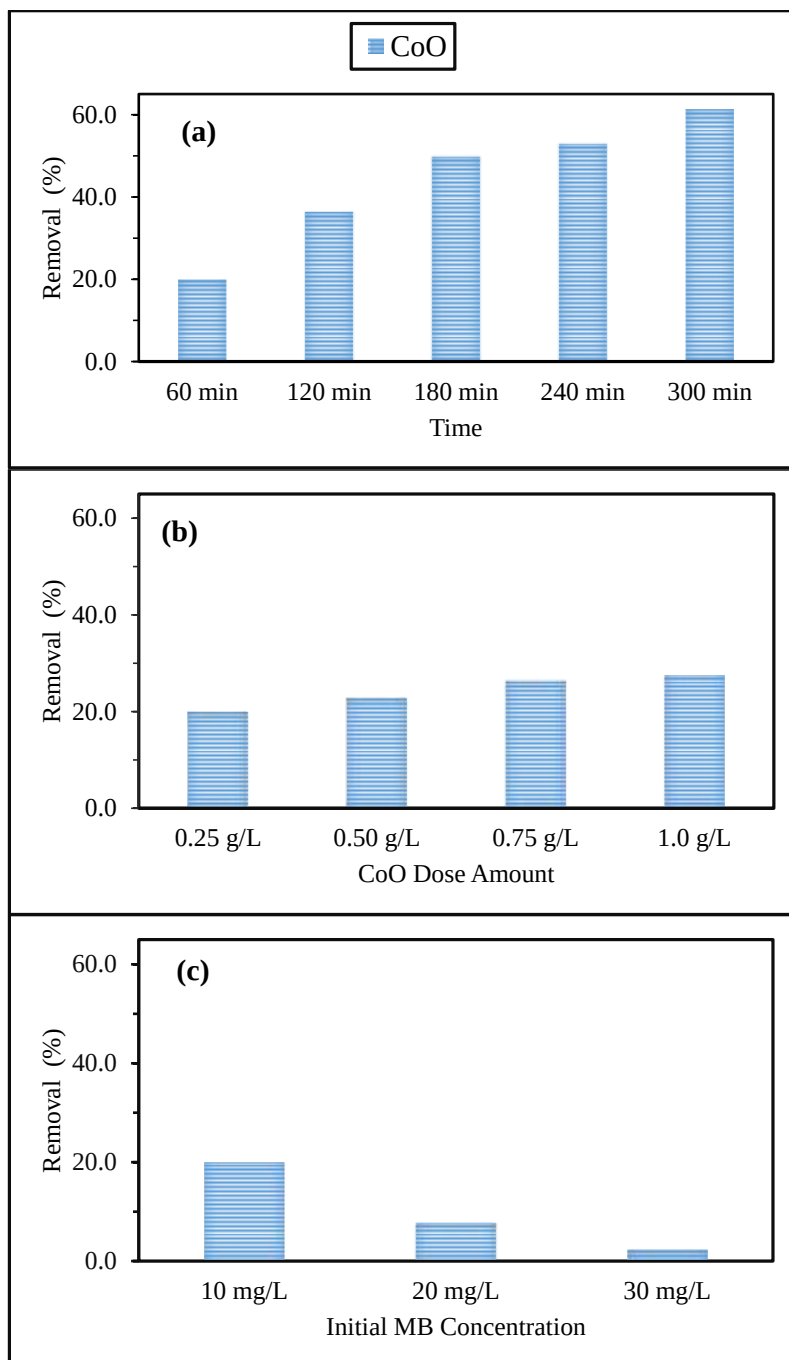


Figure 3. (a) Photocatalytic degradation of MB in the presence of CoO, (b) CoO dose effect, and (c) initial MB concentration effect.

It was observed that CoO nanoparticles revealed a photocatalytic removal efficiency of 61.3% for MB dye in 300 minutes. The effect of CoO dose on MB dye concentration at 10 mg/L after 60 minutes is presented in Figure 3 (b). The degradation of MB increased with the higher catalyst amounts, achieving MB removal efficiency from 19.9% at 0.25 g/L CoO dose to 27.4% with 1.0 g/L CoO. The effect of initial MB concentration on the photocatalytic performance of CoO catalyst (0.25 g/L) by varying MB concentrations was investigated (Figure 3 (c)). The higher MB concentration led to decreased degradation due to a lower adsorption of available active sites responsible for generating hydroxyl radicals.

CONCLUSIONS

The structural properties of CoO were determined using FT-IR and Raman spectroscopy techniques. FT-IR confirmed the evidence of characteristic bands of CoO related to the metal-oxygen groups. The influence of initial MB concentration on the CoO specimen was studied, revealing that a high MB concentration solution (30 mg/L) led to decreased degradation. The photocatalytic removal efficiency of MB dye was 61.3% after 300 minutes.

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STRATEGY FOR DEVELOPING LOBSTER CULTIVATION BUSINESSES (*PANULIRUS SP*) ON BALANGLOMPO ISLAND

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ABSTRACT

This study aims to determine the business development strategy of *Panulirus sp* lobster shrimp farming on Balang Lompo Island, Pangkajene Islands Regency. The research was conducted in August - October 2023 on Balang Lompo Island, Pangkep Regency. The type of research used in this research is survey research with a sampling method carried out by purposive sampling. The number of samples in this study were 48 people. The data collection techniques used such as observation, interviews and literature studies which are then analyzed using SWOT analysis. The results of the subtotal score of the IFAS matrix and EFAS matrix of the SWOT quadrant diagram, it can be seen that the Lobster cultivation business on Balanglompo island is in quadrant I. This position is a very favorable situation. This position is a very favorable situation. And also the Lobster cultivation business has opportunities and strengths so that it can take advantage of existing opportunities. The strategy that must be applied in this condition is to support aggressive growth policies (Growth strategy) and sustainable.

Keywords: Lobster Farming Business, Growth Strategy, Strategic Partnerships, Sustainable Development

INTRODUCTION

Lobster (*Panulirus sp.*) is one of Indonesia's fisheries commodities that has high economic value. Lobster has experienced an increase in demand of around 15% in the world. The international market affects the increase in demand, especially China, which is an export destination country. According to the Fish Quarantine, Quality Control, and Fishery Product Safety Agency, the total volume of live lobster exports in January 2021 was 682,870 tails with an average export per day of 42,679 tails. Indonesia's lobster export destinations are spread to many countries in the world. This shows that Indonesian lobster has gone global so that there are many enthusiasts from abroad who want to consume Indonesian lobster. Currently, the largest importer of Indonesian lobster in the world is China (Reztrianti et al., 2023)

The distribution of lobsters in Indonesia is spread in the waters of the west coast of Sumatra, east coast of Sumatra, north and south coast of Java, Bali, Nusa Tenggara, Malacca Strait, East Kalimantan, West Kalimantan, South Kalimantan, South Sulawesi, North Sulawesi, Papua, Maluku, and Arafura waters. There are 5 types of lobsters found in Indonesian waters, namely batik lobster (*Panulirus longipes*), pearl lobster (*Panulirus omatus*), sand lobster (*Panulirus homarus*), bamboo lobster (*Panulirus versicolor*), rock lobster (*Panulirus penicillatus*). From the BKIPM KKP 2020 statistics, lobster production in Indonesia reached

239 tons. This shows that South Sulawesi has good potential to develop and utilize its marine wealth, especially lobsters (M. Ward et al., 2018).

According to Edward, aquaculture in coastal areas has significant relevance in alleviating poverty. Furthermore, environmentally friendly aquaculture in coastal areas is an important instrument to improve the economic status of small-scale fishers and seagrass conservation. From the 2020 KKP data, it shows that the coastal areas of South Sulawesi have lobster cultivators in 4 districts, namely Bone, Bulukumba, Takalar and Pangkep (Aslan et al., 2015).

Pangkep Regency is a regency in South Sulawesi whose area is directly adjacent to the Makassar Strait. This situation is the main capital of marine fisheries business to develop lobster aquaculture business is increasingly wide open. Pangkep district is one of the Lobster (*Panulirus* sp.) producing areas in South Sulawesi, this can be seen from the area and length of 264.15 Km² with craggy coastal conditions which are lobster habitat, so it is not wrong if Pangkep district is also a potential lobster (*Panulirus* sp.) producer (Fakhriyyah et al., 2020)

Balang Lompo Island is located in Liukang Tupabbiring Subdistrict, Pangkep Regency which is one of the lobster (*Panulirus* Sp.) producing areas. Lobster cultivation on Balang Lompo Island is carried out in cages, this activity has become the identity of local fishermen. Fishermen (producers) in cultivation activities want to make a profit for the sustainability of their business. Therefore, to maintain the sustainability of the lobster cultivation business, an effective and sustainable business development strategy is needed to develop lobster cultivation on Balang Lompo Island (Fakhriyyah et al., 2020). Based on this description, this study aims to determine the effective and sustainable lobster cultivation business development strategy.

DATA AND METHODS

This research was conducted in August - October 2023 in Pangkep Regency, Liukang Tupakbiring District, Mattirusompe Village. The determination of this location was chosen purposively with the consideration that the sub-district above is an area that has lobster cultivation production in Pangkep Regency.

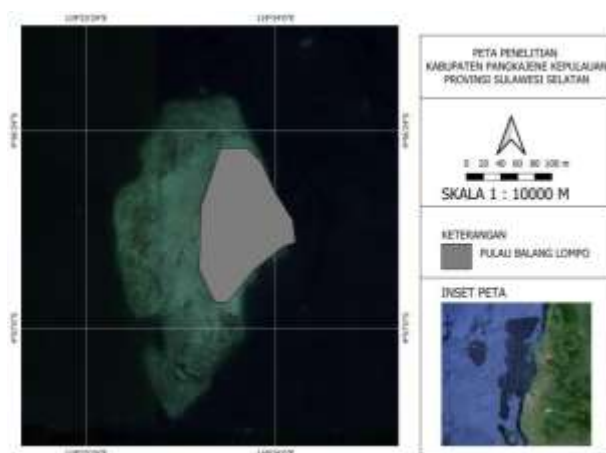


Figure 1. Research location on Balang Lompo Island, Pangkep Regency.

Data Capture Method

The sampling method was carried out by purposive sampling with a total sample size of 48 people. The data collection techniques used are observation, interview and literature study.

Data Analysis

In this study, researchers used qualitative and quantitative approaches. The analysis technique used in quantitative and qualitative approaches is the SWOT analysis technique which is used to formulate business development strategies. This analysis is carried out using data collection tools, namely observation, interview and documentation guidelines. After that, special, detailed and static management is carried out to determine the strategy for the loubstear culture business.

SWOT Matrix

This matrix uses situation analysis of internal and external conditions to develop several alternative strategies that can be used by the company.

1.1 EFAS and IFAS Matrix

The IFAS EFAS matrix is a matrix that determines the factors of the four variables or aspects (strengths, weaknesses, opportunities, threats) based on the results of previous analysis through direct field observations or surveys and the results of interviews with respondents. Then give the weight and reting value, giving the weight and reting value based on the level of influence of strategic factors ranging from important to unimportant. So that the average weight value depends on the number of strategic factors for each aspect or variable (Sammut-Bonnici & Galea, 2015).

In this IFAS and EFAS matrix, a score is also determined which is the result of multiplying the weight and rating values. The sum of the values/score of the results of the reduction between IFAS and EFAS in the two matrices will be used in the SWOT analysis diagram to determine which strategic strategies can be applied in the development of mangrove ecotourism (Sammut-Bonnici & Galea, 2015).

1.1.Quadrant of SWOT analysis

After determining the number of weights and ratings in the previous IFAS and EFAS matrices, then the number of values/score results of teduction between IFAS and EFAS in the two matrices will be used in the SWOT analysis diagram to determine which strategic strategies can be applied in the development of mangrive ecotourism (Sammut-Bonnici & Galea, 2015)

RESULTS AND DISCUSSION

Internal Factors of Lobster Farming

Internal factors are factors that come from within which consist of two points including strengths and weaknesses (Strengths and Weakness). Based on the results of observations in the field and interviews conducted with several respondents, it can be determined that the internal factors of Lobster cultivation include the following:

1.1 Power

Factors that are strengths that can be overcome in the development of crayfish farming businesses include:

1.1.1.High Nutritional Value

Lobsters have a larger body size compared to tiger prawns or king prawns so the meat content is more. The texture of the meat is chewier, has a lower fat, cholesterol and salt content so it is safe for consumption by all consumers. Lobster also has a high zinc content that can increase vitality in humans. The nutrients contained in lobster meat can also repair damaged body cells (L. . Ward et al., 2003). Based on research Pearl lobster contains crude protein of 57.82%, carbohydrates 7.95%, lipids 7.63% reported that both types of lobster contain various essential and non-essential amino acids, saturated fatty acids, and unsaturated fatty acids. Similarly, sand lobster also contains good nutrition, namely crude protein of 56.53%, carbohydrates 7.26%, lipids 7.18%, various essential and non-essential amino acids, and saturated and unsaturated fatty acids (Ya et al., 1991)

1.1.2.Strategic Location

Balang Lompo Island, Liukang Tupabbiring Sub-district, Pangkep District, South Sulawesi Province is the center of the cultured lobster commodity in Pangkep District. The marine waters of Balang Lompo Island have great potential for the development of lobster farming. The cultivated lobster is pearl lobster (*Panulirus Ornatus*). Based on data from the Pangkep Regency Fisheries Service (2023) lobster production on Balang Lompo Island reached 3 tons with a selling value of Rp 1,440,000,000 billion for 2020, 3.4 tons with a selling value of Rp 1,584,000,000 billion for 2021, and 2.84 with a selling value of Rp 1,362,200,000 for 2022.

1.1.3.Seedling Availability

One of the main factors for successful cultivation is the availability of seeds. Based on the results of the interview, seeds are bought by cultivators at collectors, and collectors buy seeds from Takalar and Bantaeng whose seeds come from nature. The form of cultivation carried out by lobster farmers is cultivation with lobster seed enlargement patterns. While the lobsters used as seeds are lobster seeds purchased from collecting traders. The seeds produced by buying from intermediary traders occur due to the lack of knowledge of the cultivators and the cultivators' experience in hatchery techniques. Although the farmers do not have the knowledge and experience in terms of the hatchery process, the farmers have knowledge in terms of the cultivation of lobster seed enlargement.

1.1.3.High Price

Lobsters can also be sold for consumption and as broodstock for hatcheries. The selling price of lobsters in the international market that have a size of 1 kilogram can reach US\$ 100 per kilogram. While the selling price of lobster with a size of 300 grams - 1 kilogram ranges from US\$ 50 - US\$ 80 per kilogram (Pereira & Josupeit, 2017). The high selling price of lobster in the international market is a big and interesting opportunity in the lobster enlargement cultivation business in Indonesia because besides being able to be done with simple technology, the availability of lobster seeds in Indonesia is also very high (M et al., 2019).

There are different prices for each type of lobster, the price of lobster depends on the type and size of the lobster. The higher the size of the lobster, the higher the price offered. Pearl lobster (*Panulirus ornatus*) has a significant price difference compared to other types of lobster. Pearl lobster (*Panulirus ornatus*) has an average price of 2 to 3 times compared to others (Muzayyin et al., 2019b).

1.1.4.Feed Availability

Feed is an important factor in the success of aquaculture. In lobster farming, the feed used by lobster farmers is bête-bête fish and small crabs (kuyu - kuyu). The price of bête-bête fish feed ranges at Rp 5,000.00 per Kg for kuyu - kuyu and Rp 35,000.00 per basket for bête-bête fish. Feeding is usually done once a day with the amount of feed as much as 4 - 6 kg per cage, the time of feeding is usually done in the morning or in the afternoon.

1.2.Weaknesses

Factors that are weaknesses that can be overcome in the development of crayfish farming business include:

1.2.1.Production continuity and quantity

Based on the analysis of the results of interviews with informants, the weakness of the lobster farming business is the lack of continuity and quantity of production. This is because the production capacity of each cultivator is still very limited so that it is unable to meet the targets needed.

1.2.2.Market access

Access to markets is a factor that can ensure market certainty for farmers. However, farmers have very little information about the market.

1.2.3.Lack of communication between cultivators and stakeholders

Based on interviews with informants, one of the weaknesses of aquaculture development is the lack of communication between farmers and all stakeholders (Mas'ud & Tenriyola, 2023). They work on their own so they cannot meet the needs of the existing market. The existence of lobster associations has not had much positive effect on the farmers.

1.2.4.Long enlargement production cycle (7-8 months/cycle)

The production cycle for the enlargement business is relatively long, ranging from 7-8 months per production cycle.

External Factors of Lobster Farming

External factors are factors that come from outside which consist of two points including opportunities and threats (Opportunities and Threats). Based on the results of observations in the field and interviews conducted, it can be determined that the external factors of Lobster cultivation include the following:

1.1.Opportunities

Factors that become opportunities that can be overcome in the development of crayfish farming businesses include:

1.1.1.Low industry growth and competitiveness

The lobster processing industry needs a stable supply and a clear target market for lobster production processing results. Lobster has great potential in the food processing industry and the culinary industry. Lobster can even be used as canned food, snacks, and so on without losing the flavor and protein content in the lobster using the latest technology before being exported out of town and/or abroad (Adha et al., 2024). In addition, lobster can also be used in the culinary industry, for example processing lobster into ready-to-eat dishes. This is supported by the demand of lobster importing countries until now still not fulfilled, therefore lobster is a commodity that has a high selling value and tends to increase. This is an opportunity for local fishermen and farmers to develop lobster catching and cultivation businesses. The price of imported lobster is highly influenced by the fishing season (Adha et al., 2023).

1.1.2.Domestic market

The domestic market is mainly on the island of Java, namely Jakarta, Surabaya, Yogyakarta and Malang. With many market destinations for lobster consumption, it is a great opportunity for the development of lobster aquaculture. With a large population, the domestic market offers a large consumer potential for domestic products. The domestic market is mainly on the island of Java namely Jakarta, Surabaya, Yogyakarta and Malang. Strong domestic market growth will also increase people's purchasing power, which in turn will drive economic growth.

1.1.3.International market

Lobster is one of the fishery products that has a high selling value. Indonesia is a major exporter of lobster seeds to various world producer countries such as Vietnam, Hong Kong, Singapore, Thailand, Brunei Darussalam and Malaysia. Vietnam has been the main destination country for Indonesian lobster seed exports in recent years (Muzayyin et al., 2019a).

1.1.4.Information technology

Advances in information technology can be utilized to improve cultivation results. Based on the results of observations, it can be concluded that the use of the underwater lobster apartment system in lobster cultivation has a very positive effect on growth and survival reaching 100%. Water quality with the underwater lobster apartment system is maintained and does not exceed the limit value of water quality for cultivation (Kleiven et al., 2022).

1.1.5.Government policy

Regulation of the Minister of Maritime Affairs and Fisheries of the Republic of Indonesia No.1 Permen-KP in 2015 prohibits the export of lobster seeds under 200 grams. So that lobster cultivation becomes one of the most promising business opportunities for fishermen (Maskun et al., 2020). The prohibition of catching and releasing lobsters, crabs and crabs is one of the legal objectives in terms of ecological benefits. The application of the ministerial regulation when examined from the aspect of expediency, economic expediency is seen from the amount of income obtained by the fishing community after the enactment of the ministerial regulation that the ministerial regulation increases income for fishermen which has become the state's obligation to improve the welfare of the entire community.

1.2.Threat

Factors that become threats that can be overcome in the development of crayfish farming business include:

1.2.1.Variety of fishery commodities/substitutes

One of the threats of lobster farming is the wide selection of fishery commodities that can be cultivated. One of the threats of lobster farming is the large selection of fishery commodities that can be cultivated. This can certainly provide alternative choices for consumers in meeting their needs. In aquaculture, there are at least 4 types of superior commodities cultivated from various types of seawater fish groups such as (tuna, tuna and skipjack), and crustaceans (crabs and shrimp).

1.2.2.Seed smuggling

This smuggling is rampant because the seafood commodity business is very lucrative, with a fairly simple way billions of rupiah transactions can be carried out illegally. Currently, the Indonesian government is making efforts to prevent the smuggling of seafood that is prohibited from export. In addition to harming the State, it can also make marine biota increasingly rare coupled with losses that reach trillions of rupiah. The modus operandi is generally to circumvent various export-import facilities provided by Customs. One example of a criminal case of lobster seed smuggling, as reported that police officers from the Subdit Tipiter Ditreskrimsus Polda Lampung together with the Fish Caratina Center and the Radin

Airport managed to thwart an attempt to smuggle lobster seeds. Intan succeeded in thwarting an attempt to smuggle shrimp fry that were about to be taken to Batam, approximately 52,884 lobster seedlings that were about to be smuggled.

1.2.3.Lack of investor interest

Investors or corporations are not yet interested in lobster farming. This is why lobster production is still very low compared to demand. Investors or corporations are not interested in lobster farming. Because in general, aquaculture is a high-risk activity and the control of external factors that are not human will is one of the reasons why investors or corporations are not interested in investing in the sector, besides that Aquaculture (lobster) requires sufficient land and can be guaranteed sustainability. This is why lobster production is still very low compared to demand.

SWOT analysis matrix

Based on the identification of internal and external factors that have been carried out consisting of strengths, weaknesses, opportunities and threats, the following SWOT analysis matrix is presented as follows:

Table 1. SWOT analysis matrix

Internal External	Strengths (S) 1. High nutritional value 2. Simple cultivation techniques 3. Seed availability 4. High selling price 5. Feed availability	Weaknesses (W) 1. Product continuity and quantity 2. Limited market access 3. Lack of communication between cultivators and stakeholders 4. Long production cycle
	Opportunities (O) 1. Low growth and competitiveness 2. Domestic market 3. International market 4. Information technology 5. Government policy	WO Strategy 1. Strengthening the collaboration of 3 agents (government, farmers, universities) in an effort to prevent crop failure and oversupply (unsold products) 2. Optimizing the use of technology as an inventory management tool for monitoring lobster stock and predict feed requirements.
Treats (T) 1. Variety of fisheries continuity 2. Seed smuggling 3. Lack of investor interest	ST Strategy 1. Exploration of collaboration opportunities with research institutions or universities that support innovation and diversification of fishery products. 2. Application of appropriate technology to increase production capacity	WT Strategy 1. Improve production efficiency by optimizing natural and artificial feeding 2. Development of an integrated supply chain as an effort to increase the efficiency of lobster farming

The following are some of the strategies obtained from the SWOT matrix analysis, including the following

1.1.S-O Strategy (Strengths- Opportunities)

Strength-Opportunity (SO) strategy, made based on the way of thinking of lobster aquaculture managers by utilizing all strengths to seize and take advantage of maximum opportunities. Some of the strategies obtained include the following:

- a. Form contract-based strategic partnerships with restaurants or the government.
- b. Focus on product improvement by capitalizing on high selling prices

This strategy is taken with the consideration that the cultivator sells a product, namely lobster, which is a superior product for export with many market networks making the company have to increase production volume to meet market demand. According to (Shields, 2011) who explained related to contract farming stated that this system is a dynamic partnership between small farmers and a large business, which provides benefits for both, without sacrificing other parties. Contract farming is also believed to be an instrument for technology transfer, creating political and economic stability through income distribution, and most importantly supporting agricultural modernization (Jensen et al., 2000)

1.2.S-T Strategy (Strengths-Threat)

Strength-Threat (ST) strategy, using the strengths possessed by the Lobster cultivation business to overcome threats. Some of the strategies obtained include the following:

- a. Exploration of collaboration opportunities with research institutions or universities that support innovation and diversification of fishery products.
- b. application of appropriate technology to improve efficiency in the supply chain and production process to reduce working capital requirements and operational costs.

One of the efforts in ensuring business sustainability is that business people must ensure 3 aspects in their business, namely people (Social Line), Planet (environmental line), and Profit (Economic line), this can be done by exploring various potential collaborations and applying appropriate technology that refers to the principle of green technology.

1.3.W-O Strategy (Weakness- Opportunity)

Weakness-Opportunity (WO) strategy, utilizing existing opportunities by minimizing existing weaknesses. Some of the strategies obtained include

- a. Strengthening the collaboration of 3 agents (government, farmers, universities) in efforts to prevent crop failure
- b. Optimizing the use of technology as an inventory management tool to monitor lobster stock and predict feed requirements.

Collaboration is an important role in every line of business this is done to ensure the sustainability of each existing business in addition to the collaboration of 3 agents makes each element hold a key role in efforts to advance the people's economy. According to Primyastanto, 2011, inland fisheries as one of the sources of income for fish farmers plays an important role in export activities. Inland fisheries production with an area and potential that allows it to be increased, namely by improving the combination of factors of production and technological renewal. The inland fisheries sector shows prospects with considerable development both from the source and potential and identification of the business (Van Der Meeren, 2005).

1.4.W-T (Weakness-Threat) Strategy

Weakness-Threat (WT) strategy, based on defensive activities and trying to minimize existing weaknesses and avoid threats. Some of the strategies obtained include the following:

- a. Improve production efficiency by optimizing natural and artificial feeding
- b. Development of an integrated supply chain as an effort to increase the efficiency of lobster farming

IFAS and EFAS Matrix

1.1.IFAS Matrix

According to (Rangkuti, 2016) the IFAS (Internal Factor Analysis Summary) matrix is a matrix that describes the arrangement of a list of internal factors that affect the performance of an organization or company, including internal factors are strengths and weaknesses. The IFAS matrix consists of columns, weights, ratings and total values which are the result of multiplying the weights and ratings. The weight and rating columns are filled in according to the value which is the result of the grouping of internal factors.

Based on the results of research and determination of weight and rating values, it is known that the highest weight value in the strength aspect is a weight value of 0.13 and a rating value of 4. After analyzing the overall value for internal factors, the strength aspect is 2.47. Meanwhile, based on the results of research and determination of weight and rating values, it is known that the highest weight value is 0.11 and a rating value of 3. After analyzing the overall value for the weakness aspect factor, namely 0.78. Based on the results of the analysis of the weight and rating of the internal factors, the total value of the strengths and weaknesses aspects is 3.25.

Table 2. IFAS matrix (internal factor analysis summary) of lobster farming business

No	Internal factors	Weight	Rating	BxR
Power				
1	Nutritional value	0.13	4	0.50
2	Simple cultivation techniques	0.13	3	0.38
3	Strategic location	0.09	3	0.28
4	Seedling availability	0.13	4	0.50
5	High price	0.13	3	0.38
6	Feed availability	0.11	4	0.44
Total		0.70		2.47
Weaknesses				
1	Product continuity and quantity	0.06	2	0.13
2	Limited market access	0.08	3	0.23
3	Lack of communication between cultivators and stakeholders	0.11	3	0.33
4	Long production cycle	0.05	2	0.09
Total		0.30		0.78
Total amount		1.00		3.25

1.2.EFAS Matrix

Table 3. EFAS matrix (external factor analysis summary) of lobster farming business

No	External factors	Weight	Rating	BxR
Opportunities				
1	Low growth and competitiveness	0.16	3	0.49
2	Domestic market	0.16	4	0.65
3	International market	0.12	4	0.49
4	Information technology	0.14	3	0.43
5	Government policy	0.14	3	0.43
Total		0.73		2.49

		Threat		
1	Variety of fishing communities	0.08	3	0.24
2	Seed smuggling	0.10	2	0.20
3	Lack of investor interest	0.08	3	0.24
Total		0.27		0.69
Total amount		1.00		3.18

SWOT Quadrant

Knowing the position of the lobster farming object can be described by the SWOT quadrant. The SWOT quadrant diagram is used to find the position of the cultivation business addressed by the quadrant point (x,y), so as to obtain the main alternative strategies that can be applied to the development of lobster farming businesses. To determine the position of the lobster farming business, calculations are made based on the difference from the subtotal score of the IFAS matrix and EFAS matrix. Based on the results of the subtotal score of the IFAS matrix and EFAS matrix SWOT quadrant diagram, it can be seen that the existing lobster farming business on Balanglombo island is in quadrant I. Lobster cultivation on Balang lombo island has opportunities and strengths so that it can take advantage of existing opportunities. The strategy that must be applied is to support aggressive growth policies (Growth strategy). To determine the position, a calculation is made based on the difference from the sub-total score of the IFAS matrix and EFAS matrix, the results can be formulated as follows:

Point Coordinate (X) = (Power-Weakness)

$$= 2,47 - 0,78$$

$$= 1,69$$

Coordina Point (Y) = (Opportunities - Threats)

$$= 2,49 - 0,69$$

$$= 1,80$$

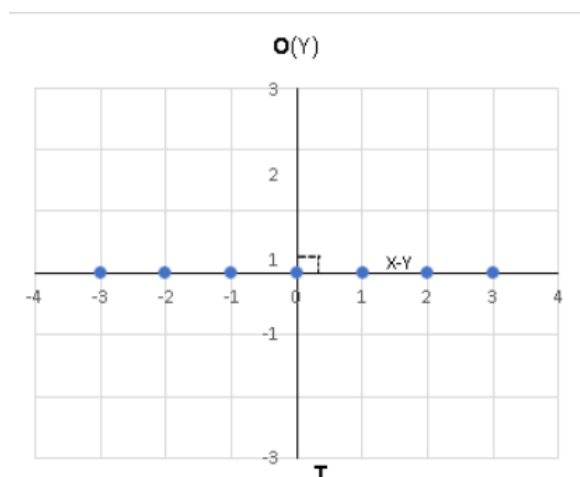


Figure 2. SWOT quadrant diagram

CONCLUSION

Based on the research results that have been presented above, the following conclusions can be drawn: The results of the subtotal score of the IFAS matrix and EFAS matrix of the SWOT quadrant diagram, it can be seen that the Lobster cultivation business on the island of Balanglombo is in quadrant I. This position is a very favorable situation. This position is a very favorable situation. And also the Lobster cultivation business has opportunities and strengths so that it can take advantage of existing opportunities. The strategy that must be applied in this condition is to support aggressive growth policies (Growth strategy) and sustainable. The strategies offered are:

1. Form contract-based strategic partnerships with restaurants or the government.
2. Focus on product improvement by utilizing high selling prices strengthening agent collaboration (government, cultivators, universities) in efforts to prevent crop failure
3. Optimizing the use of technology as an inventory management tool to monitor lobster stock and predict feed requirements

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STRATEGIES FOR HOST-SEEKING IN ENTOMOPATHOGENIC NEMATODES: MATCHING SPECIES WITH TARGET INSECT PESTS FOR EFFICIENT BIOCONTROL

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ABSTRACT

Entomopathogenic nematodes (EPNs) are endoparasitic organisms capable of actively seeking and infecting a wide range of insect hosts. Furthermore, these organisms stand out as promising future agents in the field of biological control. In this review, three commonly used EPN species, *Steinernema carpocapsae*, *S. feltiae*, and *Heterorhabditis bacteriophora* were comparatively evaluated based on their host-seeking behaviors. Based on current laboratory and field findings, the effects of different foraging strategies (ambushing, intermediate or cruiser) of each species on their effectiveness against specific insect pest groups were reviewed. As an ambush species, *S. carpocapsae* was found to be more effective against insect pests living near the soil surface or in leaf areas. *S. feltiae*, an intermediate forager, offers flexibility in use against pests of different stages and in a wide range of habitats, particularly in temperate conditions. *H. bacteriophora*, a cruiser (active mobile) species, demonstrated superior activity in deeper layers, particularly against insects that are mobile underground. These results suggest that the selection of EPN species should be compatible with the insect pest's behavior and ecological niche to maximize success in biological control.

Keywords: *Heterorhabditis bacteriophora*, *Host seeking*, *Steinernema carpocapsae*, *Steinernema feltiae*

INTRODUCTION

Entomopathogenic nematodes (EPNs) are extensively employed in both conventional and organic agriculture as a viable method for pest control in crops (Smart Jr, 1995; Shapiro-Ilan et al., 2006; Kurtz et al., 2007; Batalla-Carrera et al., 2010; Labaude & Griffin, 2018; Vashisth et al., 2013; Dede et al., 2022). These organisms are known as obligate endoparasites and require insect host tissue to complete their life cycle. The life cycle of EPNs consists of the following stages: egg, first-stage juvenile, second-stage juvenile, third-stage juvenile known as the infective juvenile (IJ), fourth-stage juvenile, and adult (Gaugler et al., 1997; Stuart et al., 2006; Kurtz et al., 2007; Bütüner & Susurluk, 2023a; Bütüner et al., 2024). These obligate endoparasitic organisms can infect their hosts only during the infective juvenile stage of their development (Jansson et al., 1990; Susurluk et al., 2001; Lewis et al., 2006; Kurtz et al., 2007; Susurluk & Ehlers, 2008).

Upon encountering a host, entomopathogenic nematodes enter the host's body through natural openings such as the mouth or anus, releasing their symbiotic gram-negative bacteria into the host's internal environment (Smart Jr, 1995; Ehlers, 2001; Lewis et al., 2006; Susurluk & Ehlers, 2008). Species belonging to the family Heterorhabditidae introduce these bacteria through regurgitation, whereas species from the Steinernematidae family do so via defecation. The gram-negative bacteria, members of the family Enterobacteriaceae, differ according to the

nematode family: Heterorhabditidae species are symbiotically associated with *Photorhabdus* spp., while Steinernematid species are associated with *Xenorhabdus* spp. Once inside the host, the bacteria multiply rapidly, causing septicemia and ultimately leading to the host's death within 24 to 72 hours (Peters & Ehlers, 1994; Ehlers, 2001; Ciche et al., 2006; Shapiro-Ilan et al., 2006; Lewis et al., 2006; Susurluk & Ehlers, 2008; Susurluk & Bütüner, 2024). Inside the cadaver, the nematodes can complete 3 to 4 generations, and upon depletion of nutrients within the host tissues, infective juveniles (IJs) emerge to seek new hosts (Ciche et al., 2006; Shapiro-Ilan et al., 2006; Susurluk & Bütüner, 2024). It is known that IJs can survive in the soil without feeding and can seek hosts for up to two years (Susurluk & Ehlers, 2008).

The selection of an EPN species for pest management in agricultural fields is of critical importance, as host-seeking behaviour tends to vary considerably between species (Campbell et al., 2003; Lewis et al., 2006; Griffin, 2012). For instance, species like *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) are generally classified as cruisers (Cohen et al., 2002; Susurluk & Ehlers, 2008; Santhi et al., 2015). *Steinernema feltiae* (Rhabditida: Steinernematidae) is classified as exhibiting an intermediate foraging strategy (Wilson et al., 2012; Santhi et al., 2015), whilst species like *S. carpocapsae* are generally recognized as ambushers (Grewal et al., 1995; Santhi et al., 2015). Therefore, in determining which EPN species to deploy against a particular pest, it is essential to consider whether the insect pest spends any part of its life cycle in the soil, as well as which part of the cultivated plant is affected by its feeding activity (Grewal et al., 1994; Lewis et al., 1995; Campbell et al., 2003). In this review, recent findings on the efficacy of various EPN species against different pests have been reviewed in relation to their host-seeking behaviours.

SELECTING A HOST FOR *Steinernema carpocapsae*

Species of *S. carpocapsae* are generally characterised by limited movement within the soil, typically remaining near the site of application. They are highly effective in the potential control of pests that cause damage on or near the soil surface, particularly around the roots and root collar. In recent years, their use in foliar applications has increased significantly (Lewis et al., 1992; Grewal et al., 1994; Wilson et al., 2012; Baiocchi et al., 2019). Due to their low mobility in soil, they may exhibit greater tolerance to environmental factors compared to other EPN species. However, a notable disadvantage is their limited efficacy in deep soil applications, where their restricted movement reduces their effectiveness against subterranean pests (Lewis et al., 1996; Gang & Hallem, 2016; Baiocchi et al., 2019). Consequently, they may not perform adequately in deep, highly moist soils. In a field study conducted by Nouh & Adly (2021), the efficacy of *H. bacteriophora* and *S. carpocapsae* isolates was evaluated against adults of *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae). The results showed that at identical application rates, *S. carpocapsae* caused 96% mortality on adult *G. gryllotalpa*, whereas *H. bacteriophora* resulted in 72% mortality. Based on these findings, it can be concluded that *S. carpocapsae*, despite its relatively limited mobility compared to *H. bacteriophora*, may be more successful in controlling *G. gryllotalpa*, particularly given the pest's damaging activity around the root and root collar regions. Similarly, in a study conducted by Morton & Garcia-del-Pino (2017), which simulated field conditions, the efficacy of various commercial and local EPN isolates was assessed against *Agriotes obscurus* (L.) (Coleoptera: Elateridae). The results indicated that *S. carpocapsae* isolates exhibited significantly higher efficacy compared to isolates of other species. In addition, the use of *S. carpocapsae* isolates is commonly preferred for the potential control of certain pests, particularly those belonging to the order Lepidoptera that spend their pupal stage within plant debris or on the upper layers of the soil, where they cause damage. In a study conducted by Gholami Ghavamabad et al. (2024), the efficacy of various isolates was evaluated against *Cydalima perspectalis* (Lepidoptera: Crambidae) and

Hyphantria cunea (Lepidoptera: Arctiidae) under both field and laboratory conditions. The results revealed that *S. carpocapsae* was particularly effective against the larvae of these pests when applied as a foliar treatment.

SELECTING A HOST FOR *Steinernema feltiae*

Species of *S. feltiae* are generally characterized by moderate mobility within the soil and a flexible host-seeking strategy that combines both ambush and cruising behaviors (Grewal et al., 1997; Susurluk, 2008). This intermediate foraging strategy allows *S. feltiae* to effectively target a wide range of insect hosts, particularly those dwelling in the upper soil layers or leaf litter, as well as in cryptic habitats such as crevices and under bark. Because of its sensitivity to chemical signals and capacity to seek out immobile or less active hosts, *S. feltiae* is particularly effective against soft-bodied insect larvae and pupae (Gaugler et al., 1989; Rahoo et al., 2017; Askary et al., 2018). *S. feltiae* is commonly utilized in temperate areas and greenhouse environments due to its tolerance for low temperatures and adaptability to various conditions (Chen & Glazer, 2005; Navaneethan et al., 2010; Rahoo et al., 2016). Nonetheless, its effectiveness sometimes declines in arid soils or at high temperatures, potentially limiting its use in specific field conditions. *S. feltiae* possesses a significant advantage due to its demonstrated efficacy in enclosed environments with increased humidity, making it appropriate for integrated pest management (IPM) initiatives in controlled agriculture (Hirao & Ehlers, 2009; Navaneethan et al., 2010; Rahoo et al., 2016).

In a study conducted by Gouge & Hague (1995), the efficacy of several EPN isolates was evaluated against larvae of *Bradysia paupera* (Diptera: Sciaridae). The experiments were carried out at two different temperatures (22 and 30°C). At both temperatures, isolates of *S. feltiae* were found to be more effective than those of other species, achieving 100% larval mortality at 22°C. Similarly, Morton & García del Pino (2007) assessed the efficacy of several commercial EPN species against larvae of *Scatella stagnalis* (Diptera: Ephydriidae). The results indicated that commercial products based on *S. feltiae* produced higher larval mortality rates compared to other species. Similarly, in a study conducted by Susurluk (2011), *Steinernema feltiae* isolates achieved an 80% mortality rate in the potential control of last-instar larvae of *Delia radicum* (Diptera: Anthomyiidae). In a separate study, Kasi et al. (2021) evaluated the potential efficacy of different EPN species against second-instar larvae of *Pieris brassicae* (L.) (Lepidoptera: Pieridae). The highest larval mortality rate, 72%, was observed in treatments involving *S. feltiae* isolates. Furthermore, in a recent study by Polat et al. (2024), the potential of EPNs to control both larvae and adults of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) was investigated in poultry house environments. The data demonstrated highly effective results for the potential control of *A. diaperinus*, indicating the strong biocontrol potential of the applied EPN species.

SELECTING A HOST FOR *Heterorhabditis bacteriophora*

Heterorhabditis bacteriophora exhibits an active cruising host-seeking strategy, facilitating significant movement throughout the soil matrix in pursuit of appropriate hosts. The IJs of this species can detect and pursue carbon dioxide gradients and other volatiles released by active soil-dwelling insect larvae (Ehlers, 1996; Susurluk & Ehlers, 2008a; Ulu & Susurluk, 2014; Dede et al., 2022). This behaviour makes *H. bacteriophora* particularly effective against mobile insect pests that live in deeper levels of soil and exhibit continuous movement in search of food sources (Susurluk, 2008).

Due to its ability in deep-soil foraging, *H. bacteriophora* is regarded as highly efficient for managing subterranean pests, including larvae of *Diabrotica* spp. (Coleoptera:

Chrysomelidae), *Phyllophaga* spp. (Coleoptera: Scarabaeidae), and *Popillia japonica* (Coleoptera: Scarabaeidae) (Wang et al., 1994; Paoli et al., 2017; Garriga et al., 2024). This species thrives in moist, warm soil conditions, exhibiting optimal activity at temperatures ranging from 20 to 30°C. Nonetheless, its effectiveness may diminish in arid or colder soils, where mobility and host-seeking efficiency are hindered (Susurluk & Ehlers, 2008b; Bütüner & Susurluk, 2023b). In a study conducted by Dede et al. (2022), the *H. bacteriophora* HBH hybrid strain was utilised for the potential control of *Loxostege sticticalis* (Lepidoptera: Pyralidae), which caused an epidemic in Türkiye in 2022. The results revealed that at 30 °C, the application of low doses resulted in up to 97% mortality in last-instar larvae. Similarly, Del Valle et al. (2017) evaluated an isolate of *Steinernema rarum* (Rhabditida: Steinernema) and an isolate of *H. bacteriophora* for their potential to control larvae of *Diloboderus abderus* (Coleoptera: Scarabaeidae). The findings indicated that the *H. bacteriophora* isolate was more effective in the control of *D. abderus* larvae. Likewise, in a study by Goudarzi et al. (2015), the potential efficacy of *S. carpocapsae* and *H. bacteriophora* isolates was assessed against larvae of *Agrotis segetum* (Lepidoptera: Noctuidae). According to the results, *H. bacteriophora* isolates achieved a mortality rate of 98% on *A. segetum* larvae.

CONCLUSIONS

An examination of the potential control of agricultural insect pests using *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* reveals that understanding the host-seeking behaviour of EPNs, along with the life cycle and ecology of the target pest, is essential. When an appropriate EPN species is matched with a suitable target organism, these endoparasitic agents can produce highly effective control outcomes. *S. carpocapsae*, through its ambusher strategy, has proven to be effective against pests that reside close to the soil surface. In contrast, *S. feltiae* demonstrates a more balanced and versatile efficacy across various pest stages and habitats, owing to its intermediate foraging behaviour. *H. bacteriophora*, on the other hand, provides a distinct advantage in the control of mobile pests dwelling in deeper soil layers due to its high mobility and active cruising behaviour. Therefore, the success of EPN-based biological control strategies relies not only on the selection of a virulent isolate but also on the compatibility between the ecological characteristics of the target pest and the behavioural ecology of the nematode. Future applications should prioritise the alignment of EPN behavioural traits with the biology of the pest species, as this approach will be fundamental in enhancing both the efficacy and reliability of biological control efforts.

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THE SPERM QUALITY PARAMETERS OF COMMONLY AQUACULTURED TILAPIA SPECIES

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ABSTRACT

Tilapia species are members of the family Cichlidae. Although this group is known as African freshwater fish, they have spread all over the world over last decades. Some advantages of tilapias such as fast growth rate, feeding on different trophic levels, ability to survive extreme aquatic conditions and reproduction performance are attributed to this wide distribution. Moreover, tilapias include also edible and aquarium species. Two species, *Oreochromis niloticus* and *O. mossambicus*, have become prominent in this group in terms of evaluation of their spermatological characteristics. Sperm densities and motility parameters (duration of sperm motility, total motility, velocity, linearity, etc.) of both stripped and testicular sperm samples have been revealed for these species. Additionally, the effects of some ions, pH and temperature on their sperm motility parameters were determined and discussed to understand how environmental and ecological factors affect these parameters. In general, spermatozoa of these species could be characterized by slower velocity and longer motility duration when compared to spermatozoa properties of the most cultured species like rainbow trout and common carp which their sperm parameters are established very well.

Keywords: Tilapia, Oreochromis, Spermatozoa, Sperm Motility

INTRODUCTION

Over last decades, aquaculture production has consistently increased and has become one of the fastest expanding sectors in global livestock production. Thus, aquaculture has gained economic importance compared to previous decades. Moreover, increases in aquaculture production remarkably contribute to meeting the growing global demand for protein (FAO, 2010). Fish species being produced in the general aquaculture production is also one of the notable issues to discuss and assess. Tilapia species constitute one of the important groups of fish in global aquaculture and more than 40% of aquaculture is derived from Tilapia production. Additionally, Tilapia species are the most produced species in some countries like Brazil Egypt, Indonesia, Philippines, etc (Gjedrem, 2012; de Oliveira et al., 2014).

Tilapia is a highly preferable species for aquaculture due to several beneficial biological and commercial properties. It exhibits flexible feeding habits, primarily as a herbivore or omnivore at a low trophic level, depending on different food sources like algae, bacteria, and detritus. In aquaculture conditions, Tilapia feeds could be prepared mainly consist of grains and agriculture by-products. This fish possesses a fast growth rate and shows easy adaptability to different conditions, including high stocking densities. Furthermore, it is highly disease resistant and tolerant of poor water quality. Tilapia species are also prolific breeders with easy breeding in captivity. Collectively, these characteristics contribute to low production costs (Prabu et al., 2019). Moreover, Tilapia species have a remarkable market demand and compete with salmon and carp species in terms of their economic values (Cai, 2025). In addition to their utilization as aquaculture species, the ecological importance of tilapia species should be

emphasized. Many *Tilapia* species have been considered as invasive species in different countries like Türkiye, distributing unintentionally through different water bodies (Çiçek, 2021; El-Sayed and Fitzsimmons, 2023).

Determining sperm quality is closely related to improving fish production conditions and is critical for obtaining targeted numbers of larvae. This determination also provides information about the ecological spread of the species. Sperm quality is typically determined by kinetic features of motility such as the percentage of motile cells, sperm velocity and the duration of motility (Alavi and Cosson, 2006). In *Tilapia*, like many fish species, spermatozoa are immotile in testes, and they activated that is gain the ability to motility, only after contacting with the aquatic environment. This sperm motility is necessary for fertilizing the egg and quickly loses this ability within minutes. Motility of spermatozoa is one of the primary factors determining the rate of successful fertilization (Cosson, 2004).

The aim of this study is to reveal some spermatological parameters of *Tilapia* species. For this purpose, osmolality and pH values of seminal plasma, sperm concentrations, total motility, motility durations and sperm velocity parameters (curvilinear velocity; VCL and straight-line velocity; VSL) were represented. Moreover, activating media used for triggering sperm motility and some factors such as pH, contents and temperature of these media were discussed.

MATERIAL AND METHOD

Sperm samples were collected from mature male *Tilapia* species. Generally, these samples were obtained from broodstock fish kept in fish farm facilities. Usually, no hormone injections are required to obtain spermatozoa from *Tilapia* species. Anesthesia could be administered to males when required. Sperm samples are taken by stripping (abdominal massage) or dissection of testes. Especially before the abdominal massage application, the urinary bladder was drained by hand pressure at the abdomen to avoid any urine contamination. Before the sampling, males the genital papilla and the abdomen were thoroughly dried. Both the stripping and the dissection samples were placed immediately on ice and immediately controlled under the microscope. To determine spermatozoa concentration, samples were evaluated haemocytometrically diluting with non-activating media (Figure 1). Motility parameters were analyzed by computer-assisted sperm analysis (CASA) or subjective evaluation under a microscope.

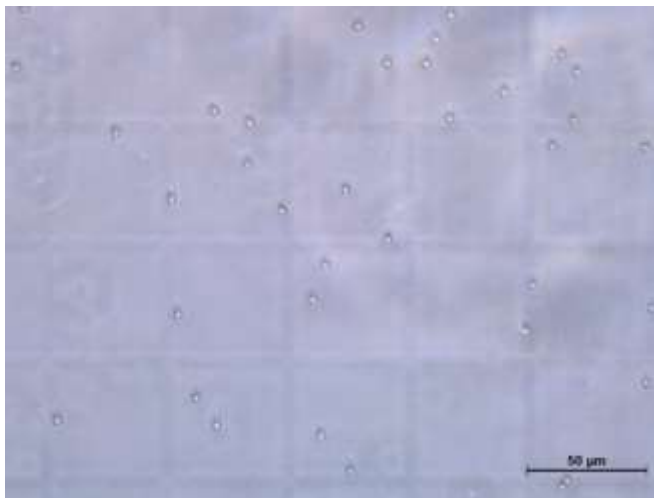


Figure 1. An image used for the calculation of sperm concentration in Nile *Tilapia* (photographed by B. E. İNANAN).

RESULTS AND DISCUSSION

Sperm sampling methods used for *Tilapia* species, and also osmolality and pH of seminal plasma and sperm concentration were found in *Tilapia* species were represented in Table 1. Even though the stripping method was more commonly used, sperm samples from testis and spermatic duct were also handed in *Tilapia* species (Linhart et al., 1999; Gennotte et al., 2012; Acar et al., 2022). Osmolarity values are around 300 mOsmol Kg⁻¹, as expected in many fish. On the other hand, pH levels of seminal plasma mainly ranged from 7.5 to 8. Generally, sperm concentrations were calculated as 2-3 x10⁹ /ml. Conceivably, higher concentrations were reported for testicular samples (Linhart et al., 1999; Acar et al., 2022). However, lower concentrations were also found for the stripped samples (Garcia-Abiado et al., 1996; Linhart et al., 1999; Ugwu et al., 2018). Besides sperm sampling methods, urine contamination could be one of the main issues on variations observed in especially pH levels and sperm concentrations. When sperm samples are taken by the stripping from fish, urine contamination is usually a hard problem to avoid (Ciereszko et al., 2010). Due to their anatomical structure, tilapia fish are also apt to exhibit this problem. The care should be necessarily taken to avoid urine contamination during collecting sperm samples from *Tilapia* fish (İnanan et al., 2025).

Activating media used for triggering sperm motility, post-activation time when motility analyses were conducted, total sperm motility, motility durations and sperm velocity parameter in *Tilapia* species were shown in Table 2. Distilled water has been used mostly for triggering sperm motility in *Tilapia* species. In addition, pond water and some ionic solutions with different contents were utilized for this purpose. Total motility duration of *Tilapia* species sperm could reach 30 min. In general, motility values were recorded > 80%. These motility values were determined at 5th, 10th, 12th, 20th, 30th and 60th of post-activation time. VCL values varied 40 to 90 µm s⁻¹ whereas VSL values 32 and 60 µm s⁻¹. It has been well established that sperm quality in farmed fish is highly affected by different factors such as Rearing photoperiod and temperature, nutrition, stress to broodstock (Rurangwa et al., 2004). Some factors also could be effective on sperm motility parameters during the analysis of them. One of the main reasons may be the activating media used for triggering motility. These media can cause variations within and between species in terms of their motility parameters. For instance, cytoplasmic irregular bulges and curling in spermatozoa could be formed due to osmotic shock due to sperm activation in media with lower osmolality like distilled water. Distilled water has been frequently used for triggering sperm motility in *Tilapia* species. In parallel with this, Utarabhand and Watanakul (1998) reported that sperm motility durations were recorded as 6', 3' and 2' when *O. niloticus* spermatozoa activated by an activating medium (10 mM Tris-HCL, 1.5 mM NaCl, 0.5 mM KCL, 1.0 mM CaCl₂, 0.5 mM MgCl, pH 7.), filtered freshwater and distilled water. Moreover, it has been shown that ≤50 mOsmol/kg of osmolality values could induce some decreases in motility and VCL values of *O. niloticus* spermatozoa (İnanan, 2025). On the other hand, some *Tilapia* species like *O. mossambicus* can be acclimatized to euryhaline water, and this acclimatization could have effects on some spermatological characteristics (Linhart et al., 1999; Morita et al., 2003).

Motility parameters of *Tilapia* spermatozoa are acutely affected by the characteristics of activation media such as pH, ionic contents, and the osmolality (ionic and non-ionic). Moreover, the temperature of activating media is a critical determinant. Considering that *Tilapia* spermatozoa have longer motility durations when compared to other commonly cultured fish species like rainbow trout and carp, maintaining a stable temperature is very critical for determining accurate motility duration for *Tilapia* species, especially when assessing motility under laboratory conditions (Dzyuba et al., 2019). Consequently, choosing an appropriate activation medium is predominant for successful fertilization.

Table 1. Sperm sampling methods, osmolality and pH of seminal plasma and sperm concentration in Tilapia species

Species	Sampling method	Osmolality (mOsmol Kg ⁻¹)	pH	Sperm concentration (x10 ⁹ /ml)	Authors
<i>O. niloticus</i>	stripped	305±9	7.4±1.2	3.59±0.72	Musa, 2010
<i>O. niloticus</i>	sperm duct			1.69±0.6	Gennotte et al., 2012
<i>O. niloticus</i>	stripped			2.7 ± 0.15	Rana and McAndrew, 1989
<i>O. niloticus</i>	stripped			~ 2	Salirrosas et al., 2017
<i>O. niloticus</i>	stripped			2,59±0,38	Acar et al., 2022
<i>O. niloticus</i>	testicular			~ 25	Acar et al., 2022
<i>O. niloticus</i>	stripped			1.33 ±0.19	Garcia-Abiado et al., 1996
<i>O. niloticus</i>	stripped		8.04	2.63	Mataveli, et al., 2007
<i>O. niloticus</i>	stripped	320-380	6.4-8.2	3.04	Chao et al., 1987
<i>O. mossambicus</i>	stripped			0.09	Linhart et al., 1999
	testicular	337±4		9.9±0.85	Linhart et al., 1999
<i>O. mossambicus</i>	sperm duct	299±24			Morita et al., 2003
<i>O. mossambicus</i>	stripped			1.81	Ugwu et al., 2018
<i>O. mossambicus</i>	stripped	310-360	6.2-8.0	27.4	Chao et al., 1987
<i>O. aureus</i>	stripped	300-370	6.6-8.2	6.25	Chao et al., 1987
<i>T. zillii</i>	stripped	370-380	6.2-8.0	0.77	Chao et al., 1987
<i>O. niloticus</i> <i>x O. aureus</i>	stripped	240-310	7.0-7.8	6.83	Chao et al., 1987

Table 2. Activating media used for triggering sperm motility, post-activation time when motility analyses were conducted, total sperm motility, motility durations and sperm velocity parameter in Tilapia species

Species	Activating media (AM)	Post-activation time (s)	Duration (min' s'')	Motility (%)	VCL ($\mu\text{m s}^{-1}$)	VSL (μm^{-1})	Authors
<i>O. niloticus</i>	Distilled water			>90			Poleo et al., 2005
<i>O. niloticus</i>	NaCl ¹ KCl ¹ Mannitol ¹		>20'				Mochida et al., 1999
<i>O. niloticus</i>	Distilled water	5 th	>540''	95±5	65±13	32±11	Musa, 2010
<i>O. niloticus</i>	AM ²	30 th	~ 800''	80-85	~ 40	~ 30	Dzyuba et al., 2019
<i>O. niloticus</i>		60 th	18'04"±6'56"	56±13	71-76	40-44	Gennotte et al., 2012
<i>O. niloticus</i>	Farming water		32.6'	~ 35			Salirrosas et al., 2017
<i>O. niloticus</i>	Distilled water		21.9'	~ 20			Salirrosas et al., 2017
<i>O. niloticus</i>	Distilled water	12 th	9'33''±57''	81±5%	76±5		İnanan, 2025
<i>O. niloticus</i>	NaCl ³	12 th	18'37''±53''	96±2%	91±5		İnanan, 2025
<i>O. niloticus</i>	AM ⁴	12 th	~ 400''	~ 80			Acar et al., 2022
<i>O. niloticus</i>	Pond water	within 60		> 90			Piamsomboon et al., (2019)
<i>O. niloticus</i>	AM ⁵			81			Mataveli et al., 2007
<i>O. niloticus</i>	0-5‰		4-5'				Chao et al., 1987
<i>O. niloticus</i>	AM ⁶	30 th	6'	73			Utarabhand and Watanakul, 1998
<i>O. niloticus</i>	Filtered freshwater		3'	43			Utarabhand and Watanakul, 1998
<i>O. niloticus</i>	Distilled water		2'	33			Utarabhand and Watanakul, 1998
<i>O. mossambicus</i>	Water		>30'		70–80		Morita et al., 2003
<i>O. mossambicus</i>	Distilled water	20 th	>8'	48±5	42±5		Linhart et al., 1999
<i>O. mossambicus</i>	AM ⁷	6-10		62	~ 80	~ 60	Ugwu et al., 2018
<i>O. mossambicus</i>	0-15‰		4-5'				Chao et al., 1987
<i>O. aureus</i>	0-5‰		4-5'				Chao et al., 1987
<i>T. zillii</i>	0-12‰		15'				Chao et al., 1987
<i>O. niloticus</i> x <i>O. aureus</i>	0-7‰		5'				Chao et al., 1987

¹ < 200 mOsm/kg; ² NaCl, 40 mM; Tris-HCl, 10 mM; pH 8.2; 0.25%, pluronic acid; and osmolality, 97 mOsm/kg

³ 100 mOsmol/kg; ⁴ 40 mM NaCl, 10 mM Tris-HCl, 0.2% BSA, pH 8.2; ⁵ 1% sodium bicarbonate; ⁶ Artificial freshwater 10 mM Tris-HCL, 1.5 mM NaCl, 0.5 mM KCL, 1.0 mM CaCl₂, 0.5 mM MgCl, pH 7.0; ⁷ 50 mM NaCl, 10 mM CaCl₂, 0.5% albumin, 10 mM Hepes-NaOH, pH 8.0

CONCLUSIONS

In Tilapia fishes, studies about sperm motility activation have been noticeably focused on *O. niloticus* and *O. mossambicus*. This is obviously a result of these species being commercially important cultured species around the world. However, in addition to novel data to be conducted on other species like *O. Aureus*, more detailed studies are needed for sperm properties of these species. In addition to their use in aquaculture, these species also exist in nature or invasively distributed in different parts of the aquatic ecosystem. For example, some

species such as *O. aureus*, *O. niloticus*, *Coptodon rendalli* and *C. zillii* (accepted name for *Tilapia zillii*) have been able to create and sustain healthy populations that reproduce in natural aquatic ecosystems in Turkey (Çiçek, 2021). These kinds of studies are required for Tilapia species, especially introduced in aquatic ecosystems.

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EFFECT OF MANURE ON SOIL MECHANICAL PROPERTIES AND CULTIVATION

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ABSTRACT

In this study, effects of farmyard manure application on Atterberg limits of a clay soil were investigated in the field study. Farmyard manure was incorporated to soil as four different rates (0, 2, 4, and 6%) with three replications in a randomized block design for eight months. Manure application increased liquid limit (LL) and plastic limit (PL) with increasing the application rates. According to control treatment, the highest increments for liquid and plastic limits were statistically determined with the highest application rate of manure. Liquid limit showed significant positive correlations with plasticity index and plastic limit. As a result, increments in the organic matter contents of soil increased the Atterberg limits. It indicates that addition of organic wastes to soil allows for field operations in high moisture contents.

Key words: Atterberg limits, organic waste, soil moisture, cultivation.

INTRODUCTION

Atterberg limits are empirical values and developed for engineering purposes. Evaluations of soil engineering properties can be useful to people interested in planning, construction and maintenance projects, along with agricultural soil management. These limits describe in approximation of the change of the soil mechanical properties under the different soil moisture contents. If water is added to dry soil, the moisture content at which the soil becomes plastic is the plastic limit. If more water is added, the soil becomes fluid. The moisture content at which the soil changes from plastic to a fluid state is the liquid limit. The moisture content between plastic and liquid limit is the plasticity index (Baumgarti, 2002). Soil moisture content is one of the most important factors for tillage operation. The optimum water content for tillage defined as the water content at which tillage produces the greatest proportion of small aggregates (Dexter and Bird 2001). The plastic limit is generally regarded as the most suitable moisture content for soil cultivation and seedbed preparation (Kirchhof, 2002). Bushan and Ghildyal (1972) found that more small aggregates produced when tillage was done at 70% of PL than either 60 or 99% of PL.

It has been known that additions of organic matter improve soil physical properties such as, soil water content, saturated hydraulic conductivity, aggregation and total porosity (Gülser et al., 2008; Gülser and Candemir, 2012; Gülser and Candemir, 2015; Demir and Gülser, 2021). Organic matter also influences the soil mechanical properties. Attom and Al-Sherif (1998) found that the addition of burned olive waste (non-plastic material) reduced the plasticity index, maximum dry density and unconfined compressive strength of the soil. Smith et al. (1985) found that organic matter content was correlated with liquid and plastic limit. In another study by Malkawi et al. (1999), organic matter content decreased plasticity index, maximum dry density and soil's compactability.

Soil workability is related with soil moisture condition which is suitable for achieving the desired effect without structural damage such as smearing or compaction. Index of consistency value

defines consistency condition of a soil at any given soil moisture content (Baumgartl, 2002). Many studies showed that optimum soil moisture contents near the field capacity are highly related with index of consistency values of soils (Gülser et al., 2009; Gülser et al., 2010; Gülser, 2023). Gülser and Candemir (2006) found that optimum soil moisture contents near field capacity for workability would not have structural deformation in soil series of Ondokuz Mayıs University. In order to improve the mechanical properties of a clay soil, the effects of manure application on Atterberg limits and soil workability were investigated under field conditions in this study.

MATERIAL AND METHODS

A field experiment was conducted at the Experimental Field of Agricultural Faculty in Ondokuz Mayıs University, Samsun. Farmyard manure (M) was incorporated within 0 - 20 cm soil depth as four different treatment rates (0, 2, 4, and 6 %) with three replications in a randomized block design. Some chemical and physical characteristics of the soil were determined in soil samples taken from 0 to 20 cm depth of the field as follows; particle size distribution by hydrometer method (Day, 1965), soil reaction, pH, in the 1:1 (w:v) soil water suspension by pH meter, electrical conductivity ($EC_{25^{\circ}C}$) in the same soil suspension by EC meter, organic matter content by Walkley-Black method (Jackson, 1962) and exchangeable cations by ammonia acetate extraction (Kacar, 1994). After eight months, soil samples were taken from each plot for chemical and mechanical analyses. Liquid limit, plastic limit and plasticity index values of the soil samples were determined according to Black (1965). Some physical and chemical properties of the soil are given in Table 1. The results can be summarized as follows; the textural class of soil is clay, neutral in pH, high in organic matter, none saline according to EC value (Soil Survey Staff., 1993).

Table 1. Some physical and chemical properties of the soil

Sand, %	18.94	NH ₄ OAc extractable	
Silt, %	24.82	K, cmol/kg	1.20
Clay, %	56.23	Na, cmol/kg	0.22
$EC_{25^{\circ}C}$, mmhos cm^{-1}	0.65	Ca, cmol/kg	34.90
pH (1:1)	6.95	Mg, cmol/kg	8.15
Organic matter, %	3.75		

Statistical analysis of the results was accomplished by standard analysis of variance, pairs of mean values compared by least significant difference (LSD) and correlations between the limit values and the other soil properties estimated using the SAS software package (SAS Institute, 1998).

RESULTS AND DISCUSSION

The effects of different organic waste application rates on liquid limit values are given in Figure 1. Liquid limit values increased with increasing the application rates of farmyard manure treatments. Increments in LL were statistically significant at 1% level. Liquid limits varied from 65,18% for the highest application rates of manure to 62.51% for the control application with a general mean of 63.96%. Liquid limits were significantly influenced especially by the highest application rates of M treatment ($p < 0.01$).

Plastic limit values were significantly influenced by the farmyard manure applications at 1% level (Figure 2). Plastic limits varied from 44.48% for the highest application rates of manure to 35.77% for the control application with a general mean of 41,26%. Plastic limit values

showed higher increases with the all application rates of manure treatments. There was not significant difference among the effects of the lowest application rates on PL. Plasticity index, which is the difference between LL and PL, was also significantly influenced by the manure treatments ($p < 0.01$). Plasticity index varied from 26,75% for the control treatment to 21.00% for the highest application rate of manure application with a general mean of 22.75% (Figure 3). Farmyard manure applications decreased the plasticity index values significantly compared to the control application. Regardless of different application rates, percent changes in the LL, PL and PI with farmyard manure according to the control are determined as follows; LL had the lowest percentage increase (2,44%), PL had the highest percentage increase (11.87%) while PI had 14,81% decrease according to the control.

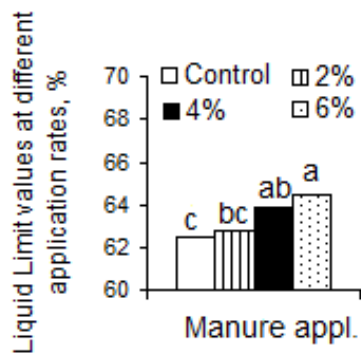


Figure 1. Changes in the LL with different application rates of farmyard manure
*there is a significant difference between the different letters statistically at 1% level.

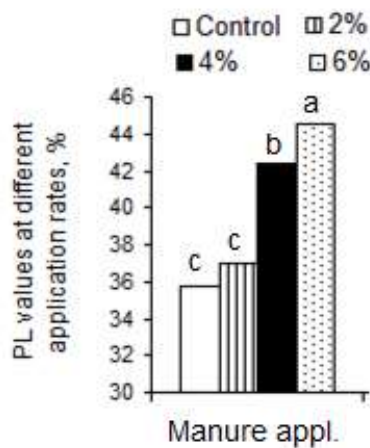


Figure 2. Changes in the PL with different application rates of farmyard manure
*there is a significant difference between the different letters statistically at 1% level.

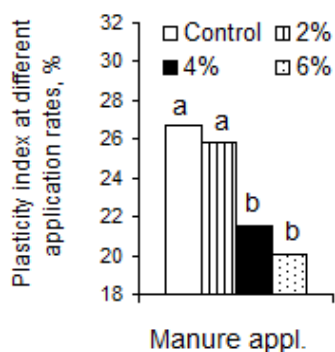


Figure 3. Changes in the PI with different application rates of farmyard manure

*there is a significant difference between the different letters statistically at 1%level.

Descriptive statistical results for the effects of organic waste treatments on organic C and exchangeable cations are given in Table 2. Generally, manure treatments increased the OC content and exchangeable cations of the soil according to the control treatment. According to mean values, control treatment showed the lowest values for each property, except Ca and Na contents. Manure application increased mean K, Na and Mg contents compared to control treatment while Ca content decreased by manure treatment. Atterberg limits indicate that increasing application rates of organic wastes increased both LL and PL. But, OC content was not correlated with PI. Kirchhof (2002) reported that soil organic matter has a high absorptive capacity for water and increases the Atterberg limits, but tends to have a little effect on the plasticity index. Demiralay and Güresinli (1979) found that LL, PL and PI were significantly correlated with organic matter (OM) contents of soils and these limits increased with increasing OM content. Atanur (1973) found that PL of montmorillonit clay increased with the cation type in the following order: Na<H<Mg<Fe<Ca<K.

Table 2. Descriptive statistical results for the effects of manure application on organic C and exchangeable cations

	Treatment	Minumum	Maximum	Mean	Std. Deviation
OC, %	Control	2.24	2.52	2.37	0.14
	Manure	2.83	5.99	3.99	1.02
K, cmol/kg	Control	0.64	1.66	1.12	0.52
	Manure	2.10	8.37	5.21	2.15
Na, cmol/kg	Control	0.20	0.21	0.21	0.01
	Manure	0.65	0.89	0.77	0.10
Mg, cmol/kg	Control	6.82	9.44	8.13	1.31
	M	11.54	17.31	14.19	2.16
Ca, cmol/kg	Control	34.62	35.14	34.88	0.26
	Manure	26.22	35.14	31.29	3.11

The lower and upper soil gravimetric water contents for optimum soil workability calculated from the index of consistency are given in Table 3 for different farmyard manure application doses. If the soil has the optimum water content for workability, tillage produces the greatest proportion of small aggregates. Dexter and Bird (2001) reported that the optimum water content for tillage was 90% of the water content at the PL. The range of water contents for tillage increases with increasing soil OM content. Increases in the PL limit can be desirable for tillage in an early spring when soil has high moisture content. However if soil shows high plasticity index, tilling the soil when it is wet causes structural damage and muddy condition instead of friable condition in soil (Demiralay and Güresinli, 1979).

Table 3. The lower and upper soil gravimetric water contents for optimum soil workability

Manure application doses	Lower gravimetric water content, %	Upper gravimetric water content, %
Control	32,19	38,37
2% M	33,24	37,85
4% M	35,30	38,12
6 % M	35,46	40,03

Farmyard manure treatment decreased the plasticity index with increasing PL according to control. Therefore manure application into clay soils has a benefit to tilling soil at higher moisture content without damaging the soil structure and having friable condition in soil. The results of this study indicate that manure application in to a clay soil allows for field operations in high moisture contents depends on the application doses.

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THE EFFICACY OF PROBIOTICS IN THE PREVENTION OF *HELICOBACTER PYLORI*

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ABSTRACT

Helicobacter pylori, a Gram-negative, microaerophilic bacterium, remains one of the most prevalent human pathogens worldwide, infecting nearly half of the global population. It is classified by the World Health Organization as a Group 1 carcinogen and is a major etiological agent of peptic ulcer disease, chronic gastritis, and gastric adenocarcinoma. Despite significant advancements in eradication strategies, conventional antibiotic-based therapies, such as the proton pump inhibitor (PPI)-based triple therapy, have seen diminishing success rates owing to the alarming rise of antibiotic resistance, particularly to clarithromycin, and frequent treatment-related side effects. The increasing global challenge of antibiotic resistance and the consequent decline in eradication rates have driven the need for alternative or complementary therapeutic approaches that can enhance treatment efficacy while minimizing adverse reactions. Within this context, probiotics, live microorganisms that confer health benefits when administered in adequate amounts, have gained prominence as potential adjunctive agents in *H. pylori* eradication therapy.

The findings presented demonstrate that probiotic supplementation offers significant advantages in *H. pylori* management through multifactorial mechanisms. Probiotics exert direct antimicrobial activity by producing bacteriocins, organic acids, and hydrogen peroxide, which inhibit *H. pylori* growth and interfere with urease activity, an essential enzyme for bacterial survival in the acidic gastric environment. Besides, probiotics compete with *H. pylori* for adhesion sites on the gastric mucosa, effectively reducing bacterial colonization. Beyond their antimicrobial action, probiotics modulate the host immune system by balancing pro- and anti-inflammatory cytokine levels, thus attenuating gastric inflammation and supporting mucosal healing. Certain strains, like *Lactobacillus rhamnosus*, *L. reuteri*, *Bifidobacterium longum*, and *S. boulardii*, have been found particularly effective in reducing virulence gene expression, such as *cagA* and *vacA*, and inhibiting toxin-mediated cellular damage.

The primary objective of this paper is to evaluate the efficacy and mechanisms of probiotic supplementation in preventing and managing *H. pylori* infection, both as a monotherapy and as an adjunct to conventional eradication regimens. This article synthesizes data from recent large-scale randomized controlled trials (RCTs) and meta-analyses that explore the therapeutic potential of probiotics in enhancing eradication rates, reducing antibiotic-induced dysbiosis, and improving patient compliance. The article also aims to identify the most effective probiotic strains, optimal dosage forms, and administration durations for maximizing therapeutic benefit and ensuring safety.

In conclusion, probiotics represent a promising adjunctive strategy for overcoming the limitations of conventional *H. pylori* eradication therapies. Their dual ability to enhance treatment efficacy and minimize side effects offers a viable path toward improved clinical outcomes and patient adherence. Given the escalating problem of antibiotic resistance and the global prevalence of *H. pylori* infection, the integration of specific probiotic formulations, particularly multi-strain preparations combining *Bifidobacterium*, *Lactobacillus*, and *Saccharomyces* species, should be considered a key component in future therapeutic protocols.

Key words: *Helicobacter pylori*, Probiotics, Antibiotic resistance, Eradication therapy, Gut microbiota

1.INTRODUCTION

Helicobacter pylori (*H. pylori*), a spiral-shaped, Gram-negative, microaerophilic bacterium, represents one of the most widespread human bacterial infections globally, colonising approximately half of the world's population. While the majority of infected individuals (estimated at 80%) remain asymptomatic, *H. pylori* plays a definitive etiological role in several severe gastrointestinal pathologies (3,4). Its presence is fundamentally linked to the development and progression of peptic ulcer disease, and it significantly elevates the lifetime risk for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (1,4).

The prevalence of this infection exhibits considerable variability across different regions. Developing nations experience higher rates, ranging from 20% to 90%, while developed nations report lower rates, spanning from 10% to 60% (3).

Beyond its well-established role in digestive tract diseases, *H. pylori* has been implicated in a spectrum of systemic, extragastric disorders. These associated conditions include chronic urticaria, ischemic heart disease, autoimmune thyroid diseases, idiopathic

thrombocytopenic purpura, and certain neurologic diseases. Furthermore, its association with iron deficiency anaemia and an increased risk for metabolic syndrome and diabetes underscores its pervasive impact on systemic health and the substantial burden it places on public health systems (4).

Effective eradication is paramount, not merely for curing active gastritis and peptic ulcers, but also for its prophylactic potential. Successful *H. pylori* clearance may reduce the risk of gastric cancer development and mitigate subsequent healthcare costs associated with chronic *H. pylori*-related illnesses (3,5).

Despite its discovery and utilisation by Warren and Marshall nearly four decades ago, the reliable eradication of *H. pylori* remains a substantial clinical challenge. Current standard treatment regimens, particularly proton pump inhibitor (PPI)-based triple therapy, are notably diminishing in effectiveness, with reported cure rates declining to between 50% and 70% (3, 5, 6).

This decreasing success rate is predominantly attributable to several interconnected factors, among which the high prevalence of antibiotic resistance is paramount. The global escalation in antibiotic resistance significantly impairs treatment efficacy. Resistance patterns in bacteria vary geographically and are frequently correlated with the widespread utilisation of specific antibiotics within the general population. Patient compliance issues constitute a significant concern. Standard treatment protocols frequently involve intricate medication schedules and are often accompanied by common antibiotic-associated adverse effects, including nausea, vomiting, or diarrhoea. These factors collectively diminish patient adherence, thereby complicating the attainment of successful bacterial eradication. In response to these resistance challenges, more aggressive regimens, such as bismuth quadruple therapy (BQT), have been proposed to enhance bacterial susceptibility. Nevertheless, even these intensified therapeutic approaches are confronted with analogous issues related to adverse effects and patient compliance (2, 7).

Consequently, there exists an urgent and paramount necessity for alternative therapeutic approaches that can alleviate the adverse disruption of the gastrointestinal microbiota induced by prolonged, high-dose antibiotic administration, while concurrently enhancing efficacy and safety (1, 2, 4).

The foundational concept of probiotics, microorganisms that confer health benefits to the host, has its origins in the early 20th-century research conducted by Ilya Ilyich Mechnikov. He initially explored the therapeutic potential of Lactic Acid-Producing Bacteria (LAB), positing that their ingestion could enhance human well-being (8). The term "probiotic" was

formally coined by Werner Kollath in 1953. Etymologically, the term derives from the Latin prefix "pro" and the Greek root "bios," meaning "for life." Kollath classified these substances as active biological agents vital for promoting various aspects of health. The contemporary, internationally accepted definition, established by the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO), more precisely stipulates that probiotics are "live microbes when administered in adequate quantities, confer health benefits on host organisms" (9).

The most extensively studied and commercially utilised probiotic strains primarily belong to the diverse groups of *Bifidobacterium* and *Lactobacillus*. Additional strains, including *S. boulardii* and *Escherichia coli* Nissle 1917, are also widely employed. Furthermore, other genera such as *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Propionibacterium*, *Bacillus*, and *Leuconostoc* are recognised for their potential probiotic benefits. These advantageous microbes exert their salutary effects through multiple mechanisms, including limiting the colonisation of pathogenic bacteria by restricting available binding sites on the mucosal epithelial cells, thereby enhancing the gut's defensive barrier. Probiotics actively modulate the host immune response, contributing to overall immunological balance. Additionally, they play a vital role in improving the integrity of the intestinal barrier, which is essential for preventing systemic exposure to luminal antigens and toxins (9, 10).

The colonisation of *H. pylori* within the gastroduodenal environment induces significant ecological disturbance to the host's indigenous microbial community. The presence of this pathogen conventionally results in a reduction of microbial diversity, culminating in a condition termed dysbiosis. This disruption not only undermines the host's intrinsic defence mechanisms but also precipitates heightened localised inflammation. Therefore, therapeutic approaches aimed at *H. pylori* must extend beyond mere pathogen eradication to encompass the requisite restoration of the disrupted microbial ecology (1,7).

The integration of specific probiotic strains into the management of *H. pylori* infection offers multifaceted advantages, including immunomodulatory effects and direct anti-pathogenic actions. These benefits effectively complement the inherent limitations of conventional antibiotic therapies. Probiotics actively compete with *H. pylori* for critical adhesion sites and essential nutrients within the mucosa. Furthermore, these beneficial microorganisms possess the capacity to synthesise metabolites with potent antimicrobial activity, thereby directly inhibiting pathogen growth and viability. Additionally, probiotics stimulate mucus production, thereby enhancing the protective mucosal barrier of the stomach and preventing the invasive progression of infection (1, 7, 11,).

Probiotic administration assists in optimising the host's immune response. It facilitates the necessary rebalancing of proinflammatory and anti-inflammatory cytokine profiles and concurrently enhances general host defence mechanisms against microbial threats. The incorporation of probiotics into antibiotic-based eradication protocols is instrumental in mitigating the iatrogenic side effects of treatment. They are efficacious against intestinal dysbiosis induced by broad-spectrum antibiotics, resulting in a marked reduction in treatment-related adverse effects such as diarrhoea and nausea. This tangible improvement in tolerance is directly manifested in improved patient compliance with rigorous treatment regimens (2, 3). Clinical evidence, particularly from recent comprehensive meta-analyses, strongly validates this approach, confirming that the adjunctive use of probiotics increases the overall eradication rate while simultaneously reducing the incidence of side effects associated with anti-*H. pylori* therapies. The potent, multi-targeted effects of probiotics, from direct bacterial antagonism and barrier reinforcement to systemic immune regulation, position their strategic integration as a vital therapeutic tool to overcome the challenges of *H. pylori* infection management (1,2,3).

2. Pathogenesis of *H. pylori*

2.1. Mechanisms of Colonisation and Persistence

H. pylori employs a sophisticated array of bacterial factors to successfully colonise the harsh, acidic gastric environment and establish chronic infection (12-14). Acidic adaptation and urease activity are fundamental to the survival of *H. pylori* within the gastric environment. The bacterium's capacity to establish and sustain colonisation in the highly acidic stomach relies on a sophisticated acid adaptation mechanism, with the urease enzyme serving as the central component. Urease activity is precisely regulated to ensure the bacteria's continued viability in such a challenging environment (4,5,12). The enzyme's function is regulated by the proton-gated channel ureI, a transmembrane protein that functions as an environmental sensor. This channel remains closed under neutral conditions; however, it opens promptly when the pH decreases to an acidic level (approximately pH 5.0). Such an opening permits the rapid uptake of urea from the surrounding gastric fluid into the bacterial periplasm. Upon internalisation, urease swiftly hydrolyses urea into ammonia (NH₃) and carbon dioxide (CO₂). Ammonia immediately reacts with water to form ammonium hydroxide (NH₄OH), which serves as a strong base, effectively neutralising the microenvironment immediately adjacent to the bacterial surface. This mechanism of neutralisation is vital for the persistence of infection, as evidenced by the observation that urease-deficient mutants are unable to colonise the stomach for extended periods (4,12). Considering its indisputable role in bacterial survival, the urease pathway

constitutes a pivotal target for the development of non-antibiotic therapeutic agents. The blockade of this crucial virulence factor offers significant therapeutic prospects for the eradication of *H. pylori* (12).

H. pylori uses 4–7 polar, sheathed flagella to move through the thick gastric mucus and reach the more neutral pH near the epithelial cell surface. The ability to move using flagella is crucial for starting an infection; if mutations occur in the majority of genes associated with motility or chemotaxis, the bacterium is unable to colonise (4,5,12). Flagellar function is quite complex, involving over 40 proteins and controlled by a multi-step transcription process involving factors like σ_{80} , σ_{54} , and σ_{28} . A major part of motility is the posttranslational glycosylation of flagellin with pseudaminic acid (Pse); stopping the Pse biosynthesis pathway could be a promising target for new anti-virulence drugs (12,15).

The colonisation and persistent presence of *H. pylori* are critically reliant on its array of Outer Membrane Proteins (OMPs), which primarily serve as surface adhesins. These proteins facilitate the initial contact and secure attachment of the bacterium to the gastric epithelial cells of the host, thereby ensuring its retention against the mechanical forces within the stomach and initiating pathological processes. Significant adhesins involved in this mechanism include BabA, SabA, AlpA/B, and OipA (4,5). Their shared role is to facilitate how bacteria stick to specific receptors on the surface of human epithelial cells, helping to lay the groundwork for long-lasting infections.

These proteins are implicated in the induction of chronic inflammation through the stimulation of IL-8 secretion. This chemokine release initiates the localised recruitment of immune cells, a hallmark of *H. pylori*-associated gastritis. OipA (outer inflammatory protein) is a potent virulence factor, capable of driving inflammation by enhancing IL-8 expression. Furthermore, its pathological impact includes promoting adherence and inducing apoptosis (programmed cell death) within host cells, demonstrating a key role in epithelial damage independent of its copy number (4,5,13,14,16). BabA (blood group antigen-binding adhesin) interacts with the Lewis B (Le^b) blood-group antigen on host cells, and its presence is associated with a heightened risk of peptic ulcer and gastric cancer within Western populations. SabA (sialic acid-binding adhesin) binds to the inflammation-induced sialyl-Lewis x (sLe^x) glycosphingolipid antigen, facilitating colonisation during active gastritis. The expression of *sabA* is regulated through phase variation via CT dinucleotide repeats, enabling the bacterium to adapt to evolving host environments swiftly (4,5,12,14). **Table 1** shows that pathogenesis and Clinical Sequelae of *Helicobacter pylori* Infection.

Table 1: Pathogenesis and Clinical Sequelae of *Helicobacter pylori* Infection

Stage	Process / Mechanism	Affected Site / Cellular Effect	Clinical Sequelae (Outcomes)
Colonization and Adaptation	Transmission via oral-oral or fecal-oral routes.	Gastric Mucosa	Initial Infection
	The bacterium hydrolyzes urea into ammonia and carbon dioxide, locally neutralizing the acidic microenvironment for survival.	Mucosal Surface of the Stomach (Acidic Niche)	Persistent Colonization of the Gastric Niche
Mucosal Injury and Chronic Inflammation	Attachment to epithelial cells via adhesins.	Gastric Epithelial Cells	Superficial Mucosal Irritation
	Translocation of and toxins into host cells, disrupting cell signaling (CagA) and inducing vacuolation/apoptosis (VacA).	Gastric Epithelium and Lamina Propria	Acute and Chronic Gastritis
	Activation of pro-inflammatory signaling pathways and secretion of cytokines.	Immune Cells	Sustained Inflammation and Tissue Damage
Chronic Progression	Ongoing inflammation leads to tissue damage and cell loss.	Gastric Mucosa and Submucosa	Peptic Ulcer Disease (Gastric and Duodenal Ulcers)
	Transformation of gastric epithelium into tissue resembling intestinal cells	Gastric Mucosal Glands	MALT Lymphoma
Carcinogenesis	Accumulation of genetic instability and cellular irregularities driven by chronic inflammation.	Epithelial Cells	Gastric Adenocarcinoma (Gastric Cancer)

Abbreviation: MALT (Mucosa-Associated Lymphoid Tissue)

2.2. Host immune response and inflammation

The *Cytotoxin-associated gene A* (*CagA*) serves as a virulence factor that is strongly and consistently linked to severe clinical outcomes following *H. pylori* infection. Strains identified as *CagA*-positive are epidemiologically associated with the progression of acute gastritis, gastric ulceration, and the development of gastric carcinoma (GC). This direct correlation indicates that the production capability of *CagA* is frequently utilised as a metric for assessing the pathogenic potential of individual *H. pylori* isolates. Geographically, *CagA*-positive strains are highly prevalent, influencing approximately 60% of infections in Western nations and reaching prevalence rates as high as 90% in East Asian countries (12,14).

CagA is encoded within the *cag* Pathogenicity Island (*cagPAI*), a 35–40 kb chromosomal segment comprising over 30 genes. Notably, the *cagPAI* encompasses genes homologous to a Type IV Secretion System (T4SS). Upon direct contact with gastric epithelial cells, this complex molecular syringe translocates the *CagA* protein into the host cell cytoplasm. Key components of the T4SS, such as *CagL* and *CagY*, facilitate this process by interacting with host cell receptors like β 1-integrin (4,5,12). The *CagA* protein is further classified into

distinct variants based on the repeated Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs located at its N-terminus. East Asian-type CagA demonstrates a significantly greater affinity for the host phosphatase SHP-2 compared to the Western-type CagA. This enhanced binding capability is believed to account for the more significant cytoskeletal alterations induced by East Asian strains, thereby contributing to their stronger association with gastric carcinogenesis (5,12,13). Once introduced into the host cytoplasm, CagA localises to the inner plasma membrane through interaction with phosphatidylserine. It exerts its disruptive effects via two principal signalling pathways, including both phosphorylation-dependent and phosphorylation-independent mechanisms (12).

Phosphorylation-Dependent Effects: The protein is subjected to tyrosine phosphorylation by host Src family protein tyrosine kinases. Subsequently, the phosphorylated CagA interacts with the host phosphatase SHP-2, an interaction that significantly influences cell adhesion, spreading, and migration (5,12,13). **Phosphorylation-Independent Effects:** Non-phosphorylated CagA also possesses autonomous signalling functions. A conserved motif in the C-terminus facilitates interaction with the host's hepatocyte growth factor receptor, Met. This interaction contributes to heightened cellular proliferation and inflammation via the Akt signalling cascade, which subsequently activates downstream factors such as NF- κ B and β -catenin. Collectively, CagA actively remodels host cellular behaviour, inducing morphological changes (e.g., cell pedestal formation), disrupting the cytoskeleton, stimulating epithelial cell proliferation, and driving the inflammatory response through IL-8 secretion (12, 17).

The Vacuolating Cytotoxin A (VacA) protein is a critical virulence factor produced by *H. pylori* that instigates significant host cellular pathology through its multifaceted toxic effects. The primary cytotoxic function of VacA stems from its ability to form an anion-selective channel in the host cell membrane. This channel, once embedded, facilitates the release of bicarbonate and organic anions into the host cytoplasm, potentially aiding bacterial growth by supplying metabolic substrates (12,17). More notoriously, VacA is internalised via endocytosis, where the toxin channel permits anions to permeate the late endosomes. This action induces the accumulation of weak bases, leading to substantial water influx and the characteristic formation of large cytoplasmic vacuoles. Additionally, VacA exerts disruptive effects on vital cellular components. The toxin can directly target host mitochondria, triggering the release of cytochrome C and initiating the pathways of apoptosis (programmed cell death). VacA disrupts the natural balance of cell proliferation and death by interfering with genes that regulate the cell cycle. The presence of VacA elicits acute inflammatory responses from the host, particularly by inducing host cells to release the pro-inflammatory chemokine IL-8 (4,5,12,16).

While all *H. pylori* strains possess the *vacA* gene, the toxic activity of the resulting protein varies considerably, determined by the specific genopattern of the strain. The *vacA* gene comprises three principal polymorphic regions (signal sequence (s-region), mid-region (m-region) and intermediate region (i-region)), and the combination of subtypes (s1a, s1b, s1c, s2, m1, m1T, m2, i1, i2, and i3) influences the level of virulence. s1/m1 subtypes are more virulent than s2/m2 (12).

Neutrophil-activating protein (NAP), which is an adhesin, stimulates neutrophils to produce reactive oxygen species (ROS) and promotes the release of inflammatory mediators (IL-8, MIP-1 α/β). NAP is directly linked to the hallmark neutrophil infiltration observed in chronic gastritis. Intriguingly, NAP also functions as a DNA-protecting protein and is thought to be essential for bacterial survival within macrophages by modulating phagosome pH (12,18). Heat Shock Protein 60 (Hsp60) is an adhesin that is prominently expressed under acidic stress conditions and acts as a potent immunogen. It promotes the release of pro-inflammatory cytokines (IL-6, IL-8, TNF- α) from host cells through TLR2 activation. Anti-Hsp60 antibody titers frequently correlate with the progression of gastritis and gastric cancer (Table 1) (12,14,18).

2.3. Risk factors for infection and reinfection.

The persistent clinical issue presented by *H. pylori* results from a convergence of factors that influence the initiation of infection (acquisition) and the propensity for chronic colonisation, therapeutic failure, and subsequent disease progression. Therefore, a comprehensive understanding of these key variables, spanning macro-level socioeconomic factors to micro-level bacterial genetic determinants, is crucial for developing effective global health management and therapeutic strategies (4,12). The risk of contracting *H. pylori* is not uniformly distributed, exhibiting a marked correlation with socioeconomic status and hygiene standards. Regions suffering from low socioeconomic development and poor communal sanitation consistently report significantly elevated prevalence rates. For instance, historical data indicate incidence exceeding 70% in certain African nations, in stark contrast to rates below 25% in countries like those in Oceania. This robust epidemiological gradient highlights that factors such as public health coverage, access to clean water, and ambient environmental conditions are key predictors of infection burden (4,5,12). Acquisition generally occurs early in life, often during childhood, thereby establishing the potential for lifelong carriage. Transmission pathways are diverse but frequently encompass faecal–oral and oral–oral routes, as well as gastric–oral transfer through contaminated food, water, or medical instruments.

Additionally, vertical transmission among genetically related individuals is an acknowledged mode of dissemination, indicating that household or familial clustering constitutes a significant risk factor (5). Once colonisation is established, the risk of developing severe pathology and unsuccessful eradication therapy is primarily determined by the pathogen's molecular arsenal and its interaction with the host (5,12,16). The presence of specific virulence genes constitutes a significant factor in risk stratification. Strains expressing the East Asian-type CagA protein are associated with an increased risk of gastric cancer, a phenomenon attributed to its enhanced binding affinity for the host's SHP-2 phosphatase. Similarly, the *vacA s1/m1* genotypes are closely linked to elevated gastric atrophy and carcinoma risk due to their potent cytotoxic activity (4,12,14). The outcome of the initial encounter is also influenced by host genetics. For example, the host's expression of the Leb blood group antigen functions as a host-side determinant, specifically regulating bacterial colonization density in strains that employ the BabA adhesin(4,5,12).

The capacity of *H. pylori* to maintain infection is derived from multiple evasion mechanisms. The bacterium employs urease and flagellar motility for immediate survival and penetration of the mucosa. For enduring persistence, it develops biofilms, structural consortia that confer protection against both the host's immune defences and therapeutic interventions. Moreover, the pathogen actively fosters a deleterious chronic inflammatory condition characterised by an excessive release of cytokines such as IL-8 and TNF- α , while concurrently utilising strategies like antigenic variation to secure its persistent presence by evading recognition by T and B lymphocytes (4,14).

Critically, the microbe's ability to invade and proliferate within epithelial cells offers a safeguarded intracellular environment that protects it from conventional antibacterial agents. This mechanism, together with the escalating global concern of antibiotic resistance, especially relating to clarithromycin-resistant strains, constitutes a significant risk factor that directly results in therapeutic failure and subsequent relapse (4,5).

3.Probiotics: General Concepts

3.1. Definition and classification of probiotics

The most widely accepted definition of probiotics, which are live microorganisms, was established by the WHO and FAO in 2001. While microorganisms exhibiting probiotic properties are mostly bacteria, some yeasts also display probiotic properties. Probiotics are closely associated with fermented foods, which have been used for thousands of years. However, the concept of using these beneficial microorganisms to promote and maintain health

is a century-old idea. The Russian scientist Élie Metchnikov was the first to discover probiotics. In the years following this discovery, microbiological and immunological studies aimed to improve health not only through the consumption of fermented foods but also through the use of probiotic strains isolated from these foods. Probiotics are classified into two categories: endogenous probiotics and exogenous probiotics, according to their origin and interaction with the host (19).

Endogenous probiotics comprise the community of beneficial microorganisms that naturally reside within the human host, particularly colonising critical sites such as the gastrointestinal tract, skin, respiratory tract, and oral cavity. These microbes, primarily consisting of various strains of *Lactobacillus* and *Bifidobacterium*, constitute an essential component of the native microbiota, coexisting in a state of mutualistic symbiosis with the human body (9,19). These organisms are integral to the maintenance of physiological balance. Their functional contributions are multifaceted, encompassing the facilitation of digestion, robust immune system modulation, and offering ongoing protection against pathogens (19,20). More importantly, these microbial populations are not obtained from external sources later in life; rather, they develop endogenously from early infancy. The initial colonisation and subsequent maturation of this flora are substantially affected by various host-specific and environmental factors, including the mode of birth, breastfeeding practices, host genetics, dietary composition, and environmental exposure (21). For sustained health, the stability and inherent resilience of these native probiotic communities are vital. They undergo a process of adaptation to the host's unique physiological environment, thereby contributing reliably to long-term well-being. Nevertheless, this finely tuned microbial balance can be compromised or depleted by external pressures, such as the use of antibiotics, the onset of illness, or abrupt dietary shifts. Such perturbations lead to dysbiosis, a disruption of the normal microbial equilibrium, which can severely impair host health (19,23). Endogenous probiotics that include *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Escherichia coli* (non-pathogenic strains) maintains digestive health and nutrient absorption. *Lactobacillus rhamnosus* and *Bifidobacterium longum* produce vitamins such as B2, B6, B9, B12 and K2, additionally potentially enhancing respiratory health by modulating the gut microbiota, reducing inflammation, and improving immune responses (24).

Exogenous probiotics are defined as beneficial microbial strains that are introduced into the body from external sources, serving as temporary supplements to the host's native flora. Unlike the permanent, resident populations, these organisms are not indigenous but are strategically consumed to influence microbial balance and enhance specific health parameters

(25). These transient microbial supplements are typically acquired through two main avenues. The someone is fermented foods. Consumption of probiotic-rich foods, such as yoghurt, kefir, kimchi, and various other fermented products. Another one dietary supplements. Highly concentrated, specifically formulated dietary supplements provide targeted delivery of identified beneficial strains (19,24,25). A fundamental distinction of exogenous probiotics is their transient nature. While effective upon administration, they generally do not permanently colonize the gastrointestinal tract. Their effectiveness is therefore conditional on their ability to survive the harsh conditions of the upper gastrointestinal tract, including the destructive effects of stomach acid and bile salts, to successfully reach their target location in the gut where they can exert their benefit (19,26). Exogenous probiotics are employed in a focused manner to address specific health concerns or to actively restore microbial equilibrium. They are frequently utilized to restore balance following instances where the natural microbiota has been compromised, such as after antibiotic use or in cases of gastrointestinal surgery, which often leads to dysbiosis (27). It used targeted for health enhancement. Their use is strategic, providing temporary but powerful benefits for various aspects of wellness, including digestive support, immune modulation, and mental well-being. For example, they can help temporarily balance gut flora and compete with harmful bacteria. In clinical settings, they are commonly employed in targeted therapies for acute or short-term conditions. Clinicians may prescribe specific, proven strains, such as *S. boulardii* for gastrointestinal infections or certain *Lactobacillus* strains for managing antibiotic-associated diarrhoea, to address immediate disruptions (9,19). The evolving field of personalised medicine is further refining this application, with certain commercial and clinical strategies offering customised exogenous formulations tailored to a patient's unique microbiome profile to address individual health goals, including metabolic health or immune support. The strategic classification of these microorganisms as exogenous guides clinical decision-making regarding their selection, delivery methods, and expected duration of effect. The temporary but powerful action of these externally sourced microbes is their complementary role to the permanent, sustaining function of endogenous probiotics (19,24). Exogenous probiotics, including *Lactobacillus plantarum* and *S. boulardii*, assist in restoring disrupted intestinal flora following antibiotic administration. *Bifidobacterium animalis*, present in yoghurt, and *Lactobacillus casei* Shirota, found in fermented dairy products, promote healthy digestion, while *Lactobacillus reuteri* modulates the immune response of the host (19).

3.2. Mechanisms of probiotic action in the gastrointestinal tract

The demonstrable health benefits conferred by probiotic supplementation are the result of diverse and sophisticated mechanisms of action that collectively serve to enrich the intestinal microbiota, fortify host defence, and mitigate infection risk. These functional pathways extend beyond mere microbial population replacement to encompass direct competition, barrier enhancement, and comprehensive immune modulation (9,19).

Probiotics execute their salutary effects through multiple, interconnected biological pathways within the gastrointestinal lumen and mucosa (20). **Competitive Exclusion:** Probiotic organisms actively engage in competition with pathogenic bacteria for vital resources. This involves preemptive occupancy of the intestinal-lumen interface by adhering to epithelial receptors, thus restricting the binding sites available to pathogens. Furthermore, they compete directly for nutrients essential for colonisation (21). **Mucosal Barrier Fortification:** They directly contribute to the enhancement of mucosal barrier function. This involves strengthening the tight junctions between epithelial cells, thereby decreasing intestinal permeability and preventing the translocation of harmful antigens and toxins into the systemic circulation (22). **Antimicrobial Production:** Certain strains elaborate specific bacteriocins, proteinaceous toxins that exhibit targeted antimicrobial activity against competing, often pathogenic, microorganisms (19). **Immune System Modulation:** Probiotics actively promote balanced innate and adaptive immune responses. They interact with immune cells in the gut-associated lymphoid tissue (GALT), influencing the local production of antibodies and cytokines, which ultimately strengthens the host's overall resistance to infection (22). **Regulation of Cellular Kinetics:** These beneficial microbes also influence cell kinetics, affecting epithelial cell proliferation, differentiation, and apoptosis, processes that are critical for continuous mucosal turnover and tissue repair (23).

3.3. Safety and regulatory considerations

Probiotics are single or mixed cultures of live microorganisms that enhance the characteristics of the natural microflora of humans or animals and, when used at specific doses, lead to improved host health through beneficial effects in the mouth, GI tract, upper respiratory tract, or urogenital tract (28). It is well established that probiotics exert a powerful mechanism of action against other microorganisms in the host and provide benefits to the host (9).

Lactobacillus spp. and *Bifidobacterium spp.*, *Enterococcus*, *Bacillus*, and some yeasts (*S. boulardii*) are used as probiotics. Despite these benefits, because they are live microorganisms, probiotics carry safety risks that must be carefully managed from both clinical and regulatory

perspectives. Probiotics can theoretically cause complications such as systemic infections, toxic metabolic complications, allergic responses in hypersensitive individuals, and gene transfer associated with antibiotic resistance (9,30).

While the use of probiotics is generally considered safe in healthy individuals, serious risks exist, particularly in patients with compromised immune systems, organ or immune deficiencies, or conditions such as severe acute pancreatitis and compromised intestinal barriers. In patients with compromised gastrointestinal barrier function, probiotic strains may translocate from the intestinal lumen into the bloodstream, causing ischemic infections such as sepsis and bacteremia. While studies indicate a very low incidence of lactobacillus infections in the healthy population (approximately 1 in 100,000), this risk increases in at-risk groups (29). The pharmacokinetic and metabolic activities of probiotic strains should be examined. It is essential that the bacteria do not convert food components or biological secretions into harmful secondary substances in the host. For example, potentially harmful effects of enzymatic properties, such as excessive deconjugation of secondary bile acids or mucolytic activity, have been identified. However, there are no widespread reports of LAB and *Bifidobacterium* species producing particularly harmful compounds. Additionally, the potential to trigger allergic reactions in susceptible individuals is another possibility that requires attention (29,30,31). One of the most significant safety concerns is the potential for probiotic strains to harbour antibiotic resistance genes and to transfer these genes to other intestinal bacteria (including pathogens) via mobile genetic elements (plasmids, transposons). Vancomycin resistance is the most concerning type of resistance. Therefore, genotypic methods should be used to confirm that strains used as probiotics are susceptible to antibiotics used in clinical treatment and do not carry transferable resistance genes (30).

Probiotic activity is strain-specific. Therefore, identifying strains and determining their health effects, as well as conducting accurate screening and epidemiological studies, are crucial. It is essential to complement classical biochemical tests with molecular methods to accurately identify the probiotic strain at the genus, species, and strain level, and to submit these identified strains to an internationally recognised culture collection. Furthermore, probiotic candidates should be thoroughly examined for the presence of known pathogenic or virulence factors such as invasion, translocation, and toxin production (D-lactate, hemolytic activity, lecithinase activity). In addition, acid and bile resistance, epithelial cell adhesion capacity, antimicrobial activity against potential pathogens, and viability under production and storage conditions should also be examined (9,30,31).

4. Mechanisms of Probiotic Action Against *H. pylori*

Conventional antimicrobial agents, despite their efficacy, face significant limitations, namely considerable economic expense, the propensity for side effects, and the recurrent emergence of antimicrobial resistance during prolonged administration. To circumvent these drawbacks, a synergistic strategy involving the co-administration of antimicrobial pharmaceuticals with probiotics has been established. This combined approach not only enhances therapeutic outcomes but also permits a reduction in the requisite drug dosage. Ultimately, this method yields superior eradication rates for pathogens at the infection site and diminishes the potential adverse reactions typically associated with full-strength regimens (11,20,32).

Specifically, in the treatment of gastric ulcers induced by *H. pylori*, prescribed antimicrobials carry a risk of generating antibiotic resistance in the causative bacterium, in addition to their inherent side-effect profile. While the eradication success rate for antibiotic monotherapy hovers around 71%, this efficacy is significantly boosted to approximately 81% when probiotics are concurrently introduced. Furthermore, this combined protocol demonstrates a marked attenuation of treatment-related side effects(20).

4.1. Competition for adhesion sites

The initiation of *H. pylori* infection relies upon the pathogen's adhesion to the gastric mucosa, mediated by adhesins such as Outer Membrane Proteins (OMPs). This initial anchoring step constitutes a prerequisite for subsequent bacterial colonisation and the secretion of virulence factors. Probiotic supplementation protects by antagonising this critical phase of attachment.

Specifically, probiotic strains—notably members of the *Lactobacillus* genus and *S. boulardii*—engage in direct competition with *H. pylori* for shared or analogous epithelial binding sites, including glycolipid and mucin receptors. This competitive exclusion effectively reduces the available surface area for *H. pylori* colonisation (33). For instance, strains such as *Lactobacillus rhamnosus*, *L. acidophilus*, and *L. reuteri* have been empirically shown to inhibit the attachment of *H. pylori* to gastric epithelial cells (2, 11,33).

Furthermore, some probiotic agents employ a Receptor Modification mechanism. *S. boulardii*, for example, can produce ceramidase. This enzyme acts to enzymatically alter *H. pylori* binding ligands on the host cell surface, thereby functionally preventing adhesion to the epithelial cells (34). Beyond these mechanisms, probiotics possess the capacity to modulate the

expression of epithelial junction proteins and mucins, releasing bioactive compounds that safeguard the mucosal barrier integrity and consequently impede *H. pylori* colonisation (33).

4.2. Production of antimicrobial substances (bacteriocins, organic acids)

Probiotics possess the metabolic capacity to generate diverse compounds with potent antimicrobial efficacy. Lactic acid bacteria ferment carbohydrates to synthesise short-chain fatty acids, such as acetic acid, propionic acid, and butyric acid, alongside other organic acids(19,24). The *Bifidobacterium* genus contributes to the gut environment by synthesising acetic acid and lactic acid as key metabolic by-products. This organism exhibits a therapeutic capability to mitigate the pathological consequences of *H. pylori* infection, particularly when co-administered with either *Saccharomyces cerevisiae* (a common name for *S. boulardii*) or *Lactobacilli* species. This combined application is postulated to yield synergistic benefits, enhancing the overall protective effect against the pathogen(20). Furthermore, they are known to elaborate bacteriocins and hydrogen peroxide (H₂O₂), all of which contribute to significant pathogen suppression (19,24). The organic acids produced by lactobacilli not only induce a drop in gastric pH but also directly impede *H. pylori* urease activity, consequently curtailing its propagation (33). The H₂O₂ secreted by probiotics damages *H. pylori* cells through oxidative mechanisms, specifically by promoting the formation of peroxide ions that interfere with bacterial functions. Although *H. pylori* counteracts host defences with its own antioxidant machinery, such as superoxide dismutase, these probiotic-derived compounds pose a substantial threat. Additionally, bacteriocins function by compromising the structural integrity of the *H. pylori* cell wall and membrane (24,35). Specific *Lactobacillus* strains possess the capability to generate antimicrobial agents categorised as bacteriocins. These protein-based toxins demonstrate a significant anti-*H. pylori* capacity. Bacteriocins are characterised as small, dialysable peptide structures that confer their antimicrobial activity. The efficacy of these toxins against the target pathogen is highly variable, depending on the specific *H. pylori* strain encountered and the particular class of bacteriocin synthesised by the *Lactobacillus* species. Furthermore, studies have documented that, even among different bacteriocins produced by various *Lactobacillus* species, not all exhibit uniform potency; specific variants demonstrate a substantially stronger inhibitory effect against *H. pylori* isolates than others (9,29,36).

4.3. Modulation of gastric pH and microbiota balance

The urease secreted by *H. pylori* breaks down urea into ammonia, locally neutralising the acidic gastric environment to facilitate its survival. Probiotics, on the other hand, counteract this by producing lactic acid or inhibiting urease activity, thereby preventing an increase in

gastric pH and weakening *H. pylori*'s survival mechanisms within the stomach (33). The survival of *H. pylori* is crucially supported by the secretion of urease, an enzyme that catalyses the breakdown of urea into ammonia. This process locally neutralises the acidic milieu of the stomach, thereby sustaining the pathogen's viability within the harsh gastric environment (33,36). After adding probiotics (*L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *B. infantis*, and *B. longum*) to the *H. pylori* treatment, *H. pylori* urease activity was reduced, significantly reducing its adhesion to gastric epithelial cells (37).

4.4. Immune system modulation

The host immune system's response to *H. pylori* colonisation in the stomach initiates the mobilisation of a broad spectrum of inflammatory cells and associated mediators, leading to the activation of nuclear factor- κ B (NF- κ B) and the subsequent synthesis of various pro- and anti-inflammatory cytokines (36). In comparison with uninfected individuals, patients suffering from *H. pylori* infection typically display elevated gastric concentrations of several key cytokines, including TNF- α , IFN- γ , IL-6, IL-7, IL-8, IL-10, IL-17, and IL-18. Furthermore, *H. pylori*-specific CD4 + T cells are exclusively detectable in the peripheral blood and mucosal lining of infected humans (38). Probiotics demonstrate a proven ability to attenuate the cytokine cascade triggered by *H. pylori*. The inflammatory environment arising from gastric infection is characterised by the production of various immune signalling molecules, including chemokines and cytokines. Probiotic intervention alters this immunological profile, mainly by enhancing the secretion of anti-inflammatory cytokines. This modulation ultimately reduces gastric inflammatory activity and decreases the overall inflammatory burden (29). A composite probiotic product containing *Enterococcus faecalis*, *Bifidobacterium longum*, and *L. acidophilus* was proven to ameliorate *H. pylori*-induced gastritis by curbing the inflammatory cascade within gastric epithelial cells. This specific formulation achieved its effect by significantly inhibiting the NF- κ B and MAPK signalling pathways, which resulted in a decrease in the expression of cytokines, including TNF- α , IL-1 β , IL-10, IL-6, G-CSF and MIP-2 (38).

4.5. Reduction of virulence factors

The pathogenesis of *H. pylori* is mediated primarily by the toxins CagA and VacA, which are strongly correlated with severe gastric conditions and the development of neoplasia (12).

The antagonistic activities of probiotics not only reduce bacterial numbers but also inhibit the synthesis and activity of these critical pathogenicity markers. Some probiotic strains act directly on bacterial metabolism, reducing the expression of virulence genes at the mRNA

level. Reuterin, a compound produced by *L. reuteri*, led to a significant reduction in the relative mRNA expression levels of *H. pylori* virulence genes such as *flaA* (flagella gene) and *vacA* in in vitro studies (39). Cell-free supernatant from the *L. rhamnosus* JB3 strain reduced the expression levels of several *H. pylori* virulence genes, including *babA*, *sabA*, *fucT*, *vacA*, *cagA*, and *cagL* (40). Probiotics not only reduce gene expression but also the expression of virulence genes. It also inhibits the functions of its proteins within the host cell. The gastric-specific *Lactobacillus gasseri* Kx110 A1 strain has been shown to suppress the phosphorylation of the CagA oncoprotein injected into host cells via the interaction of the Src homology-2 protein tyrosine phosphatase (41). This suppression inhibits CagA signalling, which leads to cytoskeletal rearrangement. Cell-free supernatant obtained from *L. rhamnosus* JB3 has been found to reduce VacA entry into host cells via the lipid raft mechanism and VacA-induced cell damage (40). These findings strongly suggest that the therapeutic potential of probiotics against *H. pylori* is more complex than simply reducing the bacterial load but also involves neutralizing the bacteria's most pathogenic weapons at the molecular level.

5.Clinical Evidence for Probiotic Use in *H. pylori* Prevention

The integration of probiotics into therapeutic regimens for *H. pylori* infection has garnered substantial clinical attention. Evidence from randomised controlled trials and comprehensive meta-analyses demonstrates that specific live microbial strains offer significant benefits, primarily by augmenting antibiotic efficacy and mitigating associated adverse reactions (42,43).

5.1. Probiotics as Monotherapy for Infection Prevention

While conventional triple or quadruple antibiotic regimens remain the primary approach for treating *H. pylori* infections, exploring probiotics as monotherapy offers an intriguing alternative, primarily aimed at infection management rather than preventive measures. Monotherapy involves the exclusive administration of probiotics, without concomitant antibiotics, to treat diseases associated with *H. pylori*. Evidence indicates that probiotics administered alone can attain successful eradication rates, occasionally reaching up to 39%. This clearly demonstrates their inherent anti-*H. pylori* activity and potential as a standalone agent (1,49).

This approach, however, is not recommended as a first-line treatment owing to its lower efficacy compared with standard antibiotic protocols. Consequently, the use of probiotic monotherapy is predominantly confined to specific patient groups for whom antibiotics are either contraindicated or problematic. This encompasses patients with a documented history of

allergic reactions to antibiotics within standard regimens or those who categorically refuse antibiotic treatment. It may also be considered for asymptomatic young children for whom aggressive antibiotic therapy might be unnecessary or undesirable(1,44).

For individuals undergoing probiotic monotherapy, the recommended duration is at least one month. This extended period leverages the diverse mechanisms of action of probiotic strains, including their direct antagonistic activities (such as bacteriocins, acids, and hydrogen peroxide), their capacity to restore gastric and gut microbiota, and their essential immunomodulatory effects (20).

Newer forms, such as strains of indigenous microbiota, are emerging as a novel and promising monotherapy, with potential to enhance anti-*H. pylori* efficacy. However, as with many probiotic applications, the lack of definitive, large-scale, randomised, placebo-controlled, multicenter studies hinders the full confirmation of the effectiveness and safety of both standard probiotic and strains of indigenous microbiota monotherapy (1,24).

Ultimately, probiotic monotherapy demonstrates potential as an alternative treatment modality, particularly for individuals who are intolerant to antibiotics. Nevertheless, its comparatively lower success rate in eradication warrants ongoing research to identify the most efficacious strains or combinations. This initiative aims to optimise clinical outcomes and broaden its application. Presently, it functions as a specialised option rather than a standard first-line intervention for infection prevention (1,45).

5.2. Efficacy of Probiotics as Adjunctive Therapy for *H. Pylori*

The integration of probiotics into established anti-*H. pylori* regimens, such as BQT, have become a crucial strategy to overcome the challenges of antibiotic resistance and adverse effects. Given the declining efficacy of traditional therapies due to increasing clarithromycin resistance, combining standard drugs with specific live microbial strains provides a robust pathway to optimising treatment outcomes. Evidence from meta-analyses robustly supports this combined approach, reliably demonstrating that probiotics significantly boost eradication success rates. Probiotics contribute to enhanced therapy primarily through a dual mechanism: augmentation of efficacy and mitigation of side effects (42,43,7). On one hand, probiotics exert direct and indirect anti-*H. pylori* effects. These include competitive inhibition of colonisation, production of bacteriostatic substances (like antibacterial compounds), reduction of gastric pH, and inhibition of the pathogen's urease activity. Probiotics also modulate the immune system and enhance the stability of the stomach's protective barrier, collectively creating an environment less favourable for *H. pylori* survival. The importance of the microbiome is further

underscored by reports that even faecal microbiota transplantation (FMT) has cleared the pathogen in some cases (43,46,48).

On the other hand, a critical role of probiotics is the alleviation of adverse reactions associated with antibiotics. By modulating the gut microbiome across the digestive tract, strains like *Lactobacillus* and *Bifidobacterium* help maintain microbial balance. This leads to better patient adherence, which is vital for achieving high cure rates. Beyond the immediate eradication phase, long-term follow-up studies suggest that sustained probiotic supplementation helps prevent *H. pylori* recurrence (reinfection). By restoring the healthy indigenous gastrointestinal flora damaged by the aggressive antibiotic course, probiotics fortify the mucosal barrier against the reestablishment of the pathogen (2,48).

Given the accumulated evidence highlighting improved eradication rates and reduced side effects, current guidelines increasingly recommend incorporating specific probiotic strains (*Lactobacilli*, *Bifidobacterium*, *Saccharomyces*, etc.) as adjunctive agents. Despite the consensus on their beneficial adjunctive role, definitive evidence regarding the precise impact of every probiotic strain on eradication rates remains hindered by heterogeneity across different studies, including variations in study designs and inconsistent conclusions. Therefore, ongoing systematic reviews and network meta-analyses are essential to definitively assess the effectiveness and safety of integrating different probiotic formulations into the complex anti-*H. pylori* regimen (7-49). **Table 2** illustrate dual benefits of probiotic supplementation on *H. pylori* eradication outcomes.

Table 2: Dual Benefits of Probiotic Supplementation on *H. pylori* Eradication Outcomes

Category	Specific Mechanism of Action	Clinical Effect on Eradication	Supporting Meta-Analysis Evidence (RR / OR)	Practical Implication
Enhanced Efficacy (Direct and Indirect)	Direct Antimicrobial Activity	Inhibition of <i>H. pylori</i> growth and adherence in the gastric mucosa, reducing bacterial load.	Eradication Rate Increase	Supplements act as an <i>adjuvant</i> to the antibiotic regimen, directly improving success rates.
	Immune Modulation	Down-regulation of pro-inflammatory cytokines, mitigating local gastric inflammation.	Improved therapeutic environment, enhancing antibiotic access and activity.	Particularly beneficial against chronic gastritis associated with <i>H. pylori</i> .
Improved Tolerability (Safety and Compliance)	Restoration of Gut Microbiota Balance	Prevention and remediation of antibiotic-associated dysbiosis.	Total Adverse Events Reduction: RR 0.47 - 0.61 (Most robust and consistent finding).	Addresses the primary cause of treatment discontinuation and failure (patient non-compliance).
	Symptom Attenuation	Reduction of specific GI side effects associated with high-dose antibiotic regimens.	Significant reduction in Diarrhea (RR 0.41 - 0.49) and Taste Disturbance.	Enhances quality of life during therapy, ensuring patients complete the full course.
Optimal Regimen Characteristics	Superior Formulations	Synergistic or complementary mechanisms provided by multiple strains.	Best Comprehensive Performance: Bifidobacterium-Lactobacillus and triple-strain combinations.	Multistrain formulations are generally preferred over single strains for maximum benefit.
	Strategic Integration	Optimal timing and duration of probiotic administration.	High Success Probability: Probiotics added to BQT (SUCRA 99.9%) for durations >2 weeks.	Integration with potent regimens (BQT) and prolonged use yield the highest clinical benefit.

RR (Relative Risk), OR (Odds Ratio), SUCRA (Surface Under the Cumulative Ranking Curve), BQT (Bismuth Quadruple Therapy), GI (Gastrointestinal)

5.4. Differences in efficacy by probiotic strain

The literature offers compelling evidence, derived from in vitro, in vivo (animal model), and human clinical studies, supporting the utility of specific probiotic strains in combating *H. pylori* infection. This evidence underscores their dual function: improving eradication rates when used in conjunction with antibiotics and reducing associated adverse effects. Laboratory and animal model research has consistently validated the direct inhibitory capacity of numerous lactic acid bacteria against *H. pylori*. Isolates such as *Lactobacillus sake*, *L. plantarum*, *L. rhamnosus*, and *Lactobacillus. brevis* have demonstrated varying degrees of growth inhibition. This anti-pathogen activity is often mediated through the production of bacteriocins or organic acids, providing a direct antagonistic effect (7,48).

Specific strains show multi-faceted benefits. *L. casei T1* and its supernatant exhibited potent inhibitory activity, also preventing the inflammation and dysbiosis typically caused by the infection. *Lactiplantibacillus pentosus SLC13* not only inhibits growth but also significantly reduces *H. pylori*-induced interleukin-8 (IL-8) expression, suggesting its anti-inflammatory potential. *Lactobacillus paragasseri LPG-9* was shown to repair the gastric mucosal barrier by upregulating protective proteins, such as occludin and ZO-1, in in vivo models, thereby

alleviating gastritis (33). These preclinical findings confirm that the anti-*H. pylori* effect is strain-dependent and results from complex mechanisms, including direct killing, competitive exclusion, and mucosal barrier enhancement (2). Clinical meta-analyses largely confirm the benefits of using probiotics as an adjunctive therapy. A comprehensive meta-analysis demonstrated that probiotic supplementation significantly improved eradication rates compared to standard antibiotic therapy alone. Notably, the study by Shi et al., encompassing nearly 9,000 patients, found that combined probiotic treatment achieved an 81.5% eradication rate, markedly surpassing the 71.6% rate in control groups (2,33). While the efficacy is supported, the optimal strain for eradication remains an area of ongoing study. *Bifidobacterium spp.* has been highlighted in a recent meta-analysis as exhibiting the highest eradication potential, though this requires validation through further high-quality studies (7). The effectiveness of specific strains, such as *L. reuteri*, has produced conflicting results across clinical trials, likely due to heterogeneity in study design and variations in the doses of the specific strain used (33). The timing of probiotic administration is also crucial; superior outcomes are often observed when probiotics are used before and throughout the eradication treatment, especially for regimens lasting more than two weeks or those involving BQT (2,33,7).

One of the most consistently observed clinical advantages of probiotics is their role in mitigating treatment-associated adverse effects such as abdominal pain, diarrhoea, epigastric pain, bloating, headache, dizziness, nausea, and vomiting. Numerous meta-analyses have demonstrated that probiotic supplementation notably decreases the overall risk of these adverse effects. This benefit is directly attributable to the capacity of probiotics to prevent antibiotic-induced dysbiosis (microbial imbalance), consequently reducing common gastrointestinal complications (33,7,48,49).

A comprehensive network meta-analysis encompassing 34 Randomised Controlled Trials (RCTs) published between 2000 and 2022 concluded that the inclusion of probiotics in triple antibiotic therapy notably enhances the eradication rate of *H. pylori* (Risk Ratio 1.14) and decreases the incidence of adverse events (Risk Ratio 0.61). The probiotic consortium comprising *Bifidobacterium* and *Lactobacillus* demonstrated superior overall performance, with higher eradication success and reduced adverse events, surpassing the efficacy of the combination of *Bifidobacterium*, *Lactobacillus*, and *Saccharomyces* (Table 2) (48).

6. *H. pylori* eradication therapy

H. pylori remains the world's most widespread chronic bacterial infection, colonising approximately half of the global population and posing a substantial public health threat. Its

pervasive presence, which dates back millennia, is definitively linked to serious upper gastrointestinal diseases, including chronic gastritis, peptic ulcers, and the development of gastric cancer—a Group 1 carcinogen classification affirmed by the WHO/IARC (International Agency for Research on Cancer). Eradicating this Gram-negative bacillus is recognised globally (e.g., by the Maastricht and Kyoto agreements) as a cornerstone strategy for treating these morbidities and preventing carcinogenic progression (50,51). Despite the critical need for effective treatment, the global efficacy of *H. pylori* eradication therapies is demonstrably declining. The main driver of this therapeutic failure is the rapid, region-specific increase in antibiotic resistance, particularly to clarithromycin. The traditional first-line, triple-drug regimen (Proton Pump Inhibitor (PPI), clarithromycin, and amoxicillin), once achieving eradication rates over 80%, is no longer universally accepted due to clarithromycin resistance rates reaching 40% in countries like Turkey and 50% in China (52).

This situation has necessitated a shift in clinical practice toward more potent options. BQT, typically comprising a PPI, bismuth, tetracycline, and metronidazole, is now the internationally recommended first-line treatment, capable of achieving 85%-90% success under ideal adherence. However, even BQT faces clinical challenges: localised resistance patterns can drop eradication rates below 70%, and nearly half of all patients experience adverse events, such as gastrointestinal discomfort and diarrhoea, leading to poor adherence. Failures after BQT necessitate sequential second and third-line therapies, often involving levofloxacin or rifabutin, ideally guided by antimicrobial susceptibility testing, which is often costly and time-consuming. In response to the limitations of increasing resistance and reduced tolerability, the incorporation of probiotics into first-line BQT has emerged as a promising adjunctive strategy. Clinical evidence suggests that supplementing BQT with specific probiotic strains, notably *S. boulardii*, significantly enhances eradication rates (50,51).

7. Meta-Analyses and Systematic Reviews

Probiotics have emerged as an alternative treatment option due to the development of resistance to or adverse effects caused by the use of conventional triple and quadruple therapy (PPI, clarithromycin, amoxicillin, bismuth subsalicylate, tetracycline, metronidazole) protocols and alternative antibiotics (furazolidin, rifabutin, tetracycline, fluoroquinolones) for *H. pylori* eradication. Numerous meta-analyses have reported that adding probiotics before, after, or simultaneously to conventional treatment protocols increases treatment efficacy and reduces adverse events (3,11,50,51).

Liu et al. conducted a meta-analysis of 19 RCTs in 2025 and compared *H. pylori* eradication rates and adverse event incidence between BQT and probiotic-supported BQT (PBQT) treatments. The PBQT eradication rate was 85.5%, while the BQT eradication rate was 78.6%. The highest eradication rates were observed in the five studies that added *S. boulardii* as an add-on treatment (OR = 1.62, 95% CI: 1.03-2.54, P = 0.04). Conversely, studies that added multi-strain probiotics showed relatively lower eradication rates. Probiotic efficacy appears to be critically dependent on strain composition. Clinical evidence suggests that multi-strain products offer synergistic or complementary benefits. Conversely, studies evaluating single-strain preparations, including those containing essential genera such as *Lactobacillus* and *Bifidobacterium*, generally fail to demonstrate significant clinical advantages. The addition of probiotics to the BQT regimen substantially reduced the total incidence of treatment-related adverse events compared with the control group that received only the standard approach (50). Shi et al. conducted a comprehensive meta-analysis of 8,924 patients and 40 eligible studies. Compared with control groups, the probiotic intervention group demonstrated a superior eradication rate (Relative Risk (RR) 1.140, P<.001) and a significantly reduced incidence of total adverse events (RR 0.470, P<.001) based on intention-to-treat (ITT). According to Surface Under the Total Ranking Curve (SUCRA) scoring, the probiotic plus quadruple regimen performed almost flawlessly (SUCRA 99.9%), and treatment durations exceeding two weeks were highly favoured (SUCRA 92.6%). Specific adverse events were also effectively managed, with the highest efficacy noted in reducing taste disturbance (SUCRA 99.6%) and managing nausea (SUCRA 78.8%) (2).

A network meta-analysis conducted by Wang and colleagues examined 34 eligible RCTs encompassing 9,004 patients and evaluating ten different treatment strategies. Their key finding was that most probiotic-supplemented regimens outperformed standard triple therapy. In particular, *Bifidobacterium*-*Lactobacillus* and *Bifidobacterium*-*Lactobacillus*-*Saccharomyces* combination therapies were found to provide the best overall clinical outcomes, balancing high eradication rates (78.3% and 88.2%, respectively) with a low incidence of side effects. Mixing different probiotic strains, extending supplementation duration, and administering probiotics before or after triple therapy significantly enhanced the overall therapeutic effect (48).

Yao et al. focused their meta-analysis of 10 RCTs on the efficacy and safety of combining probiotics with BQT. The pooled results showed that probiotic supplementation provided a modest but statistically significant increase in eradication success, with relative risks of 1.07 (ITT) and 1.04 (PP). Notably, the addition of probiotics was highly effective in reducing

side effects, significantly reducing the overall frequency of side effects (RR 0.58), particularly diarrhoea (RR 0.41) and bitter taste (RR 0.63) (49).

Tanashat's team conducted an extensive review that included 91 RCTs involving 13,680 patients. They reported that adding probiotics significantly increased eradication rates in both ITT and PP analyses (OR 1.62 and 1.60, respectively). A detailed analysis of side effects confirmed substantial reductions across nearly all measured symptoms, including diarrhoea (RR 0.49), abdominal pain, bad taste, headache/dizziness, and nausea/vomiting. Their network meta-analysis identified *Bifidobacterium longum* as having the highest comparative efficacy for eradication against a placebo (OR 2.52), thereby highlighting the strain-specific potential within the *Bifidobacterium* genus (43).

Lau and colleagues focused on the benefits of probiotics when combined with concurrent triple therapy. Their analysis of 30 RCTs showed that the addition of probiotics significantly increased eradication rates by 12.2% (PP) and 14.1% (ITT). The review affirmed that these benefits were consistent across children and adults, as well as both Asian and non-Asian populations. While a reduction in side effects such as diarrhoea, nausea, and epigastric pain was noted, the study found no significant difference in efficacy among the various types of probiotics (*Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, or mixtures). They concluded that, given improvements in eradication and reductions in side effects, probiotic supplementation should be considered for all patients receiving the concurrent triple therapy regimen (47).

However, contrary to these studies, some studies have concluded that probiotics are ineffective for *H. pylori* eradication. These conflicting results are due to inconsistencies in experimental design, strain types, and treatment methods across different clinical studies (48).

Table 3: Summary of Key Meta-Analyses on Probiotics in *H. pylori* Eradication

Study (Authors, Year)	Number of Included Studies (Patients)	Primary Eradication Outcome	Key Adverse Event Outcome	Notable Specific Findings / Recommendations
Shi et al. (2019)	40 (8924)	Significant Increase: Control Group 71.6% Probiotics group 81.5% (RR: 1.140 (P<.001))	Substantial Reduction: Total Side Effects (RR: 0.470 (P<.001))	Optimal Regimen: Probiotics + BQT (SUCRA 99.9%). Duration: Treatment >2 weeks. Most effective in reducing taste disturbance.
Wang et al. (2023)	34 (9004)	Most effective: Bifidobacterium-Lactobacillus (78.3%) and Bifidobacterium-Lactobacillus-Saccharomyces (88.2%).	Lowest Side Effects: Bifidobacterium-Lactobacillus combinations.	Combined probiotics, longer duration, and pre/post-therapy administration enhance effect. Lactobacillus-Propionibacterium associated with highest side effects.
Yao et al. (2023)	10 (1397)	Modest Increase: RR: 1.07 (ITT)	Significant Reduction: Total Side Effects RR 0.58. Diarrhea RR 0.41.	Endorses the combination of probiotics with for clinical management.
Tanashat et al. (2024)	91 (13680)	Strong Enhancement: OR 1.62 (ITT)	Diarrhea RR 0.49, Bad Taste RR 0.64, Headache/Dizziness RR 0.46.	Most Efficacious Strain: <i>Bifidobacterium longum</i> (OR 2.52 vs. placebo).
Lau et al. (2016)	30 (APP-4,302 ITT- 4,515)	Notable Increase: APP- 12.2% ITT- 14.1% increase in eradication rate.	Reduction in diarrhea, nausea, vomiting, and epigastric pain.	Benefits are consistent across adults and children, Asian and non-Asian populations. No significant difference in efficacy among various probiotic types.
Yang et al. (2024)	28 Meta-Analysis (534 RCTs)	Consistent Improvement: RR 1.10.	Strong Reduction: Total Side Effects RR 0.54.	Highest Potential: <i>Bifidobacterium spp.</i> (requires further high-quality research). Single-strain and multi-strain preparations showed similar results.

Abbreviation: RCT (Randomized Controlled Trial), BQT (Bismuth Quadruple Therapy), ITT (Intention-To-Treat) , APP (As-Per-Protocol), SUCRA (Surface Under the Cumulative Ranking Curve), RR (Relative Risk)

CONCLUSIONS

Data from numerous large-scale systematic reviews and network meta-analyses demonstrate that probiotic integration is an essential adjunctive measure in the clinical treatment of *H. pylori* infection. This strategy effectively addresses two key challenges of modern eradication therapy: reduced efficacy due to antibiotic resistance and low patient compliance due to serious side effects.

In particular, the addition of probiotics to standard treatment regimens is associated with a statistically significant improvement in cure rates and a significant reduction in adverse

events, particularly diarrhoea, vomiting, nausea, headache, dizziness, and taste disturbances. This protective effect is crucial because improved patient tolerance directly increases compliance, a key determinant of successful eradication. Furthermore, strategic administration protocols, such as administering compound probiotic preparations (e.g., *Bifidobacterium-Lactobacillus* combinations) for periods exceeding two weeks, yield the most satisfactory clinical results.

Given the increasing global prevalence of antibiotic resistance and the severity of *H. pylori*-associated diseases, current clinical guidelines should strongly support the routine inclusion of appropriate probiotic supplementation in eradication protocols. Future research should prioritise standardised, high-quality clinical trials to definitively define single- or multi-strain formulations and their precise administration schedules to fully realise the therapeutic potential of probiotics in this critical area of gastroenterology.

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EFFECT OF NaCl STRESS ON THE YIELD, PHENOLIC CONTENT, AND PIGMENT COMPOSITION OF BROCCOLI, WHEAT, AND RADISH MICROGREENS

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ABSTRACT

Microgreens are nutrient-dense, young vegetable seedlings that represent an emerging functional food category. However, salinity stress can significantly impact their yield and nutritional composition. This study investigated the effects of salinity stress on the yield, moisture, phenolic content, and pigment composition of broccoli (*Brassica oleracea* var. *italica*), wheat (*Triticum aestivum*), and radish (*Raphanus sativus*) microgreens. Microgreens were cultivated under four salinity treatments: 0 mM (control), 25 mM, 50 mM, and 100 mM NaCl. Due to severe growth inhibition and poor germination, the 100 mM treatment was excluded from analysis. Results revealed species-specific responses to NaCl stress. Broccoli exhibited a hormetic effect, with increased biomass at 25 mM NaCl, while wheat maintained stable biomass and pigment levels, indicating strong innate tolerance. Radish, despite its high baseline biomass and pigment content, showed high sensitivity, with linear declines in yield and pigment concentrations under salinity stress. Total phenolic content decreased at both 25 and 50 mM NaCl across all species, suggesting the growth-defense tradeoff hypothesis. Photosynthetic pigments remained stable in broccoli and wheat but declined significantly in radish. Multivariate analyses confirmed that species identity was the primary driver of physiological and biochemical variation. These findings underscore the importance of species selection and water quality management in microgreen cultivation, particularly in urban and home gardening contexts.

Keywords: Microgreens, Salinity Stress, Yield, Phenolic content, Photosynthetic pigments

INTRODUCTION

Microgreens are tender, edible young seedlings of various vegetables, grains, and herbs, including wild edible species, harvested at an immature stage of growth, usually after 7 to 21 days, depending on variety (Bhaswant et al., 2023; Dereje et al., 2023). This specific growth stage is characterized by the full development of their cotyledon leaves, often with the initial emergence or partial expansion of their first true leaves, but before these true leaves are fully formed (Ebert, 2022). This is what differs them from sprouts and baby greens. Sprouts are defined as germinated seeds harvested for consumption prior to the development of their true leaves (Gupta et al., 2023), while baby greens are young plants with tender true leaves, typically harvested after 20 to 40 days (Bhaswant et al., 2023).

Microgreens are recognized as superfood or functional foods due to their densely packed nutrients and health-promoting phytochemicals, which are believed to enhance immunity and prevent diseases. They are rich in various beneficial compounds, including antioxidants, vitamins (A, C, E, K), minerals (such as calcium, iron, zinc, potassium, magnesium, and phosphorus), phenolic compounds, amino acids, glucosinolates, and glycerophospholipids (Bhaswant et al., 2023; Dereje et al., 2023; Gupta et al., 2023). Besides their nutritional and

health benefits, they are valued for their intense sensory characteristics, including a diverse range of flavors, textures, aromas, vibrant colors, and an appealing appearance (Sagar et al., 2025), while their minimal requirements for space, water, and other inputs make them suitable for home gardening, urban farming, and commercial agriculture, contributing to sustainable food systems (Bhaswant et al., 2023; González and Vargas, 2025; Seth et al., 2025).

Salinity is a critical abiotic stressor in agriculture that significantly limits plant growth and productivity worldwide (Abd elkader et al., 2024). Salt accumulation in soils is a consistent problem in arid and semi-arid regions, increased by various factors like irregular irrigation, inadequate drainage, excessive fertilization, and desertification processes, which is further exacerbated by the excessive use of saline irrigation water (Yildirim et al., 2008; Zuzunaga-Rosas et al., 2024).

Salinity primarily impacts plants through two main mechanisms: osmotic stress and ionic toxicity. First, high salt concentrations in the soil solution decrease the water potential of the soil, making it difficult for plants to absorb water, leading to a state of drought-like stress. Second, high concentrations of ions, particularly Na^+ and Cl^- , become toxic to plant tissues once absorbed. This toxic accumulation can inhibit the uptake of essential mineral nutrients, leading to nutrient imbalances (Yildirim et al., 2008).

Microgreens, being young and tender, are often more sensitive and susceptible to stress than mature plants because they are harvested at young stage, and the plants do not have enough time to build tolerance (Plocek et al., 2023). Since the quality of tap water is often influenced by the presence of high levels of NaCl (Toscano et al., 2025), microgreens grown at home can easily suffer from salinity stress, which impairs their growth and reduces their nutritional value.

Salinity is generally considered a severe stress that slows down plant growth and productivity by inducing osmotic and ionic imbalances (Abd elkader et al., 2024; Zuzunaga-Rosas et al., 2024). However, the tolerance level is species-specific, and low concentrations may not negatively impact growth, or may even promote it (Plocek et al., 2023). Particularly at low or moderate concentrations, salinity stress is often used as an elicitor to enhance the synthesis of secondary metabolites like phenolic compounds, which act as antioxidants to cope with the stress (Ebert, 2022; Giménez Martínez et al., 2023). On the contrary, salinity commonly decreases photosynthetic pigment levels due to negative impacts on photosynthetic activity and subsequent damage to cellular structures (Zuzunaga-Rosas et al., 2024).

Since salinity is a common stressor for microgreens, particularly in home and urban settings, and plant responses are highly species-specific, this study aimed to investigate and compare the effects of low-to-moderate NaCl stress (0, 25, 50, and 100 mM) on the yield, total phenolic content, and photosynthetic pigment composition of three commercially important and distinct microgreen species: *Brassica oleracea* var. *italica* (broccoli), *Triticum aestivum* (wheat), and *Raphanus sativus* (radish).

MATERIALS AND METHODS

The experiment was a 3 x 4 factorial design with four replicates. The factors were three microgreen species (broccoli, wheat, and radish) and four target NaCl concentrations (0, 25, 50, and 100 mM). This design resulted in 48 experimental units (containers). The containers were distributed across three separate shelves, with 16 containers placed on each shelf. To account for potential environmental micro-variations, shelf was treated as a random effect in the statistical analysis. LED lights with color temperature of 6000 K were mounted to the underside of each shelf to provide illumination. Seeds (GEO Sprouts, Bavicchi s.p.a., Italy) were sown in 10x10 cm plastic containers filled with a moisture-retentive substrate. Mass of seed sown per container was 2 g for radish, 3,5 g for broccoli, and 6 g for wheat. The shelves were placed in a container with a controlled photoperiod of 16 hours of light and 8 hours of dark. During the

growth period, the temperature and relative humidity varied between 23.6–25.6 °C and 52–84%, respectively.

Four salinity levels were used in the experiment: 0 mM (control), 25 mM, 50 mM, and 100 mM NaCl. The treatment solutions were prepared using sodium chloride (Kemika, Croatia, purity $\geq 99,5\%$). Seeds were sown directly onto the substrate without any pre-soaking. Salinity treatments began immediately at sowing. The microgreens were irrigated by spraying their respective treatment solutions twice daily to ensure consistent moisture and salt concentration.

The 100 mM NaCl treatment resulted in extremely poor germination and severe growth inhibition across all three species. Consequently, there was insufficient biomass for any reliable analytical measurements. Therefore, this treatment group was discarded from the study and is not included in any subsequent analysis.

Microgreens were harvested six days after sowing by cutting them approximately 3 mm above the substrate. The fresh mass for each replicate was weighed using a precision laboratory scale (Kern PFB 1200-2, KERN & SOHN GmbH, Germany). Following the fresh mass measurement, the moisture content was determined using a moisture analyzer (PCE-MA 110, PCE Instruments, Germany). Approximately 1 gram of finely chopped plant material from each replicate was dried with a gradual temperature increase up to 120°C.

Photosynthetic pigments were extracted from fresh plant tissue. A sample of 0.1 g was homogenized with an ULTRA-TURRAX T 10 basic (IKA, Germany) in 10 mL of 99.8% methanol. The homogenate was then centrifuged at 3000 rpm for 4 minutes. Quantification was performed in triplicate using a GENESYS 50 UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). The absorbance of the supernatant was measured at 665 nm (chlorophyll *a*), 652 nm (chlorophyll *b*), and 470 nm (total carotenoids). Pigment concentrations were calculated using the equations established by Lichtenthaler (1987):

$$\text{Chl } a \text{ (}\mu\text{g/ml)} = 16.72 \times A_{665} - 9.16 \times A_{652}$$

$$\text{Chl } b \text{ (}\mu\text{g/ml)} = 34.09 \times A_{652} - 15.28 \times A_{665}$$

$$C_{x+c} \text{ (}\mu\text{g/ml)} = (1000 \times A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b) / 221$$

The final pigment content was expressed as mg/g of fresh mass (FM) using the formula: $\text{Pigment (mg/100 g FM)} = (C \times V) / (10 \times M)$, where *C* = concentration ($\mu\text{g/ml}$), *V* = total volume of methanol (mL), and *M* = fresh mass of the sample (g).

Total phenolic content was determined using an adapted method from Peršić et al. (2019). Approximately 1 g of chopped plant material was extracted in 6 mL of an 80:20 (v/v) methanol-water solution. The extraction was performed in a cold ultrasonic bath for 60 minutes, after which samples were centrifuged for 10 minutes at 8000 rpm. The analysis was performed in triplicate. The reaction mixture was prepared by combining 100 μL of supernatant with 0.25 mL of Folin-Ciocalteu reagent (diluted 1:1 with water), 0.75 mL of 20% sodium carbonate (Na_2CO_3) solution, and 3.9 mL of distilled water. The mixture was then heated at 40°C for 30 minutes. Absorbance was measured at 765 nm (GENESYS 50 UV-VIS). Total phenolic content was calculated from a calibration curve prepared with gallic acid, and results were expressed as milligrams of gallic acid equivalents per 100 grams of fresh mass (mg GAE/100 g FM).

All statistical analyses were performed using R statistical software (version 4.3.3). To account for the experimental design, the effects of NaCl treatment and microgreen species on the measured parameters were analyzed using linear mixed-effects models (LMM). In the models, species and treatment (0, 25, and 50 mM NaCl) and their interaction were treated as fixed effects. Shelf and replicate were included as nested random effects to account for potential variability among shelves and the containers nested within them. The significance of the fixed effects was determined using a Type III Analysis of Variance (ANOVA) with Satterthwaite's method for degrees of freedom. When a significant interaction effect was found, post-hoc pairwise comparisons of estimated marginal means were conducted using the emmeans package, with p-values adjusted by the Tukey HSD method. To further investigate the dose-

response relationship, a polynomial regression analysis was conducted by modeling the numeric NaCl concentrations (0, 25, 50) as a second-order polynomial term. This tested for significant linear and quadratic trends in the response of each species to increasing salt stress. Additionally, a Pearson correlation analysis was performed to evaluate the relationships between all measured dependent variables. A Principal Component Analysis (PCA) was also conducted on the scaled data to visualize the overall variation and clustering among the different species and treatments. For all analyses, differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The linear mixed-effects models revealed that the microgreens species, NaCl concentration, and their interaction had significant effects on various measured parameters. The results of the Type III ANOVA tests are summarized in Table 1.

Species of microgreens showed highly significant main effect on all measured parameters ($p \leq 0.009$) demonstrating substantial inherent differences among wheat, broccoli, and radish microgreens. NaCl concentration showed significant main effects on fresh mass ($p < 0.001$), moisture content ($p < 0.001$), total phenols ($p = 0.008$), and the chlorophyll *a/b* ratio ($p < 0.001$). Significant interaction effects between species and treatment were detected for fresh mass ($p < 0.001$), moisture content ($p = 0.003$), and the chlorophyll *a/b* ratio ($p = 0.012$) which indicates that the microgreens species responded differently to stress for these specific parameters. No significant treatment or interaction effects were observed for chlorophyll or carotenoid concentrations.

Given the significant interactions between species and treatment, the specific responses of each microgreen species to the NaCl concentration were examined for fresh mass, moisture content, and the chlorophyll *a/b* ratio (Figure 1).

Table 1. Summary of ANOVA (Type III) results from the linear mixed-effects models for all measured parameters.

Dependent Variable	Factor	F-value	p-value
Fresh Mass	Species	162.72	< 0.001***
	Treatment	13.58	< 0.001***
	Species:Treatment	7.69	< 0.001***
Moisture Content	Species	56.45	< 0.001***
	Treatment	20.07	< 0.001***
	Species:Treatment	5.42	0.003**
Total Phenols	Species	71.26	< 0.001***
	Treatment	5.88	0.008**
	Species:Treatment	0.26	0.903
Chlorophyll <i>a</i>	Species	11.02	< 0.001***
	Treatment	1.89	0.170
	Species:Treatment	1.75	0.168
Chlorophyll <i>b</i>	Species	5.62	0.009**
	Treatment	2.37	0.113
	Species:Treatment	1.50	0.230
Total chlorophylls	Species	9.29	< 0.001***
	Treatment	2.01	0.153
	Species:Treatment	1.68	0.184
Carotenoids	Species	15.53	< 0.001***
	Treatment	2.35	0.115
	Species:Treatment	1.98	0.126
Chlorophyll <i>a</i> / Chlorophyll <i>b</i> Ratio	Species	66.32	< 0.001***
	Treatment	10.60	< 0.001***
	Species:Treatment	4.34	0.012*

Significance codes: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

Values represent estimated marginal means $\pm$ standard error. Different letters denote significant differences ($p < 0.05$) among treatments within each species.

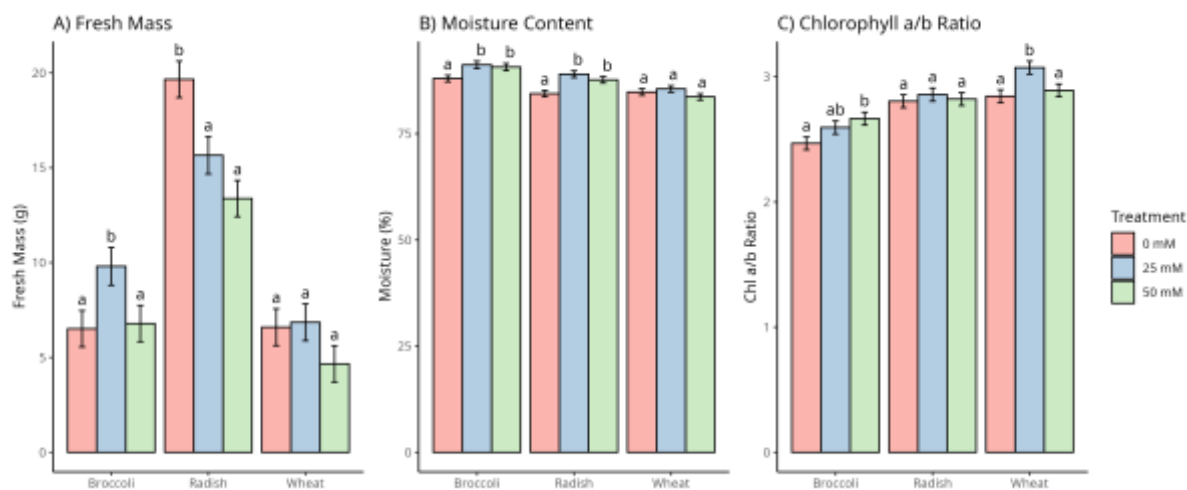


Figure 1. Effects of NaCl treatments on (A) fresh mass, (B) moisture content, and (C) chlorophyll *a/b* ratio in broccoli, wheat, and radish microgreens.

Broccoli exhibited a potential hormetic effect. The 25 mM NaCl treatment significantly increased fresh mass (Figure 1A) compared to the 0 mM control ($p = 0.014$). However, at 50 mM, the fresh mass was not significantly different from the control ($p = 0.961$) but was significantly lower than the 25 mM NaCl ($p = 0.025$). While radish microgreens produced the highest biomass overall, significantly exceeding both broccoli and wheat across all treatments, they also showed high sensitivity to salinity. Both the 25 mM and 50 mM NaCl treatments caused a significant decrease in fresh mass compared to the 0 mM control ($p = 0.005$ and $p < 0.001$, respectively). Wheat appeared tolerant to the tested concentrations. Although there was a decline in fresh mass at 50 mM concentration the difference was not statistically significant ($p > 0.19$).

NaCl treatments had varying effects on moisture content (Figure 1B). In both broccoli and radish, the 25 mM and 50 mM treatments significantly increased moisture content compared to their respective controls (Broccoli: $p = 0.004$ and $p = 0.013$; Radish: $p < 0.001$ and $p = 0.001$). In contrast, wheat moisture content remained unaffected by the NaCl treatments, with all groups showing no statistical difference.

The response of the chlorophyll *a/b* ratio to salinity also differed among species (Figure 1C). For broccoli, the ratio significantly increased at the 50 mM concentration compared to the control ($p = 0.003$), while the 25 mM treatment was not significantly different from the control. Wheat showed a non-linear response. The ratio significantly increased from 0 mM to 25 mM NaCl ($p = 0.0016$), but then significantly decreased from 25 mM to 50 mM ($p = 0.0057$), returning to a level not different from the control. The chlorophyll *a/b* ratio in radish was not significantly affected by any NaCl treatment.

To further characterize the dose-response relationship between NaCl concentration and the measured parameters, a polynomial regression analysis was conducted. The results confirmed that the trend of the response differed significantly among species for fresh mass, moisture, and the chlorophyll *a/b* ratio, as indicated by the significant interaction between the species and the treatment in the mixed model. The specific trends for each species are detailed below (Table 2).

Table 2. Results of polynomial regression models for fresh mass, moisture, and chlorophyll *a/b* ratio in response to increasing NaCl concentrations.

Dependent Variable	Species	R ²	F-statistic (DF)	Model p-value	Linear Term	Quadratic Term
Fresh Mass	Radish	0.578	F _(2,9) = 6.17	0.021	**	ns
	Wheat	0.603	F _(2,9) = 6.84	0.016	*	.
	Broccoli	0.577	F _(2,9) = 6.13	0.021	ns	**
Moisture	Radish	0.668	F _(2,9) = 9.05	0.007	*	*
	Wheat	0.390	F _(2,9) = 2.87	0.108	ns	.
	Broccoli	0.727	F _(2,7) = 9.33	0.011	**	.
Chlorophyll a / Chlorophyll b Ratio	Radish	0.280	F _(2,9) = 1.75	0.228	ns	.
	Wheat	0.523	F _(2,9) = 4.93	0.036	ns	*
	Broccoli	0.537	F _(2,9) = 5.24	0.031	*	ns

Significance codes: ns not significant; . $p < 0.1$; * $p < 0.05$; ** $p < 0.01$

For fresh mass, the species showed distinct response patterns to increasing salinity. Broccoli exhibited a significant quadratic relationship ($p=0.0067$), where mass peaked at the intermediate 25 mM concentration before declining, while the linear term was not significant ($p=0.969$). This indicates a hormetic or stimulatory effect at low salinity. Radish displayed a strong and significant linear decrease in fresh mass as NaCl concentration increased ($p=0.007$), with no significant quadratic component ($p=0.607$), showing a consistent negative impact from salt stress. Wheat also showed a significant linear decrease ($p=0.0135$), although the overall effect was less pronounced than in radish, consistent with the post-hoc analysis.

For moisture content, radish and broccoli both exhibited significant positive linear trends ($p=0.012$ and $p=0.0099$, respectively), with radish also showing a significant negative quadratic term ($p=0.018$), indicating that moisture content increased with salt concentration but at a decelerating rate. Quadratic component in broccoli was only marginally significant ($p=0.06$). Wheat showed no significant moisture response to NaCl treatment (overall model $p=0.108$), consistent with its intermediate salt tolerance.

The chlorophyll *a/b* ratio also responded differently depending on the species. Broccoli exhibited a significant linear increase following the increase in NaCl concentration ($p=0.011$). Wheat showed a significant quadratic relationship ($p=0.012$), where the ratio peaked at the intermediate 25 mM concentration. Radish displayed no significant trend in its chlorophyll *a/b* ratio in response to salt stress ($p>0.05$).

The genetic identity of the species dictates the physiological and morphological strategy employed to manage salt concentrations as low as 25 mM and 50 mM NaCl. This aligns with a growing body of literature demonstrating that plant responses to abiotic stress are highly genotype-dependent (Noreen & Ashraf, 2008; Yildirim et al., 2008; Giménez Martínez et al., 2021; Pavlović et al., 2024; Toscano et al., 2025). The results suggest three distinct strategies among the tested species: eustress-induced hormesis in broccoli, innate tolerance in wheat, and high sensitivity in radish.

Broccoli's significant quadratic fresh mass response (Table 2), characterized by a significant increase in biomass at 25 mM NaCl followed by a decline at 50 mM (Figure 1A), suggest that the 25 mM NaCl concentration acted as a mild eustressor, a low-level stressor that stimulates a positive or beneficial response, rather than a distressor that impairs development

(Plocek et al., 2023). Other studies on *Brassicaceae* microgreens confirm this hormetic effect. Plocek et al. (2023) investigated eustress in broccoli microgreens and found that yields were unaffected or, in one trial, significantly increased at low salinity levels ($1 \text{ dS} \cdot \text{m}^{-1}$, which is approximately 10 mM NaCl). Similar results were obtained by Wang et al. (2019) when broccoli sprouts had increased yields at 40 and 80 mM of NaCl but declined at higher salinities (160 , 200 mM). Other studies have shown that low salinity can increase germination percentage, germination rate, root and shoot lengths, and fresh root and shoot weight in different *Brassicaceae* sprouts (Zielińska-Dawidziak, 2021).

While radish produced the highest overall biomass, it displayed a significant and linear decrease in fresh mass in response to both 25 mM and 50 mM NaCl (Figure 1A, Table 2). This indicates that, despite being vigorous and widely classified as a moderately sensitive crop to salinity (Yildirim et al., 2008), radish in this study behaved as a glycophyte, a plant that is not adapted to saline environments (Zhao et al., 2021). The strong linear negative trend suggests that even 25 mM NaCl is beyond its eustress threshold. This aligns with germination studies on radish, which show that while it can germinate in saline media, its germination speed and success are progressively depressed as salt concentration increases (Toscano et al., 2025).

Wheat fresh mass was statistically unaffected by either 25 mM or 50 mM NaCl in post-hoc comparisons (Figure 1A), demonstrating significant resilience compared to radish. Wheat is a monocot from a *Poaceae* family, known for broad agricultural adaptation, and possesses more robust innate mechanisms to manage low-level salinity (Noreen & Ashraf, 2008; Zhao et al., 2021). The mechanism of this tolerance is explained by the moisture content data. Wheat's moisture content did not change under salt treatment (Figure 1B). Contrarily, both broccoli and radish showed a significant increase in moisture content (Figure 1B). This water accumulation is a primary osmotic adjustment strategy. The plant absorbs more water to dilute the high intracellular ion concentrations. In Plocek et al. (2023) trial broccoli microgreens percent moisture content (% MC) significantly increased under the $1 \text{ dS} \cdot \text{m}^{-1} \text{ NaCl}$ treatment, followed by 1.5 and 0.5 dS m^{-1} treatments, when compared to the control ($0 \text{ dS m}^{-1} \text{ NaCl}$). Wheat's ability to maintain stable moisture and stable biomass suggests it is not resorting to simple dilution. Instead, it likely employs different mechanisms, such as ion exclusion at the root, compartmentalization of ions in the vacuole, or the synthesis of compatible osmolytes, which are well-documented strategies in *Triticum* (Taybi & Alyahya, 2025).

The chlorophyll *a/b* ratio is an important indicator of photosynthetic apparatus adaptation and stress tolerance mechanisms under saline conditions (Muradoğlu et al., 2025). The response of the ratio is highly variable and is dependent upon various factors including plant species, genotype, salt concentration, and duration of exposure. Research shows that salt stress can lead to either an increase or a decrease in the chlorophyll *a/b* ratio depending on the specific biological and environmental context (Parida et al., 2003; Ghanem et al., 2021). As the chlorophyll *a/b* ratio is correlated with the structural and functional adaptations of the photosynthetic machinery within plants, its shift suggests an adaptive response where plants alter their pigment composition to optimize photosynthesis under adverse conditions. In stress conditions, chlorophyll *b* levels often decline more rapidly than chlorophyll *a*, resulting in a higher chlorophyll *a/b* ratio (Muradoğlu et al., 2025). This increase suggests a reduction in antenna size due to a decrease in the light-harvesting complex of PSII, which is more affected by salinity than PSI (Stefanov et al., 2021). A significant linear rise in the chlorophyll *a/b* ratio in broccoli suggests a reduction in the effective antenna size (Stefanov et al., 2021), a photoprotective strategy that limits PSII excitation under stress (Stefanov et al., 2024). This likely results from accelerated chlorophyll *b* degradation or suppressed synthesis. Stable chlorophyll *a/b* ratio in radish means that although total chlorophyll content decreased (discussed below), the reduction affected chlorophyll *a* and chlorophyll *b* proportionally, which implies that radish might primarily rely on other defensive or coping strategies that do not

involve major structural reorganization of the photosynthetic antenna at the tested concentrations. These alternative strategies often include reducing growth and leaf area expansion to reduce metabolic cost (Jamil et al., 2007), and osmotic adjustment and maintenance of water status (Toscano et al., 2025). Fresh mass decline and moisture increase with the NaCl concentration suggest this was the case in this study. Wheat genotypes display significant variations in their response to salinity, and photosynthetic parameters are strongly correlated with the susceptibility of the genotype (Kumar et al., 2017). The quadratic response in wheat indicates that the specific physiological tolerance mechanism of this genotype is maximized at the 25 mM threshold before non-adaptive degradation dominates.

For parameters where no significant interaction was detected, the main effects of species and treatment were examined (Table 3).

Table 3. Main effects of Species and NaCl Treatment on total phenols and photosynthetic pigments

Parameter	Factor	Level	Mean Value $\pm$ SE
Total Phenols (mg/ 100 g FW)	Species	Radish	194.6 $\pm$ 6.91a
		Wheat	114.7 $\pm$ 7.30b
		Broccoli	89.7 $\pm$ 8.08b
	Treatment	0 mM	151.0 $\pm$ 6.82a
		25 mM	124.0 $\pm$ 6.96b
		50 mM	124.0 $\pm$ 6.79b
Chlorophyll a (mg/ 100 g FW)	Species	Radish	40.6 $\pm$ 2.04a
		Wheat	36.1 $\pm$ 2.16ab
		Broccoli	27.9 $\pm$ 2.42b
Chlorophyll b (mg/ 100 g FW)	Species	Radish	14.4 $\pm$ 0.77a
		Wheat	12.3 $\pm$ 0.81ab
		Broccoli	11.0 $\pm$ 0.91b
Total chlorophylls (mg/ 100 g FW)	Species	Radish	55.0 $\pm$ 2.81a
		Wheat	48.4 $\pm$ 2.97ab
		Broccoli	38.9 $\pm$ 3.32b
Carotenoids (mg/ 100 g FW)	Species	Radish	10.24 $\pm$ 0.49a
		Wheat	7.91 $\pm$ 0.58b
		Broccoli	6.67 $\pm$ 0.51b

Means within the same parameter and factor that do not share a letter are significantly different ($p < 0.05$) according to Tukey's HSD test. SE = Standard Error. For all pigments, the main effect of treatment was not significant ($p > 0.05$).

The concentration of total phenols was significantly influenced by both species and treatment (Table 3). Averaged across all treatments, radish contained significantly higher levels of phenols (194.6 $\pm$ 6.91 mg GAE/100g FW) than both broccoli (89.7 $\pm$ 8.08 mg GAE/100g FW) and wheat (114.7 $\pm$ 7.30 mg GAE/100g FW), which did not differ significantly from each other. Regarding the treatment effects, the control group (0 mM) had a significantly higher concentration of total phenols (151.0 $\pm$ 6.82 mg GAE/100g FW) than both the 25 mM and 50 mM NaCl treatments (124.0 $\pm$ 6.96 and 124.0 $\pm$ 6.79 mg GAE/100g FW, respectively), which were statistically similar to each other.

These results contradict the common expectation that salt stress should induce the synthesis and accumulation of secondary metabolites like phenolics as a defense mechanism (Ebert, 2022). However, there is evidence supporting this dose-dependent reduction at moderate salinity levels. In study involving broccoli sprouts, treatment with 40 mM and 80 mM NaCl significantly decreased compared to the control, while 160 mM NaCl treatment led to a significant increase in the total phenolic content (Guo et al., 2014). In radish, 10 and 50 mM NaCl decreased, while 100 mM NaCl treatment increased total phenolic content (Yuan et al., 2010; Guo et al., 2014). This can be interpreted through a growth-defense tradeoff hypothesis. Plant metabolism operates on a finite budget of carbon and energy, creating competition between primary metabolism (growth) and secondary metabolism (defense, e.g., phenols) (Huot et al., 2014). But as high salt concentrations dramatically inhibit growth (Guo et al., 2014; Pavlović et al., 2024) the plant is actively slowing the growth rate in response to salt stress to increase survival (Zhao et al., 2021). This severe growth inhibition signals the definitive prioritization of secondary metabolism over primary metabolism (Huot et al., 2014).

In contrast to phenols, there was not significant main effect of NaCl treatment on the concentration of any of the photosynthetic pigments (all $p > 0.05$), indicating that salinity did not alter the overall pigment levels when averaged across species. However, species-specific analysis (Figure 2) revealed strong negative correlation between pigment and NaCl concentration in radish (discussed below), that was also confirmed in post-hoc analysis (data not shown). Post-hoc analysis showed statistically significant decrease in chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content in radish microgreens treated with 50 mM NaCl compared to control, while the 25 mM NaCl concentration intermediate between the two.

Although photosynthetic pigment concentrations were generally unaffected by salt treatments, except in radish where they had significantly decreased, they also showed significant differences between species. Despite decrease radish microgreens consistently exhibited the highest concentrations across all pigment fractions. For chlorophyll *a*, radish (40.6 ± 2.04 mg/100g FW) differed significantly from broccoli (27.9 ± 2.4 mg/100g FW), with wheat (36.1 ± 2.1 mg/100g FW) intermediate between the two. A similar pattern was observed for chlorophyll *b*, where radish (14.4 ± 0.77 mg/100g FW) significantly exceeded broccoli (11.0 ± 0.91 mg/100g FW), with wheat (12.3 ± 0.81 mg/100g FW) occupying an intermediate position. Total chlorophyll content followed the same trend, with radish (55.0 ± 2.8 mg/100g FW) significantly higher than broccoli (38.9 ± 3.32 mg/100g FW) and wheat (48.4 ± 2.97 mg/100g FW) intermediate. For carotenoids, radish (10.24 ± 0.49 mg/100g FW) was significantly higher than both broccoli and wheat (6.67 ± 0.51 and 7.91 ± 0.58 mg/100g FW, respectively), which did not differ from each other.

Salt stress leads to excessive formation of reactive oxygen species (ROS), which can cause oxidative damage to different biomolecules and complex cellular structures, thus compromising their stability. It also negatively affects the photosynthetic activity, limiting chlorophyll biosynthesis and reducing the concentration of other components such as carotenoids and xanthophylls (Zuzunaga-Rosas et al., 2024). The stability of these pigments in this study suggests the photosynthetic apparatus in broccoli and wheat was not experiencing significant photo-oxidative damage and the plants' innate antioxidant systems were sufficient to cope, while the radish was probably suffering from ion toxicity which led to decrease of pigments. Similarly, in the Jamil et al. (2007) study, salinity, ranging from 4.7 to 14.1 dS m⁻¹ NaCl concentrations reduced the leaf area and chlorophyll content as the salinity level increased with no significant effect on leaf water content in radish seedlings. Yildirim et al. (2008) reported significant decrease in chlorophyll content in radish plants exposed to 80 mM NaCl, in comparison to control. Contrarily, study on cauliflower microgreens found that a 50 mM NaCl concentration allowed for plant growth and stress tolerance (Pavlović et al., 2024). Similar

findings were reported for broccoli sprouts, where treatment with low salinity concentrations (40 and 80 mM NaCl) significantly promoted growth, which was linked to elevated chlorophyll content, improved Photosystem II (PSII) activity, and more developed chloroplasts, indicating enhanced photosynthesis (Wang et al., 2019). However, higher salt concentrations (100 mM or 150 mM) are almost invariably shown to significantly reduce chlorophyll and carotenoid content in salt-sensitive plants (Hossain et al., 2022).

To explore the inter-relationships between the measured parameters and the effect of salt stress, a Pearson correlation analysis was performed separately for each species (Figure 2).

A common feature across all three species was the strong, significant positive inter-correlation among all photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, total chlorophylls, and carotenoids), with correlation coefficients (*r*) often exceeding 0.95 ($p < 0.001$), which indicates that chlorophyll and carotenoid biosynthesis pathways are tightly coordinated, remaining synchronized even when overall pigment levels decline in response to stress.

The relationships between NaCl concentration, growth, and physiology were highly species-specific, corroborating the ANOVA and regression findings. Radish (Figure 2A) showed the most significant correlations with salt. NaCl concentration was strongly and negatively correlated with fresh mass ($r = -0.75$, $p = 0.005$) and all pigment concentrations, including chlorophyll *a* ($r = -0.68$, $p = 0.015$), chlorophyll *b* ($r = -0.69$, $p = 0.014$), and carotenoids ($r = -0.72$, $p = 0.009$). This suggests a direct negative impact of salt on both biomass synthesis and a direct detrimental effect of salt concentration on the production of photosynthetic pigments in radish. In contrast, NaCl concentration was positively correlated with moisture content ($r = 0.60$, $p = 0.039$). In wheat (Figure 2B) the effect of salt was more specific. NaCl concentration was significantly negatively correlated with fresh mass ($r = -0.64$, $p = 0.024$) but showed no significant relationship with pigments or moisture content. Fresh mass was very strongly and positively linked to moisture content ($r = 0.84$, $p < 0.001$). Broccoli (Figure 2C) displayed a unique response. Unlike the other species, NaCl concentration was not significantly correlated with fresh mass or any of the pigment concentrations. Instead, it showed strong positive correlations with both moisture content ($r = 0.73$, $p = 0.017$) and the Chlorophyll *a/b* ratio ($r = 0.72$, $p = 0.008$). This pattern aligns with the distinct adaptive response and potential hormetic effect observed in the previous analyses.

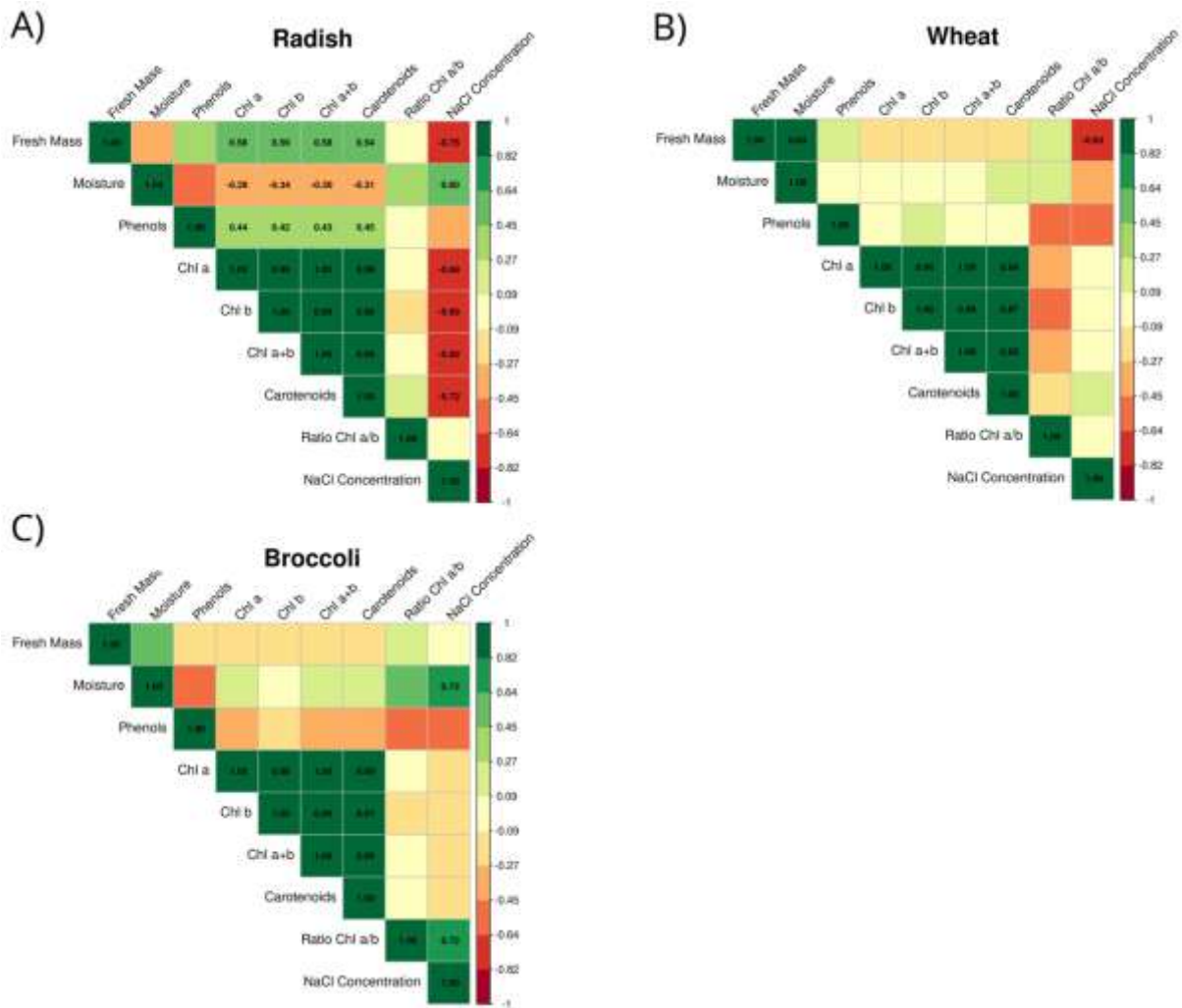


Figure 2. Pearson correlation heatmaps of physiological and biochemical parameters under NaCl treatment in microgreens of radish (A), wheat (B), and broccoli (C) showing only statistically significant ($p < 0.05$) correlations

A Principal Component Analysis (PCA) was performed to visualize the overall relationships among the measured variables and how the samples clustered by species and treatment (Figure 3). The first two principal components (PC1 and PC2) explained a combined 77% of the total variance in the dataset (56.9% and 20.1%, respectively). PC3 contributed an additional 13.3%, bringing the cumulative variance explained to 90.3% with three components. PC1 was primarily driven by positive loadings from photosynthetic pigments, carotenoids, fresh mass, and total phenols, while PC2 was defined by a strong positive loading for moisture content and a strong negative loading for the chlorophyll *a/b* ratio. The third component, PC3 was also significant and primarily represented a contrast, with strong negative loadings for fresh mass and phenols.

The PCA biplot revealed a clear separation of the three microgreen species into distinct clusters (Figure 3A). Radish samples clustered on the positive side of PC1, strongly associated with higher fresh mass, total phenols, and all photosynthetic pigments. Broccoli samples were separated along PC2, primarily driven by higher moisture content. Wheat formed a distinct cluster in the upper-left quadrant, characterized by a higher chlorophyll *a/b* ratio.

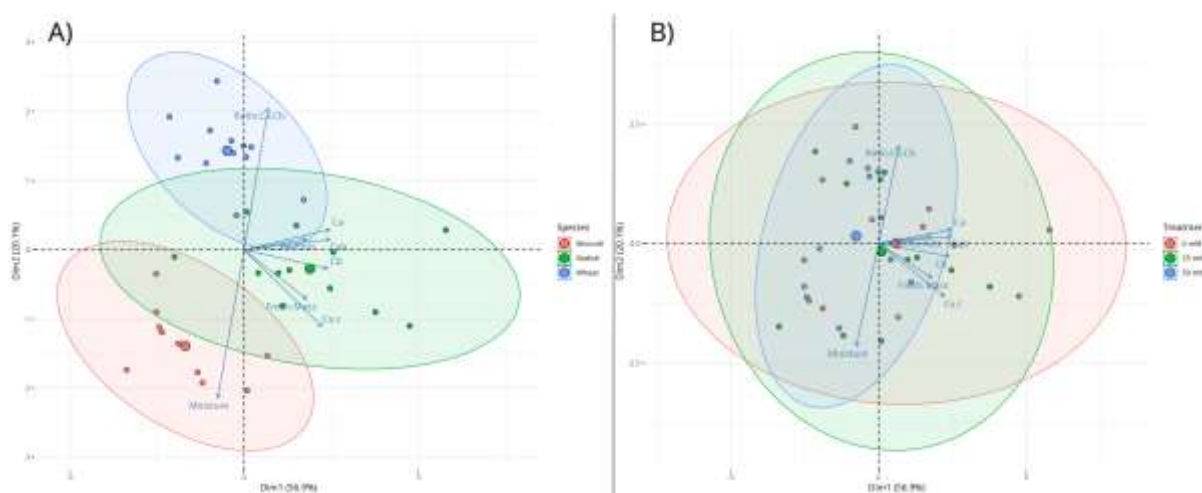


Figure 3. Principal Component Analysis (PCA) biplot of the measured physiological and biochemical parameters. Samples are grouped by (A) Species (Broccoli, Wheat, and Radish) and (B) Treatment (0, 25, and 50 mM NaCl), with 95% confidence ellipses shown.

When grouped by treatment, the clusters showed considerable overlap, indicating that the effect of species was a much stronger driver of variation than the effect of NaCl treatment (Figure 3B). However, a subtle trend was visible, with the 0 mM control samples generally shifted towards the positive side of PC1 and the 50 mM samples shifted towards the negative side, reflecting the overall stress response.

CONCLUSIONS

The response of microgreens to low to moderate NaCl stress (25 – 50 mM) is highly species-specific. Broccoli exhibited a hormetic effect and adaptive changes in chlorophyll *a/b* ratio at 25 mM NaCl. Wheat was highly tolerant, and maintained stable biomass and photosynthetic pigment levels across all treatments. In contrast, radish was highly sensitive to salinity stress, displaying linear declines in both yield and pigment content as NaCl concentration increased, suggesting a low threshold for salt tolerance despite its high productivity. The species also employed different physiological strategies. Broccoli and radish increased their moisture content as an osmotic adjustment mechanism, while wheat maintained stable moisture, suggesting different tolerance mechanisms. Total phenolic content decreased under both 25 mM and 50 mM NaCl across all species, suggesting growth-defense tradeoff hypothesis at these stress levels.

The genetic identity of the species is the primary factor determining its response to salinity, influencing both physiological and biochemical responses. These findings have practical implications for microgreens cultivation, particularly in regions with compromised water quality or in urban and controlled-environment agriculture settings. Wheat is a resilient choice for cultivation under saline conditions, low-salinity water (~25 mM) may be used to boost broccoli yields, while radish requires strict control of water salinity to maintain acceptable yield and nutritional value.

Future research should explore genotype variability within species to identify salt-tolerant cultivars, molecular mechanisms of salt tolerance, and the effects of combined environmental stressors to optimize microgreen production for both yield and nutritional quality.

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APPLICATIONS OF *ESCHERICHIA COLI* GENOME SCALE METABOLIC MODELS IN BIOTECHNOLOGY

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ABSTRACT

Genome-scale metabolic models (GEMs) provide a computational framework linking genes, proteins, and reactions across an organism's entire metabolism and can be used to simulate metabolic fluxes for diverse systems-level analyses. Since the first GEM was created for *Haemophilus influenzae* in 1999, significant progress has been made in developing and simulating GEMs for an expanding range of organisms, including bacteria, archaea, and eukaryotes. GEMs have applications, such as engineering strains for chemical and material production, identifying drug targets in pathogens, predicting enzyme functions, analyzing pan-reactomes, modeling interactions between multiple cells or species, and investigating human diseases (Gu et al., 2019). The genome-scale metabolic model (GEM) of *Escherichia coli* K-12 has been under development for more than ten years and is now widely utilized. Research using GEMs in *E. coli* has largely focused on six main areas: (1) metabolic engineering, (2) model-guided discovery, (3) prediction of cellular phenotypes, (4) analysis of biological network characteristics, (5) investigations of evolutionary dynamics, and (6) modeling interspecies interactions (McCloskey et al., 2013). This study summarizes these applications, evaluating both their achievements and limitations, while offering insight into potential future directions in modeling *Escherichia coli* metabolism. Collectively, studies over the past decade have built a genome-scale mechanistic understanding of the links between genotype and phenotype in *E. coli* metabolism, serving as a foundation for similar work in other microorganisms. Future challenges involve expanding GEMs to incorporate additional cellular processes beyond metabolism, identifying critical constraints from emerging data types, and developing computational approaches capable of accurately managing such large-scale network models.

Keywords: Genome scale metabolic model (GEM), *Escherichia coli*

INTRODUCTION

Most biopharmaceuticals approved for therapeutic use by regulatory agencies are proteins produced through recombinant DNA technology in different expression systems.

These products represent about one-sixth of the global pharmaceutical market and are its most rapidly expanding sector. Generally, biopharmaceuticals serve to replace or supplement essential proteins that the body lacks for normal physiological function. They can be broadly classified into categories such as blood factors, thrombolytics and anticoagulants, hormones, enzymes, growth factors, interferons, interleukins, vaccines, and monoclonal antibodies. By the end of 2011, out of 211 biopharmaceuticals approved, 66 (31%) were produced in *Escherichia coli*, 31 (15%) in yeast, and 91 (43%) in mammalian cells. Altogether, *E. coli*, yeast, and mammalian cells accounted for 89% of all approved biopharmaceuticals (Berlec and Štrukelj 2013).

The Gram-negative rod-shaped bacterium *Escherichia coli* has been widely utilized for producing heterologous proteins and is often the preferred host for recombinant protein expression due to several advantages. It is cost-effective, grows rapidly, supports high-cell-density fermentation, and has well-characterized biochemistry, physiology, and genetics, making it highly suitable for genetic manipulation. Numerous expression vectors and engineered strains have been developed to address diverse experimental requirements, significantly streamlining protein production. However, *E. coli* is not always the ideal host and sometimes requires optimization to meet specific needs. In particular, the expression of proteins that depend on disulfide bond formation for proper folding necessitates special attention, and modifying *E. coli* strains can substantially enhance the yield of functional, disulfide-containing proteins (Salinas et al. 2011). Systems biology, genome-scale metabolic modeling, and, in particular, metabolic engineering have proven to be powerful tools for the rational design and optimization of microbial strains. By integrating comprehensive biological data with computational modeling, these approaches allow researchers to predict and manipulate cellular metabolism in a targeted manner. This enables the development of microorganisms tailored for the efficient production of a wide array of valuable compounds, ranging from biofuels and industrial chemicals to pharmaceuticals and specialty biomolecules. Such strategies not only enhance production yields and process efficiency but also reduce the time and resources required for strain development compared to traditional trial-and-error methods. So far, modeling approaches have mostly applied constrained-based models, which seem to work well in the design of strains for the improved production of chemicals that belong to the central carbon metabolism (Carneiro et al., 2013).

Flux Balance Analysis (FBA) is a mathematical framework used to study the flow of metabolites through a metabolic network. It has become one of the most widely applied methods in systems biology, particularly for analyzing genome-scale metabolic models that

capture all known biochemical reactions and their associated genes in an organism. By converting metabolic reactions into a stoichiometric matrix and applying mass balance and flux constraints, FBA predicts how metabolites flow under steady-state conditions. The method allows researchers to estimate important biological outcomes such as growth rate or metabolite production without requiring kinetic parameters, which are often difficult to measure. Using linear programming, FBA identifies the optimal flux distribution that satisfies both the network constraints and a biological objective, such as maximizing biomass formation. FBA has proven effective in predicting microbial growth under different environmental and genetic conditions, such as aerobic versus anaerobic metabolism in *Escherichia coli*. It enables large-scale simulations of metabolic perturbations, including single and double gene knockouts, and provides insights into pathway redundancy and system robustness. Although FBA cannot predict metabolite concentrations or capture dynamic regulatory mechanisms, its simplicity and computational efficiency make it a cornerstone of metabolic modeling. Beyond basic physiological studies, it underpins applications in metabolic engineering, such as identifying genetic modifications for enhanced production of target compounds using algorithms like OptKnock. With tools such as the COBRA Toolbox, FBA continues to support the development and utilization of genome-scale metabolic reconstructions across a growing range of organisms (Orth et al., 2010).

This study provides an overview of *Escherichia coli* K12 genome scale metabolic models with their applications, assessing their successes and shortcomings, and highlighting possible avenues for future research. Over the past decade, such efforts have contributed to a genome-scale, mechanistic understanding of how genotype relates to phenotype in *E. coli* metabolism, forming a foundation for comparable studies in other microorganisms. Being important for pharmaceutical industry, *Escherichia coli* GEMs are important for metabolic engineering studies as well. Looking ahead, key challenges include extending GEMs to encompass cellular processes beyond metabolism, integrating constraints derived from new data types, and creating computational methods capable of efficiently handling these complex, large-scale network models.

1. *Escherichia coli* and its industrial importance

Escherichia coli is widely favored as a model organism and industrial workhorse due to its rapid growth in defined, inexpensive media, non-aggregating behavior, scalability, extensive genetic and molecular toolkits, and well-characterized transcriptome, proteome, and

metabolome. Many strains are classified as biosafety level 1, making them suitable even for educational purposes, such as school transformation kits. Typically, *E. coli* is a Gram-negative, rod-shaped bacterium about 1 μm long and 0.35 μm wide, though size can vary depending on strain and environmental conditions. Ecologically, *E. coli* is a facultative aerobe, capable of respiration in the presence of oxygen or fermentation in its absence. This adaptability allows it to colonize the infant gut first, consuming oxygen and enabling the establishment of strict anaerobes in the microbiota. Phylogenetically, it belongs to the Enterobacteriaceae family and is related to pathogens such as *Salmonella*, *Klebsiella*, and *Yersinia pestis*. While most *E. coli* strains are harmless, certain pathogenic strains contain genetic islands that confer tissue-specific virulence. From a biochemical perspective, *E. coli* cells are composed of roughly 55% protein, 25% nucleic acids, 9% lipids, 6% cell wall material, 2.5% glycogen, and 3% other metabolites. This composition is crucial for biotechnological applications because carbon flux—the flow of carbon through metabolic pathways—is tightly regulated by complex networks. Understanding and modeling these regulatory systems is essential for engineering novel metabolic pathways or optimizing existing ones (Idalia and Bernardo 2017). Among the various model organisms and genetic engineering tools available, *Escherichia coli* stands out both as a model organism and as what can be described as a “molecular biologist’s toolkit.” (Idalia and Bernardo 2017).

Blattner et al. (1997) determined the sequence of 4.64 Mbp genome of *E. coli* K-12 that encodes 4,288 proteins, with 38% of unknown function, and contains multiple paralogous gene families, including 80 ABC transporters. Its genome is highly organized with respect to replication and shows evidence of plasticity through insertion sequences, phage remnants, and horizontal gene transfer. Comparative analysis with other microbes highlights both widely shared and unique gene families (Blattner et al., 1997). *Escherichia coli* is one of the most widely utilized microorganisms for producing recombinant proteins. Its cost-effective scalability and flexibility as a host system make it an appealing alternative to non-microbial production methods for protein-based therapeutics. In fact, several FDA-approved pharmaceutical products—such as human insulin, somatotropin, human parathyroid hormone, and interferon alfa-2b—have been successfully produced using *E. coli*. The success of these products largely stems from the organism’s ability to serve as a microbial platform capable of rapidly and efficiently generating high-quality proteins while preserving the economic feasibility of large-scale industrial production (Carneiro et al. 2013). Beyond its widespread use in recombinant protein production, *Escherichia coli* is among the most extensively studied organisms, with its physiology well characterized. In *E. coli*, recombinant proteins are typically

synthesized in the cytoplasm, where they may either remain or be directed to other cellular compartments, most commonly the periplasm. They can also be localized to the inner or outer membranes, or in rare cases, secreted into the surrounding medium. Each of these compartments possesses distinct features that can be advantageous for recombinant protein expression and are often leveraged for that purpose. However, certain characteristics may hinder expression efficiency, prompting the development of strategies to minimize or overcome these challenges (Berlec and Štrukelj 2013). Using *E. coli* as a host for producing human therapeutic glycoproteins is generally more suitable for smaller and less complex proteins. Nevertheless, the advancements made over the past 12 years suggest that *E. coli* holds strong potential as a viable platform for industrial-scale glycoprotein production in the future (Jaffe et al. 2014). *E. coli* expression systems offer numerous advantages, such as fast growth, high protein expression levels, extensive understanding of its genetics, a wide variety of mutant host strains and expression vectors, and low-cost cultivation conditions. For these reasons, it is a popular choice for recombinant protein production (Huleani et al. 2021). For 40 years, *E. coli* has been a leading platform for recombinant protein production due to its efficiency, cost-effectiveness, and well-understood biology. While challenges remain for large or heavily glycosylated proteins, advances in strain engineering, secretion systems, and protein-folding strategies have expanded its capabilities. These improvements have allowed *E. coli* to compete with mammalian systems, exemplified by FDA-approved drugs like Beovu and Cablivi (McElwain et al., 2022). *Escherichia coli* is one of the most thoroughly studied and well-characterized bacteria, attracting interest for industrial, genetic, and pathological research; consequently, computational modeling approaches have been developed to describe and predict its cellular behavior (Reed et al., 2003). *Escherichia coli* central carbon metabolism that is vital for computational modeling is briefly depicted in Figure 1.

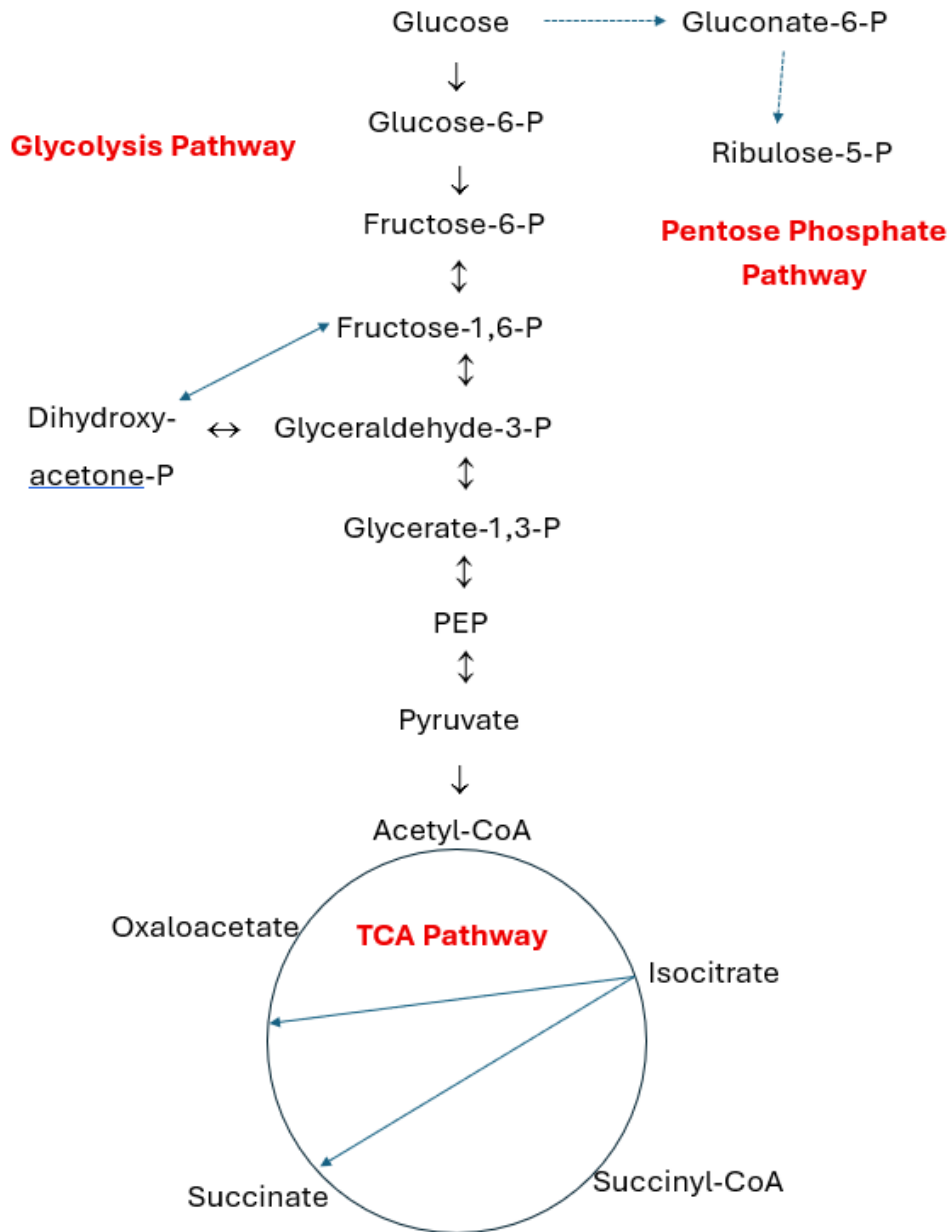


Figure 1. Brief representation of *Escherichia coli* central carbon metabolism (Adapted from Leatham-Jensen et al. 2016)

2. Genome scale metabolic models

Genome-scale metabolic models (GEMs) provide a computational framework linking genes, proteins, and reactions across an organism's complete metabolic network, allowing simulation-based prediction of metabolic fluxes for systems-level analyses (Gu et al., 2019). To reconstruct a genome-scale metabolic model (GEM), access to the organism's complete genome sequence is essential. Metabolic genes identified in the genome are linked to

biochemical reactions either manually or through automated annotation tools, with reactions connected by their shared metabolites. The resulting GEM is then represented mathematically as a stoichiometric matrix (S), which defines the proportions of metabolites in each reaction, and a flux vector (v), indicating the rate of each reaction. This mathematical formulation enables a quantitative analysis of the metabolic network, where the rate of metabolite accumulation is expressed as $S \times v$. However, it is often challenging to directly measure variations in internal metabolite concentrations experimentally, making simulation a practical alternative. To estimate reaction fluxes, researchers often apply the steady-state assumption, meaning that internal metabolite concentrations remain constant during the simulation ($S \times v = 0$). Despite this assumption, the system remains underdetermined, necessitating additional constraints. Typically, lower and upper bounds are set for reactions, which, for internal reactions, generally restrict fluxes to thermodynamically favorable directions within GEMs. For exchange reactions, experimental measurements—such as nutrient uptake and byproduct secretion rates—can be used to define flux bounds, adding further constraints. Even with these limits, the system is usually still underdetermined. To resolve this, flux balance analysis (FBA) assumes that cells optimize a specific objective function, defined as a linear combination of reaction fluxes. Linear programming can then be used to determine a flux distribution that maximizes or minimizes the objective function under the given constraints. While maximizing growth is commonly applied in microbial and cancer cell models, alternative objective functions can be used depending on the scientific question. By incorporating additional constraints, such as transcriptomic data and nutrient exchange rates, different flux distributions can be calculated, enabling comparisons of metabolic states under specific conditions. Therefore, GEM reconstruction and simulation provide a quantitative, mechanistic framework to study genotype–phenotype relationships. High-quality GEMs are essential for systematically understanding the metabolism of a target organism (Li et al., 2023).

Since the first GEM for *Haemophilus influenzae* was reported in 1999 (Gu et al., 2019), over 7,000 organisms from all three domains have had their genome-scale metabolic networks reconstructed (Heinken et al., 2021): GEMs initially created for model organisms, including *Escherichia coli* and *Saccharomyces cerevisiae*, have been extended to numerous microorganisms and multicellular systems, such as humans and plants (Gu et al., 2019). Advances in GEM reconstruction have enabled a broad spectrum of metabolic studies by allowing model-driven hypothesis generation and context-specific simulations (Gu et al., 2019). Key applications benefiting from these developments include, but are not limited to, microbial

strain engineering for bio-based chemical and material production, drug target identification in pathogens, enzyme function prediction, pan-reactome analyses, modeling intercellular or interspecies interactions, and elucidating human disease mechanisms (Gu et al., 2019). The scope of GEM applications is anticipated to grow even further in the coming years (Gu et al., 2019). Future applications are expected to include studying complex interactions among multiple cell types, microbial communities, and host–microbiota systems, enabling GEMs to address increasingly intricate biological questions (Gu et al., 2019). Traditional biochemical approaches have provided valuable insights but remain limited to specific pathways (Chung et al., 2021). Systems biology, particularly through genome-scale metabolic models (GEMs), now enables comprehensive *in silico* exploration of metabolism (Chung et al., 2021). By combining stoichiometric data with gene–protein–reaction associations, GEMs can simulate cellular function across diverse organisms (Chung et al., 2021). To improve their predictive accuracy, current efforts focus on integrating key regulatory layers, including transcriptional, post-translational, epigenetic, protein interaction, allosteric, and signaling mechanisms (Chung et al., 2021). Over the past two decades, genome-scale metabolic network models (GEMs) have evolved from first-generation models with limited predictive accuracy to next-generation models incorporating omics data, additional constraints, integrated networks, and whole-cell models (Ye et al, 2022). These advances have enabled applications in industrial biotechnology, such as phenotype prediction and metabolic engineering, as well as in human health for biomarker and drug target discovery (Ye et al, 2022). Recent integration of machine learning with GEMs allows dynamic parameter optimization and precise prediction of metabolic fluxes, while mechanistic “white-box” approaches provide insights into underlying cellular processes (Ye et al, 2022). Together, these developments enhance both the predictive power and biological interpretability of GEMs (Ye et al, 2022). Constraint-based metabolic modeling and genome-scale metabolic models (GEMs) are widely used to investigate metabolic processes, particularly in cancer research, where they help reveal metabolic rewiring mechanisms and identify potential drug repurposing opportunities and off-target effects (Kishk et al., 2022). A GEM provides an *in silico* representation of cellular metabolism, capturing metabolite–reaction interactions through a sparse stoichiometric matrix and linking genes to reactions using Boolean gene–protein–reaction (GPR) associations (Kishk et al., 2022). Beyond cancer, GEMs have been applied to investigate the microbiome’s role in Parkinson’s disease, psychiatric and neurodegenerative disorders like Alzheimer’s, and Parkinson’s-like conditions in mice (Kishk et al., 2022). Over the past 15 years, GEMs have been reconstructed for humans and various model animals (e.g., mouse, rat, zebrafish, CHO cells) to quantitatively simulate metabolic

networks, study diseases, and guide biopharmaceutical design (Li et al., 2023). These models help uncover tissue-specific metabolic functions, predict disease mechanisms, identify biomarkers and drug targets, and optimize therapeutic protein production (Li et al., 2023). Despite major advances—from early human models like Recon1 to the highly curated Human1—challenges remain in integrating regulatory and omics data, refining model accuracy, and developing personalized, whole-body simulations that could ultimately support precision medicine (Li et al., 2023).

3. Genome scale metabolic models for *Escherichia coli* K-12

Reconstruction of the metabolic models of *E. coli* has been progressing since 1990 (Reed and Palsson 2003). Edwards and Palsson (2000) integrated biochemical literature, annotated genome sequence data, and strain-specific information to construct the first genome-scale *in silico* model representing the metabolic functions of *Escherichia coli* MG1655 with 720 reactions and 436 metabolites. Using flux balance analysis (FBA), Edwards and Palsson (2000) simulated the effects of gene deletions in central metabolism and compared the computational predictions with experimental results. The *in silico* model accurately predicted growth outcomes for about 86% of mutant strains, demonstrating that genome-based metabolic reconstructions can effectively link genetic information to metabolic behavior through systems-level analysis. The first genome-scale metabolic (GEM) model for *Escherichia coli* accounting for 660 gene products was named as *iJE660* (Edwards and Palsson 2000, Reeds et al., 2003). *iJE660* was used to perform *in silico* gene deletion studies, to predict both optimal growth behavior and the outcome of adaptive evolution (Reeds et al. 2003).

An updated annotation of the *E. coli* K-12 genome was published in 2001 (Serres et al., 2001), which motivated Reeds et al. (2003) to generate the *iJR904* GEM/GPR model expanding on the earlier *iJE660a* by incorporating 904 genes, 931 unique reactions, 625 metabolites, and direct gene–protein–reaction (GPR) associations. Gene–protein–reaction associations (GPRs) were included for the first time to connect reactions with genes, and all reactions were elementally and charged balanced through the inclusion of protons. New genes and reactions improved the model’s predictive accuracy. This study also identified potential ORFs that could fill gaps in the metabolic network (Reed et al. 2003).

Thereafter Feist et al. (2007) expanded the scope of network and enhanced the reconstruction of the *E. coli* metabolic network *iAF1260* based on *iJR904* (Reed et al, 2003), the current functional annotation of the *E. coli* genome (Riley et al, 2006), content characterized in the EcoCyc Database and specific biochemical characterization studies on the metabolic

machinery and capabilities of *E. coli*. The major areas of expansion of iAF1260 over previous *E. coli* network reconstructions come under the following five categories: (i) increased scope, (ii) compartmentalization, (iii) increased pathway detail, (iv) incorporation of reaction thermodynamics and (v) alignment with EcoCyc. Feist et al. (2007) included many reactions for the synthesis of cell wall components, and all metabolites were assigned to the cytoplasm, periplasm, or extracellular space. Applications demonstrating the capabilities of the genome-scale metabolic model to predict high-throughput experimental growth and gene deletion phenotypic screens were presented. A core model version of iAF1260, useful for testing and debugging new constraint-based algorithms and for educational use, was also published (Orth et al, 2010).

Orth et al. (2011) presented iJO1366, which accounts for 1366 genes, 2251 metabolic reactions, and 1136 unique metabolites. Although *E. coli* metabolism has been extensively studied for many years, the updates incorporated into the iJO1366 reconstruction stem from ongoing discoveries that continue to advance understanding of this organism. The iJO1366 model is an updated reconstruction of the *E. coli* metabolic network, expanding on the previous iAF1260 version (Table 1). It incorporates newly identified genes and reactions discovered since 2007, including data from knockout strain experiments in various media. By addressing gaps in the earlier model, iJO1366 offers a more complete and accurate representation of *E. coli* metabolism. It also serves as a foundation for reconstructing metabolic networks of other *E. coli* strains and related species, providing insights that can support future research and discoveries.

Recent advances in DNA sequencing provided multiple *Escherichia coli* genome sequences, raising questions about the definitions of “strain” and “species.” Therefore, Monk et al. (2013) constructed genome-scale metabolic models (GEMs) for 55 *E. coli* strains to map both species-wide and strain-specific metabolic networks. These models were used to compare core and pan-metabolic functions, predict growth on over 650 nutrients under aerobic and anaerobic conditions, and explore the genetic basis of strain-specific auxotrophies. Experimental validation of selected strains and compounds confirmed the models’ predictions with 80% accuracy, highlighting functional differences between strains and identifying niche-specific growth capabilities.

Weaver et al. (2014) presented EcoCyc–18.0–GEM, a genome-scale model of the *E. coli* K–12 MG1655 metabolic network with 1445 genes (Table 1). The *E. coli* model was developed using the EcoCyc bioinformatics database. The model was validated through a three-part approach: (i) Simulated growth on glucose under aerobic and anaerobic conditions was

compared with experimental chemostat data and previous *E. coli* modeling results. (ii) Gene essentiality was assessed for 1,445 genes, with the EcoCyc–18.0–GEM model reaching 95.2% accuracy in predicting growth outcomes for experimental gene knockouts. (iii) Nutrient utilization was predicted across 431 different media conditions, with the model achieving an overall accuracy of 80.7%.

Monk et al. (2017) developed iML1515 model that presents the most comprehensive genome-scale reconstruction of the *E. coli* K-12 MG1655 metabolic network to date. Covering 1,515 open reading frames, 2,719 metabolic reactions, and 1,192 unique metabolites (Table 1), iML1515 integrates transcriptomic, proteomic, and metabolomic data and links 1,515 protein structures, providing a bridge between systems and structural biology. The model has been applied to construct metabolic networks for *E. coli* strains from the human gut microbiome, predict metabolic capabilities of clinical isolates, and perform comparative structural proteome analyses across 1,122 strains to identify multi-strain sequence variations. Ye et al. (2020) used the GECKO approach to develop an enzyme-constrained *Escherichia coli* model, ec_iML1515. After validation with experimental data, ec_iML1515 demonstrated greater accuracy than conventional genome-scale metabolic models. Analysis with this model revealed that protein demand and synthesis rates are critical for lysine production. To optimize lysine yield, modular engineering was applied to enhance expression of highly demanded proteins, while synthesis of energy-related proteins was controlled via NH_4^+ and dissolved oxygen (DO) levels. These strategies together resulted in a lysine titer of 193.6 ± 1.8 g/L and a yield of 0.74 g/g glucose, showing that incorporating enzymatic constraints enables accurate intracellular phenotype predictions and rational design for targeted metabolite production. Thereafter, Mao et al. (2022) developed a Python-based workflow, ECMpy, to create enzyme-constrained metabolic models, leading to the construction of eciML1515 for *E. coli* based on its latest metabolic model iML1515. By adding enzyme amount constraints and accounting for protein composition, the model accurately predicted overflow metabolism, improved growth rate predictions on 24 carbon sources, and revealed a tradeoff between enzyme efficiency and biomass yield under varying substrate consumption.

Table 1. *Escherichia coli* Genome Scale Metabolic Models

GEM	Gene #	Reaction #	Metabolite #	Reference
<i>iJE660</i>	660	720	436	Edwards and Palsson (2000)
<i>iJR904</i>	904	931*	625	Reeds et al. (2003)
<i>iAF1260</i>	1260	2077	1039*	Feist et al. (2007)
<i>iJO1366</i>	1366	2251	1136	Orth et al. (2011)
EcoCyc–18.0–GEM	1445	2286*	1453	Weaver et al. (2014)
<i>iML1515</i>	1515	2719	1192*	Monk et al. (2017)
ec_ <i>iML1515</i>	1515	6101	3610	Ye et al. (2020)
eciML1515	1515	-	-	Mao et al. (2022)

*unique

DISCUSSION

Unraveling the intricate connection between genotype (an organism's genetic makeup) and phenotype (its observable traits) requires an integrated, interdisciplinary approach. Genome-scale metabolic models (GEMs) are a prime example of this integration, as they combine genomic information with biochemical knowledge to mathematically simulate cellular metabolism. This process cannot be achieved through biology alone — it demands the combined efforts of biological sciences to understand molecular mechanisms, computer science to handle and model large-scale biological data, and quantitative analysis to predict how genetic or environmental changes affect cellular behavior (Edwards and Palsson, 2000). Together, these fields enable GEMs to serve as powerful tools in modern bioengineering, shaping how scientists design, analyze, and manipulate living systems for applications in biotechnology, medicine, and synthetic biology.

E. coli genome-scale metabolic models (GEMs) link genes, enzymes, and reactions to predict metabolism and guide research. They are used for metabolic engineering, discovering gene functions, predicting phenotypes, analyzing networks, studying evolution, and modeling interspecies interactions. In this work, all genome-scale metabolic models (GEMs) of *Escherichia coli* that have been constructed since 2000 were comprehensively analyzed, focusing on the progression of model complexity, data integration strategies, and their contributions to understanding *E. coli* metabolism. Future progress depends on integrating additional cellular processes and improving computational tools to better connect genotype to phenotype (McCloskey et al., 2013). Many *E. coli* K-12 MG1655 genes remain

uncharacterized, with several predicted to play roles in metabolism (Riley et al., 2006). As research progresses, further updates to the *E. coli* metabolic network will be necessary. Future expansions are expected to involve integrating this metabolic network with other cellular systems, such as transcription, translation, and transcriptional regulation (Orth et al., 2011).

CONCLUSIONS

The future of *Escherichia coli* genome-scale metabolic models (GEMs) lies in creating highly integrative and predictive frameworks. Next-generation GEMs will combine multi-omics data—including genomics, transcriptomics, proteomics, and metabolomics—with regulatory and signaling networks to capture context-dependent cellular behavior. Enzyme-constrained approaches will refine predictions by accounting for protein allocation and kinetics, while strain-specific and microbiome-level models will enable analysis of niche adaptations and interspecies interactions. Advances in machine learning and AI will further improve model accuracy, help fill gaps in metabolic networks, and guide rational strain design for biotechnology applications such as biofuels, pharmaceuticals, and high-value chemicals. Ultimately, these developments aim to mechanistically link genotype to phenotype, allowing precise predictions of growth, metabolic fluxes, and evolutionary responses under diverse conditions.

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ADVANCES IN GENOME SCALE METABOLIC MODELING OF *CHLAMYDOMONAS REINHARDTII*

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Algae and marine microorganisms contribute nearly half of global inorganic carbon fixation. As atmospheric CO₂ levels increase, understanding how photosynthetic organisms convert CO₂ into essential metabolites and biomolecules is becoming increasingly important. These organisms not only capture CO₂ but can also serve as sustainable platforms for producing bulk chemicals, since their key inputs—light and CO₂—are freely available. To exploit this potential, a detailed understanding of photosynthetic metabolism is required (Boyle and Morgan 2009). Flux Balance Analysis (FBA) is a computational approach used to study the flow of metabolites through metabolic networks. It models metabolism as a set of stoichiometric equations representing the balance between reactants and products in each biochemical reaction, forming a stoichiometric matrix (*S*). By applying mass balance and thermodynamic constraints, FBA defines the feasible space of possible metabolic flux distributions. A biological objective, such as maximizing biomass or metabolite production, is then optimized using linear programming to predict cellular phenotypes like growth rates or product yields. Unlike kinetic models, FBA does not require rate constants, allowing rapid and large-scale simulations of genome-scale networks. It has been widely applied to predict metabolic behavior under genetic or environmental perturbations, identify essential genes, and guide metabolic engineering strategies for improved production of desired compounds (Orth et al. 2010). Flux balance analysis (FBA), a constraint-based modeling approach, enables prediction of metabolic fluxes using stoichiometric information and physiological constraints. It has been successfully applied to multiple model organisms to predict gene knockout viability, theoretical yields, and metabolic bottlenecks. Previous studies have used FBA to investigate photosynthetic metabolism in *Synechocystis* and *Arthrospira platensis* (Boyle and Morgan 2009). In this study, the green alga *Chlamydomonas reinhardtii* was selected as a representative eukaryotic model for photosynthetic metabolism due to its well-characterized genome, experimental tractability, and relevance as an intermediate between cyanobacteria and higher plants. Developments of genome scale metabolic models for *Chlamydomonas reinhardtii* are summarized in this study.

Keywords: Genome scale metabolic model (GEM), *Chlamydomonas reinhardtii*

INTRODUCTION

Human actions are constantly altering the global ecosystem. A major concern is the rising concentration of atmospheric and dissolved CO₂, largely resulting from our reliance on fossil fuels for energy production. The anticipated outcomes of these activities—such as global warming, climate change, and ocean acidification—pose serious threats to both terrestrial and aquatic environments. Because photosynthetic organisms can capture solar energy and convert CO₂, they play a crucial role in efforts to develop sustainable societies for the future (Imam et al. 2015). Algae and other marine organisms contribute to fixing nearly half of the Earth's atmospheric inorganic carbon. As carbon dioxide levels continue to rise, understanding how photosynthetic organisms transform CO₂ into metabolites and other essential compounds has become increasingly critical. Beyond their natural carbon-fixing ability, these organisms hold great promise for producing low-cost bulk chemicals, as their main inputs—light and carbon dioxide—are freely available. Nevertheless, realizing this potential through metabolic engineering requires a more comprehensive understanding of photosynthetic metabolism (Boyle and Morgan 2009).

Microalgae-based technologies that integrate air emission bioremediation with biomass production offer a promising alternative for reducing atmospheric pollutants while generating renewable biomass suitable for bioenergy and other valuable bioproducts. Through photosynthesis, microalgae can capture CO₂ and accumulate biomass, thereby lowering the net CO₂ emissions associated with biofuel production and promoting environmentally sustainable solutions such as third-generation biofuels. As a result, microalgae have gained increasing attention as sustainable sources of bioactive compounds with applications across multiple fields, including nutrition, aquaculture, pharmaceuticals, and biofuels (Winck et al. 2016). Microalgae can synthesize a diverse array of compounds, including carotenoids, lipids, hydrogen, proteins, and starch (Kliphuis et al. 2012). These substances have a wide range of uses, from fine chemicals and biofuels to food additives (Gomes de Oliveira Dal'Molin et al. 2011; Kliphuis et al. 2012). Compounds such as hydrogen, methane, triacylglycerols, and ethanol produced from algae could serve as biofuels, though significant challenges remain (Gomes de Oliveira Dal'Molin et al. 2011). For large-scale commercial production to be economically viable, it is essential to maximize algal biomass yield and optimize its composition. A thorough understanding of how these compounds are generated within algal

metabolism is crucial to fully harness the potential of microalgae and their products (Kliphuis et al. 2012).

Chlamydomonas reinhardtii is the best-studied organism in terms of genome annotation and molecular mechanisms, within all microalgae, making it the reference organism for studying algal lipid metabolism (Yao et al. 2023). *Chlamydomonas reinhardtii* is the focus of extensive research over recent decades. It is considered a model organism for green microalgae due to its versatile metabolism and its capacity to grow both photoautotrophically and heterotrophically on acetate (Kliphuis et al. 2012). Furthermore, *C. reinhardtii* can accumulate starch and generate hydrogen under anaerobic conditions. The sequencing of its genome (Merchant et al., 2007) offers a valuable foundation for constructing metabolic network models (Kliphuis et al. 2012). Achieving the production rates and concentrations needed for commercial viability will require system-level metabolic engineering for *Chlamydomonas reinhardtii* (Gomes de Oliveira Dal'Molin et al. 2011). In this context, genome-scale metabolic reconstructions (GEMs) are employed to integrate and analyze large omics datasets and to test metabolic designs computationally (Gomes de Oliveira Dal'Molin et al. 2011). GEMs link annotated metabolic genes and proteins to reactions based on current knowledge of the organism (Gomes de Oliveira Dal'Molin et al. 2011). Numerous studies have shown that carefully curated GEMs can guide the creation of engineered strains in ways that are often non-intuitive (Gomes de Oliveira Dal'Molin et al. 2011).

In this study, a comprehensive overview of genome-scale metabolic modeling efforts for *Chlamydomonas reinhardtii*, a model green microalga, is provided. These modeling studies aim to systematically capture the organism's complex metabolic network, integrating information from genomics, transcriptomics, and biochemical data. By summarizing the various models developed to date, including their construction methods, scope, and applications, this work highlights how genome-scale models have advanced our understanding of *C. reinhardtii* metabolism. Moreover, these models provide valuable insights for optimizing the production of biotechnologically relevant compounds such as starch, lipids, and hydrogen, ultimately supporting efforts to harness the full potential of this versatile microalga for research and industrial purposes.

***Chlamydomonas reinhardtii* and its importance**

Chlamydomonas reinhardtii which is a ~10- μ m, unicellular, soil-dwelling green alga with multiple mitochondria, two anterior flagella for motility and mating, and a chloroplast that houses the photosynthetic apparatus and critical metabolic pathways is a single-celled green alga that separated from the evolutionary line of land plants more than a billion years ago. It

serves as a model organism for investigating chloroplast-dependent photosynthesis and the structure, development, and operation of eukaryotic flagella—cellular features inherited from the common ancestor of plants and animals but lost in terrestrial plants. The sequencing of its ~120-megabase nuclear genome, along with comparative phylogenomic studies, identified numerous previously uncharacterized genes likely involved in chloroplast and flagellar formation and activity. These genomic insights enhance understanding of early eukaryotic cells, uncover novel genes related to photosynthetic and flagellar processes, resulting in the prediction of 15,000 protein-coding genes (Merchant et al. 2007; Boyle and Morgan 2009). The availability of a full genome sequence for this species (Merchant et al., 2007) means that scientists can now adopt a systems level approach to understanding metabolism (Chapman et al. 2015). *Chlamydomonas reinhardtii* has long been utilized as a model organism for investigating photosynthesis. In recent years, however, it has also gained interest as a promising platform for biofuel production. This microalga is a facultative phototroph, capable of using light and atmospheric CO₂ as its sole carbon source, but it can also grow mixotrophically when provided with additional organic carbon sources, such as acetate (Chapman et al. 2015). Additionally, *C. reinhardtii* can grow without photosynthesis by utilizing acetate as a carbon source, although its heterotrophic growth is limited to acetate and other two-carbon compounds in the absence of light (Boyle and Morgan 2009). Its relatively simple structure and metabolism make it an ideal link between higher plants and cyanobacteria in photosynthetic research. As the first algal species with a fully sequenced genome, it has provided valuable insights into gene functions (Boyle and Morgan 2009). *Chlamydomonas reinhardtii* gained considerable attention for its potential applications in biofuel and hydrogen production (May et al. 2009).

Genome scale metabolic models

Genome-scale metabolic model (GEM) is a species-specific biological reaction network (Figure 1), usually reconstructed by researchers from an annotated genome of the organism (Yao et al. 2023). GEMs provide a computational framework linking genes, proteins, and reactions across an organism's complete metabolic network, allowing simulation-based prediction of metabolic fluxes for systems-level analyses (Gu et al., 2019). To reconstruct a genome-scale metabolic model (GEM), access to the organism's complete genome sequence is essential. Metabolic genes identified in the genome are linked to biochemical reactions either manually or through automated annotation tools, with reactions connected by their shared metabolites. The resulting GEM is then represented mathematically as a stoichiometric matrix

(S), which defines the proportions of metabolites in each reaction, and a flux vector (v), indicating the rate of each reaction. This mathematical formulation enables a quantitative analysis of the metabolic network, where the rate of metabolite accumulation is expressed as $S \times v$. However, it is often challenging to directly measure variations in internal metabolite concentrations experimentally, making simulation a practical alternative. To estimate reaction fluxes, researchers often apply the steady-state assumption, meaning that internal metabolite concentrations remain constant during the simulation ($S \times v = 0$). Despite this assumption, the system remains underdetermined, necessitating additional constraints. Typically, lower and upper bounds are set for reactions, which, for internal reactions, generally restrict fluxes to thermodynamically favorable directions within GEMs. For exchange reactions, experimental measurements—such as nutrient uptake and byproduct secretion rates—can be used to define flux bounds, adding further constraints. Even with these limits, the system is usually still underdetermined. To resolve this, flux balance analysis (FBA) assumes that cells optimize a specific objective function, defined as a linear combination of reaction fluxes. Linear programming can then be used to determine a flux distribution that maximizes or minimizes the objective function under the given constraints. While maximizing growth is commonly applied in microbial and cancer cell models, alternative objective functions can be used depending on the scientific question. By incorporating additional constraints, such as transcriptomic data and nutrient exchange rates, different flux distributions can be calculated, enabling comparisons of metabolic states under specific conditions. Therefore, GEM reconstruction and simulation provide a quantitative, mechanistic framework to study genotype–phenotype relationships. High-quality GEMs are essential for systematically understanding the metabolism of a target organism (Li et al., 2023). A genome-scale metabolic model, which focuses solely on predicting metabolic fluxes, is therefore formulated as a linear optimization problem and can typically be solved in about 0.1 seconds using flux balance analysis within the COBRA Toolbox (Yao et al. 2023).

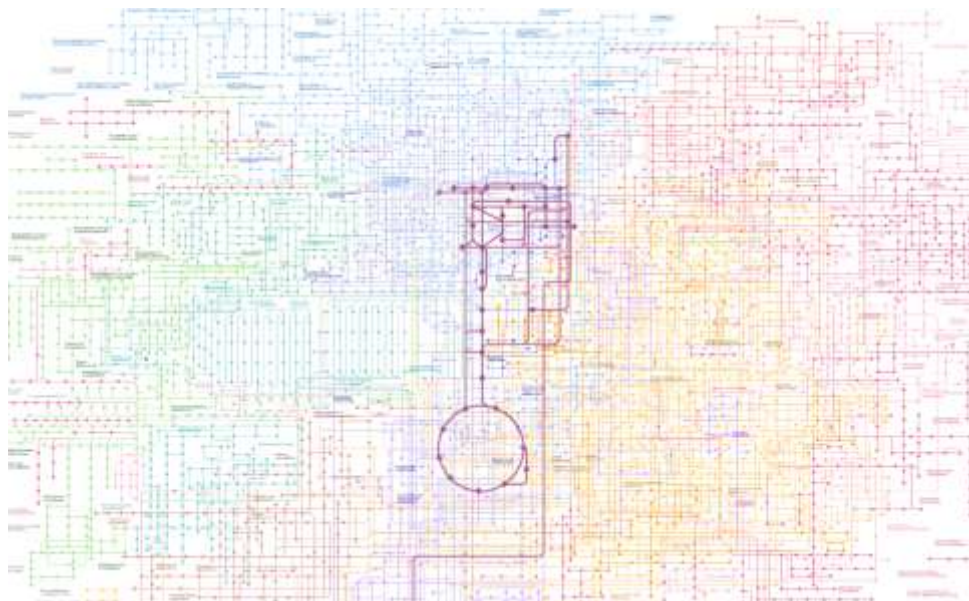


Figure 1. Metabolic pathways (<https://www.kegg.jp/pathway/map01100>, Accessed 23.10.2025)

Since the first GEM for *Haemophilus influenzae* was reported in 1999 (Gu et al., 2019), over 7,000 organisms from all three domains have had their genome-scale metabolic networks reconstructed (Heinken et al., 2021): GEMs initially created for model organisms, including *Escherichia coli* and *Saccharomyces cerevisiae*, have been extended to numerous microorganisms and multicellular systems, such as humans and plants (Gu et al., 2019). Advances in GEM reconstruction have enabled a broad spectrum of metabolic studies by allowing model-driven hypothesis generation and context-specific simulations (Gu et al., 2019). Key applications benefiting from these developments include, but are not limited to, microbial strain engineering for bio-based chemical and material production, drug target identification in pathogens, enzyme function prediction, pan-reactome analyses, modeling intercellular or interspecies interactions, and elucidating human disease mechanisms (Gu et al., 2019). The scope of GEM applications is anticipated to grow even further in the coming years (Gu et al., 2019). Future applications are expected to include studying complex interactions among multiple cell types, microbial communities, and host–microbiota systems, enabling GEMs to address increasingly intricate biological questions (Gu et al., 2019). Traditional biochemical approaches have provided valuable insights but remain limited to specific pathways (Chung et al., 2021). Systems biology, particularly through genome-scale metabolic models (GEMs), now enables comprehensive *in silico* exploration of metabolism (Chung et al., 2021). By combining stoichiometric data with gene–protein–reaction associations, GEMs can simulate cellular

function across diverse organisms (Chung et al., 2021). To improve their predictive accuracy, current efforts focus on integrating key regulatory layers, including transcriptional, post-translational, epigenetic, protein interaction, allosteric, and signaling mechanisms (Chung et al., 2021). Over the past two decades, genome-scale metabolic network models (GEMs) have evolved from first-generation models with limited predictive accuracy to next-generation models incorporating omics data, additional constraints, integrated networks, and whole-cell models (Ye et al, 2022). These advances have enabled applications in industrial biotechnology, such as phenotype prediction and metabolic engineering, as well as in human health for biomarker and drug target discovery (Ye et al, 2022). Recent integration of machine learning with GEMs allows dynamic parameter optimization and precise prediction of metabolic fluxes, while mechanistic “white-box” approaches provide insights into underlying cellular processes (Ye et al, 2022). Together, these developments enhance both the predictive power and biological interpretability of GEMs (Ye et al, 2022). Constraint-based metabolic modeling and genome-scale metabolic models (GEMs) are widely used to investigate metabolic processes, particularly in cancer research, where they help reveal metabolic rewiring mechanisms and identify potential drug repurposing opportunities and off-target effects (Kishk et al., 2022). A GEM provides an *in silico* representation of cellular metabolism, capturing metabolite–reaction interactions through a sparse stoichiometric matrix and linking genes to reactions using Boolean gene–protein–reaction (GPR) associations (Kishk et al., 2022). Beyond cancer, GEMs have been applied to investigate the microbiome’s role in Parkinson’s disease, psychiatric and neurodegenerative disorders like Alzheimer’s, and Parkinson’s-like conditions in mice (Kishk et al., 2022). Over the past 15 years, GEMs have been reconstructed for humans and various model animals (e.g., mouse, rat, zebrafish, CHO cells) to quantitatively simulate metabolic networks, study diseases, and guide biopharmaceutical design (Li et al., 2023). These models help uncover tissue-specific metabolic functions, predict disease mechanisms, identify biomarkers and drug targets, and optimize therapeutic protein production (Li et al., 2023). Despite major advances—from early human models like Recon1 to the highly curated Human1—challenges remain in integrating regulatory and omics data, refining model accuracy, and developing personalized, whole-body simulations that could ultimately support precision medicine (Li et al., 2023).

Genome scale metabolic models for *Chlamydomonas reinhardtii*

May et al. (2008) combined bioinformatics with metabolomics and proteomics techniques to identify 1069 proteins and 159 metabolites; providing an approach in which computational metabolic modeling is complemented by proteomics and metabolomics data for *Chlamydomonas reinhardtii*. Thereafter, Boyle and Morgan (2009) reconstructed a compartmental metabolic network for primary metabolism in the green alga *C. reinhardtii*. Authors developed a stoichiometric model representing the primary metabolism of *C. reinhardtii* using information from genomic and pathway databases along with published literature using compartments cytosol, chloroplast and mitochondria. This network with 458 metabolites and 484 reactions (Table 1) encompasses key routes of central metabolism—including glycolysis, the TCA cycle, and both oxidative and reductive pentose phosphate pathways—as well as the biosynthetic pathways for amino acids, nucleotides, chlorophyll, lipids, and starch. Intracellular fluxes were calculated using FBA for three growth conditions, autotrophic, heterotrophic, and mixotrophic. A mixed integer linear program was formulated in the GAMS environment (GAMS Development Corporation, Washington, DC) and the optimum solution was found using the ILOG CPLEX 8.100 solver (ILOG, Inc. Mountain View, CA) (Boyle and Morgan 2009). Meanwhile, Manichaikul et al. (2009) generated a central metabolic network reconstruction of *C. reinhardtii* accounting with reactions localized to the cytosol, mitochondria, chloroplast (including the lumen as a subcompartment for photosynthesis) glyoxysome and flagellum. The resulting network reconstruction, named iAM303, accounted for 259 reactions and 267 metabolites (Manichaikul et al. 2009). Although these two models are valuable, their incorporation of cellular compartmentalization and genomic data was largely restricted to central metabolic pathways.

Gomes de Oliveira Dal'Molin et al. (2011) developed a genome-scale metabolic model called AlgaGEM for *Chlamydomonas reinhardtii*, representing the metabolism of a compartmentalized algal cell. The model integrates 866 unique genes, 1,862 metabolites, 1,725 reactions, over 2,200 gene-enzyme-reaction associations and four cellular compartments. It organizes metabolism across the cytoplasm, mitochondrion, plastid, and microbody, using both algal data and compartment information from *Arabidopsis thaliana*. AlgaGEM, which does rely on AraGEM for the compartmentalisation of reactions for which no data exist for *C. reinhardtii*, accurately represents primary metabolism and predicts specific algal behaviors, such as phosphoglycolate catabolism rather than recycling during photorespiration. The model was validated against growth and metabolic data under autotrophic, heterotrophic, and mixotrophic

conditions. It also predicts increased hydrogen production when cyclic electron flow is disrupted, reproducing observations from high-producing mutants, and highlights pathways and targets to enhance H₂ yield. AlgaGEM was created and curated in Excel for easy annotation. A 2D reaction-centered SBML version was generated using a custom Java application, with gene–protein–reaction associations added as notes since SBML lacks a dedicated element for them. Constraint-based modeling was performed using the COBRA Toolbox in MATLAB, and simulated flux distributions were visualized on a compartmentalized algal cell metabolic map drawn in Excel. Chang et al. (2011) developed *iRC1080* which is the genome-scale *C. reinhardtii* metabolic network accounting for 1080 genes, associated with 2190 reactions and 1068 unique metabolites, distributed across 10 compartments (Table 1); devising a novel light-modeling approach that enables quantitative growth prediction for a given light source, resolving wavelength and photon flux. *iRC1080* was reported to be the first network to account for detailed photon absorption permitting growth simulations under different light sources; validated in content and function, and its application for detailed modeling of diverse light sources (Chang et al. 2011).

Kliphuis et al. (2012) constructed a metabolic network describing the primary metabolism of *Chlamydomonas reinhardtii* with 160 reactions and 164 compounds and two cell compartments, the chloroplast and the cytosol, to be able to uncouple the Calvin cycle, the PPP, and the production and consumption of NADPH. For the light reaction, only linear electron transport was modeled. Energy parameters for maintenance and biomass formation were obtained by performing chemostat experiments. Significant improvements in annotation resulting from re-sequencing of the *C. reinhardtii* genome combined with better gene models (Blaby et al., 2014) motivated Imam et al. (2015) to introduce an updated and substantially enhanced genome-scale metabolic model for the extensively studied microalga *Chlamydomonas reinhardtii*. This model, named *iCre1355*, marks a significant improvement over earlier versions in terms of both scope and predictive capability. It integrates a wide array of metabolic processes encoded in the nuclear, chloroplast, and mitochondrial genomes, incorporating 1355 genes (1460 transcripts), 2394 reactions, and 1133 metabolites (Table 1). *iCre1355* was shown to be a better predictor of gene essentiality, growth rate and lipid yield than the previous genome-scale metabolic model, representing a high-quality, experimentally validated GEM and a powerful resource for performing systems-level analysis of *C. reinhardtii* and related microalgae. The previously developed genome-scale model *iRC1080* for *Chlamydomonas reinhardtii* was used as the foundation for reconstructing the updated model. Refinement began by collecting *C. reinhardtii*-specific metabolic and genomic data from four

major databases: ChlamyCyc 4.0 and KEGG (for metabolic pathways), NCBI (for chloroplast and mitochondrial genomes), and Phytozome 10.1 (for nuclear genome data, version 5.5). From this integrated dataset, 586 potential metabolic genes that were not represented in *iRC1080* were identified for manual review. The curation process included literature verification of gene functions, domain analysis using Pfam and BLAST to confirm enzymatic activity and gene–protein–reaction (GPR) associations, and evaluation of network connectivity to remove isolated genes. This systematic process yielded a validated set of 312 genes (320 transcripts) and 153 corresponding metabolic reactions. Simulations were conducted in the GAMS programming environment (GAMS Development Corporation, <https://www.gams.com/>) using the CPLEX solver. *iCre1355* in GAMS and SBML formats were provided. Winck et al. (2016) enhanced the *Chlamydomonas reinhardtii* *iRC1080* metabolic model by integrating genomic data, identifying 1,632 enzymes from 14,414 protein sequences, and extracting their corresponding reactions from KEGG. The model was refined through manual curation to add missing reactions, define reaction directionality, ensure connectivity, and assign proper compartmentalization, with special attention to lipid and carbohydrate pathways. Using this improved genome-scale model, they studied the effects of high CO₂ levels on metabolism, identifying key genes and pathways involved in CO₂ uptake and fixation. Biomass production was analyzed under varying CO₂ concentrations, sensitive genes were experimentally validated, and metabolome profiling was performed. Their findings highlighted the roles of carbon-concentrating mechanisms, photorespiration, and mitochondrial processes in controlling biomass, while also revealing potential pathways and genes for optimizing microalgal biomass production.

Bjerkelund Røkke et al. (2020) developed a standardized chloroplast model *iGR774* was developed, containing 788 reactions, 764 metabolites, and 774 genes since the chloroplast is a vital organelle for photosynthesis, carbon fixation, and key biosynthetic processes. It can simulate chloroplast metabolism in three microalgae species including *Chlamydomonas reinhardtii* and is supported by COBRA Toolbox tools that enable integration into larger models, switching between organism-specific modes, and expansion with additional reactions. Yao et al. (2023) merged *iCre1355* and *iGR774* (chloroplast model) by first deleting all chloroplast metabolites and reactions in *iCre1355*. The newly merged GEM has 1354 genes, 2641 reactions, and 2240 metabolites. The gene-reaction rules were curated using complex data from ChlamyCyc 8.0 database. The starch metabolism pathway was also added to the merged model with gene-reaction rules. Thereafter, a protein-constrained (PC) model was constructed by integrating protein and enzyme information into the merged model. Finally, the expression

data were overlaid onto the PC-model for novel context-specific modelling, which can predict the respective metabolic state using FBA and flux variability analysis (FVA). The resulting model computes proteomes and intracellular flux states that are consistent with the measured transcriptomes. Arend et al. (2023) used advanced mass spectrometry and the NIDLE method to estimate in vivo k_{max_app} values for 568 reactions in *Chlamydomonas reinhardtii*, including hard-to-measure transport reactions, expanding coverage ~10-fold over previous in vitro data. They found low correlation between k_{cat} and k_{max_app} in eukaryotes and showed that replacing k_{cat} with k_{max_app} in protein-constrained genome-scale metabolic models (pcGEMs) improved predictions of enzyme usage. Raw GECKO models generated using *iCre1355* underestimated growth, which was corrected by adjusting k_{cat} values and enzyme saturation coefficients. This work enhances understanding of uncharacterized enzymes and supports more accurate and effective biotechnological applications of *Chlamydomonas*.

Table 1. Basic properties of *Chlamydomonas reinhardtii* GEMs

GEM	Reaction #	Metabolite #	Gene #	Reference
-	484	458	-	1. Boyle and Morgan (2009)
<i>iAM303</i>	259	267	303	2. Manichaikul et al. (2009)
AlgaGEM	1725	1862	866	3. Gomes de Oliveira Dal'Molin et al. (2011)
<i>iRC1080</i>	2190	1068*	1080	4. Chang et al. (2011)
-	160	164	-	5. Kliphuis et al. (2012)
<i>iCre1355</i>	2394	1133	1355	6. Imam et al. (2015)
<i>iGR774**</i>	788	764	774	7. Bjerkelund Røkke et al. (2020)
-	2641	2240	1354	8. Yao et al. (2023)

*unique

**chloroplast model for *Chlamydomonas reinhardtii* GEMs

DISCUSSION

Microalgae are capable of producing a variety of high-value compounds and biofuel precursors by utilizing industrial waste and light energy, making them important biotechnological tools for advancing a net-zero carbon economy. However, improving the economic viability of photosynthetic bioreactors requires further optimization of specific biotechnological goals, such as lipid, pigment, or hydrogen production. Our capacity to rationally engineer microbial metabolism depends on how well we understand the metabolic networks of these cell factories. Genome-scale metabolic models (GEMs), which mathematically capture metabolic knowledge, together with constraint-based modeling, have

become valuable tools for guiding metabolic engineering strategies (Arend et al. 2023). With the growing focus on algae-based biofuel production, the reconstructed metabolic networks of *C. reinhardtii* provide foundation for engineering algae to enhance lipid or starch synthesis (Boyle and Morgan 2009).

In this study, eight genome-scale metabolic models (GEMs) of *Chlamydomonas reinhardtii* were compiled and systematically analyzed to evaluate their structure, content, and predictive capabilities. The models were compared in terms of reaction coverage, metabolite representation, gene associations, and compartmentalization to identify similarities, differences, and potential gaps in metabolic knowledge. By analyzing these GEMs side by side, the study aimed to assess their reliability for simulating growth and metabolic fluxes under various conditions, as well as their utility for guiding metabolic engineering and biotechnological applications. This comprehensive analysis provides insights into the current state of metabolic modeling for *C. reinhardtii* and highlights areas where further refinement and standardization are needed.

CONCLUSION

The future of *Chlamydomonas reinhardtii* genome-scale metabolic models (GEMs) is expected to focus on increasing their predictive accuracy and biotechnological utility. Advances in multi-omics integration, including transcriptomics, proteomics, metabolomics, and fluxomics, will allow the development of more condition-specific and realistic models. Enzyme-constrained models (pcGEMs) incorporating in vivo turnover numbers and protein abundances will enable precise predictions of metabolic fluxes, growth rates, and enzyme allocation. Additionally, future models are likely to incorporate dynamic and regulatory features, capturing cellular responses to changing environmental conditions such as light, nutrients, and stress. Standardization and improved annotations across multiple GEMs will enhance interoperability and usability, while integration with machine learning approaches will aid in identifying metabolic bottlenecks and optimizing pathways for biofuel, lipid, pigment, and hydrogen production. Overall, *C. reinhardtii* GEMs are expected to evolve into highly predictive, enzyme-aware tools that support both fundamental research and industrial biotechnology applications.

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DETERMINATION OF ANTIBIOTIC RESISTANCE PROFILES AND EFFECTIVE ANTIBIOTICS IN *SALMONELLA* ISOLATES FROM TURKIYE

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ABSTRACT

Salmonella species are foodborne pathogens that cause various infections in humans and animals, such as gastroenteritis, typhoid fever, and paratyphoid fever. Increasing antibiotic resistance in recent years poses a major threat to public health and limits current treatments. In this study, the antibiotic resistance profiles of six different *Salmonella* serovars (Enteritidis, Typhimurium, Virchow, Infantis, Kentucky, and Thompson) isolated from Türkiye were examined using disk diffusion and microdilution methods, and the most effective antibiotics were determined. In the trial, which was conducted using a total of 19 different antibiotics, the antibiotic resistance profiles of the isolates were determined, and the resistance patterns between serovars were compared. Our research revealed that resistance levels to some antibiotics showed significant differences between serovars. Typhimurium and Virchow showed susceptibility to sulfonamide antibiotics, while other isolates were found to be highly resistant. All serovars remained susceptible to trimethoprim and ciprofloxacin antibiotics, while widespread resistance to vancomycin was detected. All isolates were found to be resistant to at least one antibiotic, and multidrug resistance was common. Following resistance analysis, the data were normalized, and the three most effective antibiotics were selected to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Ciprofloxacin showed susceptibility in all isolates, with only *S. Virchow* and *S. Infantis* (0.19 µg/mL) and *S. Kentucky* (0.3 µg/mL) exhibiting low MIC values. The lowest MIC values for neomycin ranged from 1.5 to 3.1 µg/mL, while a marked resistance was observed in *S. Infantis* at 50 µg/mL. While most isolates were susceptible to trimethoprim at 0.19 µg/mL, only *S. Infantis* showed high resistance at 12.5 µg/mL. MBC results supported the MIC findings. Ciprofloxacin showed very low MBC values of <0.024 µg/mL in most isolates, but *S. Virchow* (3.125 µg/mL), *S. Infantis* (0.390 µg/mL), and *S. Kentucky* (0.781 µg/mL) exhibited higher values. While MIC values for neomycin were generally between 3.125-6.25 µg/mL, >50 µg/mL was found in *S. Infantis*. For trimethoprim, all serovars showed an MIC value of 12.5 µg/mL, with only *S. Infantis* having a higher bactericidal threshold value of 50 µg/mL. The data obtained show that *Salmonella enterica* serovars from cheese sources are multidrug resistant, but ciprofloxacin and trimethoprim retain their effectiveness against most isolates. In contrast, the high resistance of *S. Infantis* to both neomycin and trimethoprim emphasizes that serovar-level differences are critical in resistance monitoring. Our study shows that the different *Salmonella* serovars isolated have high resistance rates to important antibiotics, which may pose a risk in terms of economics and public health. Therefore, the resistance rates determined in our data should be considered in both epidemiological surveillance and treatment strategies.

Keywords: *Salmonella*, isolates, antibiotic resistance, ciprofloxacin, neomycin, trimethoprim.

INTRODUCTION

Salmonella enterica is a food- and waterborne pathogen that causes serious gastroenteritis outbreaks and invasive systemic diseases worldwide. This pathogen has high morbidity and mortality rates in developing countries. According to World Health Organization (WHO) data, salmonellosis cases, which affect millions of people each year, seriously threaten global food safety and the economy (WHO 2025). This pathogen, a Gram-negative bacterium, has more than 2,600 serovars (Xin et al. 2021). Serovar differentiation plays an important role in combating this pathogen. Increasing antibiotic resistance is negatively affecting the fight against the pathogen. *Salmonella* isolates show increasing multidrug resistance (MDR) to many drugs used in animal husbandry and human health. Resistance genes are spread mainly through mobile genetic components such as plasmids and transposons, facilitating the spread of resistant *Salmonella* within the food chain (Liu et al. 2020, Li et al. 2021, Mthembu et al. 2021, Yan et al. 2024). This situation poses significant challenges in the empirical treatment of invasive *Salmonella* infections, particularly due to increasing resistance to key antibiotics such as ceftriaxone, fluoroquinolones, and azithromycin (Sjölund-Karlsson et al. 2010, Lee et al. 2021a, Pietsch et al. 2021, Shi et al. 2021, Ivanova et al. 2024, Zhang et al. 2024). Differences in adaptation mechanisms and genetic material transfer among *S. enterica* serovars lead to distinct differences in antibiotic resistance profiles. The prevalence of various serovars among different animal species and their exposure to different antibiotics in different geographical regions are the main factors contributing to this variation in resistance development (Kuang et al. 2015). Understanding these serovar-level differences in resistance profiles is critical for implementing accurate and targeted treatment strategies during outbreaks.

This study aims to determine the resistance status of six *S. enterica* serovar isolates (Table 2) –isolated from cheese and identified at the serovar level– to a range of antibiotics (Table 1) commonly used in clinical and veterinary settings and to compare the differences in resistance profiles among these six isolates. The data obtained aims to reveal the current antibiotic resistance status of local *Salmonella* strains, thereby assisting in the more effective planning of *Salmonella* infection treatment and the establishment of local resistance monitoring studies.

Table 4. Antibiotic class and mechanisms of action of the antibiotics used in the study.

Antibiotic Class	Primary Mechanism of Action	Antibiotics
Aminoglycosides	Inhibition of Protein Synthesis	Gentamicin, Kanamycin, Neomycin, Spectinomycin, Streptomycin
Amphenicols	Inhibition of Protein Synthesis	Chloramphenicol, Florfenicol
Cephalosporins (β -Lactams)	Inhibition of Cell Wall Synthesis	Ceftiofur (3rd Generation Cephalosporin)
Diaminopyrimidines (Folate Antagonists)	Inhibition of Bacterial Folic Acid Synthesis	Trimethoprim
Fluoroquinolones (Quinolones)	Inhibition of Nucleic Acid (DNA) Synthesis	Ciprofloxacin
Glycopeptides	Inhibition of Cell Wall Synthesis	Vancomycin
Macrolides	Inhibition of Protein Synthesis	Erythromycin
Penicillins (β -Lactams)	Inhibition of Cell Wall Synthesis	Amoxicillin, Ampicillin
Quinolones	Inhibition of Nucleic Acid (DNA) Synthesis	Nalidixic Acid
Rifamycins	Inhibition of Nucleic Acid (RNA) Synthesis	Rifampicin
Sulfonamides	Inhibition of Folic Acid Synthesis	Sulfadiazine, Sulphonamides
Tetracyclines	Inhibition of Protein Synthesis	Tetracycline

MATERIAL & METHODS

Bacterial Strains and Culture Conditions

Salmonella enterica strains isolated from cheese and identified at the serovar level by molecular analysis were obtained from the culture collection established by the Prokaryotic Genetics Research Group (Ankara University, Faculty of Science, Department of Biology) (Table 2). *Salmonella* isolates were incubated at 37°C for 18 hours in Luria Bertani (LB) medium. Stock cultures have been stored at -80°C in a medium containing 60% glycerol.

Table 5. *Salmonella* isolates isolated from cheese and the laboratory codes.

Serovar	Laboratory Code
S. Enteritidis	DMC3
S. Infantis	DMC7
S. Kentucky	DMC34
S. Thompson	DMC39
S. Typhimurium	DMC4
S. Virchow	DMC5

Disc Diffusion

The antibiotic susceptibility profiles of six *S. enterica* serovars were assessed through the disc diffusion method on Mueller-Hinton Agar (MHA), following the standards set by the Clinical and Laboratory Standards Institute (CLSI). Each isolate was taken from a fresh culture and prepared as a suspension equivalent to the McFarland standard. The prepared bacterial suspensions were spread evenly and homogeneously over the entire surface of MHA plates. Following the plating process, the plates were permitted to dry for a duration of 5 to 15 minutes. Commercial discs containing a total of 19 different antibiotics (Table 3) were placed on the plate surface using sterile forceps. The plates were placed upside down and incubated at 37°C for a duration of 16 to 20 hours. Upon completion of the incubation period, the diameters of the inhibition zones that developed around the discs were quantified using a micrometer.

Table 6. Antibiotics.

Antibiotic Name	Antibiotic Code	Disc Content (µg)
Amoxicillin	AMC	30
Ampicillin	AM	10
Ceftiofur	EFT	30
Chloramphenicol	C	30
Ciprofloxacin	CIP	5
Erythromycin	E	15
Florfenicol	FFC	30
Gentamicin	GM	10
Kanamycin	K	30
Nalidixic Acid	NA	30
Neomycin	N	5
Rifampicin	RD	5
Spectinomycin	SPT	100
Streptomycin	S	10
Sulfadiazine	SD	250
Sulphonamides	S3	300
Tetracycline	TE	30
Trimethoprim	TMP	5
Vancomycin	VA	30

Minimum Inhibitory Concentration (MIC)

A Minimum Inhibitory Concentration (MIC) test was performed to determine the susceptibility levels of isolates belonging to six different *S. enterica* serovars to trimethoprim, neomycin, and ciprofloxacin. In the test, solutions prepared with stock concentrations of the three antibiotics at 100 µg/mL were used. The MIC test was performed according to standard methods based on the liquid microdilution principle. *S. enterica* isolates were incubated in Mueller-Hilton Broth at 37°C for 18 hours. Active cultures were adjusted to a 0.5 McFarland turbidity standard. This initial suspension was then diluted with MHB and inoculated into 96-well F-bottom microplates to ensure a final bacterial concentration of approximately 5×10^5 CFU/mL in the wells. After distributing the antibiotic dilutions and bacterial inoculum to the microplates, the plates were incubated at 37°C for 20-24 hours. The MIC value was determined as the lowest antibiotic concentration at which no visible growth was observed.

Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) values of *S. enterica* isolates against antibiotics were determined following MIC testing. The MBC test was performed using the cultural counting technique from liquid microdilution plates in accordance with CLSI standards. After the 20-24-hour incubation period during which the MIC values were determined, 10 µL samples were taken from all wells where no visible growth was observed and were streaked onto antibiotic-free MHA. The agar plates were incubated at 37°C. After incubation, the colonies on the agar plates were counted. The MIC value was defined as the

lowest antibiotic concentration ($\mu\text{g/mL}$) resulting in the death of more than 99% of the initial inoculum.

RESULTS & CONCLUSION

Antibiotic Susceptibility Profiles of Foodborne *S. enterica* Isolates

The antibiotic resistance profiles of six different *S. enterica* serovars isolated from cheese were evaluated using a disk diffusion test with 19 different antibiotics. Significant disparities in resistance levels to specific antibiotics were noted when comparing the resistance patterns among the serovars (Table 3). The controls of the experiments were carried out with ATCC strains.

Isolate	Antibiotics							
DMC3	VA	NA	S3	TE	AM	K	N	CIP
	R	23 mm	R	20 mm	25 mm	23 mm	16 mm	35 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	26 mm	28 mm	30 mm	16 mm	R	23 mm	25 mm	31 mm
								
	E		S			GM		
	12 mm		20 mm			23 mm		
								

Figure 1. Antibiotic susceptibility profiles of *S. Enteritidis* (DMC3) isolate determined by disk diffusion.

The antibiotic susceptibility profile of the *S. Enteritidis* (DMC3) isolate tested by the disk diffusion method against 19 different antibiotics was analyzed (Figure 1, Table 4). The DMC3 isolate was susceptible to 12 antibiotics, including critical therapeutic agents such as ciprofloxacin, ampicillin, ceftiofur, chloramphenicol, and gentamicin. However, the said isolate exhibited resistance against neomycin, erythromycin, sulfadiazine, sulfonamides, rifampicin, and vancomycin.

Table 7. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Enteritidis* (DMC3) isolate.

<i>S. Enteritidis</i> (DMC3)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	23 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	25 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	25 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	28 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	35 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	12 mm	≥ 23 (S)	Resistant (R)
Florfenicol	31 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	23 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	23 mm	≥ 18 (S)	Susceptible (S)
Nalidixic Acid	23 mm	≥ 19 (S)	Susceptible (S)
Neomycin	16 mm	≥ 17 (S)	Resistant (R)
Rifampicin	R	≤ 16 (R)	Resistant (R)
Spectinomycin	30 mm	N/A	Susceptible (S)
Streptomycin	20 mm	≥ 15 (S)	Susceptible (S)
Sulfadiazine	16 mm	≥ 17 (S)	Resistant (R)
Sulphonamides	R	≤ 12 (R)	Resistant (R)
Tetracycline	20 mm	≥ 19 (S)	Susceptible (S)
Trimethoprim	26 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

The phenotype of the DMC3 isolate, which was found to be multidrug resistant (MDR) in trials, poses a potential public health risk. However, DMC3 also provides an important clinical benefit, as it retains sensitivity to key antibiotics used to treat systemic infections, including fluoroquinolones (ciprofloxacin) and third-generation cephalosporins (ceftiofur) (McDermott et al. 2018). This suggests that infections are controllable with current treatment strategies. At the same time, the resistance of the isolate to vancomycin and erythromycin is a natural resistance attributable to the Gram-negative characteristics of *Salmonella*, which is an expected result and is in line with the literature (Harakeh et al. 2005, Gurler et al. 2015, Thung et al. 2016). Resistance to critical veterinary and public health drugs such as aminoglycosides (neomycin) and sulfonamides (sulfadiazine) suggests that the bacterium harbors resistance genes acquired via plasmids that can be easily transferred to other bacteria (Emond-Rheault et al. 2020, McMillan et al. 2020, Wang et al. 2022). Thus, to fully understand the potential for transfer of resistance genes of the MDR phenotype and its impact on public health, it is crucial to characterize the phenotypic resistance profile at the genetic level, validated by molecular techniques such as Next Generation Sequencing (NGS) or PCR.

Isolate	Antibiotics							
DMC4	VA	NA	S3	TE	AM	K	N	CIP
	R	22 mm	20 mm	17 mm	21 mm	23 mm	14 mm	31 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	23 mm	28 mm	26 mm	12 mm	7 mm	20 mm	25 mm	27 mm
								
	E		S			GM		
	12 mm		15 mm			23 mm		
								

Figure 2. Antibiotic susceptibility profiles of *S. Typhimurium* (DMC4) isolate determined by disk diffusion.

The DMC4 isolate showed susceptibility to 13 of the 19 antibiotics evaluated. DMC4 isolate showed resistance to neomycin, erythromycin, tetracycline, sulfadiazine, rifampicin, and vancomycin antibiotics (Figure 2, Table 5). The isolate was classified as Multi-Drug Resistant (MDR) as it was resistant to a total of five different classes of antibiotics: aminoglycosides (neomycin), macrolides (erythromycin), tetracyclines (tetracycline), sulfonamides (sulfadiazine), and rifampicins/glycopeptides (rifampicin and vancomycin). The susceptibility profile of the DMC4 isolate, similar to the previous isolate, shows high susceptibility to important human antibiotics such as ciprofloxacin, ampicillin, ceftiofur, and chloramphenicol. This provides an advantage in clinical treatment. The resistance profile of the DMC4 isolate is particularly noteworthy with resistance to tetracycline and sulfadiazine.

Table 8. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Typhimurium* (DMC4) isolate.

<i>S. Typhimurium</i> (DMC4)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	20 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	21 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	25 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	28 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	31 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	12 mm	≥ 23 (S)	Resistant (R)
Florfenicol	27 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	23 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	23 mm	≥ 18 (S)	Susceptible (S)
Nalidixic Acid	22 mm	≥ 19 (S)	Susceptible (S)
Neomycin	14 mm	≥ 17 (S)	Resistant (R)
Rifampicin	7 mm	≤ 16 (R)	Resistant (R)
Spectinomycin	26 mm	N/A	Susceptible (S)
Streptomycin	15 mm	≥ 15 (S)	Susceptible (S)
Sulfadiazine	12 mm	≥ 17 (S)	Resistant (R)
Sulphonamides	20 mm	≥ 12 (S)	Susceptible (S)
Tetracycline	17 mm	≥ 19 (S)	Resistant (R)
Trimethoprim	23 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

Tetracycline resistance associated with the presence of *tet* genes and sulfadiazine resistance associated with the presence of *sul* genes are quite common in *Salmonella* strains of food and animal origin and are often found on plasmids carrying other resistance genes (Aarestrup et al. 2003, McMillan et al. 2020). The presence of resistance indicates acquired resistance mechanisms that should be monitored from a veterinary and public health perspective.

Like the DMC3 isolate, vancomycin and erythromycin resistance in the DMC4 isolate can be interpreted as the expected natural resistance in *Salmonella*, while neomycin, tetracycline, sulfadiazine, and rifampicin resistance are indicative of acquired multidrug resistance. The profiling of this isolate provides important information on the prevalence and evolution of antimicrobial resistance in *S. Typhimurium* strains. Molecular characterization of resistance genes is critical for understanding resistance mechanisms and the potential for spread by plasmids.

Isolate	Antibiotics							
DMC5	VA	NA	S3	TE	AM	K	N	CIP
	R	R	20 mm	16 mm	24 mm	22 mm	13 mm	26 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	23 mm	26 mm	26 mm	19 mm	7 mm	21 mm	24 mm	29 mm
								
	E		S			GM		
	12 mm		14 mm			22 mm		
								

Figure 3. Antibiotic susceptibility profiles of *S. Virchow* (DMC5) isolate determined by disk diffusion.

The *S. Virchow* isolate tested was susceptible to 12 out of 19 antibiotics. The DMC5 isolate showed intermediate susceptibility (I) to streptomycin and resistance to 6 different antibiotics: erythromycin, nalidixic acid, neomycin, rifampicin, tetracycline and vancomycin (Figure 3, Table 6). Since the isolate was resistant to agents belonging to five different classes of antibiotics, it was classified as MDR.

Table 9. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Virchow* (DMC5) isolate.

<i>S. Virchow</i> (DMC5)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	21 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	24 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	24 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	26 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	26 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	12 mm	≥ 23 (S)	Resistant (R)
Florfenicol	29 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	22 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	22 mm	≥ 18 (S)	Susceptible (S)
Nalidixic Acid	R	≤ 13 (R)	Resistant (R)
Neomycin	13 mm	≥ 17 (S)	Resistant (R)
Rifampicin	7 mm	≤ 16 (R)	Resistant (R)
Spectinomycin	26 mm	N/A	Susceptible (S)
Streptomycin	14 mm	≥ 15 (S)	Intermediate (I)
Sulfadiazine	19 mm	≥ 17 (S)	Susceptible (S)
Sulphonamides	20 mm	≥ 12 (S)	Susceptible (S)
Tetracycline	16 mm	≥ 19 (S)	Resistant (R)
Trimethoprim	23 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

DMC5 isolate shows a significant resistance development compared to other isolates. The resistance of the isolate to nalidixic acid indicates the presence of a potential resistance mechanism within the quinolone/fluoroquinolone group. Resistance to nalidixic acid is usually associated with decreased susceptibility to critical drugs such as ciprofloxacin (Albayrak et al. 2004, Redgrave et al. 2014, Guzmán-Martín et al. 2018). While it is positive that the isolate remains susceptible to ciprofloxacin, nalidixic acid resistance has serious public health significance, as it may signal the onset of quinolone resistance determining region (QRDR) mutations and should be closely monitored (Adhikari et al. 2022). Tetracycline and neomycin resistances support the presence of acquired resistance genes that are easily spread via plasmids. Like previous isolates, vancomycin and erythromycin resistances are part of the natural resistance phenotype of *Salmonella*. The fact that this isolate showed Intermediate Susceptibility (I) to streptomycin reduces the likelihood of successful treatment with this agent and may represent a transition towards resistant strains.

In conclusion, the MDR profile of DMC5, characterized by nalidixic acid resistance, provides critical data for monitoring the development of antimicrobial resistance in *Salmonella* strains. Identification of the genetic mechanisms underlying this resistance phenotype, especially by molecular methods such as *gyrA* mutations (Redgrave et al. 2014, Kongsoi et al. 2016), is essential to fully understand the epidemiologic and clinical significance of resistance.

Isolate	Antibiotics							
DMC7	VA	NA	S3	TE	AM	K	N	CIP
	R	R	R	R	24 mm	R	R	25 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	15 mm	25 mm	9 mm	R	9 mm	21 mm	23 mm	30 mm
								
	E		S			GM		
	10 mm		9 mm			24 mm		
								

Figure 4. Antibiotic susceptibility profiles of *S. Infantis* (DMC7) isolate determined by disk diffusion.

The *S. Infantis* isolate was found susceptible to only 7 of the 19 antibiotics tested. The isolate showed resistance to 12 different antibiotics (Figure 4, Table 7). This isolate strongly meets the definition of MDR because it is resistant to agents belonging to a total of seven different antibiotic classes: quinolones/fluoroquinolones (nalidixic acid), aminoglycosides (kanamycin, neomycin, streptomycin, spectinomycin), macrolides (erythromycin), tetracyclines (tetracycline), sulfonamides (sulfadiazine, sulphonamides), rifampicins (rifampicin), and glycopeptides (vancomycin).

Table 10. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Infantis* (DMC7) isolate.

<i>S. Infantis</i> (DMC7)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	21 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	24 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	23 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	25 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	25 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	10 mm	≥ 23 (S)	Resistant (R)
Florfenicol	30 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	24 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	R	≤ 13 (R)	Resistant (R)
Nalidixic Acid	R	≤ 13 (R)	Resistant (R)
Neomycin	R	≤ 13 (R)	Resistant (R)
Rifampicin	9 mm	≤ 16 (R)	Resistant (R)
Spectinomycin	9 mm	N/A	Resistant (R)
Streptomycin	9 mm	≤ 11 (R)	Resistant (R)
Sulfadiazine	R	≤ 12 (R)	Resistant (R)
Sulphonamides	R	≤ 12 (R)	Resistant (R)
Tetracycline	R	≤ 14 (R)	Resistant (R)
Trimethoprim	15 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

Isolate DMC7 had the highest resistance profile among the strains examined in this study. The widespread resistance strongly suggests that the isolate carries a broad spectrum of acquired resistance genes. The persistence of ciprofloxacin susceptibility, while resistance to nalidixic acid was observed in DMC5, may indicate the presence of low-level quinolone resistance mutations, such as *qnr* or *gyrA* mutations, in isolate DMC7 (Kehrenberg et al. 2007, Redgrave et al. 2014, Kongsoi et al. 2016, Li et al. 2021). This could be interpreted as a potential precursor to full ciprofloxacin resistance. The simultaneous development of resistance to kanamycin, neomycin, and streptomycin suggests the presence of aminoglycoside-inactivating enzymes, typically encoded by the *aadA* gene on multiple resistance plasmids (Stern et al. 2018, Lee et al. 2021b). The resistance of DMC7 to tetracycline, sulfonamides (sulfadiazine/sulphonamides), and rifampicin increases the potential risk of treatment failure for this isolate against key antibiotic classes used in both veterinary and human medicine (Campbell 1999, Chopra & Roberts 2001, WHO 2024). The isolate's resistance to vancomycin and erythromycin further confirms the expected natural resistance phenotype in *Salmonella*.

In conclusion, DMC7's highly MDR profile necessitates molecular genetic analysis of this strain. Plasmid-mediated spread of genetic material poses serious risks to public health and food safety, and this isolate is critical for epidemiological monitoring of resistance mechanisms.

Isolate	Antibiotics							
DMC34	VA	NA	S3	TE	AM	K	N	CIP
	R	R	R	16 mm	21 mm	19 mm	14 mm	26 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	27 mm	27 mm	12 mm	R	8 mm	24 mm	24 mm	29 mm
								
	E		S			GM		
	15 mm		12 mm			20 mm		
								

Figure 5. Antibiotic susceptibility profiles of *S. Kentucky* (DMC34) isolate determined by disk diffusion.

S. Kentucky isolate was susceptible to 8 of the 19 antibiotics tested (amoxicillin, ampicillin, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, kanamycin, and trimethoprim) and resistant to 9 different antibiotics (erythromycin, nalidixic acid, neomycin, rifampicin, sulfadiazine, sulphonamides, tetracycline, and vancomycin). It showed Intermediate Susceptibility (I) to streptomycin (Figure 5, Table 8). DMC34 was strongly classified as MDR as it was resistant to agents belonging to six different classes of antibiotics.

Table 11. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Kentucky* (DMC34) isolate.

<i>S. Kentucky</i> (DMC34)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	24 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	21 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	24 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	27 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	26 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	15 mm	≥ 23 (S)	Resistant (R)
Florfenicol	29 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	20 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	19 mm	≥ 18 (S)	Susceptible (S)
Nalidixic Acid	R	≤ 13 (R)	Resistant (R)
Neomycin	14 mm	≥ 17 (S)	Resistant (R)
Rifampicin	8 mm	≤ 16 (R)	Resistant (R)
Spectinomycin	12 mm	N/A	Resistant (R)
Streptomycin	12 mm	≤ 11 (R)	Intermediate (I)
Sulfadiazine	R	≤ 12 (R)	Resistant (R)
Sulphonamides	R	≤ 12 (R)	Resistant (R)
Tetracycline	16 mm	≤ 14 (R)	Resistant (R)
Trimethoprim	27 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

DMC34 demonstrates a less extensive resistance profile compared to earlier isolates, particularly DMC7, yet maintains certain essential resistance characteristics. DMC34 is characterized by the lack of kanamycin resistance, a feature commonly observed in other multidrug-resistant strains. This indicates variability in the presence of aminoglycoside-inactivating enzyme genes and gene combinations on plasmids among different *Salmonella* strains (Petrin et al. 2023, Ishihara et al. 2025). However, resistance to neomycin persists. Intermediate susceptibility to streptomycin indicates a potential increase in resistance. Like all the isolates analyzed, DMC34 maintained its susceptibility to the main therapeutic options such as ampicillin, ceftiofur, chloramphenicol, and ciprofloxacin. This suggests that there are currently effective options for the systemic treatment of *Salmonella* infections. Resistance to nalidixic acid, tetracycline, and sulfonamides emphasizes that the isolate carries multiple acquired resistance genes and potentially has the risk of spreading resistance to other bacteria via plasmids (Emond-Rheault et al. 2020, McMillan et al. 2020).

In conclusion, the multidrug resistance phenotype of the DMC34 carries significant risks for public health due to the persistence of nalidixic acid resistance and the presence of sulfonamide/tetracycline resistance. Supporting the phenotypic data obtained with molecular genetic analyses will reveal the spread potential and epidemiologic importance of this strain more clearly.

Isolate	Antibiotics							
DMC39	VA	NA	S3	TE	AM	K	N	CIP
	R	22 mm	R	16 mm	20 mm	23 mm	13 mm	33 mm
								
	TMP	C	SPT	SD	RD	AMC	EFT	FFC
	25 mm	26 mm	24 mm	14 mm	7 mm	21 mm	23 mm	26 mm
								
	E		S			GM		
	10 mm		14 mm			22 mm		
								

Figure 6. Antibiotic susceptibility profiles of *S. Thompson* (DMC39) isolate determined by disk diffusion.

The *S. Thompson* isolate (DMC39) was susceptible to 11 of the 19 antibiotics tested. It showed Intermediate Susceptibility (I) to streptomycin. DMC39 exhibited resistance to 7 different antibiotics (erythromycin, neomycin, rifampicin, sulfadiazine, sulphonamides, tetracycline, and vancomycin). As with the other isolates, DMC39 was classified as MDR, as it was resistant to agents belonging to at least five different antibiotic classes (Figure 6, Table 9).

The profile of DMC39 differs from other MDR strains in that it retains full susceptibility to quinolones/fluoroquinolones, particularly ciprofloxacin (33 mm) and nalidixic acid (22 mm). This suggests that DMC39 has not yet acquired the critical quinolone resistance mechanisms seen in DMC5, DMC7, and DMC34. While the DMC39 strain retains sensitivity to antibiotics that are extremely important in human treatment, it exhibits resistance to older-generation agents (neomycin, sulfonamides, and tetracycline) commonly used in agriculture and animal husbandry. Resistance to aminoglycosides (neomycin), tetracyclines, and sulfonamides indicates the presence of common plasmid-mediated acquired resistance genes (*tet*, *sul*, and *aadA*). Moderate susceptibility to streptomycin (14 mm) suggests the presence of a resistance mechanism but not yet leading to full phenotypic resistance.

Table 12. Zone diameters and CLSI classification of antibiotic susceptibility results of *S. Thompson* (DMC39) isolate.

<i>S. Thompson</i> (DMC39)			
Antibiotics	Zone Diameter (mm)	CLSI Breakpoint (mm)	Interpretation
Amoxycillin	21 mm	≥ 14 (S)	Susceptible (S)
Ampicillin	20 mm	≥ 16 (S)	Susceptible (S)
Ceftiofur	23 mm	≥ 21 (S)	Susceptible (S)
Chloramphenicol	26 mm	≥ 18 (S)	Susceptible (S)
Ciprofloxacin	33 mm	≥ 21 (S)	Susceptible (S)
Erythromycin	10 mm	≥ 23 (S)	Resistant (R)
Florfenicol	26 mm	≥ 18 (S)	Susceptible (S)
Gentamicin	22 mm	≥ 15 (S)	Susceptible (S)
Kanamycin	23 mm	≥ 18 (S)	Susceptible (S)
Nalidixic Acid	22 mm	≥ 19 (S)	Susceptible (S)
Neomycin	13 mm	≥ 17 (S)	Resistant (R)
Rifampicin	7 mm	≤ 16 (R)	Resistant (R)
Spectinomycin	24 mm	N/A	Susceptible (S)
Streptomycin	14 mm	≤ 11 (R)	Intermediate (I)
Sulfadiazine	14 mm	≥ 17 (S)	Resistant (R)
Sulphonamides	R	≤ 12 (R)	Resistant (R)
Tetracycline	16 mm	≤ 14 (R)	Resistant (R)
Trimethoprim	25 mm	≥ 11 (S)	Susceptible (S)
Vancomycin	R	≤ 9 (R)	Resistant (R)

In conclusion, the *S. Thompson* isolate is still an MDR strain due to its sulfonamide, tetracycline, and neomycin resistances, although it presents a positive picture with its continued susceptibility to quinolones. The presence of these resistances is important for the strain's potential to be of food or animal origin and to spread resistance along the food chain. Epidemiologic surveillance of this isolate and detection of resistance genes are critical to manage food safety and public health risks.

Determination of MIC and MBC Values of Multidrug-Resistant *Salmonella enterica* Isolates Against Three Selected Antibiotics

MIC values were determined according to standard methods based on the principle of liquid microdilution, and the results were evaluated according to the criteria specified in the Clinical and Laboratory Standards Institute (CLSI) guidelines. The disk diffusion assay results obtained in preliminary studies were normalized to eliminate differences in the disk loads of antibiotics and to standardize the comparison. These normalized results revealed that neomycin, trimethoprim, and ciprofloxacin were the three agents that showed the highest in vitro activity against *S. enterica* isolates (Table 10).

Table 13. Minimum Inhibitory Concentration profiles of *S. enterica* isolates.

Antibiotics	Stock Conc.	Enteritidis (DMC3)	Typhimurium (DMC4)	Virchow (DMC5)	Infantis (DMC7)	Kentucky (DMC34)	Thompson (DMC39)
Trimethoprim	100 µg/mL	0.195 µg/mL	0.195 µg/mL	0.195 µg/mL	12.5 µg/mL	0.195 µg/mL	0.195 µg/mL
Ciprofloxacin	100 µg/mL	>0.024 µg/mL	>0.024 µg/mL	0.195 µg/mL	0.195 µg/mL	0.390 µg/mL	>0.024 µg/mL
Neomycin	100 µg/mL	1.562 µg/mL	3.125 µg/mL	3.125 µg/mL	50 µg/mL	3.125 µg/mL	3.125 µg/mL

Except for DMC7 (12.5 µg/mL), all other isolates exhibited low MIC values (0.195 µg/mL) against trimethoprim. Analysis of the obtained data showed that DMC7 was either close

to the CLSI limit of susceptibility or showed a resistant phenotype. Isolates DMC3, DMC4, and DMC39 were found to be highly susceptible to ciprofloxacin with the lowest MIC value ($>0.024 \mu\text{g/mL}$). DMC34 showed decreased susceptibility with a higher MIC ($0.390 \mu\text{g/mL}$).

The high MIC values of the *S. Infantis* isolate to trimethoprim and neomycin definitively demonstrate that this serotype has acquired specific resistance mechanisms not found in most other serotypes. This limits treatment strategies for *S. Infantis*-associated infections. The elevated ciprofloxacin MIC in *S. Kentucky* indicates that this isolate has developed low-level resistance to quinolones. While isolates such as *S. Enteritidis* maintain high susceptibility ($<0.024 \mu\text{g/mL}$), this increase in *S. Kentucky* signals evolution toward fluoroquinolone resistance under the pressure of antibiotic use.

Table 14. Minimum Bactericidal Concentration profiles of *S. enterica* isolates.

Antibiotics	Enteritidis (DMC3)	Typhimurium (DMC4)	Virchow (DMC5)	Infantis (DMC7)	Kentucky (DMC34)	Thompson (DMC39)
Trimethoprim	12.5 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$
Ciprofloxacin	$>0.024 \mu\text{g/mL}$	$>0.024 \mu\text{g/mL}$	3.125 $\mu\text{g/mL}$	0.390 $\mu\text{g/mL}$	0.781 $\mu\text{g/mL}$	$>0.024 \mu\text{g/mL}$
Neomycin	3.125 $\mu\text{g/mL}$	3.125 $\mu\text{g/mL}$	6.25 $\mu\text{g/mL}$	$>50 \mu\text{g/mL}$	6.25 $\mu\text{g/mL}$	3.125 $\mu\text{g/mL}$

The MBC determines the lowest concentration of an antibiotic needed to kill 99% of bacteria. In this study conducted on six *S. enterica* isolates, MBC values determined for trimethoprim, ciprofloxacin, and neomycin were examined together with MIC, and bactericidal requirements showed significant differences among strains (Table 11). In isolates DMC3, DMC4, DMC5, DMC34, and DMC39, the MIC value for trimethoprim was $0.195 \mu\text{g/mL}$, while the MBC was $12.5 \mu\text{g/mL}$ ($\text{MBC/MIC} = 64$). This indicates the requirement for a lethal dose well above the inhibitory concentration. In isolate DMC7, the MIC was $12.5 \mu\text{g/mL}$ and MBC was $50 \mu\text{g/mL}$, with an MBC/MIC ratio of 4. Isolates DMC3, DMC4, and DMC39 had MIC and MBC values against ciprofloxacin that were very close to or equal to the lowest concentration tested, suggesting that bactericidal activity may occur even at the MIC level. Three other isolates (DMC5, DMC7, and DMC34) had MICs ranging from 0.195 - $0.390 \mu\text{g/mL}$ and MBCs ranging from 0.390 - $3.125 \mu\text{g/mL}$. Five of the six isolates had MBC/MIC ratios of 1-2 against neomycin, indicating a strong bactericidal profile. However, isolate DMC7 exhibited significantly higher resistance or tolerance with MIC of $50 \mu\text{g/mL}$ and $\text{MBC} > 50 \mu\text{g/mL}$.

The MBC data obtained revealed that *S. enterica* strains exhibited different bactericidal concentration requirements against trimethoprim, ciprofloxacin, and neomycin. The high MBC/MIC ratios observed for trimethoprim suggest that the antibiotic inhibits growth in most *Salmonella* strains solely by inhibiting folate synthesis, with bactericidal activity occurring at much higher concentrations. This is consistent with strain-specific structural variations in dihydrofolate reductase and differences in binding affinity for trimethoprim; therefore, in our study, trimethoprim activity is thought to vary significantly depending on enzyme polymorphisms (Huovinen 2001, Sköld 2001). Ciprofloxacin, on the other hand, exhibited a classic bactericidal profile with close MIC and MBC values in many strains, but increased ratios in some strains suggested the involvement of the SOS response or DNA repair (Sridhar et al. 2021). Central carbon metabolism has also been reported to enhance fluoroquinolone killing activity, and synergy has been demonstrated between LL-37, an antimicrobial peptide produced by the human immune system, and ciprofloxacin (Sakoulas et al. 2017, Braetz et al. 2022). As

additional evidence for persister formation, *Salmonella* has been reported to form small colony variants after intensive ciprofloxacin exposure (Drescher et al. 2019). While the low MBC/MIC ratios for neomycin are consistent with classical aminoglycoside bactericidal activity, the high MIC and MBC values >50 µg/mL in isolate DMC7 suggest resistance mechanisms such as aminoglycoside-modifying enzymes.

CONCLUSION

All six *S. enterica* isolates studied retained susceptibility to key antibiotics critical for the treatment of *Salmonella* infections, including fluoroquinolones (ciprofloxacin), third-generation cephalosporins (ceftiofur), and penicillin (ampicillin). This suggests that invasive systemic infections caused by these strains can still be successfully treated with current clinical protocols. All isolates were classified as Multidrug Resistant (MDR) because they showed resistance to agents belonging to at least three different antibiotic classes. Resistance to nalidixic acid among *S. Infantis*, *S. Kentucky*, and *S. Thompson* isolates is a major public health concern. Nalidixic acid resistance is an indicator of acquired or on-the-verge-of-acquiring resistance mechanisms that could lead to full resistance to more potent fluoroquinolones such as ciprofloxacin (Murray et al. 2005, Redgrave et al. 2014, Guzmán-Martín et al. 2018). The widespread resistance to sulfonamides, tetracyclines, and neomycin strongly suggests that these *S. enterica* strains carry acquired resistance genes, which are commonly used in animal health and readily transferred via plasmids. This increases the potential for resistance to spread through the food chain and into the environment. The *S. Infantis* isolate has the broadest resistance profile, resisting 12 antibiotics tested and seven different classes, including quinolones. This isolate exemplifies the severe high-grade MDR phenotype seen in *S. enterica* strains.

All six different *S. enterica* isolates analyzed remained susceptible to the main therapeutic agents (ciprofloxacin and ceftiofur) but were classified as MDR due to their common sulfonamide, tetracycline, and neomycin resistance profiles; nalidixic acid resistance observed in half of the isolates is the most critical public health finding, indicating the potential for these strains to transition to full resistance to quinolones and requiring urgent molecular genetic follow-up.

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EFFECTS OF FOREST FIRES ON WILDLIFE

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ABSTRACT

Forest fires are among the most impactful natural disturbances, causing both immediate and lasting effects on biodiversity and ecosystem processes. This review consolidates current understanding of how wildfires affect vertebrate and invertebrate species, focusing on population changes, ecological roles, and recovery pathways after fires. Invertebrates—such as soil microfauna like nematodes and protozoa, mesofauna like Collembola and mites, and macrofauna including earthworms, insect larvae, and ants—are highly vulnerable to the heat from fires and the loss of litter cover. These groups often see sharp population declines within a year or two post-fire, disrupting decomposition and nutrient cycling. However, many invertebrate communities tend to recover within three to five years when vegetation regrows and soil moisture is restored. Conversely, vertebrates face direct death and long-term ecological challenges. Small mammals, reptiles, amphibians, and cavity-nesting birds often experience population drops due to limited movement and the destruction of vital nesting and foraging sites. Larger mammals may escape death initially through dispersal but later encounter increased predation and competition in fragmented landscapes. Unlike invertebrates, vertebrate populations usually take decades to rebound, owing to their reliance on complex habitats and stable food webs.

Beyond species-level effects, post-fire declines in animal populations significantly harm ecosystem functions. Decreases in invertebrates' roles in decomposition and soil fertility, along with vertebrates' roles in seed dispersal and pollination, slow down vegetation renewal and weaken ecosystem resilience. Recovery processes differ greatly among ecosystems, influenced by fire severity, habitat features, and climate conditions. Including both vertebrate and invertebrate indicators in monitoring and restoration efforts is crucial for a comprehensive understanding of post-fire ecosystem changes. In the face of accelerating climate change, adaptive management should focus on preserving biological legacies, encouraging habitat diversity, and using native plants that support detrital pathways. These strategies improve the chances of functional recovery, boost ecosystem resistance to repeated fires, and protect biodiversity amid increasing disturbance.

Keywords: Wild fires; forest fauna; ecosystem recovery; biodiversity; ecological restoration.

INTRODUCTION

Forest fires are among the most destructive natural disturbances, affecting not only the tree cover but also the vertebrate and invertebrate fauna inhabiting forest ecosystems. These fires cause both habitat loss and direct mortality, thereby profoundly altering ecosystem dynamics (Certini, 2005; Doerr & Santín, 2016). The intensity, duration, and spread of fire are the primary factors determining the extent of its impact on fauna.

During fire events, the forest floor litter layer, soil moisture, and microhabitats are extensively degraded, leading to a significant loss of habitat for soil-dwelling invertebrate groups. Microfauna (e.g., nematodes, protozoa), mesofauna (e.g., Collembola, mites), and macrofauna (e.g., earthworms, insect larvae, ants) are among the most severely affected

organisms after wildfires. High temperatures destroy the organic layer, eliminating both the physical habitat and the food resources essential for these organisms.

At the same time, smoke and extreme heat pose severe threats to mobile vertebrate groups such as birds and mammals. While some species can escape the fire zone, reptiles, amphibians, and small mammals that cannot abandon their burrows often face high mortality rates (Neary et al., 1999). Post-fire ecosystems also experience disruptions in food webs, producing cascading effects across multiple trophic levels, not just at the level of individual species.

Today, climate change has become one of the most significant drivers, increasing both the frequency and severity of forest fires. Prolonged droughts, intensified heatwaves, and extreme weather events elevate the probability of fire ignition and exacerbate the risk of uncontrollable fire spread (Pellegrini et al., 2018). Consequently, fire no longer represents a temporary disturbance for faunal communities but rather a persistent ecological pressure. Understanding the impacts of fire on fauna is therefore essential for developing effective conservation strategies in response to the increasingly frequent fire regimes projected under future climate change scenarios.

EFFECTS ON INVERTEBRATE FAUNA

Invertebrate fauna play critical roles in forest ecosystems by driving decomposition, nutrient cycling, and soil fertility. Soil-dwelling microfauna (e.g., nematodes, protozoa), mesofauna (e.g., Collembola, mites), and macrofauna (e.g., earthworms, insect larvae, ants) are directly affected by wildfires. Post-fire high temperatures and the loss of the organic layer result in substantial population declines within these groups (Haimi et al., 2000; Vasconcelos et al., 2009; Santorufo et al., 2024). In particular, decomposer groups such as Collembola and mites exhibit dramatic reductions in abundance during the first one to two years following fire events. The losses observed in soil fauna after fires are critical not only in terms of species richness but also in relation to ecosystem processes. The functions of microarthropods such as Collembola and mites in enhancing decomposition rates and supporting microbial activity decline markedly, leading to disruptions in carbon and nitrogen cycling (Lavelle et al., 2006). Similarly, the absence of earthworms and ants can result in the deterioration of soil structure and a reduction in water-holding capacity.

Nevertheless, several studies have reported that faunal communities in certain habitats begin to recover within three to five years after fire. For example, in controlled burning experiments in Finnish spruce forests, Collembola populations were found to return to levels comparable to those in unburned sites within three years (Haimi et al., 2000). Likewise, research conducted in Italy demonstrated that the regeneration of vegetation was accompanied by increases in soil microfaunal densities (Santorufu et al., 2024). Long-term observations in tropical and subtropical ecosystems have further shown that as soil moisture is restored and the litter layer reaccumulates, nematodes and other microfaunal groups undergo gradual increases in abundance (Paul et al., 2011).

However, the severity and frequency of fire remain among the most decisive factors shaping recovery trajectories. While soil fauna generally recovers more rapidly after low-intensity, short-duration fires, large-scale fires with prolonged high temperatures have been reported to suppress faunal diversity for decades (Doerr & Santín, 2016). This evidence highlights that invertebrate fauna are not merely passive components of post-fire ecological restoration but also serve as active biological indicators that determine the overall success of ecosystem recovery.

EFFECTS ON VERTEBRATE FAUNA

Vertebrate fauna are affected by forest fires through both direct and indirect mechanisms. The high temperatures and dense smoke generated during fire events can be lethal, particularly for species with limited mobility. Small mammals (e.g., rodents), reptiles, amphibians, and birds are all subject to direct mortality. Species inhabiting underground burrows, such as some rodents and reptiles, are at especially high risk due to oxygen depletion and extreme heat exposure during fires (Neary et al., 1999). Individuals that survive the immediate effects of fire subsequently face indirect pressures, including habitat loss, food scarcity, and increased predation risk.

Post-fire habitat fragmentation negatively influences vertebrate reproduction, foraging, and migration behaviors. As habitat heterogeneity declines, the availability of shelter and nesting sites is reduced, placing genetic diversity within populations at risk (Banks et al., 2011). Moreover, the disruption of food webs following fire can lead to declines in herbivore populations due to reduced forage availability, which in turn cascades to affect predator populations.

Avian species exhibit variable responses to fire. The exposure of seeds in burned areas may provide temporary benefits to granivorous birds (Brawn et al., 2001). However, forest-dependent species often suffer significant population losses as a result of reduced nesting opportunities and diminished food resources. In particular, cavity-nesting birds face increased threats after fire events because of the reduced availability of suitable tree cavities for reproduction (Lindenmayer et al., 2014).

Large mammals generally escape direct mortality by fleeing fire zones, but in the post-fire landscape they frequently encounter reduced food availability and fragmented habitats, forcing them to migrate to new areas. Such migrations elevate interspecific competition and impose additional stress on life-history processes (Pellegrini et al., 2018). Furthermore, the increase in open habitats following fire raises predation risks for some species. For example, large herbivores such as deer and wild boar may exploit new plant growth emerging after fire, but in open areas they become more vulnerable to predators (Engstrom, 2010).

Overall, the impacts of forest fires on vertebrate fauna vary according to species' ecological niches, mobility, and the rate at which habitats regenerate after fire. While some species may temporarily benefit from altered post-fire conditions, most experience significant short- and long-term declines. As such, vertebrate fauna should be regarded as a critical component in post-fire ecosystem restoration assessments.

COMPARATIVE RESPONSES OF VERTEBRATE AND INVERTEBRATE FAUNA TO FOREST FIRES

While both vertebrate and invertebrate fauna are negatively affected by forest fires, the mechanisms and timescales of their responses differ considerably. Invertebrates, particularly soil-dwelling groups such as Collembola, mites, nematodes, and earthworms, are highly sensitive to the immediate thermal effects of fire and the loss of the litter layer. Their populations often collapse within the first one to two years post-fire, leading to disruptions in decomposition and nutrient cycling processes (Haimi et al., 2000; Vasconcelos et al., 2009; Santorufo et al., 2024). Nevertheless, many invertebrate groups demonstrate relatively rapid recovery trajectories, with some communities reaching pre-fire densities within three to five years, particularly in ecosystems where vegetation regeneration and soil moisture restoration occur (Paul et al., 2011).

In contrast, vertebrate fauna are affected not only by direct mortality but also by long-term ecological pressures such as habitat fragmentation, reduced food availability, and altered

trophic interactions. Small mammals, reptiles, amphibians, and cavity-nesting birds often experience severe population declines due to their limited ability to escape fire or loss of critical nesting and foraging habitats (Neary et al., 1999; Lindenmayer et al., 2014). Large mammals may survive the immediate disturbance by dispersing but subsequently face increased competition and predation risk in fragmented landscapes (Pellegrini et al., 2018; Engstrom, 2010). Unlike invertebrates, which can rebound relatively quickly under favorable conditions, vertebrate populations often require longer recovery timescales, extending over decades, due to their dependence on complex habitat structures and stable trophic networks (Banks et al., 2011). Taken together, these findings underscore that invertebrates primarily reflect short- to medium-term soil and microhabitat dynamics following fire, while vertebrates embody broader and longer-term ecosystem consequences, including habitat regeneration, landscape connectivity, and food web stability. Recognizing these differential responses highlights the importance of integrating both vertebrate and invertebrate indicators into post-fire monitoring and restoration strategies.

ECOSYSTEM FUNCTION REHABILITATION AND RECOVERY

Post-fire faunal losses are critical not only in terms of species diversity but also for the continuity of ecosystem functions. When the contributions of invertebrates to decomposition and nutrient cycling decline, soil carbon and nitrogen cycles are disrupted; the rate of organic matter breakdown decreases, and soil fertility is diminished (Lavelle et al., 2006; Bardgett & van der Putten, 2014). Temporary reductions in groups such as earthworms, mites, and Collembola slow down humus formation and directly affect soil–water relationships.

Similarly, vertebrate fauna play pivotal roles in post-fire ecosystem functioning. The reduced contributions of birds and mammals to seed dispersal and pollination processes delay natural vegetation regeneration. This, in turn, weakens the mosaic structure of post-fire habitats and can lead to long-term declines in biodiversity (Engstrom, 2010). Moreover, declines in predator populations disrupt predator–prey dynamics, increasing the fragility of trophic networks.

The recovery process varies substantially depending on habitat structure, fire severity, and climatic conditions. In some ecosystems, recovery of particular faunal groups can be observed within three to five years, while in others the process may take decades (Balletto et al., 2023; Fusco et al., 2024). For example, soil fauna in boreal forests have been reported to recolonize relatively quickly (Haimi et al., 2000), whereas in Mediterranean ecosystems, vegetation recovery and faunal diversity restoration require considerably longer timescales (Santorufu et al., 2024).

For this reason, post-fire monitoring programs must include long-term assessments of both invertebrate and vertebrate groups. Evaluations should not be limited to population-level changes but should also consider ecosystem functions such as decomposition, nutrient cycling, and seed dispersal. Such an approach would allow post-fire ecological restoration practices to be assessed more comprehensively, providing a holistic measure of ecosystem recovery success.

In the context of accelerating climate change, restoring ecosystem functions after fire should be embedded within adaptive, landscape-scale management that explicitly leverages faunal indicators. Priority actions include retaining biological legacies and structural complexity (e.g., unburned refugia, coarse woody debris, litter) to facilitate rapid recolonization by soil invertebrates and to provide nesting substrates for vertebrates; promoting habitat heterogeneity and connectivity to buffer trophic networks against recurrent burns; and deploying ecologically informed fuel-management (e.g., patchy prescribed fire, targeted thinning) that maintains soil moisture and organic horizons critical for decomposition and nutrient cycling. Post-fire interventions should favor native vegetation that supports detrital

pathways (thereby accelerating C–N recovery) and minimize practices that remove legacies essential for functional rebound. Monitoring should pair population metrics across trophic levels with process-based indicators (decomposition rates, soil respiration, seed rain) and remote sensing of canopy/ground-layer recovery, enabling adaptive adjustments under shifting fire regimes (Doerr & Santín, 2016; Pellegrini et al., 2018). Collectively, these strategies align functional recovery with climate-adaptation goals by increasing ecosystem resistance to extreme events and shortening the time to regain key services.

CONCLUSION AND RECOMMENDATIONS

Forest fires exert destructive impacts on both vertebrate and invertebrate fauna, fundamentally altering ecosystem functioning. Direct mortality, habitat loss, and food web disruption during fire events lead to severe short-term population declines. In particular, invertebrates with low mobility and vertebrates such as reptiles, amphibians, and small mammals that are unable to abandon their burrows are among the most heavily affected groups. In the post-fire period, the loss of organic matter and changes in microclimatic conditions interrupt the roles of soil fauna in decomposition and nutrient cycling. Similarly, the contributions of vertebrate fauna to seed dispersal, pollination, and trophic balance diminish, thereby weakening the natural regeneration capacity of ecosystems. Nevertheless, certain groups are capable of recovery over the medium to long term as habitats regenerate. The rate of recovery is strongly influenced by factors such as fire severity, ecosystem structure, and prevailing climatic conditions.

To ensure the restoration of ecosystem functions following fire, the development of long-term fauna monitoring programs is of critical importance. Such programs can provide insights into the population dynamics of both vertebrate and invertebrate species, thereby guiding ecological restoration practices. Reforestation and habitat restoration should consider not only the regeneration of aboveground vegetation but also the preservation and enhancement of belowground biodiversity. Selecting plant species that promote decomposition and litter accumulation can accelerate the recovery of soil fauna.

Furthermore, faunal diversity should be integrated into forest management and fire mitigation plans, with soil fauna recognized as key bioindicators of ecosystem health. In the context of climate change, which is intensifying the risk of wildfires, proactive approaches and adaptive forest management strategies at the landscape scale must be prioritized. Such strategies will be essential to building fire-resilient ecosystems and safeguarding faunal diversity in the face of increasingly frequent and severe fire regimes.

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EVALUATION OF HEMATOLOGICAL AND BIOCHEMICAL BLOOD PROFILES IN CLINICALLY HEALTHY SPORT HORSES

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ABSTRACT

The aim of this study was to evaluate the main hematological and biochemical parameters in clinically healthy sport horses in order to assess their health status and metabolic performance. Five horses of different breeds and ages, belonging to two riding clubs, were subjected to clinical and paraclinical examinations, including complete blood count and biochemical blood analysis. The results indicated that hematological parameters were within physiological limits, confirming the good health condition of the animals (Weiss & Wardrop, 2010). Biochemical determinations revealed slight variations in hepatic enzyme activity (GOT, GPT, ALP) and LDH, correlated with the muscular effort specific to sport horses (Hodgson et al., 2014; Kaneko et al., 2008; Lekeux, 2007). These findings emphasize the importance of periodic monitoring of hematological and biochemical profiles for preventing metabolic disorders and optimizing athletic performance in competition horses (Hodgson et al., 2014; Kaneko et al., 2008; Snow & Mason, 2002).

Keywords: sport horse, blood count, biochemical profile, performance, metabolic monitoring

INTRODUCTION

Equestrian sports represent a major branch of the modern equine industry, where both performance and health are essential. Hematological and biochemical evaluations provide fundamental information on the physiological state and adaptation to physical effort (Rose and Hodgson, 2000; McGowan, 2008). Blood tests allow the early identification of metabolic disorders, muscular fatigue, and hepatic imbalances, thus contributing to maintaining health and improving performance (Smith, 2015). Given the increasing competitive demands, hematological and biochemical monitoring has become an indispensable tool for both veterinarians and trainers (Valberg, 2015; Persson, 1997).

MATERIALS AND METHODS

The research was conducted on a group of five clinically healthy sport horses originating from two riding clubs. Individual data were as follows: Carisma (female, 13 years), Colorado (male, Hungarian sport horse, 14 years), Andaluz (female, 17 years), Diana (female, Arabian purebred, 15 years), and Shagya (female, 8 years). Each horse underwent a complete clinical examination, followed by blood collection from the jugular vein under aseptic conditions (Tennant, 2018). Paraclinical determinations included a complete blood count and biochemical blood analysis (urea, creatinine, total proteins, albumin, globulins, A/G ratio, glucose, uric acid, ALP, GOT/ASAT, GPT/ALAT, LDH, cholesterol). The obtained values were compared to reference ranges for sport horses, according to specialized literature (Kaneko et al., 2008; Lumsden, 2000).

RESULTS AND DISCUSSION

Clinical examination showed that all horses were healthy, with no signs of systemic disease. The hematological profile revealed values within physiological limits for erythrocytes, leukocytes, platelets, hemoglobin, and hematocrit, confirming a normal hematological balance (Hodgson et al., 2014; Weiss & Wardrop, 2010). These results are consistent with those reported by Rose and Hodgson (2000), who demonstrated that regular training maintains high hematological stability in sport horses.

Table 1. Hematological parameters in clinically healthy sport horses

Parametru	U/M	Val. fiziol.	1	2	3	4	5
RBC	$10^{12}/\text{mm}^3$	6,8-12,9	8,56	6,95	6,94	8,95	8,93
Hb	g/dl	11-19	14,6	13,2	12,9	16,6	16,1
Ht	%	32-53	37,48	34,54	34,8	39,93	39,14
MCV	fl	37-59	44	50	50	45	44
MCH	pg	12,3-19,7	17,0	18,9	18,6	18,5	18,1
MCHC	g/dl	31-39	38,9	38,1	37,1	41,5	41,2
WBC	$10^9/\text{mm}^3$	5,4-14,3	6,89	8,39	9,37	7,87	7,96
PLT	$10^9/\text{mm}^3$	100-400	129	106	107	99	98
LYM	$10^9/\text{mm}^3$	1,5-7,7	2,73	2,05	2,51	2,26	2,94
MON	$10^9/\text{mm}^3$	0-1,5	0,04	0,47	0,72	0,04	0,36
NEU	$10^9/\text{mm}^3$	2,3-9,5	3,84	5,52	5,81	5,47	4,36
EOS	$10^9/\text{mm}^3$	0,1-1	0,35	0,32	0,30	0,09	0,27
BAS	$10^9/\text{mm}^3$	0-0,5	0,03	0,03	0,03	0,01	0,02
LYM	%	17-68	39,7	33,8	26,8	28,7	36,9
MON	%	0-14	0,6	6,1	7,7	0,6	4,6
NEU	%	22-80	55,8	58,6	62,0	69,4	54,8
EOS	%	1-8	3,6	1,30,2	3,2	1,2	3,4
BAS	%	0-3	0,4		0,3	0,1	0,3

Regarding biochemical parameters, uric acid levels were slightly below the physiological limit in all horses, without clinical significance. According to Kaneko et al. (2008), low uric acid levels can be associated with efficient purine metabolism and moderate protein turnover. The slight increase in alkaline phosphatase (ALP) observed in some individuals may be explained by stress during blood collection (Tennant, 2018). Hepatic enzymes GOT (ASAT/TGO) and GPT (ALAT/TGP) were at the upper physiological limit, possibly reflecting muscular effort (Valberg, 2015; McKenzie et al., 2013).

Table 2. Biochemical blood parameters in clinically healthy sport horses

Parametru	U/M	Valoarea fiziologică	1 carisma	2 colorado	3 andaluz	4 diana	5 shagya
Prot. total	g/dl	5,2-7,9	8,20	7,40	5,90	5,70	5,10
Albumina	g/dl	2,7-3,7	3,30	3,20	2,90	3,86	3,20
Globuline	g/dl	2,5-4,2	4,90	4,20	3,00	3,50	2,90
Album/glob	/	0,95-1,65	0,67	0,76	0,96	1,10	1,10
T-Bil	mg/dl	0,3-3,0	3,7	1,9	0,6	0,7	0,9
BUN	mg/dl	10-25	25	26	16	18	18
UA	mg/dl	2,4-4,7	0,6	0,5	0,5	0,5	0,6
Cre	mg/dl	1,5-2,7	1,1	2,9	0,9	1,2	1,1
TG	mg/dl	25-500	29	29	29	30	34
T-Cho	mg/dl	75-150	81	107	99	78	77
ALP	IU/l	70-230	136	181	177	214	166
GOT	IU/l	200-300	354	340	398	320	345
GPT	IU/l	3-23	12	17	6	23	7
LDH	IU/l	162-412	432	467	445	445	432

LDH (lactate dehydrogenase) showed a mild increase, suggesting transient post-exercise lactic acidosis, a condition frequently described in the literature (Popescu, 2019; Snow & Mason, 2002). Such moderate variations are typical in active sport horses, representing a physiological adaptation to sustained exercise. The combined analysis of these parameters provides valuable insights for assessing performance capacity and preventing overtraining syndromes (Persson, 1997; Lekeux, 2007).



Img. 1 Carisma, female, 13 years

Nr. crt	Parametrii biochimici	Valori obținute	Valori de referință
1	T-prot	8,20	5,2-7,9 g/dl
2	Albumine	3,30	2,7-3,7 g/dl
3	Globuline	4,90	2,5-4,2 g/dl
4	BUN	27,00	10-25 mg/dl
5	Creatinină	2,90	1,5-2,7 mg/dl
6	Bilirubină totală	3,70	0,3-3,0 mg/dl
7	T-CHO	81,00	75-150 mg/dl
8	HDL-c	57,00	10-150 mg/dl
9	TG	29,00	25-500 mg/dl
10	GOT	354,00	200-300 UI/l
11	GPT	7,00	3-23 UI/l
12	GGT	29,00	4-13 UI/l
13	ALP	136,00	70-230 UI/l
14	LDH	432,00	162-412 UI/l
15	CPK	195,00	2-23 UI/l

Table 3. Biochemical blood parameters Carisma, female, 13yr

Biochemical analysis revealed slight deviations in some parameters, including cases of hyperglobulinemia in two horses. These changes may reflect subclinical physiological adaptations to physical exertion or early signs of metabolic imbalance related to muscle activity, liver function, or digestion, even in the absence of clinical symptoms (McKenzie et al., 2013; Smith, 2015).



Nr. crt	Parametrii biochimici	Valori obținute	Valori de referință
1	T-prot	7,40	5,2-7,9 g/dl
2	Albumine	3,20	2,7-3,7 g/dl
3	Globuline	4,20	2,5-4,2 g/dl
4	BUN	22,30	10-25 mg/dl
5	Creatinină	2,50	1,5-2,7 mg/dl
6	Bilirubină totală	1,90	0,3-3,0 mg/dl
7	T-CHO	107,00	75-150 mg/dl
8	HDL-c	69,00	10-150 mg/dl
9	TG	27,00	25-500 mg/dl
10	GOT	340,00	200-300 UI/l
11	GPT	7,00	3-23 UI/l
12	GGT	28,00	4-13 UI/l
13	ALP	181,00	70-230 UI/l
14	LDH	467,00	162-412 UI/l
15	CPK	273,00	2-23 UI/l

Table 4. Biochemical blood parameters Colorado, male, 14 yrs

The results confirm that clinical examination alone is not sufficient for a complete evaluation of sport horses' health status. Periodic biochemical determinations allow early detection of metabolic dysfunctions, ensuring proper preparation for competitions (Rose and Hodgson, 2000; Kaneko et al., 2008). Moreover, these findings underline the importance of routine blood profiling in performance horses, not only for diagnostic purposes but also as a preventive tool. Regular monitoring can help detect subtle imbalances early, guiding training adjustments and supporting the long-term health and performance of sport horses (Valberg, 2015; McGowan, 2008).

CONCLUSIONS

The investigated horses exhibited hematological and biochemical values within physiological limits, indicating an overall good health condition. The mild variations in hepatic and muscular enzyme levels reflect physiological adaptation to physical effort, without pathological relevance (McKenzie et al., 2013). Periodic monitoring of hematological and biochemical profiles is essential for maintaining performance and preventing chronic fatigue. Blood testing provides objective information that complements clinical examination and represents an indispensable tool in veterinary sports medicine (Lekeux, 2007; Smith, 2015). Continuous evaluation allows early detection of physiological imbalances caused by intense training, dietary changes or competition stress. Implementing preventive health programs and individualized nutritional and recovery plans can help maintain optimal performance levels and ensure the long-term well-being of these valuable animals.

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AQUACULTURE AND INLAND FISHERIES IN ALBANIA: TRENDS, PRODUCTION, AND POLICY FRAMEWORKS

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ABSTRACT

Aquaculture and inland fisheries are central components of Albania's food security, rural development, and biodiversity conservation strategies, despite contributing only about 0.3% to national GDP. These sectors play an outsized role in sustaining rural livelihoods, particularly in lakeside communities, and in safeguarding endemic species such as the Ohrid trout (*Salmo letnica*). This study provides an integrated assessment of Albania's inland fisheries and aquaculture, combining national statistics and policy review. Inland capture fisheries, operating across Albania's natural lakes, lagoons, and rivers, have shown significant growth—expanding from 1,688 tonnes in 2016 to 4,858 tonnes in 2023—though lagoon fisheries face severe pressures from pollution, habitat degradation, and invasive species. Aquaculture production has tripled over the past decade, driven by freshwater trout and carp farming alongside marine species such as seabass and seabream, supported by hatchery programs producing millions of juveniles annually for both farming and restocking. Regional comparisons position Albania as a rising leader in the Western Balkans, second only to Croatia in aquaculture value, reflecting strong growth dynamics and policy-driven modernization. However, persistent challenges remain, including weak enforcement capacity, insufficient data collection (especially for riverine fisheries), infrastructural deficits in processing and markets, and the collapse of mussel farming in coastal lagoons. The findings highlight Albania's progress toward a sustainable blue economy, while underscoring the need for stronger institutions, climate-resilient management, and enhanced regional cooperation to secure long-term sustainability and competitiveness.

Keywords: Aquaculture; Inland fisheries; Sustainability; Policy frameworks; Fish production; Regional comparison

INTRODUCTION

Aquaculture and inland fisheries are key pillars of Albania's strategy for food security, rural employment, and biodiversity conservation. Although their direct contribution to national GDP is modest (around 0.3%), these sectors exert a disproportionate impact on local economies, particularly in rural and lakeside communities where alternative livelihoods are limited. In recent years, aquaculture has emerged as a dynamic and expanding component of the fisheries sector, offering sustainable alternatives to wild capture and contributing to economic diversification. Inland capture fisheries also remain essential, operating across Albania's extensive freshwater systems—including natural lakes and coastal lagoons—which together account for more than one-quarter of the country's reported capture fish production. Beyond their nutritional and economic roles, these inland waters support endemic species of high

conservation value, such as the Ohrid trout (*Salmo letnica*) and belvica (*Salmo ohridanus*), underscoring the need to balance utilization with ecological stewardship.

Inland fisheries in Albania are characterized by multi-use freshwater systems, where exploitation overlaps with conservation, agriculture, hydropower, and tourism. This complexity highlights the need for integrated management approaches that ensure sustainable resource use and better coordination across sectors (Shumka et al., 2024).

Albania hosts 101 freshwater fish species across 11 hydrographic basins, with a remarkable level of diversity and endemism shaped by its unique geological and climatic conditions. However, these ecosystems face increasing pressures from habitat modification, invasive species, and human activities, underscoring the need for integrated management and conservation (Shumka et al., 2023).

The Italian and Balkan peninsulas are recognized as hotspots of endemic freshwater fish diversity, with about 86 endemic species recorded in Italy, Croatia, Albania, and Montenegro. In Albania, aquaculture is dominated by common carp and rainbow trout, alongside farming of the endemic Ohrid trout (*Salmo letnica*), which illustrates both the country's biodiversity value and its production potential (Bianco & Ketmaier, 2015).

This study provides a focused assessment of Albania's aquaculture and inland fisheries sectors. It examines socio-economic significance, production and catch trends, hatchery output, and the policy frameworks shaping the sector's trajectory. A national perspective is combined with a regional comparison across the Western Balkans, where fisheries and aquaculture development vary widely due to ecological resources, policy environments, and investment capacities. Using FAO FishStat and national statistical data, Albania's production volumes and aquaculture values are compared with those of Bosnia and Herzegovina, Montenegro, Croatia, North Macedonia, Serbia, and Slovenia for the period 2019–2023. This comparative lens situates Albania within the regional context and highlights both best practices and persistent challenges in achieving sustainable and inclusive growth.

By integrating ecological, economic, and policy perspectives, the study aims to inform national strategies and regional cooperation efforts that support a sustainable, low-carbon, and resilient blue economy grounded in the responsible use of inland aquatic resources. Key questions addressed include: how production and catch trends have evolved in the last decade; what challenges and opportunities the sectors face, particularly in freshwater and lagoon fisheries; and how policy and management interventions can strengthen long-term sustainability.

METHODOLOGY

This study applied a multidisciplinary approach, combining quantitative data analysis with a review of policy frameworks and relevant literature. Statistical data were collected primarily from the Albanian Institute of Statistics (INSTAT) and the Ministry of Agriculture and Rural Development, supplemented by international datasets such as FAO FishStat. The analysis covers the period from 2016 to 2024, with particular focus on national production trends. Inland catch data for 2016–2024 were examined to assess production dynamics and interannual variability. Where available, catches were disaggregated by water body type (lakes, lagoons, rivers). The geographic and ecological characteristics of key fishing areas, including Lakes Shkodra, Ohrid, and Prespa, were considered to contextualize resource use and conservation needs.

Aquaculture output was analyzed in terms of annual production volumes and hatchery performance. Hatchery and nursery production figures were compiled by species and year to evaluate the scale of juvenile production for trout, carp varieties, and other species, and to assess the contribution of these facilities to aquaculture growth and restocking programs.

Regional comparisons were conducted using FAO FishStat data for seven Western Balkan countries: Albania, Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia, and Slovenia. Indicators included total fisheries production (capture plus aquaculture in live weight), aquaculture production volume (tonnes), and aquaculture production value (USD). The comparative timeframe (2019–2023) enabled analysis of growth trajectories, fluctuations, and structural differences among countries, allowing Albania's performance to be positioned relative to its neighbors.

To complement the statistical analysis, national fisheries legislation, policy documents, and investment programs were reviewed, together with relevant EU alignment processes. Secondary literature on Albania's blue economy and inland fisheries was also consulted to provide socio-economic and environmental context, and to support the discussion of structural constraints and development opportunities.

RESULTS AND DISCUSSION

Socio-Economic Importance

Aquaculture and inland fisheries contribute only about 0.3% to Albania's GDP, yet their socio-economic importance far exceeds this narrow measure. These sectors provide vital sources of employment, nutrition, and income, especially for communities located near large lakes and river systems. Inland fisheries alone, operating across numerous lakes and lagoons, account for more than one-quarter of Albania's total capture fisheries production, highlighting their central role in the national fish supply. For many rural households, fishing represents a primary or supplementary livelihood, while fish products are an essential component of local food security. Traditional practices in areas such as Lake Shkodra, Lake Ohrid, and the Prespa Lakes also reinforce community identity and cultural heritage.

Beyond their economic role, inland waters and aquaculture support important environmental and biodiversity functions. The transboundary lakes of Shkodra, Ohrid, and Prespa are recognized biodiversity hotspots, home to endemic species found nowhere else. Their sustainable management therefore has regional conservation significance. The Ohrid trout (*Salmo letnica*), for example, is valued not only for its commercial importance but also for its conservation status; its recovery has relied on hatchery programs and cross-border coordination in fisheries management. In sum, although modest in macroeconomic terms, aquaculture and inland fisheries form a foundation for Albania's rural development, food security, and environmental stewardship.

Inland Fisheries: Resources and Catch Trends

Albania's inland fisheries are anchored in extensive freshwater resources, most notably its major natural lakes—Shkodra, Ohrid, and the two Prespa lakes—which are of exceptional ecological and economic significance. These transboundary water bodies support diverse fish communities and productive fisheries but also require careful management to avoid overexploitation. Sustaining their yields and conserving their unique biodiversity depends heavily on cross-border cooperation with Montenegro (Lake Shkodra) and with North Macedonia and Greece (Ohrid and Prespa). Beyond the lakes, Albania's hydrographic network of over 11,000 km of rivers, including the Drin, Vjosa, Seman, Shkumbin, and Mat, has potential for significant inland fisheries. However, systematic data collection for riverine catches remains scarce, suggesting that the actual contribution of rivers and streams is underreported. This data gap poses a dual challenge: it may reflect unregulated fishing pressure or reveal underutilized resources that could be more effectively managed.

Catch statistics for inland waters show a clear upward trend between 2016 and 2024. Reported production rose from 1,688 tons in 2016 to a peak of 4,858 tons in 2023, before a slight decline to 4,505 tons in 2024 (Figure 1). This growth highlights the increasing role of inland waters in supporting Albania's fish supply, food security, and rural livelihoods. More than half of the recorded catches are composed of cyprinid species (Cyprinidae), with growth driven by improved management practices and effective restocking programs in the major lakes.

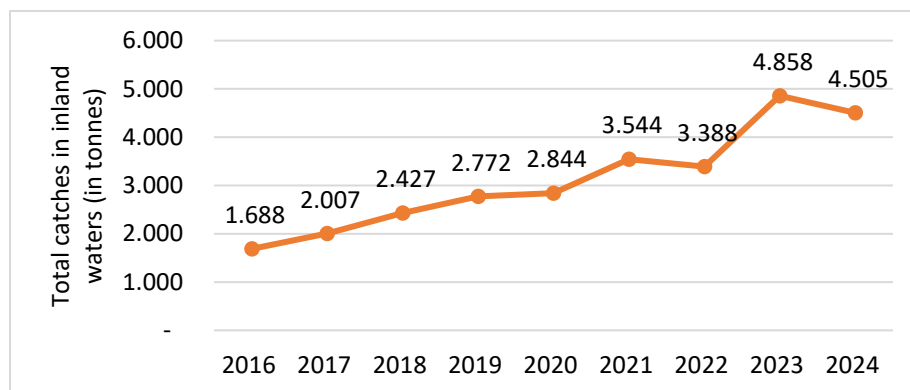


Figure 1. Total catches in inland water during 2016-2024. (In tons).

Source: Authors Calculations, Based on INSTAT Data

Catch records from Lake Ohrid indicate that native species such as *Alburnus scoranza*, *Rutilus prespensis*, and *Salmo letnica* remain central to local fisheries, although shifts in catch per unit effort point to both ecological pressures and socio-economic challenge (Bllaca & Kolaneci, 2023). These findings underscore the need for long-term sustainability strategies in Albania and across the Balkans. Similarly, a 2022 socio-economic assessment of Albania's lagoon fisheries documented declining catches, an aging fisher population, and mounting pressures from illegal fishing and invasive species such as the blue crab (*Callinectes sapidus*). The study further emphasized the lack of systematic monitoring and highlighted the urgent need for reliable statistical surveys to manage these vulnerable ecosystems effectively (Bakiu et al., 2022).

Aquaculture Production and Hatchery Efforts

Aquaculture in Albania has shown dynamic growth over the past decade, becoming an increasingly important complement to wild fisheries. Total production nearly tripled between 2016 and 2023, rising from about 3,200 tonnes to more than 9,100 tonnes. This expansion has been driven mainly by finfish culture. Rainbow Trout farming in cold freshwater systems has grown rapidly, while marine aquaculture continues to produce seabass and seabream in cages. Mediterranean mussel (*Mytilus galloprovincialis*) farming in coastal lagoons also contributes, though production has declined sharply in recent years. In 2023, aquaculture output included approximately 9,141 tonnes of finfish (freshwater and marine combined) and 186 tonnes of mussels. Despite the decline in mussel farming, overall aquaculture trends remain strongly positive.

A key foundation of this growth is Albania's network of hatcheries and nurseries, which serve both commercial production and biodiversity conservation. Hatcheries release millions of juveniles annually for species such as Rainbow Trout, Ohrid trout, and carp, which are either grown in farms or stocked into natural waters to support capture fisheries. In 2023, hatchery output included 3.3 million Rainbow Trout fry, 1.8 million Ohrid trout fry, and more than 1 million common carp fry (Table 1). The surge in Rainbow Trout production in 2022 (9.0 million

juveniles, up from zero in 2021), followed by a reduction in 2023, illustrates an adaptive management approach. Hatchery output is adjusted in response to broodstock availability, market demand, survival rates, and the success of previous stocking efforts.

Table 1. Hatchery and Nursery Production in Albania (Million Juveniles)

Species	2021	2022	2023
Ohrid Trout	1.4	1.6	1.8
Common Carp	1.0	1.6	1.2
Silver Carp	1.3	0.5	0.4
Bighead Carp	0.2	0.2	0.1
White Amur	0.5	0.2	0.2
Rudd	0.1	0.0	0.0
Rainbow Trout	0.0	9.0	3.3

Source: INSTAT, annual fisheries reports.

The dual role of hatcheries—enhancing commercial aquaculture and conserving native species—is particularly significant in Albania. For endemic species such as the Ohrid trout, hatchery-based stock enhancement is carried out in coordination with conservation authorities to strengthen wild populations and preserve genetic diversity. At the same time, the availability of hatchery-raised fry for carp and trout reduces pressure on wild stocks by making aquaculture more self-sufficient. Continued investment in hatchery infrastructure, coupled with rigorous genetic management and disease control, will be essential to sustain aquaculture expansion while safeguarding natural fisheries. Although Albania already operates hatcheries for carp and other freshwater species, further investment and research are needed to expand capacity and improve resilience (Jimenez, 2015).

National Fishing Trends (2016–2023)

Across all fishing activities in Albania—marine capture, inland capture, coastal lagoon fisheries, and aquaculture—a marked shift in sector composition has taken place over the 2016–2024 period (Figure 2). Total capture production (marine, coastal, lagoon, and inland waters) fluctuated between 7,800 and 10,000 tonnes, reaching a low point in 2020 due to the COVID-19 pandemic’s disruption of labor and markets, before peaking in 2023. Marine fisheries remained the largest contributor within capture production but showed a steady decline after 2018, reflecting either overexploitation or the effects of stricter management measures. Coastal line catches were highly volatile, with steep drops after 2017 and only partial recoveries, while coastal lagoon fisheries displayed a long-term downward trend, underlining their ecological fragility. In contrast, inland waters showed strong growth, with catches rising from 1,688 tonnes in 2016 to 4,858 tonnes in 2023, more than doubling within seven years and highlighting their growing importance for national fish supply.

Aquaculture has emerged as the dominant driver of overall production. Output expanded from 3,200 tonnes in 2016 to over 9,000 tonnes in 2023, before declining slightly to 7,165 tonnes in 2024. Despite this recent drop, aquaculture remains the backbone of the sector and its primary source of growth. Mussel (*Mytilus galloprovincialis*) production, by contrast, has been highly unstable: after collapsing post-2016, it showed a brief recovery but fell again in 2023–2024, largely due to environmental and market pressures. When total production is considered—including aquaculture and mussel farming—Albania’s output grew steadily from

12,534 tonnes in 2016 to a peak of 19,338 tonnes in 2023, before declining to 16,452 tonnes in 2024.

These patterns point to a structural transformation: traditional marine and lagoon fisheries are in decline, while inland waters and aquaculture are assuming an increasingly central role in sustaining Albania's fish supply, food security, and rural livelihoods.

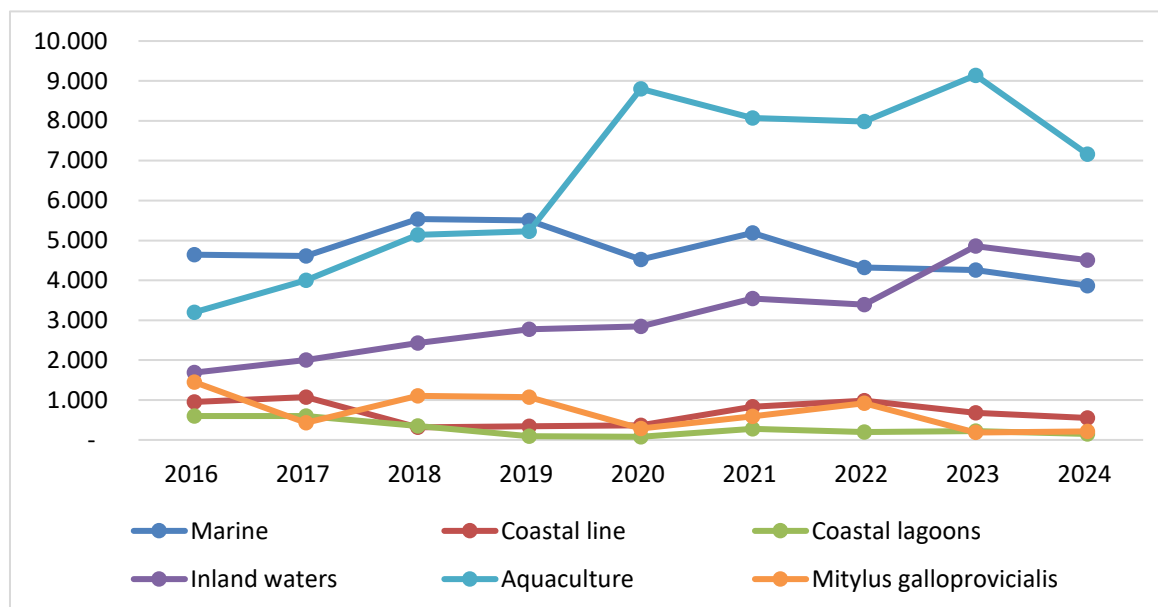


Figure 2. Trends in fishing categories over the years
Source: Authors Calculations, Based on INSTAT Data

Challenges in Freshwater and Lagoon Fisheries

Despite notable progress in recent years, Albania's fisheries sector continues to face significant challenges and structural constraints, particularly in inland and lagoon systems. One of the most pressing issues is the environmental and infrastructural degradation of coastal lagoons. Mussel farming, once a leading sub-sector, has suffered a dramatic collapse, with production falling from 1,450 tonnes in 2016 to just 186 tonnes in 2023. This decline has been linked to water quality deterioration in the Butrint Lagoon, disease outbreaks, and persistent market and export barriers. Reviving this industry will require targeted interventions such as strengthened water quality monitoring, investment in modernized farming infrastructure (including rafts and depuration facilities), and stricter quality control to meet international export standards. More broadly, lagoon ecosystems face cumulative pressures from upstream pollution, habitat alteration, and climate change, including rising temperatures and altered salinity regimes, all of which undermine the sustainability of aquaculture and wild lagoon fisheries.

Inland fisheries also confront critical management challenges, most notably the lack of reliable data on riverine fisheries. Commercial and subsistence catches from rivers are often unreported, leading to an underestimation of the sector's economic value and obscuring the risks of overharvesting sensitive stocks such as trout and eel. Addressing this gap will require improved monitoring systems, licensing and catch reporting mechanisms, and greater involvement of local communities through co-management initiatives.

Structural weaknesses are further compounded by the fragmentation and limited capacity of fishing organizations. Many inland fisheries are managed by small associations or cooperatives with insufficient financial and technical resources, limiting their ability to enforce regulations, restore habitats, and participate effectively in decision-making. Transboundary

coordination also remains a challenge, particularly for the large lakes shared with neighboring countries. Harmonizing conservation efforts and combating illegal fishing require stronger bilateral agreements, joint scientific monitoring, and enhanced collaboration under EU-supported or regional frameworks.

These challenges are consistent with wider regional patterns. Lagoon fisheries across the Balkans face declines due to illegal fishing, invasive species, and weak organizational capacity (Bakiu et al., 2022), while riverine fisheries remain underreported and poorly monitored (Shumka et al., 2023). Regional studies also highlight the vulnerability of endemic lake fisheries to pollution, overexploitation, and fragmented governance (Bianco & Ketmaier, 2015).

Regional Comparisons in the Western Balkans (2012–2022)

A comparative analysis of fisheries and aquaculture indicators across the Western Balkans shows that Albania has emerged as one of the regional leaders in fish production. According to FAO FishStat data, total production (capture plus aquaculture, in live-weight tonnes) increased from 7,810 tonnes in 2020 to 10,011 tonnes in 2023, reflecting a nearly 30% rise in just four years. This growth reflects expansion in both capture fisheries and aquaculture. By contrast, Bosnia and Herzegovina has consistently reported only 305 tonnes annually, indicating either stable but very limited production or unchanging reporting practices. Croatia remains the undisputed regional leader, with production volumes exceeding 50,000 tonnes each year. Although output peaked at more than 71,600 tonnes in 2020 before falling back to 56,200 tonnes in 2023, Croatia continues to dominate Adriatic fisheries. Its strong position is underpinned by a highly developed mariculture sector, which accounts for 87% of national aquaculture output by volume and expanded by nearly 40% between 2013 and 2022, concentrated in coastal counties such as Zadar and Dubrovnik-Neretva (Milošević et al., 2024).

Other countries display much smaller but revealing trends. Montenegro maintains relatively modest production, averaging around 800 tonnes with a peak of 1,276 tonnes in 2019. North Macedonia produces between 399 and 514 tonnes annually, reflecting a limited but stable inland fishery. Serbia consistently reports higher production than most of its neighbors (excluding Croatia), ranging from 1,900 to 2,547 tonnes, with a recovery to 2,470 tonnes in 2023. These patterns align with country-level studies, which emphasize how Serbia's fisheries reflect broader political, social, and environmental pressures. Current stocks vary from “at the limit of sustainability” in parts of the Danube and Sava to relatively sustainable levels in reservoirs and protected areas, illustrating the cumulative effects of hydropower development, agriculture, pollution, and overfishing (Simić et al., 2024). **Slovenia** contributes the smallest amounts, between 260 and 312 tonnes, remaining relatively stable over the years.

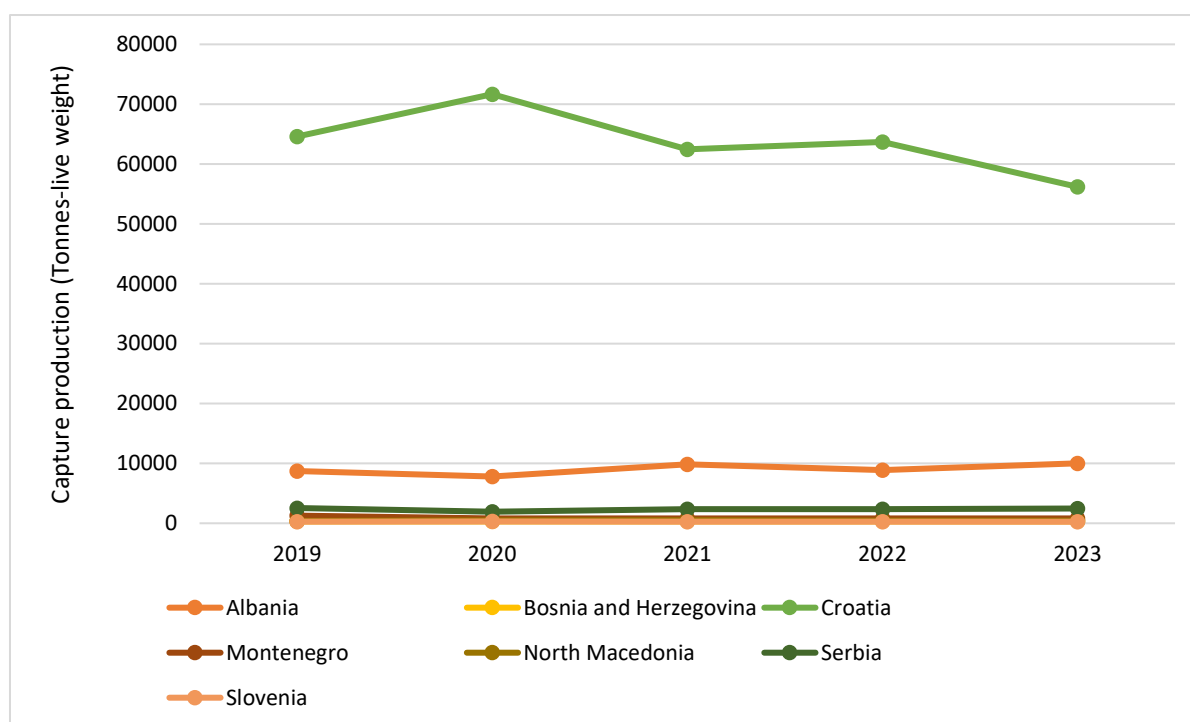


Figure 3. Fish production in live weight (tonnes) in Balkan countries (2019–2023)
Source: Authors calculations based on FAO FishStat

Aquaculture-specific data further underscore Albania's progress in recent years. **Figure 4** shows that between 2019 and 2023, production volumes increased steadily from 6,300 tonnes to 9,327 tonnes, with only minor year-to-year variation. This trajectory reflects the sector's stable expansion, particularly in marine fish farming and inland trout culture. By contrast, Bosnia and Herzegovina remained stable at around 3,800–4,000 tonnes, suggesting a mature but limited inland system dominated by carp and trout. Croatia continues to lead the region by a wide margin: production rose from approximately 20,443 tonnes in 2019 to nearly 28,000 tonnes in 2021, before stabilizing at around 27,000 tonnes in 2022–2023. Its dominance is driven by large-scale marine aquaculture, supplying high-value species such as sea bass and sea bream to EU markets. Montenegro, though small in absolute terms, shows recovery and growth; following a decline below 900 tonnes in 2020–2021, output reached 1,364 tonnes in 2023, reflecting renewed investment in marine farming. North Macedonia maintains modest but stable levels (1,600–1,900 tonnes annually), almost entirely from freshwater carp and trout. Serbia supports one of the region's larger inland aquaculture sectors, with production fluctuating between 5,800 and 7,300 tonnes; after peaking in 2021, it stabilized around 5,800 tonnes by 2023, demonstrating resilience despite some volatility.

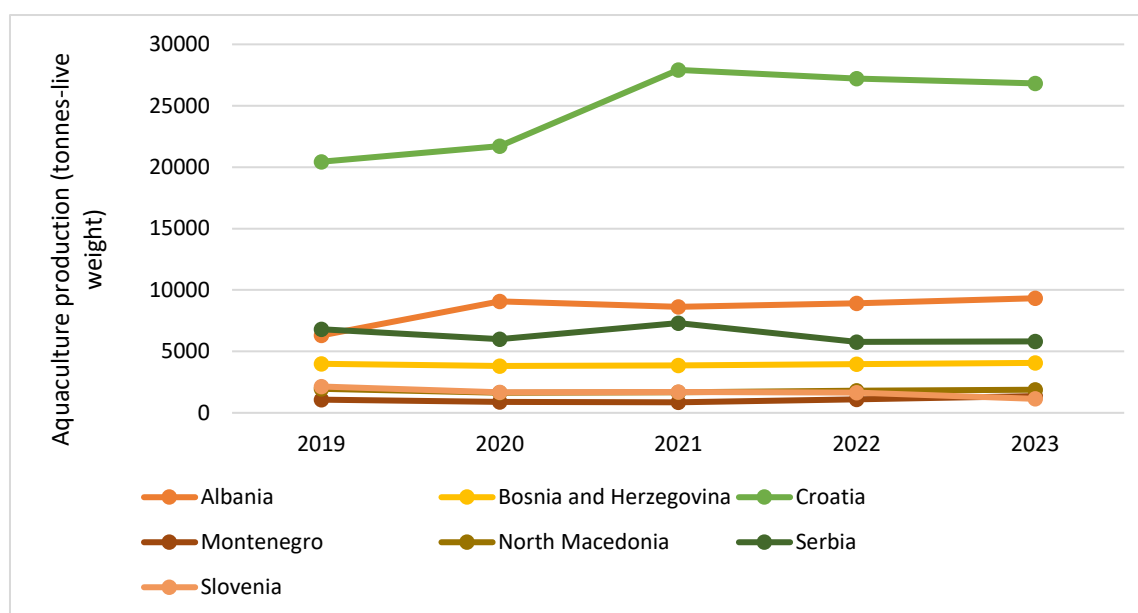


Figure 4. Aquaculture production in tonnes (2019-2023)

Source: Authors calculations based on FAO FishStat

The aquaculture production value between 2019 and 2023 are shown in figure 5, highlighting both growth and disparities across the Balkans. Albania recorded one of the strongest upward trends, nearly doubling from USD 22,374 thousand in 2019 to more than USD 56,500 thousand in 2023, reflecting robust expansion in marine aquaculture (sea bass, sea bream, mussels) and inland trout farming. This performance places Albania second only to Croatia. Bosnia and Herzegovina, by contrast, showed only modest fluctuations between USD 12,500 and 16,700 thousand, while North Macedonia rose moderately from USD 9,536 to 11,242 thousand. Croatia dominates the region, with values climbing from USD 135,700 thousand in 2019 to more than USD 220,300 thousand in 2023, representing over half of all regional aquaculture output. Montenegro's values almost doubled from 2019 to 2023 (from 4,254 to 7,207 thousand), confirming proportional growth despite small absolute scale. Serbia also performed strongly, with production value rising from USD 20,241 to 27,321 thousand, reflecting its robust carp-based aquaculture. Slovenia was the only country to register a decline, from USD 7,246 to 6,205 thousand, pointing to stagnation and limited expansion capacity. Taken together, **Figures 4 and 5** demonstrate that aquaculture in the Balkans expanded significantly between 2019 and 2023, led overwhelmingly by Croatia and, increasingly, Albania. Serbia also showed strong growth, while Bosnia, North Macedonia, and Montenegro maintained modest but stable sectors. Slovenia's decline underscores the uneven development of aquaculture across the region. These contrasts highlight both the diverse aquaculture potentials of the Balkans and the critical role of coastal resources and market access in driving sectoral growth.

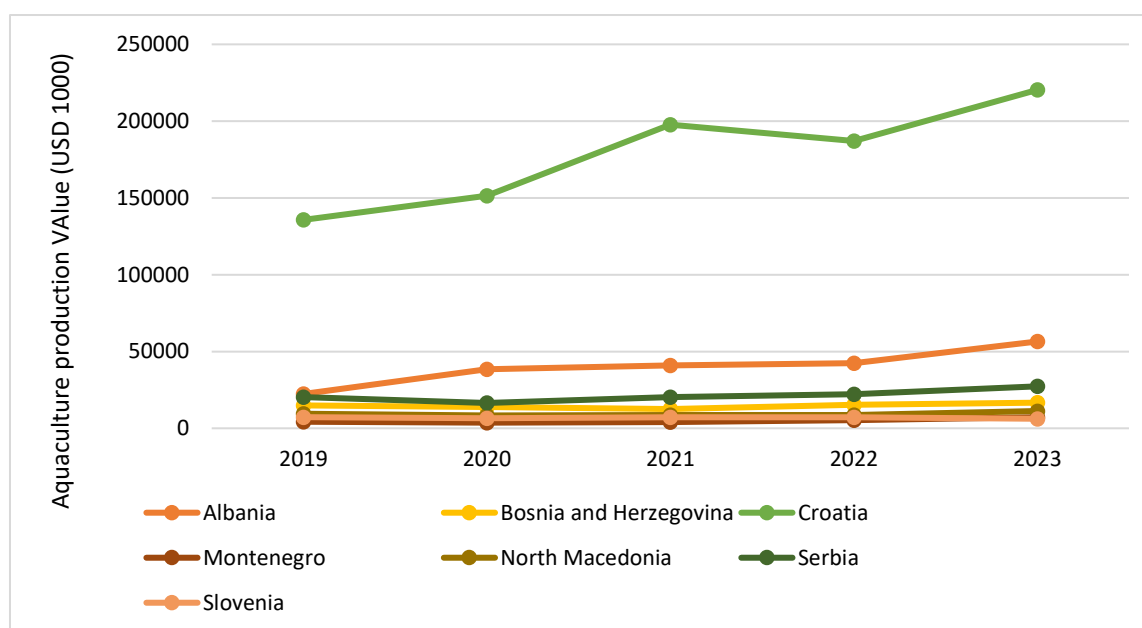


Figure 5. Aquaculture production value in Balkan countries (2019–2023)

Source: Authors calculations based on FAO FishStat

The dynamics observed in aquaculture production value across the Balkans between 2019 and 2023 are consistent with broader national and regional analyses. FAO country profiles emphasize that Albania's aquaculture sector has expanded rapidly in both marine (sea bass, sea bream, mussels) and inland trout farming, supported by targeted investments and an increasingly export-oriented focus (FAO, 2019). A dedicated FAO assessment of Albania's aquaculture potential further highlights the sector's capacity to nearly double its value within a short period, illustrating the transition from small-scale freshwater farming to a diversified marine and inland aquaculture industry (FAO, 2020). Croatia's dominance in regional aquaculture value mirrors Mediterranean-wide trends, where growth has been concentrated in high-value species such as sea bass and sea bream, supported by well-developed mariculture infrastructure and strong export orientation (Sicuro, 2024). At the European level, Eurostat aquaculture statistics confirm that sector value is increasingly concentrated in fewer, higher-priced species, underscoring how Croatia's and, increasingly, Albania's growth trajectories are tied to market access and consumer demand (Eurostat, 2025). Collectively, these findings explain the observed disparities across the Western Balkans, where Albania and Croatia emerge as regional leaders, Bosnia and Herzegovina, North Macedonia, and Montenegro sustain more modest but stable sectors, and Slovenia shows signs of stagnation..

These comparisons also highlight how policy support and investment directly influence outcomes. Albania and Croatia, which have prioritized fisheries and aquaculture development through subsidies, infrastructure upgrades, and alignment with EU regulations, have consistently outperformed neighbors facing stagnation or decline. North Macedonia's recent growth demonstrates the potential impact of targeted measures such as hatchery programs or lake co-management reforms, while Bosnia and Herzegovina's stagnation reflects unresolved structural barriers. Albania's position—second in the region in both aquaculture output and value—demonstrates its growing potential as a regional hub for sustainable aquaculture. This progress underscores the importance of continued emphasis on best practices, institutional strengthening, and knowledge transfer among Balkan countries. Enhanced regional cooperation, whether through multilateral platforms such as the General Fisheries Commission for the Mediterranean (GFCM) or bilateral partnerships, could help lagging countries catch up and foster more balanced progress in sustainable fisheries across the Western Balkans.

Trade data and market trends

From 2012 to 2021, Albania's aquatic product exports grew more than fourfold—from \$36.7 million to \$152.6 million—driven by improved production, certification standards, and access to European markets. Imports also rose to \$124.2 million, reflecting increased domestic demand. Despite this, Albania maintained a positive trade balance, with exports consistently exceeding imports. Total trade value reached \$276.9 million in 2021, underscoring the sector's growing economic significance (Figure 6).

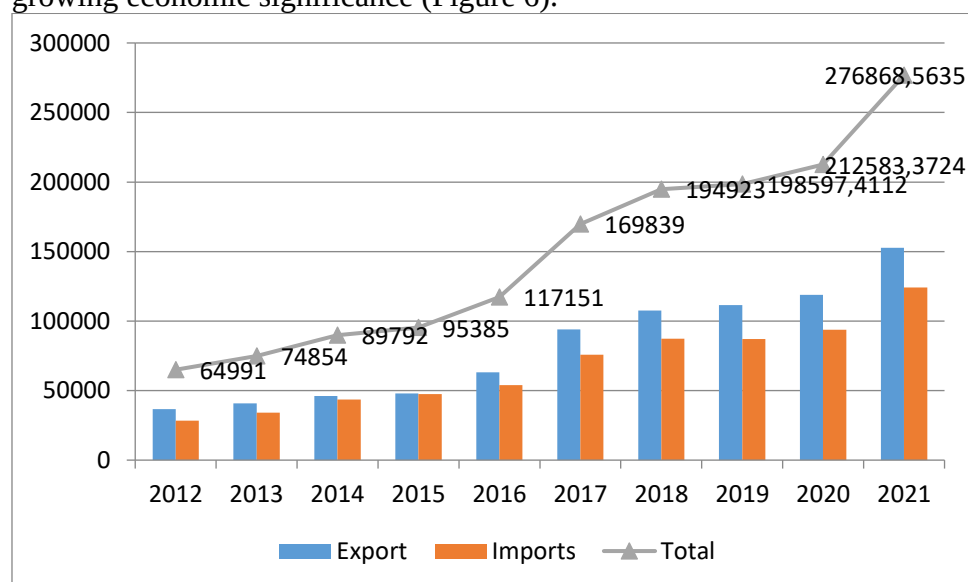


Figure 6: Albania's aquatic products trade (2012–2021)

Source Authors calculations based on FAO, FishStat

Policy Frameworks and Structural Constraints

Albania's fisheries and aquaculture sectors operate within an evolving policy framework that seeks to balance growth with sustainability. In recent years, national legislation has been revised to align more closely with European Union standards as part of the EU integration process. Current policies emphasize the sustainable use of aquatic resources, the implementation of science-based management plans for key fisheries, and the development of aquaculture under environmentally responsible guidelines. Through the Ministry of Agriculture and Rural Development, the government has supported initiatives such as the creation of fishery management organizations for inland waters, restocking programs in transboundary lakes, and investment schemes to modernize aquaculture facilities. These efforts have provided the foundation for positive production trends observed in recent years.

Nonetheless, several structural constraints continue to hinder the sector's full potential. Monitoring and enforcement capacity remains limited, making it difficult to ensure compliance with regulations on gear use, seasonal bans, and quotas. The dispersed nature of inland fisheries and the predominance of small-scale operators exacerbate these challenges. Strengthening inspection and enforcement agencies, while engaging local communities in co-management arrangements, would improve adherence to sustainable practices. Reliable data collection systems are also urgently needed, as gaps in riverine catch statistics and other fisheries data undermine effective resource management and evidence-based policymaking.

Infrastructure and market access represent another significant constraint. Many inland fishers lack cold storage, processing facilities, and consistent markets, which limits both income and growth opportunities. Investment in value chain infrastructure—such as processing plants, transportation networks, and market development for value-added products—could improve

the economic resilience of fishing communities. For aquaculture, persistent challenges include feed supply, biosecurity, and environmental licensing. Clearer and more efficient procedures for farm concessions in coastal and inland waters are required to attract investment while protecting ecosystems.

Transboundary and ecosystem considerations are also central to Albania's policy framework. Participation in regional bodies and agreements, including the General Fisheries Commission for the Mediterranean (GFCM) and joint lake management committees with neighboring countries, is vital for addressing shared concerns such as migratory fish stock management, invasive species control, and biodiversity protection. Climate change adds another layer of complexity, as fisheries are increasingly vulnerable to droughts, rising temperatures, and extreme weather events that affect aquatic habitats.

Recent assessments show that while Albanian fisheries and aquaculture legislation has been substantially updated to align with the EU acquis, critical gaps remain in enforcement, monitoring control, and surveillance (Vuksani et al., 2025). Structural issues such as illegal and unreported fishing, overcapacity of the fleet, lack of modern wholesale fish markets, and weak waste management systems continue to undermine sector sustainability. Addressing these challenges will require investments in processing infrastructure, market development, and stronger institutional enforcement mechanisms (Alite & Çeka, 2024).

Robust policy alignment and targeted investment will be essential to overcoming these hurdles. Albania's progress compared with some of its regional neighbors can be attributed in part to proactive measures and strategic projects, such as hatcheries and port improvements for fish trade. Going forward, maintaining this momentum will depend on continuous policy adaptation to emerging challenges, stronger institutional frameworks for implementation and enforcement, and enhanced regional cooperation to ensure that progress in one country is not undermined by inaction in another.

CONCLUSION

Fisheries and aquaculture in Albania represent a sector of modest GDP contribution but significant socio-economic and environmental importance. The analysis shows that while inland capture fisheries remain vital for food security and rural livelihoods, aquaculture has emerged as the principal driver of growth, with production nearly tripling between 2016 and 2023. This structural transformation reflects both increased reliance on inland waters and the expansion of marine aquaculture, though challenges persist in lagoon ecosystems, where mussel farming has collapsed due to pollution, disease, and market barriers.

Regional comparisons highlight Albania's progress relative to its Western Balkan neighbors: the country now ranks second only to Croatia in aquaculture value, underscoring its potential to serve as a regional hub for sustainable production. Strong policy alignment with EU standards, hatchery-based restocking programs, and targeted investments have supported this trajectory. However, structural constraints—including weak monitoring and enforcement, gaps in river fisheries data, limited infrastructure, and fragmented management organizations—continue to hinder full sectoral development.

Going forward, strengthening institutions, modernizing infrastructure, and improving data collection will be essential to ensure sustainability. Enhanced regional cooperation, particularly in managing shared water bodies and harmonizing conservation measures, will also be critical. By balancing production growth with biodiversity protection, Albania can consolidate its position as a regional leader and contribute to a resilient blue economy in the Western Balkans.

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THE IMPORTANCE OF INTERCROPPING IN FORAGE PRODUCTION

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ABSTRACT

Intercropping systems that combine cereals with legumes or other forage species have gained increasing attention due to their potential to improve forage yield and nutritive value. Integrating cereals with protein-enhancing species offers both nutritional and economic benefits, particularly in silage production. Numerous studies have demonstrated that intercropping maize with legumes such as soybean, cowpea, or common vetch results in higher dry matter yield and crude protein content compared to sole cropping. For instance, mixed maize-soybean systems produced forage with 11–51% greater crude protein content than pure stands. Similarly, intercropping faba bean with wheat improved dry matter yield and enhanced crude protein, neutral detergent fiber (NDF), and water-soluble carbohydrate (WSC) levels compared to sole crops. Pea-barley and pea-oat mixtures have also been reported to increase forage yield and quality, with land equivalent ratio (LER) values of 1.05–1.24 for biomass and 1.05–1.26 for crude protein, confirming the advantage of intercropping. Maize-legume mixtures consistently show higher digestibility, greater ash content, and reduced NDF and acid detergent fiber (ADF) compared to sole maize. Furthermore, vetch-cereal combinations and specific seeding ratios, such as 65:35 vetch: barley, have been found to optimize forage quality. Overall, intercropping systems effectively enhance forage production by increasing dry matter yield, improving crude protein levels, and enriching digestibility, thereby meeting livestock nutritional needs while promoting sustainable and efficient use of arable land.

Keywords: Intercropping, Forage crops, Crude protein, Dry matter yield, Silage quality, Legume-cereal mixtures, Nutritional improvement

INTRODUCTION

Increasing global population and demand for animal-derived protein have made efficient forage production a central concern in sustainable livestock systems. Forage crops represent the foundation of ruminant nutrition, and the rising feed costs have encouraged alternative cultivation methods that optimize both yield and quality. Intercropping, defined as the cultivation of two or more species in the same field simultaneously, has re-emerged as a promising strategy to enhance resource utilization and stabilize production (Lithourgidis et al., 2011).

Modern agricultural practices have often relied on monoculture systems that, while efficient under high-input management, can lead to environmental degradation, soil nutrient depletion, and reduced biodiversity (Altieri, 1999). In contrast, intercropping offers a biologically intensive approach, maximizing land use through spatial and temporal complementarity among crops (Andrews & Kassam, 1976). Particularly in arid and semi-arid regions, intercropping of cereals and legumes can improve soil fertility, increase dry matter yield, and reduce the need for synthetic fertilizers (Agegnehu et al., 2008; Hauggaard-Nielsen et al., 2001).

INTERCROPPING IN FORAGE MIXTURES

Forage-based intercropping systems have been widely adopted across tropical and temperate regions. Legumes such as vetch (*Vicia sativa*), pea (*Pisum sativum* L.), cowpea (*Vigna unguiculata* L.), and mungbean (*Vigna radiata* L.) are often combined with cereals including maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), oat (*Avena sativa* L.), and barley (*Hordeum vulgare* L.). The goal is to integrate the high biomass potential of cereals with the protein-rich and nitrogen-fixing capacity of legumes.

The cereal–legume combination enhances the balance between energy and protein supply, making the mixture superior in nutritional composition. For example, maize–soybean and maize–cowpea intercropping systems yielded forage with 11–51% higher crude protein than sole maize stands (Bharti et al., 2023; Muoneke et al., 2007). Similarly, faba bean–wheat mixtures increased CP and dry matter yields by 10–30% compared to monocrops (Carrubba et al., 2008). Studies in Mediterranean climates have reported land equivalent ratio (LER) values above 1.0, confirming the greater land-use efficiency of intercropping systems (Rao & Willey, 1980; Lithourgidis et al., 2006).

INTERCROPPING SYSTEMS IN FORAGE CROPS

Several intercropping designs are employed depending on species growth habits and environmental conditions. Common patterns include alternate row, strip, and mixed seeding systems. In alternate row systems, rows of cereal and legume crops are arranged in fixed sequences (e.g., 1:1 or 2:1), as demonstrated by Ertekin (2021) in sweet sorghum–mungbean combinations under Eastern Mediterranean conditions. Narrow-row or twin-row sowing patterns increased light interception and supported better legume development, ultimately enhancing silage quality.

Other systems include relay intercropping, where a second crop is sown before the first is harvested, and strip intercropping, allowing mechanized operations and reduced competition. The success of each system depends on synchronizing growth cycles and harvesting times, as mismatched phenology may reduce forage quality and yield (Midmore, 1993).

ADVANTAGES OF INTERCROPPING IN FORAGE CROPS

The principal advantage of intercropping lies in efficient resource utilization. Complementary root architectures and canopy structures enable better use of water, light, and soil nutrients. Legumes contribute fixed nitrogen, reducing fertilizer needs and enhancing cereal growth (Hauggaard-Nielsen & Jensen, 2001).

Yield advantages are often expressed through the Land Equivalent Ratio (LER). An LER > 1.0 indicates that intercropping outperforms monocropping in biomass production per unit area. Reported LERs for cereal–legume mixtures range between 1.05 and 1.30 (Rao & Willey, 1980; Dhima et al., 2007). Furthermore, forage from intercropping systems typically exhibits lower NDF and ADF, and higher digestibility, resulting in improved animal performance (Ghanbari et al., 2010; Tolera, 2003).

Beyond yield and nutrition, intercropping increases resilience to abiotic stress (drought, frost) and biotic stress (pests, weeds, and diseases) by diversifying the agroecosystem (Lithourgidis et al., 2011). The mixture's canopy coverage reduces soil erosion and conserves moisture. Economically, it offers a form of risk insurance, ensuring at least one successful crop under variable climatic conditions.

DISADVANTAGES OF INTERCROPPING IN FORAGE CROPS

Despite its benefits, intercropping poses practical challenges. The most common issues include difficulties in mechanization, harvest timing mismatches, and competition between species when the component crops differ greatly in growth rate or canopy structure (Rao, 1986). Improper selection of species or seeding ratios can reduce yields or complicate silage making due to differences in dry matter content at harvest.

Moreover, nitrogen transfer from legumes to cereals is often limited to subsequent cropping seasons rather than within the same growth cycle, reducing the short-term fertilization effect (Jensen et al., 2005). Therefore, system optimization through experimental trials is essential before large-scale adoption.

SOME FINDINGS FROM THE LAST TWO DECADES OF INTERCROPPING STUDIES IN FORAGE PRODUCTION

During the past two decades, extensive research has highlighted intercropping's role in sustainable forage production. Studies across Asia, Africa, and the Mediterranean basin consistently show higher dry matter yield and CP content in cereal–legume mixtures than in sole crops.

Maize–legume systems: Muoneke et al. (2007) and Bharti et al. (2023) found 20–40% yield increases and enhanced CP in maize–cowpea and maize–soybean mixtures.

Barley–vetch and oat–pea systems: Lithourgidis et al. (2006) and Agegnehu et al. (2008) reported LER values of 1.1–1.25 and higher relative feed value (RFV) compared to monocrops.

Sorghum–cowpea or mungbean systems: Ertekin (2021) demonstrated that sweet sorghum–mungbean intercropping improved forage and silage quality under semi-arid Eastern Mediterranean conditions. Twin-row sowing and a 2:1 sorghum ratio increased crude protein and lactic acid content while reducing fiber fractions (NDF, ADF).

Wheat–faba bean combinations: Carrubba et al. (2008) observed improved soil nitrogen and forage nutritive value, with CP increases of up to 30%.

These studies confirm that intercropping optimizes both agronomic productivity and nutritional balance, contributing to sustainable livestock feeding systems.

CONCLUSIONS

Intercropping represents a strategic approach to improving forage productivity, nutritive value, and environmental sustainability. Combining cereals with legumes enhances protein content, fiber digestibility, and overall feed efficiency while reducing dependence on chemical fertilizers. Although system design and management remain crucial to success, evidence from the past two decades confirms intercropping as a viable alternative to monocropping, particularly under conditions of limited water and nutrient resources.

Future research should focus on developing region-specific intercropping models, optimizing sowing ratios, and integrating advanced mechanization and precision agriculture tools to make intercropping more accessible to farmers.

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PERFORMANCE EVALUATION OF GRAIN MAIZE VARIETIES UNDER THE ECOLOGICAL CONDITIONS OF THE AMIK PLAIN, HATAY

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ABSTRACT

This study was conducted in 2024 under the ecological conditions of the Amik Plain, Hatay, Türkiye, to compare yield-related traits of nine grain maize (*Zea mays* L.) varieties and to recommend high-yielding cultivars to local farmers. The experiment was arranged in a randomized complete block design with three replications. Significant differences ($p < 0.05$) were observed among varieties for ear weight, ear length, ear diameter, kernel weight per ear, and thousand-kernel weight, whereas the number of kernels per ear was not statistically significant ($p = 0.06$). The highest ear weight was recorded in P2105 (238.93 g) and P1884 (230.93 g), while P2105 also exhibited the greatest ear length (23.32 cm) and ear diameter (47.86 mm). Kernel weight per ear was highest in P2105 (219.20 g), followed by P1884 (193.27 g) and CA403 (193.00 g). Thousand-kernel weight ranged from 190.48 g (P1551) to 248.50 g (CA403), with P2105 (230.73 g) also ranking among the top performers. In contrast, P1551 consistently produced the lowest values across most traits. The results indicate that P2105, followed by P1884, demonstrated superior performance in the Amik Plain environment, suggesting their suitability for high-yield grain maize production in the region. These findings provide valuable guidance for cultivar selection, aiming to enhance productivity and economic returns for farmers under similar agro-ecological conditions.

Keywords: *Zea mays* L., Grain yield, Variety performance, Amik Plain

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important cereal crops, widely cultivated for both grain and silage. Given its high productivity potential and adaptability, maize plays a vital role in the cropping systems of many countries (Kim and Lee, 2023; Danilevicz et al., 2021). In Türkiye, grain maize production has expanded under irrigated conditions, especially in the Mediterranean and Southeastern Anatolia regions, where climate and soil conditions can vary markedly (Kizilgeçi et al., 2018).

Yield and yield-component traits such as ear weight, ear length, ear diameter, kernel number, kernel weight per ear, and thousand-kernel weight are key indicators of cultivar performance and have been used extensively in breeding and agronomic evaluation studies (Kizilgeçi et al., 2019; Ruswandi et al., 2022). For example, Kizilgeçi et al. (2018) found significant variation among 125 Turkish maize landraces in traits such as ear diameter (14.13–48.92 mm) and ear weight (10.2–285.26 g) when grown under southeastern Anatolian conditions. Such variation underlines the potential for selecting high-yielding genotypes adapted to specific agro-ecological zones.

Furthermore, the genotype $\times$ environment interaction remains critical in maize performance, especially under variable Mediterranean-type environments where water stress, high temperatures, and erratic rainfall may reduce yield stability (Kim and Lee, 2023). Studies employing stability analyses and biplot methods have demonstrated that hybrids with high and

stable yields under diverse conditions can be selected using combined analyses of grain yield and yield-component traits (Ruswandi et al., 2022).

Despite the availability of numerous maize cultivars, region-specific evaluations remain essential because local environmental conditions — such as those in the Amik Plain (Hatay) — impose unique constraints and opportunities. The Amik Plain is characterised by Mediterranean climatic influences, relatively mild winters and hot summers, and irrigated cropping systems, which may favour certain maize genotypes over others. Yet to date, few studies have explicitly focused on grain maize varietal performance under the specific ecological conditions of the Amik Plain.

Therefore, this study was conducted to evaluate nine grain maize varieties under the ecological conditions of the Amik Plain, Hatay, with the objectives of (i) comparing yield-related traits among the varieties, and (ii) recommending high-yielding cultivars suitable for the local farming community. By focusing on ear weight, ear dimensions, kernel weight per ear and thousand-kernel weight, this research aims to provide relevant cultivar selection guidance for enhanced productivity in the region.

MATERIAL AND METHOD

The field experiment was carried out in 2024 at the Agricultural Research and Application Center of Hatay Mustafa Kemal University, located in the Amik Plain (36°15' N, 36°30' E; 94 m a.s.l.). The region is characterized by a typical Mediterranean climate, with mild and rainy winters and hot, dry summers. Soil analyses of the experimental field indicated a clay-loam texture with low organic matter content (1.93%), moderate lime content (6.45%), and a slightly alkaline pH of 7.12. During the experimental period (Figure 1), total precipitation was concentrated in early spring but declined markedly towards summer, while average air temperatures remained above long-term means, indicating a predominantly dry growing season. These conditions represent the typical climatic pattern of the Amik Plain during maize cultivation.

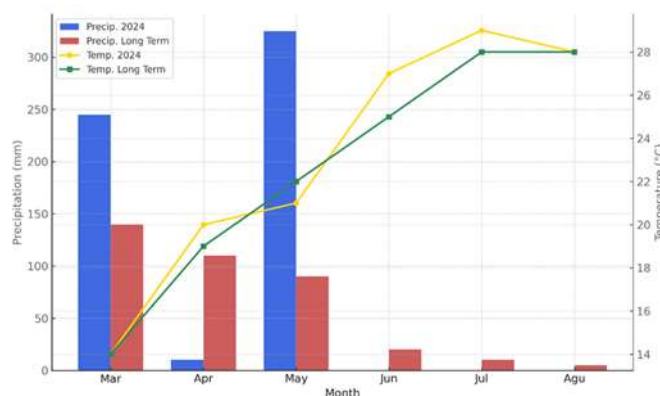


Figure 1. Climatic data obtained from the meteorological station closest to the experimental site

The study aimed to evaluate the performance of nine grain maize (*Zea mays* L.) varieties — CA401, CA402, CA403, P1410, P1541, P1551, P1884, P2085, and P2105 — under the same ecological conditions. The experiment was established on March 25, 2024, and harvesting was conducted on August 10, 2024. A randomized complete block design (RCBD) was employed with three replications. Each plot consisted of four rows, 5 m in length and 2.8 m in width, with 70 cm spacing between rows and 17 cm between plants within rows. Each maize variety represented one treatment within each replication.

Prior to sowing, the field was prepared through conventional tillage, including plowing, harrowing, and leveling. A compound fertilizer (15-15-15) was incorporated into the soil at a

rate equivalent to 8 kg N, 8 kg P, and 8 kg K per decare. When plants reached approximately 40–50 cm in height, 12 kg N da⁻¹ was applied as topdressing using urea fertilizer (46% N). Weed control was performed manually, and irrigation was supplied every 20 days to maintain adequate soil moisture. No serious pest or disease incidence was observed during the growing period.

At harvest, ten representative plants were selected from each plot to measure yield-related characteristics. The evaluated traits were:

Cob diameter (CD, mm): Measured at the midsection of the cob using a digital caliper.

Cob length (CH, cm): Measured from the base to the tip of the cob with a ruler.

Cob weight (CW, g): Recorded after husk removal using a precision balance.

Number of seeds per cob (NSC): Determined by multiplying the number of kernel rows by the number of kernels per row.

Seed weight per cob (SWC, g): Determined after threshing by weighing the total kernels per cob.

Thousand-seed weight (TSW, g): Calculated by counting and weighing four subsamples of 100 kernels, then converting to 1000-seed weight.

All collected data were subjected to analysis of variance (ANOVA) according to the RCBD model using JMP Pro 13.0 (SAS Institute Inc.). When significant differences among varieties were detected ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) test was applied for mean separation. Results were expressed as means $\pm$ standard error. Mean values within the same column followed by different superscript letters were considered statistically different.

RESULTS AND DISCUSSION

Significant differences were observed (Table 1) among maize varieties for cob diameter ($p < 0.01$). The widest cob was recorded in P2105 (47.86 mm), followed by P1884 (46.06 mm), while P1551 (42.94 mm) had the smallest diameter. The larger cob diameter in P2105 and P1884 suggests a higher sink capacity and potentially greater kernel set per cob. According to Yan et al. (2021), cob girth is directly associated with yield potential, as thicker cobs enhance kernel-bearing surface area and kernel development. Similarly, Long et al. (2024) reported that hybrids with wider cobs tend to accumulate more assimilates during the grain-filling stage, contributing to increased kernel mass. The smaller diameter observed in P1551 may indicate weaker sink strength and reduced assimilate allocation to the reproductive organs under the same growing conditions.

Varietal differences (Table 1) in cob length were also significant ($p < 0.01$). The longest cobs were produced by P2105 (23.32 cm), while P1551 (18.21 cm) had the shortest. Varieties P1884 (21.33 cm) and CA402 (21.27 cm) also exhibited relatively long cobs. Longer cobs generally reflect greater potential for kernel number and grain yield, as highlighted by Liu et al. (2022), who emphasized the role of cob length in determining the ear's total sink size. The superior cob length of P2105 likely results from a balanced vegetative and reproductive growth pattern, ensuring sufficient assimilate supply during ear formation. Conversely, shorter cobs in P1551 may indicate genetic limitations or less efficient partitioning of assimilates toward the ear during reproductive development.

Cob weight varied significantly (Table 1) among varieties ($p = 0.02$). The heaviest cobs were obtained from P2105 (238.93 g) and P1884 (230.93 g), while P1551 (165.73 g) had the lowest value. Cob weight integrates several yield components, such as kernel number, kernel size, and cob morphology. High cob weight indicates enhanced photosynthetic efficiency and assimilate translocation, both of which are essential for maximizing grain yield (Konuskan et al., 2024). The superior cob weight of P2105 and P1884 supports their adaptability to the Amik

Plain conditions and reflects their high genetic yield potential. The comparatively lower cob weight of P1551 suggests a weaker physiological capacity to convert vegetative biomass into grain yield.

Differences among varieties for the number of seeds per cob (Table 1) were not statistically significant ($p = 0.06$). Nonetheless, P1410 (794.00 seeds) and P2105 (784.47 seeds) had numerically higher values, whereas P2085 (620.80 seeds) and P1551 (620.27 seeds) produced the lowest. The variation observed, though not statistically confirmed, indicates genetic differences in kernel set efficiency. Long et al. (2024) noted that kernel number is more sensitive to environmental conditions, particularly during pollination, and often exhibits higher variability than kernel weight. The relatively consistent NSC values among most varieties suggest that environmental conditions during flowering were favorable, allowing differences in final yield to be driven mainly by kernel weight rather than kernel number.

Seed weight per cob showed highly significant differences (Table 1) among varieties ($p < 0.01$). The highest SWC was recorded in P2105 (219.20 g), followed by P1884 (193.27 g) and CA403 (193.00 g), while P1551 (137.27 g) had the lowest. This parameter represents both the number and mass of kernels per cob and is a crucial determinant of final yield. Liu et al. (2022) reported that high SWC values are associated with superior source–sink coordination, which ensures efficient assimilate translocation to developing grains. The high SWC observed in P2105 and P1884 highlights their strong sink strength and effective carbohydrate partitioning. In contrast, P1551's low SWC confirms its inferior grain-filling efficiency, consistent with its smaller cob morphology.

Table 1. Yield-related traits and comparison test results of different maize varieties

Cultivars	Properties					
	CD (mm)	CH (cm)	CW (g)	NSC (piece)	SWC (g)	TSW (g)
CA401	45.49±1.00 ^{abc}	19.66±0.28 ^{bc}	208.67±12.03 ^{ab}	702.80±37.02	180.67±13.48 ^{abc}	201.52±1.72 ^{bc}
CA402	43.95±1.03 ^{bc}	21.27±1.14 ^{abc}	198.67±10.18 ^{ab}	670.67±56.43	179.47±11.05 ^{abc}	237.40±7.96 ^{ab}
CA403	44.74±0.49 ^{bc}	19.39±0.81 ^{bc}	214.27±20.34 ^{ab}	664.53±62.50	193.00±12.07 ^{ab}	248.50±12.72 ^a
P1410	45.52±0.46 ^{abc}	20.97±0.3 ^{3abc}	222.67±14.34 ^{ab}	794.00±15.87	192.47±12.78 ^{ab}	209.67±4.77 ^{abc}
P1541	45.15±0.09 ^{abc}	19.98±0.03 ^{bc}	201.07±4.67 ^{ab}	670.13±24.93	169.07±7.82 ^{bc}	203.11±11.13 ^{bc}
P1551	42.94±0.79 ^c	18.21±0.02 ^c	165.73±8.38 ^b	620.27±22.14	137.27±6.40 ^c	190.48±16.83 ^c
P1884	46.06±0.08 ^{ab}	21.33±0.56 ^{ab}	230.93±4.26 ^a	755.60±16.20	193.27±4.34 ^{ab}	196.05±6.20 ^{bc}
P2085	44.30±0.43 ^{bc}	18.93±0.68 ^{bc}	186.80±7.01 ^{ab}	620.80±24.02	158.80±11.55 ^{bc}	201.33±1.33 ^{bc}
P2105	47.86±0.21 ^a	23.32±0.67 ^a	238.93±27.67 ^a	784.47±55.09	219.20±9.65 ^a	230.73±5.00 ^{abc}
P values	<0.01	<0.01	0.02	0.06	<0.01	<0.01

CD: Cob diameter, CH: Cob height, CW: Cob weight, NSC: Number of seed per cob, SWC: Seed weight per cob, TSW: Thousand seed weight

Values followed by different superscript letters within the same column are significantly different.

Thousand-seed weight varied significantly (Table 1) among varieties ($p < 0.01$), ranging from 190.48 g (P1551) to 248.50 g (CA403). High TSW values were also recorded in P2105 (230.73 g) and CA402 (237.40 g). The heavier kernel mass in these varieties suggests efficient grain filling and larger endosperm development. Yan et al. (2021) emphasized that TSW is a reliable indicator of assimilate accumulation during the grain-filling phase and a major contributor to yield stability. The superior TSW of CA403 and P2105 may thus be attributed to favorable genetic traits supporting prolonged grain filling and effective resource utilization. The lowest TSW in P1551 further reinforces its overall weak performance in kernel development.

CONCLUSIONS

The study clearly demonstrated substantial genotypic variation among the evaluated maize varieties in yield-related traits under the ecological conditions of the Amik Plain. Among the nine tested cultivars, P2105 consistently exhibited superior performance for cob diameter, cob length, cob and seed weights, and thousand-seed weight, while P1884 and CA403 also showed promising results. In contrast, P1551 recorded the lowest values across most traits, indicating limited adaptation to local conditions. Overall, the findings highlight that yield potential in maize is strongly determined by kernel weight and cob morphology rather than kernel number. Consequently, P2105 and P1884 can be recommended for grain maize production in the Amik Plain to achieve higher productivity and stability under similar agro-ecological environments.

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HOW CLIMATE CHANGE AFFECTS FOOD SYSTEMS

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A serious worldwide danger to food systems, climate change affects not just primary production but also post-harvest processing, preservation, and food safety. The biochemical composition, microbiological stability, and technological processing features of food are directly impacted by ocean acidification, rising seawater temperatures, and variations in water salinity and dissolved oxygen in aquatic food chains. These developments have a major impact on food processing technology, posing challenges to current practices and necessitating the creation and uptake of novel, climate-resilient strategies. Accelerated spoilage food spoilage is one of the most significant effects of climate-induced stress on food systems. Across a variety of food matrices, lipid oxidation, protein denaturation, variations in water activity, and microbial growth are all accelerated by rising ambient temperatures and changing environmental circumstances. These procedures lead to accelerated degradation of quality, heightened production of dangerous substances including biogenic amines, reduced shelf life, and higher threats to food safety. As a result, it has become increasingly challenging to preserve foods' nutritional content and microbiological safety using only traditional preservation methods. This study examines how oxidative and enzymatic deterioration, microbiological hazards, and spoiling are all directly impacted by climate change in food systems.

Overall, by changing the biological, technical, and environmental factors that support food production and preservation, climate change has a complex impact on global food systems. The availability and safety of food are made unstable by the disruption of both terrestrial and aquatic supply systems caused by rising sea levels, temperature swings, and extreme weather events. These systemic changes put current processing technologies to the test and highlight the pressing need for climate-smart, adaptive innovations to guarantee robust and sustainable food systems in the face of persistent global warming.

Keywords: Climate change, Food systems, Food safety, Global warming, Preservation strategies

INTRODUCTION

One of the biggest worldwide risks to food systems is climate change, which affects all phases of the supply chain, including primary production, processing, preservation, and distribution. Globally, the safety and stability of processed foods, livestock health, aquaculture yields, and agricultural productivity have already been impacted by the rise in atmospheric CO₂ levels, changes in temperature and precipitation patterns, and the increasing frequency of extreme weather events (FAO, 2023; IPCC, 2022). The biochemical composition, microbiological stability, and technological features of food raw materials are all directly impacted by these environmental disruptions, which also have an impact on crop development and animal physiology. As a result, there are additional difficulties in ensuring food safety and quality across the food chain.

Climate stress is increasing the dangers of food spoilage and safety. Across a variety of food matrices, including meats, dairy products, cereals, fruits, and seafood, rising ambient temperatures and changing humidity speed up lipid oxidation, protein denaturation, microbial proliferation, and enzymatic degradation (Liu et al., 2021; Cisse et al., 2023). These reactions degrade nutritional content, shorten shelf life, change sensory qualities, and encourage the production of dangerous substances including mycotoxins and biogenic amines. Food safety assurance systems are further complicated by shifting rainfall and irrigation patterns, which raise exposure to pollutants such as heavy metals, pesticide residues, and mycotoxigenic fungus (Zhao et al., 2022).

Traditional preservation methods, including as refrigeration, heat processing, and chemical additions, are becoming less and less capable of handling the instability brought about by climate change. Consumer opposition to chemical preservatives is driven by the growing demand for natural and minimally processed foods, while energy-intensive cold chains remain vulnerable to electricity supply disruptions. As a result, the global food industry has made low-carbon, climate-resilient, and sustainable preservation technologies a top focus (Rosello et al., 2021).

Novel non-thermal processing techniques like Pulsed Electric Fields (PEF), Cold Plasma, and High Hydrostatic Pressure (HHP) have demonstrated promise in recent years for preserving nutritional and sensory integrity and extending shelf life while reducing energy consumption and greenhouse gas emissions (Nadeem et al., 2024). Similarly, bio-based edible coatings and Modified Atmosphere Packaging (MAP) enhanced with natural antioxidants and antimicrobials are attracting interest due to their capacity to regulate oxidation and microbiological spoiling without the need for artificial chemicals (Singh & Chawla, 2023).

Beyond preservation, the food industry is adopting circular bioeconomy strategies, energy-efficient cold chain optimization, and digital traceability systems that turn agricultural and processing waste into high-value compounds. These actions taken together increase sustainability and resilience in the face of climate change (FAO, 2023; Moresi et al., 2022).

In order to assess adaptive and sustainable processing technologies that can protect food quality, shelf life, and safety, as well as how climate change affects oxidative, enzymatic, and microbial deterioration mechanisms across various food categories, this review synthesizes research published between 2020 and 2025. It also emphasizes the importance of systemic strategies for creating low-carbon, climate-resilient food systems, such as combining digitalization, renewable energy, and waste valorization.

CLIMATE CHANGE AND ITS INFLUENCE ON FOOD SYSTEMS

From production, processing, packing, and storage to transport, consumption, and waste, food systems cover the whole spectrum of operations. These systems rely on stable environmental, economic, and technical circumstances and are situated within socio-ecological contexts (Pereira, et al., 2020). However, fundamental factors are changing due to climate change: temperatures are rising, atmospheric CO₂ levels are rising, precipitation and humidity patterns are changing, and extreme weather events are occurring more frequently. Global food systems are under multiple layers of stress as a result of these changes (Ahmed et al., 2023).

First, it's critical to acknowledge that food systems both contribute to and are impacted by climate change. Significant greenhouse gas emissions are produced by the food and agriculture industries due to changes in land usage, livestock methane, fertilizer use, and food processing and transportation (Yu et al., 2025). The vulnerability of the entire supply chain is increased by this two-way link, in which food systems both influence and are influenced by the climate.

The effects of climate change are already seen at the manufacturing end of the chain. Increased soil and ambient temperatures, changed rainfall patterns, severe droughts, and storms degrade animal productivity, disrupt aquaculture operations, and lower crop yields (Saleem et al., 2025). In addition to lowering quantity, these disruptions have an impact on the quality of raw materials that are available for processing. To ensure safety and shelf life, downstream units—processing, preservation, and distribution—must adjust when the input material's moisture content, nutritional composition, or microbial load varies (Chen et al., 2021).

In this context, the properties of the raw materials are crucial. For instance, higher CO₂ can cause plants to produce more biomass, but it can also result in reduced levels of vital minerals like iron, zinc, and protein. Animals under heat stress may experience changes in their immune systems and lipid profiles, making them more vulnerable to infections (Chauan et al., 2021). Processing becomes more difficult as a result of these compositional variations since traditional protocols that were designed to guarantee a certain input quality may no longer do so when that quality changes (Lin et al, 2025).

In addition to raw materials, there is growing risk across the post-harvest and processing chain. Energy costs are rising, temperature abuse is becoming more likely, and greater ambient temperatures necessitate additional cooling, all of which put stress on cold-chain infrastructures. Foods are exposed to prolonged durations outside of optimal conditions during transportation or storage delays brought on by severe weather or infrastructure failure, which promotes microbial growth and spoiling (Ogwu et al., 2024; Mahunu et al., 2024).

Furthermore, climate fluctuation affects the internal dynamics of food, including lipids, proteins, enzyme systems, and microbial communities (Toor et al., 2024). Elaveted distribution and storage conditions speed up lipid oxidation, which results in off flavors and a reduction in nutritional value; they also speed up microbial growth and enzyme-mediated autolysis, which reduce shelf life and raise safety risks like the buildup of mycotoxins or biogenic amines. Foods such as grains, fruits, vegetables, meat, dairy, and processed goods are all affected (Dai et al., 2024).

There are significant social and economic repercussions. Decreases in quality or production raise food costs and make it more difficult for disadvantaged groups to obtain it. Disruptions to distribution and rapid spoiling increase waste and decrease availability, further taxing available resources (Wang et al., 2022). There is a greater chance of food-borne illness and inadequate nutrition in areas with unstable refrigeration or inadequate infrastructure (Skawińska & Zalewski, 2022).

The idea of resilience is crucial at the system level because of the interaction between food system fragility and climate risk. The ability of a food system to withstand shocks, adjust

to changing circumstances, and change if needed to preserve availability to wholesome, reasonably priced food is known as resilience (Zurek et al., 2022). According to recent studies, resilience and sustainability must coexist because merely preserving current operations under pressure could result in inequities or unsustainable resource consumption (Negri et al., 2021).

Climate-Induced Biochemical and Microbiological Changes in Food Raw Materials

Climate change is making all the materials utilized in food systems—crops, cattle, dairy, aquaculture, and fisheries—more vulnerable to changing environmental stresses (Yadav et al., 2024). These forces are causing changes in the harvested items' biochemical composition, microbiological stability, and physiological reactions, which directly affect processing, preservation, quality, and safety in the future (Sharma et al., 2024).

The impact of increased CO₂, warmer ambient temperatures, and changed water availability on the macromolecular profile and nutritional density of plant-based raw materials is one important biochemical aspect (Sola et al., 2025). Elevated atmospheric CO₂ tends to enhance plant biomass, but lowers protein, zinc, and iron concentrations in staple crops, according to a number of recent research (Table1). For instance, it has been demonstrated that climate stressors, such as coupled heat and water scarcity, reduce the quality of grain protein as determined by the digestibility of essential amino acids (IAAs) (Wang et al., 2025). These compositional differences are important because when raw materials diverge, processing settings that are tuned for specific protein, moisture, and fat levels may not produce the desired yield or useful qualities (Papchenko et al., 2023).

In addition, even prior to processing, environmental stressors like heat waves, drought, excessive salinity, and flooding can change enzyme activity and encourage microbial contamination (Das et al., 2025). For example, endogenous enzyme-mediated autolysis is accelerated by changes in water activity and moisture movement in harvested materials, and spoiling risk is increased by changing microbial ecology (e.g., more thermotolerant spoilage species) (Pintilei et al., 2025). The margin of safety for further processing and storage processes is decreased by these changes, especially in perishable raw materials (fruits, vegetables, dairy, and fish) (Pedro et al., 2025).

Additional stressors are brought about by climate change for raw resources generated from animals, such as meat, dairy, and aquaculture. In production animals, heat stress can change fat-to-lean ratios, increase oxidative stress in tissues, change post-mortem proteolytic enzyme activity, and affect the patterns of microbial colonization (Purpa, 2025). Compared to crops, this has received less attention in recent years, yet the effects on quality and shelf life are substantial. Heat-stressed cattle, for instance, develop muscle with a changed pH drop, which may lower their ability to retain water and make them more vulnerable to spoiling microbes (Shah et al., 2025).

Climate fluctuation affects not just the composition of raw materials but also the production of undesirable compounds and microbiological risks (Misiou et al., 2022). Lipid oxidation, protein denaturation, and the production of biogenic amines and other spoilage/by-product metabolites are all accelerated by high storage or transit temperatures in conjunction with changes in the biochemistry of the raw materials. These changes may jeopardize food safety as well as its nutritional and sensory qualities. The safety envelope margin is decreased by an increasing ambient temperature or a compromised cold chain under climate stress (Pajic et al., 2024).

Climate change exacerbates the dynamics of water activity, moisture migration, and storage, which are already complicated under "normal" circumstances. Due to growing under stress, harvested product may have more residual moisture, more microdamage, or different sugar/protein ratios when it is processed (Casu et al., 2024). Enzyme-driven breakdown and

microbial growth are accelerated by these circumstances. For instance, changing irrigation and rainfall schedules might leave produce with microfissures or a higher concentration of stress metabolites, which serve as targets for microbial invasion and spoiling (Toor et al., 2024).

Materials handled under previously approved conditions may suddenly perform worse due to factors like a shorter shelf life, a higher rate of spoiling, more quality variation, more waste, and a larger chance of safety departure.

Table 1. Major climate-induced changes in raw materials and their implications for processing and safety

Food raw material	group	Climate-induced stressor	Biochemical microbiological effect	Processing implication	References
Cereal grains (wheat, rice, maize)		Elevated CO ₂ , heat waves, drought	Protein content (–5 to –15%), Zn & Fe levels, altered starch gelatinization	Reduced dough strength, lower yield in extrusion & baking	Liu et al., 2021
Fruits & vegetables		Temperature extremes, irregular rainfall, post-harvest humidity	Respiration rate, ethylene production, micro-cracking, microbial spoilage flora	Shortened shelf-life, increased enzymatic browning & softening	Zhao et al., 2022
Legumes & oilseeds		High CO ₂ + heat stress	Amino acid digestibility, altered lipid ratio	Modified oil stability, need for different refining temperatures	Khan et al., 2020
Meat & livestock products		Heat stress, water scarcity, disease spread	Oxidative stress, altered muscle pH, spoilage bacteria	Faster rancidity, lower water-holding capacity texture loss	Bhat et al., 2022;
Dairy		Heat stress, pathogen shift	Fat and protein %, somatic cell count, altered microflora	Shorter stability, higher pasteurization load	Cisse et al., 2023

Additionally, processing technology faces a series of hurdles as a result of these changes. The value proposition of the final product decreases as the raw materials have reduced nutrient content (e.g., protein, micronutrients); the cost of preservation must increase as microbial or enzymatic breakdown speeds up through more extensive treatments, tighter control, faster processing. These stresses increase when supply chains experience climate-related disturbances (delays in transit, power outages, and temperature swings), which intensify deterioration even before processing starts.

Given these new developments, more attention is being paid to detecting and measuring climate-sensitive indicators in raw materials (such as protein digestibility, lipid oxidation indices, and changes in the microbial community) that can act as important catalysts for adaptive processing techniques. For instance, plant physiology, processing technology, and food safety are all involved in comprehending how elevated CO₂ impacts the amino acid content of grains. In a similar vein, new spoilage or pathogenic taxa that thrive in warmer or more humid environments are being found using microbial genomics.

Implications for Food Quality, Shelf Life, and Safety

The quality, stability, and safety of food are the areas where the biochemical and microbiological changes brought on by climate change are most noticeable. Food deterioration is a complex process that involves interactions between chemical, biological, and physical processes. These reactions are accelerated by rising temperatures, changes in humidity, and broken cold-chain systems, which lowers the nutritional and functional integrity of foods in all categories (Cisse et al., 2023; FAO & WHO, 2021).

Accelerated oxidative and enzymatic activities are the first steps in quality degradation. Increased reaction kinetics brought on by high ambient temperatures encourage pigment deterioration, protein denaturation, and lipid peroxidation. These reactions reduce customer approval by causing discolouration, softening of the texture, and the creation of an off-flavor (Liu et al., 2021). For example, for every 10 °C increase in storage temperature, the rate of oxidative rancidity in high-fat foods like nuts, meats, and dairy products doubles. Likewise, under warmer and more humid environments, enzymes such as lipase, protease, and polyphenol oxidase become more active, causing autolysis and browning responses in plant and animal tissues (Rosello et al., 2021).

Additionally, nutritional quality is weakened. Particularly during storage and distribution, temperature and oxygen variations accelerate the loss of thermolabile vitamins (A, C, E, B₁, B₆) and vital amino acids like lysine and methionine. Food safety and nutritional sufficiency are at risk due to climate-related increases in mycotoxin contamination, particularly aflatoxins and ochratoxins in cereals and nuts (Zhao et al., 2022). Furthermore, even before processing, high CO₂ levels might indirectly impact nutritional quality by decreasing the density of micronutrients including iron, zinc, and selenium in plant-based foods (Herrero et al., 2023).

One of the most obvious effects of stress brought on by the climate is a decrease in shelf life. Microbial growth rates are exponentially increased by warmer storage and transportation conditions, especially for mesophilic and psychrotrophic rotting organisms. For instance, under temperature variations of just 2–3 °C from ideal cold-chain conditions, the growth of *Pseudomonas*, *Enterobacteriaceae*, and *Listeria monocytogenes* in dairy and meat products can be accelerated by two to three times. Similarly, increased post-harvest respiration and water activity under heat stress reduce the shelf life of fruits and vegetables by increasing the metabolic consumption of acids and sugars (Nguyen & Le, 2024).

Food safety is an additional factor. The ecology of poisons and food-borne pathogens is changed by climate change. Warmer temperatures encourage the growth of molds and bacteria that produce toxins, while increased humidity and flooding facilitate the spread of water-borne infections including *Vibrio*, *Salmonella*, and *Campylobacter*. Traditional hazard analysis and critical control point (HACCP) systems are put to the test when vector populations and reservoirs change, altering the paths of contamination. Climate variability can increase the geographic range of infections like *Vibrio parahaemolyticus*, which was previously restricted to tropical regions but is now appearing in temperate coastal waters, according to the FAO and WHO (2021).

Chemical risks increase in processed foods as well. Variability in temperature and moisture content affects the production of dangerous substances such as acrylamide, 3-MCPD, and advanced glycation end products (AGEs). When raw materials undergo pre-processing stress (such as high quantities of sugar and amino acids) and are subjected to high-temperature treatments in less regulated environments, these compounds are more likely to occur (Nadeem et al., 2024). At the same time, during storage, oxidative and Maillard-derived processes cause darkening of color and the development of rancid or bitter odors, which raise both toxicological and sensory issues.

One of the most obvious effects of climate change on food systems is microbial spoiling. Bacterial growth and the synthesis of toxins are facilitated by inadequate refrigeration or power outages during heat stress. Traditional refrigeration is insufficient because psychrotrophic bacteria, which can live at low temperatures, multiply more quickly under mild temperature abuse. The synthesis of biogenic amines, such as histamine, putrescine, and tyramine, is increasing along with this trend, endangering both safety and sensory quality. Similarly, mycotoxins that are resistant to many traditional heat-based decontamination methods are introduced by fungal infection in grains and legumes, which rises with humidity (Bhat et al.,

2022).

The dynamics of water activity (a_w) offer yet another crucial connection between shelf stability and climate variability. Microbial instability results from raw materials' varying internal moisture under erratic rainfall and humidity patterns (Rifna et al., 2022). During humid seasons, items that are dried or semi-dried (such as powders, cereals, and dried fruits) can absorb ambient moisture more quickly, which increases microbial and enzymatic reactivation (Ben et al., 2023). On the other hand, oxidation and textural deterioration may result from excessive drying during heat stress. Processors must use adaptive packaging and humidity control technologies to preserve balance in the face of both extremes (Mia et al., 2023).

Reliability of the cold chain is increasingly important for preserving food safety and shelf life in the face of climate change. The likelihood of temperature abuse incidents is enhanced by energy outages, higher cooling demand, and longer travel distances. Every link in the chain exacerbates chemical deterioration and microbiological growth. Research on perishable goods shows that even brief temperature changes during distribution (two to four hours) can cause microbial loads to surpass regulatory limits, resulting in a 20–40% reduction in effective shelf life (Nguyen & Le, 2024).

These processes add up to a complex breakdown of the integrity of the food chain. Microbial spoiling, nutrient deterioration, quality loss, and chemical hazard development all work together to threaten public health and customer trust. Economic losses, waste, and increased pressure to use adaptive preservation techniques such as cold plasma, high-pressure processing, modified atmosphere packing, and bio-based coatings are the results for industry (Singh & Chawla, 2023; Rosello et al., 2021). It demands that regulators create dynamic frameworks for food safety that take into consideration changing environmental baselines and pathogen ecologies (Hamilton et al., 2023).

The parameters of quality, shelf life, and safety in food systems are redefined by climate change, which does more than just speed up spoiling. Integrating climate risk management into supply-chain monitoring, policy, and food processing design is necessary to maintain nutritional and microbiological integrity (Okafor et al., 2025). In order to ensure that food systems continue to be safe, nourishing, and sustainable in the face of the unprecedented variability brought on by global warming, future resilience hinges on coordinating technological innovation with environmental adaptation (Iqbal et al., 2025).

Technological Challenges in Food Processing and Preservation

Deep technological weaknesses in the world's food processing and preservation systems have started to be revealed by climate change (Galankis, 2024). Rising temperatures, variable humidity, energy insecurity, and resource shortages are putting systems and infrastructures that were created during a time of climatic stability under unprecedented stress (Jha and Dev, 2024). Every processing step from thermal treatment to refrigeration, dehydration, and packaging is impacted by these pressures, which radically change the results in terms of efficiency and safety (Chiozzi et al., 2022).

Although thermal processing is still the mainstay of food preservation, its use is becoming more and more limited due to the need for decarbonization and energy availability. Stable power supplies and predictable climatic conditions are necessary for processes including pasteurization, sterilization, baking, and frying (Grossmann et al., 2022). Heat exchangers and cooling systems become less effective as global temperatures rise, which raises operating expenses and increases greenhouse gas emissions (Dong et al., 2021). In dense production facilities, elevated ambient temperatures can jeopardize post-process cooling, increasing the risk of recontamination. These challenges are exacerbated by the shift to renewable energy;

continuous-flow production processes that rely on continuous thermal input are disrupted by the intermittent output of solar and wind sources (Qin et al 2024). Because they lack the financial and technological resources to protect themselves against such volatility, small and medium-sized businesses in developing nations are especially at risk.

Another crucial area of vulnerability is the freezing and refrigeration processes that support the delivery and safety of perishable goods (Pedro et al., 2023). Although they prevent microbial development and maintain freshness, cold chains are one of the food industry's biggest energy users. The energy load needed for refrigeration rises dramatically as mean world temperatures rise, decreasing system efficiency and limiting equipment lifespan (Bai and Sun, 2023). Condensation systems perform worse in hot and muggy weather, while power interruptions and heat waves disrupt cooling cycles and hasten spoiling (Attia et al., 2021).

Lack of water makes technology even more stressful. Water is essential to processes like blanching, extraction, and sterilizing since it functions as a solvent, cleaning agent, and processing medium. Processors are being forced to implement more stringent water-recycling procedures due to the growing frequency of droughts and competition for freshwater supplies (Sahoo et al., 2022). Closed-loop cleaning methods lower total consumption, but they also create additional hygienic risks: heat-resistant spores may survive insufficient sanitation cycles, and biofilm-forming microbes may multiply in pipes at high ambient temperatures (Boyko et al., 2024). New methods incorporating membrane filtering, UV sterilization, and sophisticated oxidation treatments are needed to maintain microbiological control while reducing water footprints; however, these methods involve financial investment and technical know-how that many processors still lack (Selvakumar, 2025).

Additionally, packaging technologies are become increasingly susceptible to climate change. While metal cans corrode more easily during alternating wet-dry cycles, conventional polymer films and laminates lose their barrier integrity at high humidity (Schenker et al., 2021). High storage temperatures raise questions about chemical safety since they encourage the migration of plasticizers and leftover monomers into food. However, there are also performance issues associated with the push to switch from fossil fuel-based plastics to bio-based or biodegradable alternatives (Seref and Cuhaoglu, 2025). In tropical areas, product protection is compromised since many biopolymers weaken or break down when exposed to heat and moisture (Acharya., 2025). Even while active and intelligent packaging that uses natural antioxidants or antibacterial agents has showed promise, it is still unclear how stable these coatings will be in harsh climates; in order to assure longevity, multilayer or cross-linked reinforcing is frequently needed (Chang et al., 2023).

The carbon footprint of food processing is increased by energy usage, water use, packaging deterioration, and manufacturing losses. The issue is cyclical: the industry's resilience is further weakened by climatic challenges that are made worse by its own emissions (Bhatia et al., 2023). Processing plant organic waste breaks down into the powerful greenhouse gas methane if left unchecked. Strategies for the circular economy that turn by-products like peels, husks, whey, and trimmings into high-value chemicals are becoming more popular, but many of these recovery technologies such enzymatic hydrolysis or spray-drying are energy-intensive in and of themselves (Wibisono et al., 2025). They run the danger of negating their environmental advantages if they are not fueled by renewable resources and incorporated into closed energy loops.

Socioeconomic differences exacerbate the gap in technology adaptability. Although they offer energy-efficient microbial control, advanced non-thermal technologies like Pulsed Electric Fields, Cold Plasma, and expensive-Hydrostatic-Pressure processing are not widely available due to their expensive installation and maintenance costs (Singh et al., 2022). Additionally, there is a lack of qualified workers in many areas who can run, manage, and validate these systems. The issue is exacerbated by a lack of data; few long-term studies have

measured how well such technologies work in reality climate-variable settings, making it challenging for investors or governments to prioritize financing (Tsakonas et al., 2024).

The food processing business must balance sustainability, safety, and efficiency in a setting that is changing quickly. The infrastructure for food security's maintenance might become one of its weakest points in the absence of systemic innovation and technology reform (Prasanna et al., 2025). Developing low-water and low-emission equipment, incorporating renewable energy into processing activities, and creating packaging and cold-chain systems that can function in the face of high heat and humidity are all necessary to ensure resilience (Ikram et al., 2025). Whether global food processing continues to contribute to climate stress or becomes a key component of climate adaptation will be decided in the ensuing decades.

CONCLUSION

Global food systems are increasingly at risk from climate change, which has an impact on all phases of production, processing, and preservation. Temperature increases, erratic precipitation, water scarcity, and energy changes all speed up lipid oxidation, enzymatic reactions, and microbial development, which increases the danger of food deterioration, lowers nutritional value, and compromises food safety. These environmental pressures highlight the drawbacks of traditional preservation techniques like heat processing and refrigeration, which are both energy-intensive and susceptible to climatic fluctuations. The food processing industry also makes a substantial contribution to greenhouse gas emissions, which feeds back into the system and exacerbates the effects of climate change.

The industry has to move toward sustainable and adaptable technology in order to guarantee resilient food systems. Alternatives that are safer and use less energy include non-thermal preservation techniques including pulsed electric fields, cold plasma, and high hydrostatic pressure. Digital cold-chain monitoring systems, bio-based coatings, and modified atmosphere packaging all show promise for preserving quality in the face of climatic stress. Ultimately, innovation, the integration of renewable energy, effective water usage, and more legislative support are necessary to protect food safety and nutritional integrity in a changing environment. In addition to safeguarding public health and food quality, developing climate-resilient processing systems will promote global sustainability and food security.

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BIOGENIC AMINES IN FOODS: SIGNIFICANCE, AND TOXICOLOGICAL RISKS

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Low-molecular-weight nitrogenous substances known as "biogenic amines" are mostly produced by microorganisms decarboxylating free amino acids. Because of their high protein content and water activity, they are found naturally in a wide range of meals, especially in fermented foods like cheese and also fish products. While putrescine and cadaverine are not directly poisonous, they amplify the negative effects of histamine and serve as markers of microbial contamination. Histamine and tyramine are the most important of biogenic amines because of their toxicological relevance.

Environmental and technological factors, such as temperature, pH, raw material quality, and hygiene practices, have a significant impact on the production of biogenic amines. Because of their strong decarboxylase activity, lactic acid bacteria, Enterobacteriaceae, and some strains of *Pseudomonas* and *Clostridium* are known to contribute significantly to the accumulation of amines during fermentation and storage. Foodborne intoxications like scombroid poisoning, which is marked by symptoms like nausea, vomiting, flushing, headaches, and hypertension, can result from consuming fish with high level of histamine. Using starter cultures with minimal decarboxylase activity, implementing good manufacturing practices (GMP), regulating microbial development, and preserving the cold chain are crucial for avoiding such poisoning. Furthermore, for the protection of consumers, biogenic amine levels must be regularly monitored and international safety guidelines must be followed. The aim of this review is to provide an overview of the occurrence, formation, and toxicological risks of biogenic amines in foods, with a particular focus on cheese and fishery products. Additionally, it emphasizes management methods and preventative efforts to lessen their buildup, guaranteeing food safety and quality.

Keywords: Biogenic amines, histamine, tyramine, cheese, fishery products, food safety

INTRODUCTION

Low-molecular-weight, nitrogenous chemical molecules known as biogenic amines (BAs) are found naturally in a variety of meals, especially those high in proteins like meat, fish, and fermented dairy products (Saxena et al., 2024; Yazgan et al., 2024). They are mostly created by transamination of aldehydes and ketones or by the microbial decarboxylation of free amino acids. Even while some biogenic amines are physiologically important in living things, such as precursors of hormones or neurotransmitters, their excessive buildup in meals can be harmful to both the quality of the product and the health of consumers (Blachier, 2025).

High amounts of biogenic amines are indicative of microbial contamination or improper processing and storage conditions in the context of food safety (Natrella et al., 2024). Because

of their established roles in foodborne intoxications including scombroid poisoning and cheese-induced hypertensive crises, histamine and tyramine are regarded as the most toxicologically significant of these substances (Turna et al., 2024). Other amines like putrescine and cadaverine, while not directly poisonous, can cause the unpleasant effects of histamine and act as markers of deterioration (Ramadan et al., 2025).

A number of variables, such as temperature, pH, microbiological activity, raw material quality, and production and storage hygiene standards, affect the level of biogenic amines in food (Ozogul et al., 2024). Understanding the processes of BA production and its toxicological significance has become more crucial as the consumption of fermented and minimally processed foods rises worldwide (Niyigaba et al., 2025).

With a focus on fermented dairy and fisheries products, this article attempts to give a summary of the production, prevalence, and health effects of biogenic amines in foods. It also emphasizes control methods and preventative actions that can lessen the production of biogenic amines, protecting consumers and guaranteeing food safety.

Formation and Microbial Sources of Biogenic Amines

Microbial decarboxylation of free amino acids, which involves the removal of the carboxyl group ($-\text{COOH}$) from amino acid molecules to produce the corresponding amine and carbon dioxide, is the main process responsible for the production of biogenic amines (BAs) in food (Table 1). Pyridoxal phosphate-dependent decarboxylase enzymes, which are generated by some bacteria as a component of their adaptive metabolic pathways, catalyze this process (Kirschning, 2024; Mirza Alizadeh et al., 2025). The conversion of histidine to histamine, tyrosine to tyramine, lysine to cadaverine, and ornithine to putrescine are the main biochemical transformations (Abril et al., 2024). These reactions, which are a microbial defense mechanism that aids in maintaining intracellular pH balance and energy homeostasis during stress, frequently take place in environments with low pH or restricted nutrition supply (Nair et al., 2025).

Table 1. Representative amino acid decarboxylation reaction

Amino Acid (Precursor)	Enzyme	Product (Biogenic Amine)	Formula
Histidine	Histidine decarboxylase	Histamine	$\text{C}_6\text{H}_9\text{N}_3\text{O}_2 \rightarrow \text{C}_5\text{H}_9\text{N}_3 + \text{CO}_2$
Tyrosine	Tyrosine decarboxylase	Tyramine	$\text{C}_9\text{H}_{11}\text{NO}_3 \rightarrow \text{C}_8\text{H}_{11}\text{NO} + \text{CO}_2$
Ornithine	Ornithine decarboxylase	Putrescine	$\text{C}_5\text{H}_{12}\text{N}_2\text{O}_2 \rightarrow \text{C}_4\text{H}_{12}\text{N}_2 + \text{CO}_2$
Lysine	Lysine decarboxylase	Cadaverine	$\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2 \rightarrow \text{C}_5\text{H}_{14}\text{N}_2 + \text{CO}_2$
Tryptophan	Tryptophan decarboxylase	Tryptamine	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2 \rightarrow \text{C}_{10}\text{H}_{12}\text{N}_2 + \text{CO}_2$
Tyrosine	Tyrosine decarboxylase	Tyramine	$\text{C}_9\text{H}_{11}\text{NO}_3 \rightarrow \text{C}_8\text{H}_{11}\text{NO} + \text{CO}_2$

The decarboxylation of histidine to histamine is catalyzed by the enzyme histidine decarboxylase (HDC), which requires pyridoxal phosphate (PLP) as a cofactor. The reaction involves the removal of the carboxyl group from the α -carbon of L-histidine, yielding histamine and carbon dioxide as shown below (Fig1). The formation of biogenic amines is significantly influenced by the microbial ecology of food systems. Although a variety of microorganisms can produce decarboxylases, the most pertinent categories are *Pseudomonas*, *Clostridium*

species, Enterobacteriaceae, and lactic acid bacteria (LAB) (Natrella et al., 2024). Some LAB strains, including *Enterococcus faecalis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus curvatus*, have high levels of amino acid decarboxylase activity. Although the fermentation and flavor development of cheese, sausage, and fermented fish depend on these bacteria technologically, if left unchecked, their presence can also lead to undesired amine accumulation (Ter et al., 2024). However, gram-negative bacteria that cause histamine production, especially when improperly chilled or stored for an extended period of time, such as *Morganella morganii*, *Klebsiella pneumoniae*, *Photobacterium phosphoreum*, and *Hafnia alvei*, are commonly isolated from fish and seafood (Oktarini et al., 2022)

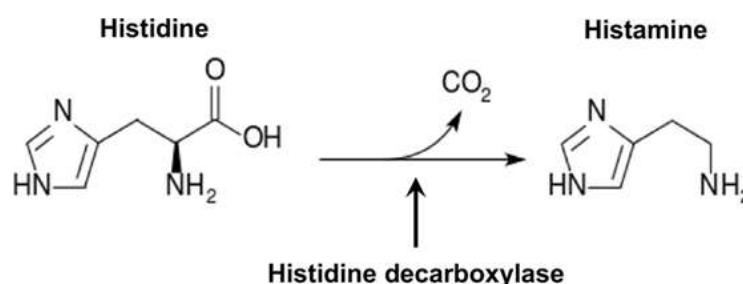


Figure 1. Histamine synthesis (Huang et al., 2018)

A number of environmental and technological factors affect the degree of amine production (Ucar & Ozogul, 2024). Although psychrotrophic species can also manufacture amines at refrigeration temperatures, temperature is one of the most important factors (Wang et al., 2025). The majority of bacteria that make amines exhibit optimal decarboxylase activity between 25 and 37 °C. Because the reaction helps bacteria resist acid stress by using protons during decarboxylation, acidic environments tend to promote decarboxylase synthesis (Anumudu et al., 2024). The pH of the food matrix has a substantial impact on substrate availability and enzyme stability. Furthermore, microbial development and the metabolic environment that controls amine accumulation are influenced by oxygen availability, salt content, water activity, and storage duration (Wang et al., 2024). Although high salt concentrations can stop bacteria from growing, they don't always stop enzyme activity after decarboxylases are created (Negm Rl-Dein et al., 2024).

The availability of precursor amino acids, which is reliant on the food's proteolytic activity, is another important consideration (Liang et al., 2025). Microbial proteases and peptidases hydrolyze proteins during fermentation and ripening, generating free amino acids that act as decarboxylation substrates. Long ripening periods or the presence of proteolytic bacteria can therefore indirectly raise BA levels. Amine production is also encouraged by the use of contaminated or improperly treated raw materials, especially milk with a high microbiological load or fish with a high histidine concentration (Bita et al., 2024).

The composition of the starting culture has an impact on the formation of amines in fermented products (Turna et al., 2024). A diverse microbiota, including strains that are decarboxylase-positive, is frequently involved in conventional or spontaneous fermentations (Zhang et al., 2025). On the other hand, amine synthesis can be severely restricted by using specific starter cultures that have little or no decarboxylase activity. Using non-decarboxylating strains of *Lactobacillus plantarum* or *Lactococcus lactis* has been to decrease the accumulation of tyramine and histamine in fermented meat and dairy products (Banicod et al., 2025).

The BA content in food is ultimately determined by the interplay of microbial metabolism, substrate composition, and processing environment. Designing preventive measures is made easier with an understanding of these mechanisms. To reduce the production

of biogenic amines, control methods such microbial selection, pH control, chilling, and good hygiene are crucial. In the dairy and fisheries industries, the use of molecular technologies, such as PCR-based detection of decarboxylase genes, has also improved the identification of microbes that produce amines, enabling better risk assessment and quality control.

Occurrence and Toxicological Implications in Foods

Although biogenic amines (BAs) are found naturally in a variety of foods, foods high in proteins and free amino acids have significantly higher quantities of BAs, particularly when they are subjected to microbial activity during processing or storage. Because of the enzymatic activity of decarboxylase-positive microbes, the most prevalent sources are fermented foods like cheese, sausages, wine, and fermented fish. The type of microorganisms involved, processing conditions, and raw material composition all affect how much BA accumulates (Turna et al., 2024; Saxana et al., 2024).

Occurrence in Dairy and Fermented Products

One of the items that has been investigated the most in relation to BA is cheese. Proteolytic enzymes from starting and non-starter microbes hydrolyze casein during ripening, yielding free amino acids that act as decarboxylation substrates (Atasever & Mazlum, 2024). The most common amine found in cheese is tyramine, which is followed by histamine (in certain types of cheese), putrescine, and cadaverine. Ripe cheeses including blue-veined, gouda, and cheddar have been found to contain high concentrations of these amines. The type of starter culture and length of ripening are important factors (Sadighara et al., 2024). For example, in artisanal and long-ripened cheeses, *Lactobacillus curvatus*, *Lactobacillus helveticus*, and *Enterococcus faecalis* have been found to be the main producers of BA (Tsanasidou et al., 2024). On the other hand, BA production can be considerably decreased by using carefully chosen starter cultures with low decarboxylase activity (Decadt et al., 2024).

Because of shorter fermentation times and reduced proteolytic activity, BA levels are often lower in fermented dairy products like kefir and yogurt. Nevertheless, detectable levels of histamine or tyramine may still result from post-processing contamination with amine-producing bacteria or from inadequate refrigeration.

Occurrence in Fish and Fishery Products

Due to their high level of free histidine, the main precursor for the synthesis of histamine, fish and seafood are especially prone to BA accumulation (Ganjeh et al., 2024). The development of particular bacteria, such *Morganella morganii*, *Photobacterium phosphoreum*, and *Klebsiella pneumoniae*, which include histidine decarboxylase enzymes, is principally linked to the generation of histamine in fish (Khatouf et al., 2024). These bacteria multiply quickly in fish that are not adequately refrigerated after capture, turning histidine into histamine at levels that could become lethal (Marzooqi et al., 2024). Species such as tuna, mackerel, sardine, and anchovy are especially prone to histamine accumulation due to their dark muscle tissues rich in histidine (Tunç et al., 2025).

One of the most prevalent types of foodborne intoxication linked to BA is scombroid poisoning, which can result from eating fish high in histamine (Saxena et al., 2025). Face flushing, headache, dizziness, nausea, vomiting, rash, and sometimes hypotension or heart palpitations are among the symptoms that usually show up minutes to hours after ingestion. Histamine is the primary cause, however other amines like putrescine and cadaverine can

increase its harmful effects by blocking the enzymes that break down histamine, such as diamine oxidase and histamine-N-methyltransferase (Kanta et al., 2025).

Toxicological Aspects and Human Health Risks

Histamine and tyramine are regarded as the most dangerous biogenic amines from a toxicological standpoint. Tyramine toxicity is linked to hypertensive crises, especially in people using monoamine oxidase inhibitors (MAOIs), while histamine toxicity is mostly caused by excessive ingestion that exceeds the body's detoxifying ability (Paparella et al., 2025). Histamine concentrations over 50 mg/kg and tyramine concentrations over 100 mg/kg are typically considered potentially hazardous, while the threshold for toxic effects differs. Additionally, cumulative health concerns may arise from long-term exposure to sub-toxic levels, particularly in sensitive people (Heidari et al., 2024).

Despite not being directly harmful, putrescine and cadaverine are significant due to their synergistic effects with tyramine and histamine (Braga et al., 2024). They can increase the toxicity and bioavailability of other amines by inhibiting detoxifying enzymes and improving intestinal permeability. Furthermore, these amines act as indicators of deterioration that reveal low quality or microbial contamination and contribute to the unpleasant taste and odor of spoiled foods.

Regulatory authorities have established specific limits for biogenic amines (BAs) in foods. According to the U.S. Food and Drug Administration (FDA, 2022), the acceptable maximum histamine level in fresh fish is 100 mg/kg. In contrast, the European Food Safety Authority (EFSA, 2023) recommends a limit of 200 mg/kg for fish products to ensure consumer safety.

In general, the presence of biogenic amines in food is a result of intricate interactions between environmental factors, microbial metabolism, and technological procedures (Ashaolu et al., 2023). Their accumulation shows product quality and marketability in addition to posing toxicological concerns to customers. Therefore, preserving food safety and protecting the public's health requires an understanding of how they are formed in different food matrices and the implementation of efficient control methods (Singh et al., 2024).

Control and Prevention Strategies

An integrated strategy including microbial control, optimal processing conditions, and good hygiene is necessary to effectively regulate the synthesis of biogenic amines (BA) in foods (Wojcik et al., 2021). The most effective preventative techniques focus on reducing microbial contamination, inhibiting amine-producing bacteria, and limiting enzyme activity during processing and storage because decarboxylase-positive microbes are the main producers of BAs (Natrella et al., 2024).

Good Manufacturing Practices and Hygiene Control

Controlling BA content is largely dependent on the application of Hazard Analysis and Critical Control Points (HACCP) and Good Manufacturing Practices (GMP) systems. Decarboxylase-positive bacteria can be avoided by upholding rigorous hygiene standards at every stage of the production process, from the selection of raw materials to packaging (Ly et al., 2021). Inhibiting microbial growth requires the use of premium raw materials with low microbial loads, quick freezing after harvest (particularly in fish), and careful handling throughout processing (Lerfall et al., 2024). Furthermore, frequent cleaning of facilities and

equipment lowers the prevalence of spoiling organisms like *Pseudomonas* and *Enterobacteriaceae* (Akinsemolu & Onyeaka et al., 2024).

Another important consideration is temperature regulation. The cold chain must be continuously maintained during storage and transportation since decarboxylase enzymes are still active at moderate refrigeration temperatures (Pajic et al., 2024). Histamine production can be considerably decreased by immediately cooling fish to below 4 °C after capture (Zincir., 2024). Similarly, limiting amine-producing bacteria populations in fermented dairy products can be achieved by managing fermentation temperature and storage conditions.

Technological and Analytical Control

Amine control is further aided by the optimization of processing factors, such as pH, salinity, and water activity (Fang et al., 2025). Enzyme induction can be decreased by keeping the pH above 5.5 as the cheese ripens or by lowering the salt concentration to inhibitory levels. Cutting down on ripening and fermentation periods may also reduce the amount of substrate available for decarboxylation (Elkot et al., 2025). Technology interventions that restrict microbial growth and enzymatic activity include high-pressure processing, vacuum sealing, and modified atmosphere packaging (MAP). A key component of preventative care is routinely checking BA levels. Liquid chromatography–mass spectrometry (LC–MS/MS) and high-performance liquid chromatography (HPLC) are the standard methods for quantifying histamine, tyramine, and related amines (Ahangari et al., 2023). On-site monitoring is now feasible because to recent developments in quick enzymatic tests and biosensor-based detection, which allow for the early identification of contaminated batches before they reach customers (Khan et al., 2024).

Integrated Risk Management

Combining microbiological, technological, and regulatory approaches represents an effective strategy for controlling biogenic amine formation. The food industry should establish critical limits for BA concentrations, routinely verify compliance with international standards, and provide adequate training for personnel handling temperature-sensitive products. Microbiological control, together with advanced analytical monitoring, ensures that biogenic amine levels remain within acceptable limits throughout the product's shelf life. Effective prevention requires a multidisciplinary and proactive approach based on proper temperature management, good hygienic practices, the selection of suitable starter cultures, and continuous monitoring. Integrating conventional food safety principles with molecular and technological innovations offers a robust framework for protecting consumers and maintaining high quality standards across the food sector.

CONCLUSION

Protein-rich foods like cheese and fisheries products invariably contain biogenic amines as byproducts of microbial metabolism. Their excessive accumulation, however, suggests inadequate temperature control, microbial contamination, or poor handling, all of which pose serious threats to consumer health and food safety. Histamine and tyramine are the most toxicologically significant of these substances since excessive consumption of them might result in allergic and hypertensive reactions. Despite not being immediately poisonous, putrescine and cadaverine are significant spoiling markers and intensify the negative effects of histamine and tyramine.

A comprehensive strategy that incorporates cold chain maintenance, proper manufacturing processes, and the use of starter cultures with low decarboxylase activity is necessary to limit the generation of biogenic amines. To reduce health hazards, rigorous adherence to international safety standards and ongoing monitoring using trustworthy analytical techniques are crucial. To ensure food safety and quality in an increasingly globalized food sector, future research should focus on the molecular mechanisms of amine formation and the development of preservation strategies.

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PREDICTION OF ANTIMICROBIAL PEPTIDES EFFECTIVE AGAINST IMPORTANT PLANT PATHOGENIC FUNGI

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ABSTRACT

Plant pathogenic fungi cause devastating crop losses worldwide and pose major challenges for sustainable agriculture. Conventional fungicides often suffer from resistance development and environmental concerns, underscoring the urgent need for novel antifungal agents. Antimicrobial peptides (AMPs) have emerged as promising candidates, yet the accurate classification of active versus inactive sequences against plant pathogenic fungi remains limited. Machine learning provides an opportunity to exploit sequence- and physicochemical-based descriptors for predictive modeling of antifungal activity. **Methods:** We compiled a dataset of peptides with known activity against major plant pathogens (*Fusarium* spp., *Botrytis cinerea*, *Verticillium dahliae*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) from the GRAMPA database. After removing highly similar sequences (>95% identity), we classified peptides as active or inactive by clustering their continuous antimicrobial activity scores using a Gaussian Mixture Model (GMM). Physicochemical properties were computed in silico using the DBAASP and Pfeature tools. The final curated dataset integrated position-specific one-hot encoding (P1–P140) with 693 numerical descriptors. Multiple ML algorithms—including Random Forest (RF), Extreme Gradient Boosting (XGB), and Support Vector Machines (SVM)—were trained and optimized via grid search cross-validation. Model performance was assessed using ROC-AUC, PR-AUC, F1-score, and threshold-optimized accuracy. **Results:** The Random Forest model achieved strong baseline performance (CV ROC-AUC = 0.858; test ROC-AUC = 0.943; accuracy = 0.88). XGB yielded comparable performance with improved calibration (test ROC-AUC = 0.936; F1 = 0.939 at optimal threshold). Feature importance analysis revealed that both physicochemical descriptors (e.g., QSO1_SC_Y, AAC_Y, SEP, CeTD_22_PO) and specific sequence positions (notably P22, P25–P31) strongly contributed to classification. Amino acids such as lysine (K), alanine (A), and valine (V) emerged as key residues associated with antifungal activity, alongside physicochemical features reflecting secondary structure and hydrophobicity. **Conclusion:** Our integrated sequence–descriptor machine learning framework successfully discriminated antifungal peptides with >0.93 ROCAUC and highlighted critical amino acids, positions, and physicochemical traits underpinning activity. This study demonstrated that combining position-specific encoding with physicochemical features improved model interpretability and predictive power. Future work would focus on expanding dataset diversity and benchmarking deep learning architectures to surpass 90% accuracy across independent validation sets.

Keywords: Antimicrobial peptide; Antifungal peptide; Plant pathogens; Pathogenic fungi

INTRODUCTION

Plant diseases pose significant problems for agricultural productivity, global food security and biodiversity in many regions of the world. Fungal plant pathogens spread via wind, water, soil, insects and other invertebrates, establishing themselves in the tissues of plant roots, stems, leaves and fruits. They cause infection by secreting enzymes that break down the cell walls of

the host plant's tissues. This results in significant losses in agricultural production and yield, as well as negatively affecting the balance of the ecosystem (Gulzar et al., 2025; Singh et al., 2023).

Chemical pesticides are commonly used in the fight against plant diseases to suppress or eliminate pathogens that cause infection. However, the excessive and indiscriminate use of pesticides disrupts the natural balance of the ecosystem and has significant adverse effects on human health (Ali et al., 2024). Since the 1950s, the use of antifungal drugs has become widespread in agricultural production to combat fungal diseases. However, the long-term and uncontrolled use of antifungal drugs has led to the development of resistance in agricultural ecosystems; increasing resistance rates, particularly to azole antifungals widely used in agricultural areas, have become a serious problem for both plant health and public health (Tang et al., 2023). Therefore, researchers are focusing on discovering new antifungal compounds that are more environmentally sensitive and safer (Marcos et al., 2008).

AMPs are bioactive peptides found in almost all organisms, from bacteria to humans. They form the first line of defence of the innate immune system (Yang et al., 2024). Many AMPs exhibit broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, viruses, single-celled protozoa and parasites (Zhang et al., 2021). Antifungal peptides (AFPs), a subclass of AMPs, are effective in controlling fungal infections by inhibiting fungal growth and reproduction. The main mechanisms of action of AFPs include disrupting membrane integrity by creating pores in the cell membrane; weakening the cell wall by inhibiting β -glucan and chitin synthesis; triggering oxidative stress and apoptosis by accumulating reactive oxygen species (ROS); suppressing DNA, RNA and protein synthesis; halting mitochondrial functions; and disrupting cytoskeletal organisation. Furthermore, some AFPs can provide indirect antifungal protection by activating defence responses in the host plant. Due to these multifaceted effects, AFPs stand out as a more advantageous and sustainable alternative to conventional antifungal drugs used in agriculture in terms of resistance development (Zhang et al., 2020; Li et al., 2021).

In this study, we developed a machine learning-based framework to classify active and inactive antimicrobial peptides against plant-pathogenic fungi. Peptide sequences compiled from the GRAMPA database were analysed using physicochemical properties and position-specific encodings, and were then classified using Random Forest, XGB and linear SVM models. Our findings demonstrate that this approach can accurately distinguish between antifungal peptides and reveal the critical amino acids, sequence positions and physicochemical properties that determine activity. These results provide a groundwork for the design of new AMP candidates against plant pathogenic fungi and the development of environmentally friendly strategies for managing plant diseases.

MATERIAL AND METHODS

Dataset Construction

A dataset of peptides with reported activity against major plant pathogens (*Fusarium* spp., *Botrytis cinerea*, *Verticillium dahliae*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) was compiled from the GRAMPA database (<https://github.com/zswitten/Antimicrobial-Peptides>). Redundant sequences exhibiting greater than 95% pairwise identity were removed dataset using Decrease Redundancy (https://web.expasy.org/decrease_redundancy). The antimicrobial activity scores of peptides available from GRAMPA were clustered into binary activity classes (active and inactive) using an unsupervised two-component Gaussian Mixture Model (GMM). Peptides assigned to the low-activity component were labeled active, whereas those in the high-activity component were labeled inactive. Physicochemical and structural descriptors were

computed *in silico* using the DBAASP (<https://dbaasp.org/home>) and Pfeature (<https://webs.iitd.edu.in/raghava/pfeature/>) platforms.

Machine learning dataset and feature representation

All sequence and descriptor information was stored in two spreadsheets containing peptides with experimentally validated antifungal activity (“positive”) and peptides lacking detectable activity (“negative”). Peptides in the positive and negative sets were assigned binary labels of 1 (active) and 0 (inactive), respectively, and merged into a single dataset. To encode primary structure information, peptide sequences were padded to a fixed maximum length of 140 residues. For each position P1–P140, the residue was represented as a categorical variable taking one of the 20 standard amino acids or a padding symbol (“–”) for positions beyond the natural sequence length. In the final table, these positional features appeared as columns P1, P2, ..., P140. In parallel, a comprehensive set of **693 physicochemical descriptors** was generated using the DBAASP and Pfeature computational tools. These descriptors encompassed amino acid and dipeptide composition (AAC, DPC), charge and hydrophobicity metrics, residue distribution patterns, structural propensities, and topological indices. Numerical descriptors were median-imputed and standardized using *StandardScaler*. The final feature matrix consisted of 140 position-specific categorical features (one-hot encoded) and 693 numerical physicochemical features yielding a total dimensionality of 833 variables.

Data Partitioning and Preprocessing Pipelines

The dataset was partitioned into training (80%) and test (20%) subsets using stratified sampling to preserve the ratio between active and inactive peptides. To account for the differing computational requirements of linear versus tree-based classifiers, two independent preprocessing pipelines were implemented.

For the linear Support Vector Machine (SVM), a sparse preprocessing pipeline was constructed. This pipeline comprised: (i) sparse one-hot encoding of position-specific amino acid features, (ii) median imputation to address missing numerical descriptors, and (iii) variance-preserving scaling using *StandardScaler* with the *with_mean=False* setting to ensure compatibility with sparse matrices.

In contrast, Random Forest and XGBoost models were trained using a dense preprocessing pipeline, which included: (i) dense one-hot encoding of sequence positions, (ii) median imputation of physicochemical variables, and (iii) conventional standardization via *StandardScaler*.

Model generalization and hyperparameter tuning were evaluated using *StratifiedKfold* cross-validation ($k = 3$, *shuffle = True*, *random_state = 42*), ensuring balanced representation of activity classes within each fold.

Model Training and Hyperparameter Optimization

Three supervised machine learning algorithms—Calibrated Linear Support Vector Machine (SVM), Random Forest (RF), and Extreme Gradient Boosting (XGBoost)—were benchmarked for antifungal peptide classification. Each model was implemented within a unified scikit-learn pipeline, incorporating the appropriate preprocessing block (sparse or dense) described above. Model hyperparameters were optimized using grid search with stratified 3-fold cross-validation, and the configuration achieving the highest mean ROC–AUC on the training folds was selected. The best estimator for each algorithm was subsequently refitted on the complete training set and evaluated on the independent test set.

1. Calibrated Linear SVM

A linear Support Vector Classifier (LinearSVC) was employed with class-weight balancing (“balanced”) and a maximum of 5000 iterations. Because LinearSVC does not provide probabilistic outputs, the classifier was wrapped in a Platt-scaled calibration model using **CalibratedClassifierCV** (method = “sigmoid”, cv = 3) to obtain well-calibrated probability estimates. Hyperparameter tuning explored the following regularization strengths: $C \in \{0.05, 0.1, 0.5, 1.0, 2.0\}$

2. Random Forest (RF)

A RandomForestClassifier with class-weight balancing and a fixed random seed (42) was used to capture nonlinear interactions between peptide descriptors. The following hyperparameters were evaluated:

Number of trees:

$n\_estimators \in \{200, 300, 500\}$

Maximum tree depth:

$max\_depth \in \{10, 20, None\}$

Minimum samples per leaf:

$min\_samples\_leaf \in \{1, 2, 4\}$

Feature subsampling strategy:

$max\_features \in \{“sqrt”, 0.3, 0.5\}$

3. Extreme Gradient Boosting (XGBoost)

An XGBClassifier (objective = “binary:logistic”, eval_metric = “auc”, tree_method = “hist”, n_estimators = 600, random_state = 42) was included as a gradient-boosted decision tree model. The grid search encompassed:

Maximum depth:

$max\_depth \in \{3, 5, 7\}$

Learning rate:

$learning\_rate \in \{0.03, 0.06, 0.1\}$

Subsampling ratio:

$subsample \in \{0.7, 0.9, 1.0\}$

Column subsampling ratio:

$colsample\_bytree \in \{0.6, 0.8, 1.0\}$

Minimum child weight:

$min\_child\_weight \in \{1, 3, 5\}$

L2 regularization:

$reg\_lambda \in \{1, 5, 10\}$

Each model–preprocessing pair was evaluated within an identical cross-validation framework using GridSearchCV(scoring=“roc_auc”). The final predictions on the held-out test set were obtained using the optimal hyperparameters identified during grid search.

All machine learning procedures, including data preprocessing, model training, and evaluation, were implemented in Python 3.13.7 using the scikit-learn and XGBoost libraries. The classification pipeline included one-hot encoding of sequence positions, standardization of numerical descriptors, and training of three models: Calibrated Linear SVM, Random Forest,

and XGBoost. Model calibration, feature importance extraction, and performance visualization were also conducted within the same Python environment. The code used in this study is available upon request.

Threshold Optimization and Performance Evaluation Metrics

Model discrimination was first assessed using threshold-independent metrics; however, binary classification requires the selection of an operating decision threshold. For each classifier, predicted probabilities on the held-out test set were initially evaluated using the conventional threshold of 0.5, from which accuracy and F1-score were derived. To obtain an optimal decision threshold, an additional threshold-optimization procedure was performed based on the precision–recall (PR) curve. Specifically, precision, recall, and F1-score were computed over all possible probability thresholds, and the threshold yielding the maximum F1-score was designated as the optimal operating point. All downstream classification metrics, including confusion matrices and detailed classification reports, were computed at this optimized threshold.

Overall model performance was quantified using a suite of complementary evaluation metrics, including the receiver operating characteristic area under the curve (ROC-AUC), precision–recall area under the curve (PR-AUC; average precision), accuracy, F1-score, and the Brier score, which captures the mean squared deviation between predicted probabilities and true class labels. To provide an interpretable view of classifier behavior, confusion matrices were generated both in raw form and as row-normalized matrices, thereby enabling visualization of the distribution of true positives, true negatives, false positives, and false negatives for each model.

To further characterise the sensitivity–specificity trade-off across the entire probability range, threshold-scanning analyses were conducted by systematically varying the decision threshold from 0 to 1 in increments of 0.005. At each threshold, accuracy, true positive rate (TPR), and false positive rate (FPR) were computed, resulting in threshold–accuracy and threshold–F1 curves. These curves provide a continuous map of model performance across the decision space and support the identification of operational thresholds suitable for experimental peptide prioritization.

Calibration and Reliability Analysis

The calibration quality of the predicted probabilities was assessed using reliability curves. For each classifier, test-set probability estimates were binned into ten equally spaced intervals, and the mean predicted probability in each bin was plotted against the empirical fraction of active peptides. Calibration performance was additionally summarised using the Brier score, which quantifies the mean squared difference between predicted probabilities and true class labels.

Feature Importance Analysis

Feature importance analysis was carried out for all tree-based models to quantify the contribution of individual features to antifungal activity prediction. For the Random Forest classifier, importance values were derived using the Gini impurity criterion. For the XGBoost model, feature contributions were computed using the gain metric obtained from the underlying gradient-boosted trees. Feature names were extracted directly from the fitted preprocessing pipeline to ensure consistent mapping between positional one-hot encoded variables and physicochemical descriptors.

RESULTS

Performance of Machine Learning Classifiers

We evaluated three supervised learning models—Random Forest (RF), XGBoost, and Calibrated Linear SVM—on the test set using ROC-AUC, PR-AUC, accuracy, and F1-score metrics. Overall, the tree-based classifiers substantially outperformed the linear SVM model. Random Forest achieved the highest discrimination ability with a test ROC-AUC of 0.943, PR-AUC of 0.963, and an F1-score of 0.909. XGBoost exhibited comparable predictive power (ROC-AUC = 0.936, PR-AUC = 0.961), but showed slightly superior calibration characteristics and a higher F1-score. In contrast, the Calibrated Linear SVM displayed lower performance (ROC-AUC = 0.866, PR-AUC = 0.895), consistent with its limited capacity to model nonlinear sequence–descriptor interactions (Table 1).

The ROC curves (Figure 1A) demonstrate that both RF and XGBoost achieve steep rises in true positive rate at low false-positive rates, indicating strong separability between active and inactive peptides. PR curves (Figure 1B) further support these observations, with both tree-based models maintaining exceptionally high precision across a wide recall range. These findings collectively indicate that nonlinear algorithms are better suited for integrating positional encodings with high-dimensional physicochemical descriptors, in agreement with previous studies on AMP classification.

Table 1. Comparative performance metrics of machine learning classifiers

Model	CV ROC-AUC	Test				Brier Score	Optimal Threshold
		ROC-AUC	PR-AUC	Accuracy	F1		
Random Forest	0.858	0.943	0.963	0.88	0.91	0.0947	0.463
XGBoost	0.842	0.936	0.961	0.92	0.94	0.0951	0.545
Linear SVM (Calibrated)	0.805	0.866	0.895	0.88	0.91	0.1264	0.505

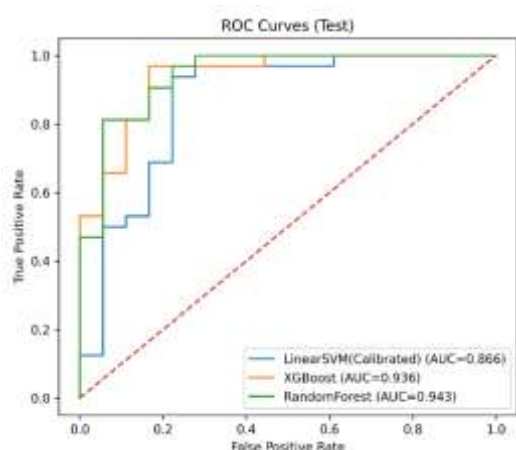


Figure 1A. ROC curves of machine learning models for antifungal peptide classification.

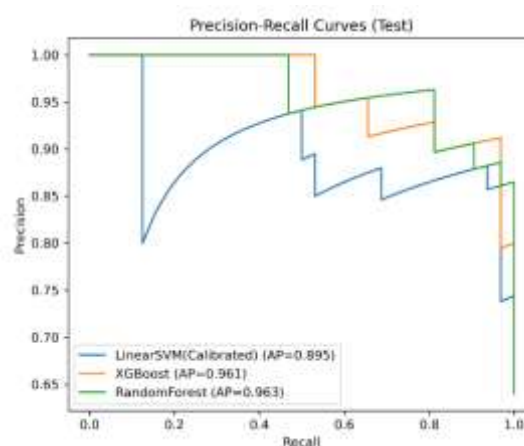


Figure 1B. Precision–Recall curves (PR-AUC) of all models showing recall vs. precision across thresholds.

Threshold-Optimized Predictions and Confusion Matrix Analysis

In binary classification tasks, selecting an appropriate decision threshold is critical to balance sensitivity and specificity. To optimize classification performance, particularly for imbalanced datasets, we applied an F1-maximizing threshold selection strategy based on precision–recall trade-offs. The resulting confusion matrices and derived performance metrics at the optimal thresholds revealed distinct behavioral profiles for each model.

At the optimal threshold of **0.463 (Table 1)**, the **Random Forest** classifier achieved a **true positive rate (TPR) of 1.00**, **false positive rate (FPR) of 0.28 (Figure 2A)**, and **overall accuracy of 0.88**. Importantly, the model produced no false negatives (FN = 0), meaning all active antifungal peptides were correctly identified. In contrast, **XGBoost**, operating at its optimal threshold of **0.545 (Table 1)**, demonstrated a more balanced performance profile. It achieved a **TPR of 0.97**, **FPR of 0.17**, and the **highest accuracy among all models (0.92) (Figure 2B)**. Compared to Random Forest, XGBoost exhibited a slightly reduced recall but considerably improved specificity. This indicates a more conservative classification boundary, effectively reducing the number of false positives while still maintaining high sensitivity—an ideal trade-off for resource-constrained experimental validation pipelines.

The **Calibrated Linear SVM**, while achieving an accuracy of 0.88 and TPR of 0.97 (Figure 2C) at an optimal threshold of **0.505 (Table 1)**, suffered from inferior calibration and decision reliability. The model generated a relatively high number of false positives and showed greater dispersion in predicted probability distributions. This underperformance reflects the limitations of linear classifiers in capturing complex, nonlinear interactions between amino acid sequences and physicochemical descriptors, which are critical in antifungal activity prediction.

Collectively, these results suggest that **XGBoost provides the most balanced classifier**, offering both high precision and recall, whereas **Random Forest is preferable in high-recall use cases** where the cost of false negatives is particularly high. The calibrated SVM, despite decent performance, is less reliable due to poorer calibration and limited model flexibility.

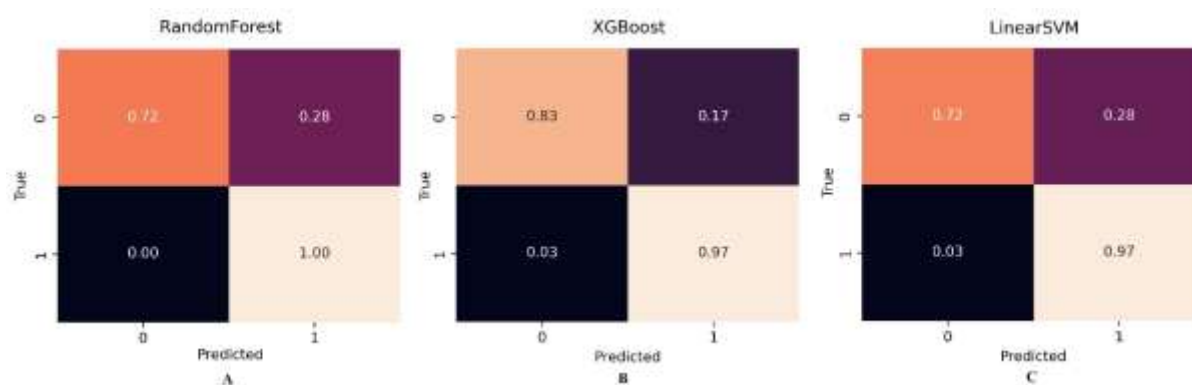


Figure 2. Normalized confusion matrix of the Random Forest (A), XGBoost (B) and Calibrated Linear SVM (C) classifier

Probability Calibration Analysis

In addition to classification accuracy, the reliability of predicted probabilities is a critical factor when deploying models for biological prioritization. Calibration analysis offers insights into how closely a model's predicted probabilities align with observed outcomes. The Brier score metric was used to evaluate the model calibration. Brier score is a metric that measures

the quality of probability estimates in terms of both calibration and sharpness. As shown in the calibration plots (**Figure 3**), there are marked differences in calibration quality across the evaluated models.

Among the three classifiers, Calibrated Linear SVM demonstrated the most reliable probability estimates, achieving the lowest Brier score (0.2723). In contrast, XGBoost and Random Forest, despite their strong classification performance, exhibited inferior calibration reliability, with Brier scores of 0.2909 and 0.3161, respectively. Given that Brier scores in the range of 0.25–0.40 are considered moderately reliable, these results suggest that while all models provide a usable level of probabilistic output, there is still a need for improvement. Overall, the predicted probabilities moderately reflect the true likelihood of antifungal activity, but further calibration refinement may enhance their reliability in practical applications.

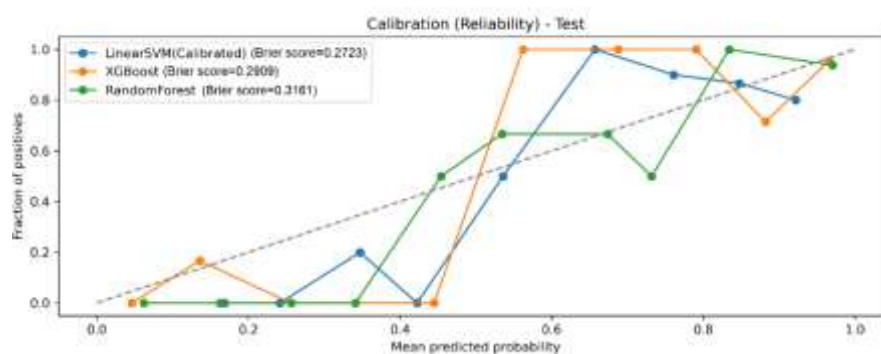


Figure 3. Calibration (reliability) curves of predicted probabilities for each classifier.

Feature Importance Analysis and Physicochemical Determinants of Antifungal Activity

Feature importance analysis was conducted to identify the key sequence positions and physicochemical descriptors that contribute most significantly to antifungal peptide classification. In the **Random Forest** model, top-ranked features included both physicochemical and sequence-based descriptors (Figure 4). Notably, **QSO1_SC_Y** and **AAC_Y** indicated that the abundance and polarity of tyrosine residues play a critical role in antifungal activity. Additional important descriptors such as **SEP**, **DDR_F**, and **PAAC1_λam1** were associated with hydrophobicity, charge distribution, and secondary structure propensity. Furthermore, position-specific one-hot encodings at **P22** and **P26** were ranked in top 20 features, suggesting that certain localized sequence regions are strongly predictive of antifungal function.

When the features were ranked according to their importance, the LinearSVM(Calibrated) model identified dipeptide composition descriptors such as **DPC1_WK**, **DPC1_FL**, and **DPC1_KL** as top contributors, suggesting that specific dipeptide patterns play a critical role in antifungal peptide classification. In addition, several features corresponding to specific amino acid positions (e.g., **P10_K**, **P15_A**, **P2_W**) were also among the most influential.

The **XGBoost** model emphasized a different but complementary set of influential features (Figure 4). High-impact physicochemical variables included **PAAC1_Y**, **SEP**, **DDR_C**, and **CeTD_23_PZ** descriptors. In terms of sequence context, the model highlighted positional

encodings at **P2_W**, and **P24_A**. These findings support the idea that specific structural and positional patterns underpin the antifungal activity of peptides.

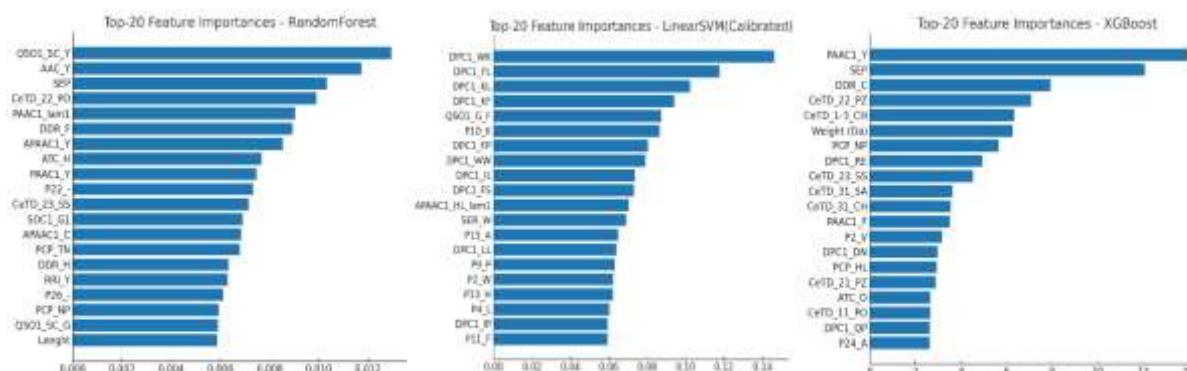


Figure 4. Top-20 most important features from the Random Forest, Linear SVM and XGBoost models

A recurring theme across all models was the importance of descriptors related to hydrophobic moment, charge asymmetry, and structural flexibility. These characteristics are well-established determinants of antimicrobial peptide (AMP)–membrane interactions and fungal cell permeability, thereby aligning the machine learning findings with known biophysical mechanisms. Notably, descriptors associated with residues such as lysine (K), alanine (A), and valine (V) consistently ranked among the most influential features. These amino acids contribute to key physicochemical properties—namely cationicity, amphipathicity, and hydrophobic interactions—which are critical for the membrane-disruptive behavior characteristic of antifungal peptides. Collectively, these results provide mechanistic insights into model decision-making and highlight a set of physicochemical and structural features that may inform the rational design of future antifungal peptide candidates (Bechinger and Gorr, 2017; Rautenbach et al., 2016; Wang et al., 2022).

CONCLUSIONS

This study demonstrates that both Random Forest and XGBoost classifiers exhibited strong predictive power, achieving ROC-AUC values exceeding 0.93 for predicting antifungal peptides effective against important plant pathogens. These tree-based models substantially outperformed the classical linear baseline and revealed biologically meaningful patterns related to amino acid composition, sequence localization, and membrane-active properties. This supports the utility of machine learning not only as a classification tool, but also as a powerful approach for exploring and leveraging peptide–function relationships. Taken together, these results present a computationally efficient and biologically valuable framework for antifungal peptide discovery.

However, looking ahead, several approaches can be proposed to further enhance model generalizability across fungal species and extend this research framework. These include: (i) expanding and diversifying the training datasets by incorporating novel antifungal sequences and activity profiles; (ii) integrating deep learning architectures, such as transformers and convolutional neural networks, to capture complex sequence–structure relationships beyond the capabilities of tree-based models; and (iii) experimentally validating high-confidence predictions in real-world bioactivity settings.

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POPULATION DYNAMICS OF THE OLIVE FRUIT FLY (*BACTROCERA OLEAE*) IN THE BERAT REGION

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ABSTRACT

Olive cultivation represents one of the most important branches of agriculture in the Berat region. This area is characterized by favorable agroclimatic conditions and a long-standing tradition in olive cultivation, a crop of great economic importance for the region. However, olive production is often affected by the damage caused by the olive fruit fly (*Bactrocera oleae*), one of the most harmful pests of this crop. This study aimed to monitor the dynamics of the olive fruit fly population through sampling of olive fruits and to assess the level of infestation by fly larvae. The study was carried out using standard methods, analyzing 100 fruits/tree (5 trees) on an area of 1 ha. It was conducted from mid-June to the end of September 2025, in the village of Qershnik, Berat district, with the cultivar "Kokermadhi i Beratit". The results showed that during 2025, the infection level of *Bactrocera oleae* in this area was about 3%. This low level of infestation is attributed to the high summer temperatures and unfavorable conditions for the development of the fly. These temperatures maintained the fly population at low levels, preventing any economic damage to the olive yield.

Keywords: Olive fruit fly (*Bactrocera oleae*), dynamics, sampling, population, infestation rate

INTRODUCTION

Olive production in Albania, as in all Mediterranean countries is an important economic activity (Bacaj, M. et.al., 2016). *Bactrocera oleae* (Rossi, 1790) (*Diptera: Tephritidae*) is the most dangerous insect pest of the olive tree in the Mediterranean region (Zineb Bourakna et.al., 2022).

Economic losses due to the olive fly are substantial, estimated at around 15% of annual olive production. (Bueno A.M, et al., 2002). The first signs are seen in early cultivars (Frantoio, Lecino and table cultivars). (attcvlore.al). The olive fly damages fruits by feeding on the mesocarp and creating oviposition sites, making them susceptible to bacterial and fungal infections. This leads to premature fruit drop, reduced quality of table olives, and increased acidity in olive oil, negatively impacting its nutritional and commercial value. (Tzanakakis. M, et.al., 2003). The predisposition to the fly attacks is tied to several factors, both intrinsic and extrinsic. The main ones are climatic (temperature and rainfall) so marked differences can occur from year to year. Wikipedia <https://en.wikipedia.org>. Monitoring is the most common operation to evaluate the population variability/size over time in cultivated fields. (Capinera, J.L., 2001), (Rossini, L, et.al., 2019). By employing a holistic approach that integrates ecological knowledge with sustainable control methods, we can ensure the continued viability of olive cultivation, protect

the environment, and produce high-quality olive oil.(Giorgos Stavrianakis et.al.,2025). To mitigate the issue of olive fly, Integrated Pest Management (IPM) models have been developed. According to the Food and Agriculture Organization of the United Nations (FAO), IPM emphasizes the growth of a healthy crop with minimal disruption to the agroecosystem and encourages natural pest control mechanisms. (FAO.,2020)

MATERIALS AND METHODS

Time and place of study

The study was conducted from the second half of June until the end of September 2025. The research site was selected in the village of Qereshnik, in the Berat district, since many olive groves are concentrated in this area. The cultivated olive variety was *Kokërrmadh i Beratit*. The area is well-suited for olive cultivation and is characterized by hilly terrain. The predominant soils are gray-brown, extending through the hilly zone up to 600 meters above sea level. The land faces west, covering an area of 1 hectare, with a slope of 13%. The climate is characterized by cold, wet winters and hot, dry summers. The hottest months of the year are July and August, with an average temperature of 28–29°C and maximum temperatures during heatwaves reaching 37–39°C. Annual precipitation ranges between 900 and 1,100 mm, falling mainly in winter.



Figure.1.The olive groves from(google earth) Figure.2.The olive grove in Qereshnik, Berat.

Study Method.

To assess the level of infestation by the olive fruit fly (*Bactrocera oleae*), the standard method of analyzing 100 olive fruits as a representative unit was used (Daane, K.M. et al., 2010).

Samples were randomly collected from several trees within the plot. The olive fruits for analysis were taken from the tree canopy at approximately human height and from the four main directions (or branches) of each tree. Sampling was carried out at weekly intervals, starting from the second half of June until the end of September 2025. From five different trees in the plot, 20 fruits were collected from each, forming a total composite sample of 100 olive fruits. Each olive fruit was carefully examined for signs of oviposition punctures, larval galleries, or the presence of larvae and pupae of the olive fruit fly. Visual inspection, magnifying lenses, and a stereomicroscope were used to detect symptoms of infestation. The infestation rate (%) was then calculated, corresponding to the number of infested olives among the 100 fruits collected from the five trees. All results were recorded weekly to analyze the progression of *Bactrocera oleae* infestation during the 2025 season in this area.



Figure 3. Analysing the extent of *Bactrocera oleae* infestation

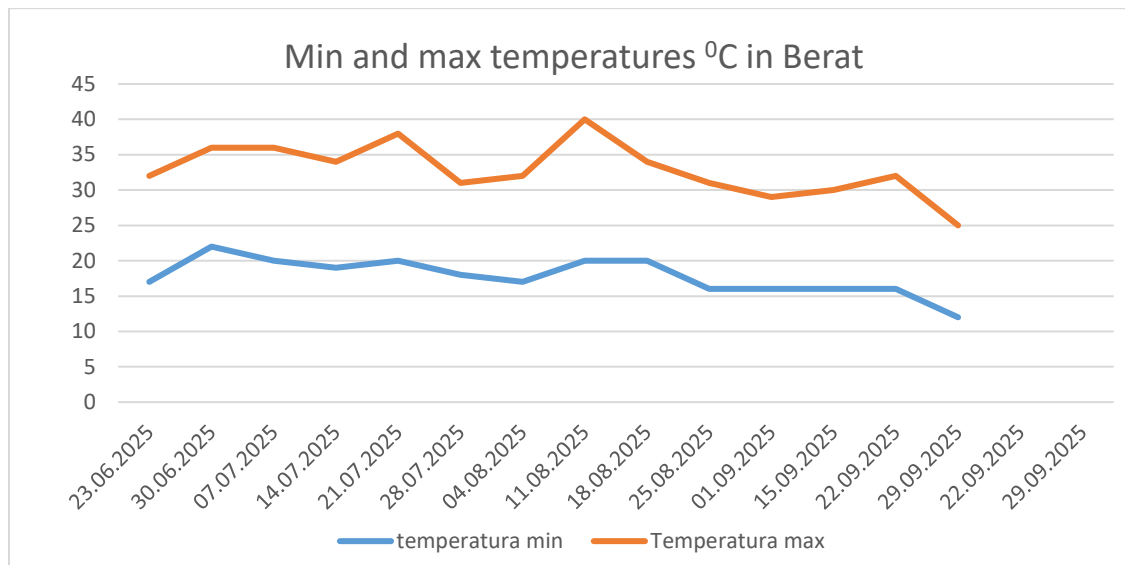
The following formula was used to calculate the infestation rate:

$$\text{Infestation rate (\%)} = \frac{\text{No. of infested fruits}}{\text{Total No. of examined fruits}} \times 100$$

Climatic conditions

Along with the data on olive fruit ripening, climatic data (temperature) were also obtained from the meteorological station in Berat. The phenology of *Bactrocera oleae* depends on environmental conditions such as temperature, precipitation, and humidity, which strongly influence the biological performance of the olive fruit fly. When temperatures exceed 30 °C for several hours during the day, they can be destructive to olive fly eggs and larvae, causing mortality rates of up to 80% (El-Salam *et al.*, 2019).

Graphic 1. Min and max temperatures for the months (June-September) in the Berat area 2025



Weather.com. <https://weather.com.sq-AL>

RESULTS AND DISCUSSION

The dynamics and aggressiveness of the olive fruit fly population are closely correlated with climatic factors, particularly temperature and humidity. Population fluctuations depend directly on the size of the previous generation, which is influenced by various ecological processes.

Table.1. Olive fly infestation rate (*Bactrocera oleae*) Qereshnik, Berat.

Sampling date	5 Tree	X 20	Olive fruit	=	100	Olive fruit
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	% of touch
23.06.2025	0	0	0	0	0	0
30.06.2025	0	0	0	0	0	0
07.07.2025	0	0	0	0	0	0
14.07.2025	0	0	0	0	0	0
21.07.2025	0	0	0	0	0	0
28.07.2025	0	0	0	0	0	0
04.08.2025	0	0	1	0	0	1
11.08.2025	0	0	0	0	0	0
18.08.2025	0	0	0	1	0	1
25.08.2025	0	1	0	0	0	1
01.09.2025	1	0	0	0	0	1
15.09.2025	0	1	0	0	0	2
22.09.2025	1	0	0	0	1	2
29.09.2025	1	0	1	0	1	3

The table shows all the sampling dates for the cultivar *Kokërrmadhi i Beratit* during the months of June to September 2025, as well as the percentage of infection determined after analysis. As can be seen from the table, no infection was detected until 28 July 2025, with a value of 0%. From the second half of July, there was an increase in the number of female fly punctures on olive fruits, as well as the presence of fly larvae, although the infection rate did not exceed 3%. This trend appears to result from the beginning of the temperature decrease.

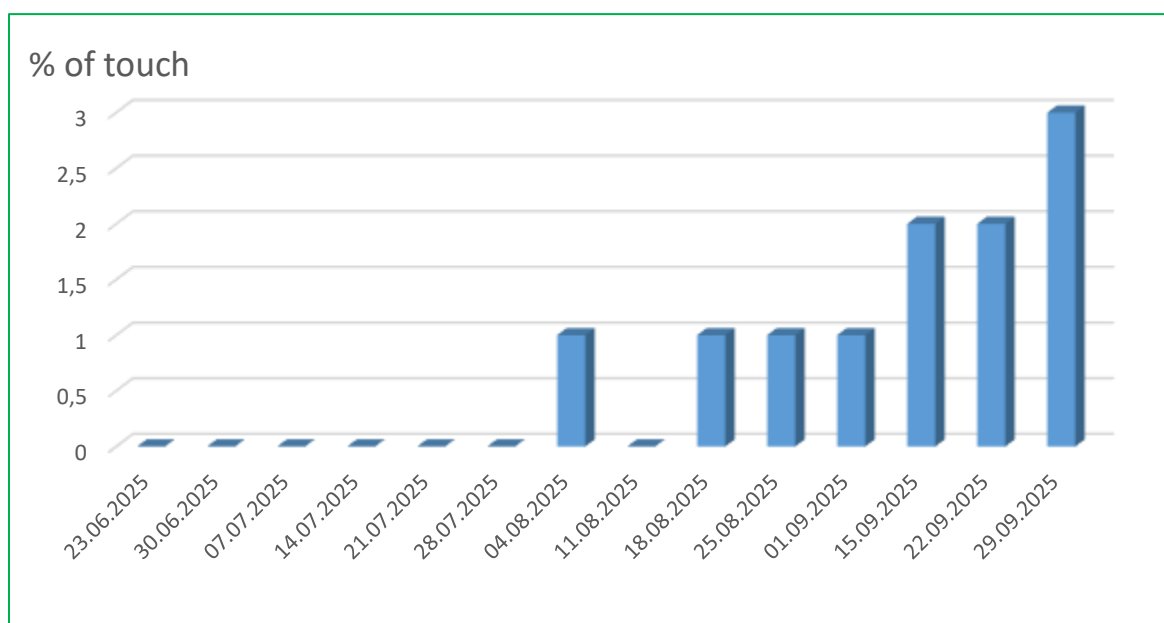
This graph reflects all the results according to the sampling dates that were taken from the second half of June to the end of September, as well as the percentage of infection after analyzing the samples taken according to the table above.

During the 2025 season, a periodic monitoring of the infestation rate by the olive fruit fly (*Bactrocera oleae*) was conducted on the cultivar “Kokërrmadhi i Beratit”. The assessment was based on 100 olive fruits randomly collected from 5 trees, at weekly intervals, from June 23, 2025, to September 29, 2025.

The results showed that until July 28, 2025, infestation was negligible, with a value of 0%. The first noticeable increase was recorded on August 4, 2025, with a light infestation rate of 1%. This percentage remained stable throughout August. In the second half of August and in September, a slight increase in fly activity was observed, with the presence of adult females and larvae in the fruits, but the overall infestation level did not exceed 3%. Therefore, for the cultivar “Kokërrmadhi i Beratit,” the infestation rate remained low during the entire observation period.

The low infestation level recorded in 2025 is closely related to the unusually high thermal conditions in the Berat area. During July and August, daily temperatures frequently exceeded 35°C, and on several days reached over 38°C. These high temperatures significantly limited the reproductive activity and larval development of *Bactrocera oleae*, preventing the completion of its full biological cycle.

Graph 2. The infestation rate of the olive fly (*Bactrocera oleae*) Qereshnik, Berat.



Sampling dates

As a result, the insect did not find optimal conditions for development, leading to naturally low infestation levels due to climatic stress. In conclusion, the low infestation rate of *Bactrocera oleae* observed in the Qereshnik area of Berat during 2025 can be attributed to high summer temperatures and unfavorable environmental conditions that restricted the biological activity and population growth of the olive fruit fly.

- For the cultivar *Kokërrmadh i Beratit*, special attention should be given because it is a table olive variety. If integrated pest management (IPM) techniques are not applied at the appropriate time, the production may lose its commercial value.
- Based on the findings of our study, if this cultivar is harvested one week earlier, no treatment is required.
- Applying treatments at the optimal time reduces costs, increases treatment efficiency, significantly limits pest population dynamics, ensures oil with minimal pesticide residues, and helps protect the environment and biodiversity in general.

Overall, the situation demonstrates that regular monitoring, combined with climate analysis, is a valuable tool for determining the optimal timing of interventions and for implementing effective IPM measures in table olives.

CONCLUSIONS

Based on the analyzed data and the climatic conditions it can be concluded that:

- The biological pressure of the olive fly has been low
- There has been a decrease in the activity of adult flight and mating;
- A reduction in the survival of eggs and larvae in the fruit;

- Limitation of fertility and overall population development;
- The economic damage threshold for table olives was not reached;
- Chemical treatment can be postponed or completely avoided while maintaining fruit quality;
- It shows that the monitoring and microclimatic conditions have been favorable for healthy production.

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STABILITY OF COMMERCIAL TREE SPECIES WOOD PRODUCTS IN EUROPE ACCORDING TO REPORTS AND STUDIES - REVIEW OF CLIMATE CHANGE AFFECTED FLUCTUATION ON THE MARKET

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ABSTRACT

Forest tree species differ greatly in their technical characteristics, on which their quality for the production of certain types of wood assortments depends, especially furniture, construction materials, and numerous other products within the wood processing industry and forestry as well. The importance of the stability of the wood market, both that needed for heating and wood for the production of other assortments, is great, because the stability of the overall market largely depends on its fluctuations, especially if we take into account the fact that some countries base their national economy on forestry and wood processing, or that forestry and wood processing represent significant potential for the further development of the national economy. In Europe, various Central European countries, such as Germany (with a focus on the Bavaria region), as well as northern countries (Scandinavia) are large producers of wood biomass. This wood biomass is used for the production of some of the most sophisticated wood assortments, and the preservation of sustainable and stable forest production is crucial for these countries. Currently, the greatest threat to countries in Europe, but also on a global level, is the intensification of the climate change process, which brings with it incalculable consequences for drought and pathogen-affected areas. The mortality rate of trees has begun to increase to almost 100% in areas attacked by the calamitous bark beetle insect. This insect, with its invasion, egg laying and forest devastation, is the number one destroyer of coniferous forest complexes, because the inoculum of infection increases very quickly, which is why the bark beetle attacks huge groups of trees. Suspicion of the appearance of bark beetles in areas around the inoculum of infection very often implies the sanitary felling of a large number of trees, which can ultimately cause a very noticeable disruption of the wood biomass market. Namely, enormous sanitary fellings, for example for spruce or pine, cause a large number of these trees to appear on the market and temporarily decrease the price of a cubic meter of wood, because in that case the market becomes overloaded with the arrival of excessive coniferous timber. Of course, there are exceptions, when certain assortments also experience price jumps, however, they are mainly related to periods after forest fires, or if there was a deficit of wood biomass of certain coniferous trees for one or two seasons, because forest stands were destroyed in fires. In this paper, we deal with price fluctuations of commercial tree species, in order to consider some of the problems potentially related to the problem of climate change and the occurrence of long-term droughts in Europe.

Keywords: *climate change, wild fires, market stability, sustainable production, forest management, bark beetle, national economy*

1.INTRODUCTION

The drying of trees is now largely linked to climate change (**Bracalini et al., 2024**). Certain types of insects in the forest are considered harmful (**Wagner et al., 2021**). Unfortunately, with the appearance of certain harmful insects, there is sometimes an inevitable appearance of pathogenic fungi, which can also harm the health of trees (**Linnakoski & Forbes, 2023**). Very often, forest stands attacked by pathogens and calamitous insects are threatened with death, and at the beginning of the infection or disease, the leaves of the trees dry up, and entire branches also dry out, and the bark falls off (**Bracalini et al., 2024**). The reduction in tree immunity significantly contributes to the disruption of the overall health and vitality of the entire forest stand (**Trumbore et al., 2015**).

For the past few decades, scientists have been studying the impact of climate change on living things, and forests in particular (**Vacek et al., 2023**). Certain commercial tree species, which are dominantly used in the timber industry, have become vulnerable in some parts of the world (**López-Tobar et al., 2024**). For example, the problem of the drying of beech forests in Europe is known, while certain pathogens, such as *Microsphaera alphitoides*, have begun to attack oak trees as well (**Bussotti et al., 2015**). The greatest threat to coniferous trees is posed by bark beetles, of which there are several species and which are difficult to identify (**Knížek & Beaver, 2007**).

In order for the wood biomass market to remain stable, it needs to be supplied with sufficient quantities of both coniferous and broadleaf wood (**Shah & Kaur, 2024**). The market in Europe is such that industry and society demand both hardwood and softwood assortments, which is why a surplus or deficit of one or the other can cause a strong imbalance in the entire market (**Wageningen University et al., 2024**). Some countries in Europe are large producers of wood, such as Germany or the Scandinavian countries (**Eurostat, 2024**), while for example one of the large producers of wood biomass in the countries of the Balkan Peninsula is Bosnia and Herzegovina (**Gvero et al., 2007**).

Depending on soil conditions, moisture content and general characteristics of vegetation cover, some lands are more or less exposed to excessive heating, which can affect the evaporation of useful materials from the soil, or the reduction of water levels (**Lozano-Parra et al., 2018**). One of the additional dangers for forest lands is the occurrence of erosion (**Borrelli et al., 2016**). The occurrence of floods and landslides is also increasingly recorded in countries affected by climate change (**Deal, 2024**), which poses damage not only to ecosystems but also to people. Remediation of the harmful consequences of climate change is extremely expensive, and the damage sometimes cannot even be estimated (**Amelang, 2022**). Climate change mitigation is a strategic and long-term priority for many countries, which have felt the consequences of global warming and whose economies have suffered damage caused by forest fires (**Reuters, 2025**).

The problem with forest fires is that they are becoming more frequent and larger every year (**Taylor, 2022**). Forest fires bring devastation to entire plant and animal habitats, as well as human settlements and factory infrastructure (**Davare, 2022**). Forest fires significantly affect the wood biomass market (**Lindenmayer et al., 2023**) and this research will also analyze data on forest fire damage to the national economy. The work should provide insight into several aspects of price fluctuations of commercial wood species.

2.MATERIAL AND METHOD

This paper will use the results of previously conducted research, since the paper is intended as a review article. In order to obtain the necessary research material for analysis, it was decided to search for relevant scientific literature using the following seven keywords: 1)

climate change, 2) wild fires, 3) market stability, 4) sustainable production, 5) forest management, 6) bark beetle, and 7) national economy. The reason for choosing these seven terms lies in the need to investigate: a) *how climate change affects the price fluctuations of wood products obtained from commercial wood species*, b) *how the occurrence of frequent forest fires affects the stability of the market for wood assortments obtained from coniferous wood*, c) *what is currently happening with the stability of the wood products market*, d) *whether wood production can currently be called sustainable*, e) *what is the relationship between forest management and the stability of the wood market, i.e. fluctuations in the prices of commercial wood on the market*, f) *to what extent the occurrence of bark beetles affects fluctuations in the prices of wood products*, and g) *how price fluctuations on the wood biomass market relate to the national economy, i.e. what impact do they have.*

In order to obtain answers to seven questions, 11 scientific and professional sources of relevant information were analyzed. The scientific articles used in this analysis are peer reviewed and published in recognized scientific journals. To obtain answers to each of the seven questions, two relevant sources of literature, or articles, were analyzed, namely: a) to obtain answers to the first question **Agarwal et al. (2025)** and **Karenlampi (2023)**, b) to obtain answers to the second question **Agarwal et al. (2025)** and **Shavazipour & Sundström (2024)**, c) to obtain answers to the third question **Compagnia del Legno (2025)** and **Houballah et al. (2024)**, d) to obtain answers to the fourth question **Shavazipour & Sundström (2024)**, **Jonsson & Sotirov (2025)** and **European Organisation of the Sawmill Industry (2024)**, e) to obtain answers to the fifth question **Karenlampi (2023)** and **Shavazipour & Sundström (2024)**, f) to obtain answers to the sixth question **Van Veen (2020)** and **International Forest Industries (2020)** and g) to obtain answers to the seventh question **Fastmarkets (2022)** and **Stora Enso (2024)**. The articles are available on: 1) *Cornell University ArXiv*, 2) *ResearchGate and market related web sites (reports)*.

The results of the analyses aimed at obtaining the necessary information are of a qualitative nature and are presented in tables for better clarity.

3.RESULTS AND DISCUSSION

The results of the analysis will be presented in seven short subchapters, for better clarity, and will contain comments in terms of synthesis of the obtained results, with possible implications for the situation in Bosnia and Herzegovina or recommendations that could be investigated in the context of the stability of the wood biomass market in Bosnia and Herzegovina.

3.1.The impact of climate change on price fluctuations of wood products from commercial tree species

In this subsection, we will review the research focuses and main results from two sources of literature, which mainly concern the examination of price fluctuations in markets, in similar and at the same time different ways. Namely, these two sources of research can help us understand how the effects of climate change affect the price fluctuations of wood products made from commercial tree species.

According to **Agarwal et al. (2025)** and **Karenlampi (2023)**, climate change has been shown to significantly affect price fluctuations with disasters. Weather events and the risk of recurring natural disasters can significantly reduce the return on investment in the timber industry. Unfortunately, the impact of climate change does not end there, and the emergence of pathogens and pests entering forest complexes with natural disasters also leads to disruptions in the percentage of harvested tree species. Thus, it may happen that in one year, due to weather

events and the appearance of pests, the market supply chain is disrupted, for example by an excessive supply of coniferous wood (or another similar scenario).

Table 1. Effects of climate change on price fluctuations of wood products made from commercial tree species.

Autor	Research Focus	Key Findings
Agarwal et al. (2025)	Researchers have been investigating the impact of catastrophic events on wildfire occurrence, as a consequence of climate change. Researchers have focused on the impact on forest least valuations and on optimal harvesting strategies under the influence of uncertain circumstances.	The research has produced results indicating that incorporating disaster risk leads to the use of more conservative strategies, which ultimately lead to the occurrence of fluctuations, in a way that the supply of wood products to the market contributes to price changes, i.e. price fluctuations.
Karenlampi (2023)	The authors analyzed the financial returns on investments in the wood industry in Austria. The analysis included the impact of disturbances such as storms and pathogen/pest outbreaks.	Weather disruptions such as storms and pathogen/pest outbreaks have been shown to be significant factors in the profitability of the wood industry. According to the authors, this leads to increased volatility in wood product prices.

3.2. The impact of frequent forest fires on the stability of the wood product market

Forest fires can be of anthropogenic origin (caused by human activity) or natural origin, i.e. directly related to climate change. Flammable material in forests, dry grass and flammable mammals pose a great risk. As forest fires spread, entire forest stands can be destroyed, and thus long-term wood production is disrupted. This subsection examines how forest fires disrupt the stability of the wood market, with a focus on coniferous wood. Literature sources indicate that forest fires are one of the most important causes of instability in the timber market.

Table 2. The impact of frequent forest fires on the stability of the timber market with a focus on conifers.

Autor	Research Focus	Key Findings
Agarwal et al. (2025)	Researchers investigated the impact of wildfires, which occur as a result of climate change, on timber harvesting and the associated circumstances that occur as a consequence during timber harvesting.	It can be seen that wildfires, through their impact on fluctuations in the prices of wood assortments, cause a certain instability, that is, a disruption of the stability of the wood products market.
Shavazipour & Sundström (2024)	In this study, the authors developed a decision-support approach to wood utilization, by considering different plans or scenarios for wood utilization. What is significant is that forest fires are included in these scenarios, which can generally cause great instability in the wood products market.	As a result of their research, the authors developed an approach that allows for more robust wood utilization to be created, in order to mitigate the destabilizing effects of forest fires on the supply of wood to the market and on prices in general.

3.3. Current state of the wood products market

This subsection examines in more detail what the literature says about the current situation on the wood market. According to **Compagnia del Legno (2025)** and **Houballah et al. (2024)**, it is observed that the coronavirus pandemic has had a significant impact on the wood market. An example of this is France. In addition, sources indicate that the growth of the construction industry and the packaging sector has significantly influenced the increase in demand for wood (paper, cardboard, wooden construction materials *etc.*).

Table 3. Current trend in the wood market.

Autor	Research Focus	Key Findings
Compagnia del Legno (2025)	This report contains an analysis of the evolution of the wood market. The focus of the analysis was the forecasts and trends related to the year 2025.	The analysis showed two most important effects on the wood market. Namely, a trend of recovery in wood production was observed, and the ocean anticipated growth was driven by increased demand in the construction and packaging sectors.
Houballah et al. (2024)	This research focused on the impact of the coronavirus (COVID-19) pandemic on the forestry sector. The research focused specifically on the Grand Est region in France.	The pandemic has caused significant disruptions in the wood biomass supply chain, leading to the emergence of new avenues for wood products and industries based on bio-principles.

3.4. Current sustainability of wood production

In this subchapter, three sources of literature indicate the current state of sustainability of the timber market and timber production from the aspect of forest exploitation methods, geopolitical situation (political trends), both at the level of timber production and at the level of sawmill operation.

According to **Shavazipour & Sundström (2024)**, **Jonsson & Sotirov (2025)** and the **European Organisation of the Sawmill Industry (2024)**, it is noted that there are various factors destabilizing production, but that at the same time solutions can be sought and sustainable production of wood biomass can be maintained for the needs of the global market.

Table 4. Sustainability of wood mass production and sustainability of sawmill operations.

Autor	Research Focus	Key Findings
Shavazipour & Sundström (2024)	In research dealing with the development of a decision-support approach to timber utilization planning, the focus on the sustainability of timber production is reflected in the inclusion of forest fires as a planning factor.	The main results of the research indicating the potential to mitigate the destabilizing effects of forest fires on wood production indicate that mitigation entails better sustainability.
Jonsson & Sotirov (2025)	The research focuses on analyzing the availability of wood in the future due to the specifics of climate policy in Europe, the specific energy requirements of countries, as well as various geopolitical influences.	The research led to the results that there are ways to support sustainability, that is, to increase sustainable wood harvesting under the influence of tensions caused by the geopolitical situation, but also that there is potential for sustainable forest utilization in parallel with sustainable wood production.
European Organisation of the Sawmill Industry (EOS) (2024)	This report focused on researching the performance of sawmills in the wood industry by analyzing influential factors such as sustainability aspects in the context of sawmill operations.	The report led to results indicating that wood in Europe is a renewable resource that generates low energy, and that productivity plays a significant role in carbon sequestration and storage. In this way, it contributes to the sustainability of this sector.

3.5. The impact of forest management on wood stability and price fluctuations of wood assortments

The literature review in this subsection relies heavily on understanding the need to mitigate negative changes in forests, which occur through climate change, and which are directly or indirectly correlated with fluctuations in the prices of wood assortments. It is noted that the occurrence of harmful consequences on trees causes problems for investments in the wood industry, and that natural disasters such as storms and insect attacks have a decisive effect on the price situation of wood products on the market. The biggest problem that arises through

the attacks of pests and pathogens in forest complexes is certainly an increase in the volume of wood mass that must be cut in order to prevent further spread of the infection, and the market is thus flooded with excessive quantities of wood mass from the same tree species.

Table 5 shows the research focus and main results of **Karenlampi (2023)** and **Shavazipour & Sundström (2024)**.

Table 5. Correlation between forest management from the aspect of timber market sustainability and price fluctuations of commercial timber products.

Autor	Research Focus	Key Findings
Karenlampi (2023)	In this study, the focus of the analysis was on the financial returns on investments in the wood industry in the context of commercial timber. The aim was to see how disturbances such as storms and outbreaks of pests and pathogens affect the financial returns on investments in Austrian commercial timber.	The research has shown that the occurrence of storms and the outbreak of pests in forests significantly affect the profitability of investments in the timber industry and commercial timber because it is correlated with price fluctuations caused by the volatility caused by these natural factors, the intensity of which can be higher or lower depending on the severity of the pest attack or the extent of the damage caused by the storms.
Shavazipour & Sundström (2024).	According to the authors, the focus of this work was to create a decision-support approach for forest exploitation, which would enable sustainable harvesting planning taking into account various scenarios, including those that involve the impact of forest fires.	In order to produce sufficient quantities of commercial timber for society and the market, the model developed by these researchers can be used, which ultimately aims to mitigate the destabilizing effects on the commercial timber supply chain and price fluctuations in a way that even includes disturbances such as forest fires, which are becoming more frequent with climate change.

3.6. The impact of bark beetle infestation on price fluctuations of wood products

In the last few decades, bark beetles have become the most common topic of research when considering damage in forest complexes, because they are insects whose spread is relatively fast. Inoculums of infection are dealt with in such a way that entire groups of trees suspected of being infected must be removed, which significantly increases the presence of coniferous wood on the wood biomass market. Bark beetles cause various damages to trees, such as technical defects, and ultimately the complete drying of the tree. The entire wood production can be destroyed if an inoculum of infection appears in the forest, because the bark beetle spreads in all directions and its suppression is an urgent measure that forest engineers must urgently take.

The author **Van Veen (2020)** and the source **International Forest** describe the effects of the appearance of bark beetles in forests on fluctuations in the prices of wood products. The sources of the literature cite examples from Europe but also from the United States.

Table 6. The impact of bark beetle infestation in forests on price fluctuations of wood products.

Autor	Research Focus	Key Findings
International Forest Industries (2020)	This report can be taken into consideration in considering the impact of bark beetle attacks on fluctuations occurring in the timber market, as the focus of this analysis is on the impact of spruce bark beetle attacks on forest trees, which caused fluctuations in the timber market in Central Europe.	The results of the analysis indicate that the widespread damage caused by the bark beetle in forests has had a significant impact on the increase in salvage logging and the subsequent decrease in timber prices due to oversupply. Oversupply occurred because it was necessary to cut down a large number of trees affected by the bark beetle to prevent the infestation from spreading further, which increased the percentage of coniferous timber on the market. The direct consequences in the form of price fluctuations are the most obvious example of the destructive power of bark beetle attacks.
Van Veen (2020)	The author of the report focused on analyzing how the European spruce bark beetle and its infestation of wood affects the softwood lumber market, both in the European Union and the United States.	The European bark beetle infestation has caused an increase in the amount of damaged wood on the market, which has to be sold after felling. This has further caused a decrease in wood prices and a shift in trading patterns, so that there has been a noticeable increase in European wood exports to the Chinese market.

3.7. The ratio of price fluctuations on the wood pulp market to the overall national economy

Fluctuations in the prices of wood products on the global wood biomass market have a major impact on national economies. This is not as noticeable in countries that do not use or produce significant amounts of wood. However, countries that export wood have as a national strategic goal the sufficient production (and also the balanced production) of sufficient quantities of wood mass from commercial tree species.

According to **Fastmarkets (2022)** and **Stora Enso (2024)** sources, we can see some main indicators of the impact of wood price fluctuations on national economies.

Table 7. Correlation of wood price fluctuations with the national economy.

Autor	Research Focus	Key Findings
Stora Enso (2024)	The article in focus of the analysis discusses the financial outlook. Namely, the financial outlook of Stora Enso plays a significant role in the wood pulp industry, which makes this research important for review.	The main results of the analysis indicate that the company anticipates continued high costs and market uncertainties. All of this reflects the way pulp price volatility moves, thus influencing broader economic indicators.
Fastmarkets (2022)	This report aims to provide an outlook on the global wood pulp market. The focus is on identifying key price drivers and risks.	The article suggests that price fluctuations in the global pulp market are influenced by disruptions in the supply chain. This has wider implications for national economies that rely on the pulp and paper industry.

4.CONCLUSION

The literature review in the article indicates a strong connection between the situation on the timber market and climate change. Climate change has already begun to have an intense impact on the fluctuation of prices of timber originating from commercial tree species, which especially refers to disturbances in the supply chain of the timber market originating from infected forest stands.

Bark beetle attacks on coniferous trees cause great technical damage, while long-term devastating consequences can occur, including the complete drying out of entire forest stands. One of the most famous harmful insects is the spruce bark beetle, which continuously causes great damage in European countries.

In addition to the damage caused by attacks by harmful insects, severe storms can also cause major problems in wood production, increasing the number of trees that must be cut down.

Uncertain climate conditions significantly affect investments in the wood industry, and significantly affect the return on investment. However, although various studies and analyses indicate that modern forestry is facing numerous challenges and problems due to the intensification of the climate change process, there is research that indicates the development of a model approach to wood harvesting that can take into account factors such as the risk of storms and pest attacks, and as the ultimate goal, create an approach that enables the mitigation of harmful consequences, in order to thereby stabilize the production of wood mass, necessary for the normal functioning of the wood products market.

The current state of the wood products market shows that, although there are disruptions in the supply chain and occasional oversupply of softwood and damaged wood on the market, these products are still being sold, even at lower prices. As a novelty, more frequent sales of wood to the Chinese market have emerged, which may eventually open up certain opportunities to see how these exports can be improved.

It is noticeable that the management of forestry companies now bears a great responsibility for forest management, because fluctuations in the prices of wood products on the global market greatly affect the national economies of countries in which wood is an important product.

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***Berberis thunbergii* DC. SUPPRESSES ANTIOXIDANT ENZYME GENES AND INCREASES REACTIVE OXYGEN STRESS, THEREBY CAUSING APOPTOSIS IN THE HeLa CERVICAL CANCER CELL LINE**

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ABSTRACT

The *Berberis thunbergii* DC., part of the Berberidaceae family, is a pharmacological plant commonly used in treating various diseases. It is recognized for its antidiabetic, antibacterial, antioxidant, antifungal, antiviral, and anticancer properties. In this study, *Berberis thunbergii* DC. leaf extract was applied to the HeLa cell line with a range of 0.077 to 1 mg/mL. The cells treated with 0.077 mg/mL of extract showed a viability of $89.79 \pm 1.13\%$ and $72.52 \pm 1.89\%$ at 24-hour and 48-hour durations, respectively. Viability was $69.14 \pm 2.18\%$ and $34.65 \pm 1.92\%$ when treated with 1 mg/mL. At a concentration of 0.5 mg/mL for 48 hours, 49.61% viability was detected. By applying 0.5 mg/mL of extract to the HeLa cells, expressions of *CytC1* (1.8 ± 0.36 -fold), *CASP3* (3.16 ± 0.26 -fold), and *CASP9* (1.62 ± 0.13 -fold) genes increased, while expressions of survivin (0.2 ± 0.05 -fold), *Bcl-2* (0.1 ± 0.016 -fold), *c-IAP1* (0.15 ± 0.02), *XIAP* (0.25 ± 0.08 -fold), *CAT* (0.09 ± 0.04 -fold), and *Mn-SOD* (0.1 ± 0.05 -fold) genes decreased. Flow cytometry analysis revealed 40.98% apoptotic and 72.99% ROS-affected cells. Changes in membrane potential and apoptotic nuclei were observed. The data indicate that the leaf extract of *Berberis thunbergii* DC induces reactive oxygen stress in HeLa cells and leads to apoptosis. Consequently, it may be beneficial in cancer treatment, particularly for cervical cancer.

Keywords: Apoptosis, *Berberis thunbergii* DC, HeLa, reactive oxygen species

INTRODUCTION

Berberine (Brb) is a phytochemical found in various medicinal Chinese plants, including *Berberis aristata*, *Berberis vulgaris*, *Berberis petiolaris*, *Berberis aquifolium*, *Berberis asiatica*, *Berberis thunbergii* DC. (*B. thunbergii*), *Coptis teeta*, *Coptis chinensis* (commonly known as goldenthread), *Hydrastis canadensis* (known as goldenseal), *Phellodendron amurense*, and *Caulis mahoniae*. It is an isoquinoline alkaloid with a range of therapeutic properties, such as antiviral, antimicrobial, antidiarrheal, anti-inflammatory, and antitumor activities (Mirhadia et al., 2018). The anticancer efficacy of Brb is delineated into four phases: reduction of proliferation, induction of apoptosis, inhibition of angiogenesis, and suppression of metastasis (Mirhadia et al., 2018). In a study conducted on the HT29 colon cancer cell line, the IC₅₀ dose of berberine was calculated to be 52.37 ± 3.45 μ M. This study shows that HeLa cells are arrested in the G2/M phase after 24 h of berberine treatment, leading to apoptosis (Li et al., 2023). In another study conducted on the hepatocellular carcinoma cell line HepG2, it was shown that the expression levels of SP1, which is responsible for cell differentiation and growth, and CCND1 and BCL-2, which are responsible for regulating the cell cycle and proliferation, were suppressed by microRNA-22-3p (miR-22-3p) following the application of 100 μ M berberine for 24 hours, leading to cell apoptosis (Chen et al., 2016). Liu et al.

demonstrated that BBR suppresses senescent human glioma cells by downregulating EGFR-MEK-ERK signaling (Liu et al., 2015). Wang et al. demonstrated that BBR suppresses liver cancer cells by triggering apoptosis and autophagic cell death (Wang et al., 2010). Chen et al. demonstrated that an average dose of 80 μ M berberine, administered for 48 hours (with specific doses of 58.33 ± 4.96 for A549 cells and 49.00 ± 2.87 for PC9 cells), increased the expression level of miR-19a in A549, PC9, H460, H1299, Beas-2b, and 293T lung cancer cells. In contrast, the expression levels of tissue factor (TF) and MAPK were suppressed, leading to apoptosis in these cells (Chen et al., 2019). It is possible to find many studies in the literature on the anti-cancer effects of berberine on various types of cancer (Chi et al., 1994; Chen et al., 2020; Cheng and Ji, 2020; Chiu et al., 2021).

Despite its anti-cancer properties, some studies indicate that berberine may also be toxic to healthy cells and tissues (Hu et al., 2014). Brb interacts with nuclear DNA and produces breaks in the DNA double helix in a dose-dependent manner (Yuan et al., 2015). Furthermore, Brb modulates gene function via interacting with TATA boxes and the polyadenylated tails of mRNA (Liu et al., 2008). In contrast, Och et al. investigated the toxic effects of an extract from the berberine-rich *B. thunbergii* on both hematopoietic cancer cells and healthy hematopoietic cells, finding that *Berberis thunbergii* had a similar cytotoxic effect on cancer cells as berberine, while its toxic effect on healthy cells was much lower compared to berberine (Och et al., 2019).

Based on these data, this study investigated the toxic effects of the extract derived from the leaves of *B. thunbergii* on HeLa cells, which are a cervical cancer cell line, as well as the molecular mechanisms underlying these toxic effects.

MATERIALS AND METHODS

The *Berberis thunbergii* DC. Collected at the Balkan Campus of Trakya University and identified by the EDTU herbarium (herbarium number: EDTU-29958).

After harvesting from the Balkan Campus of Trakya University, the leaves of *B. thunbergii* were initially cut into small pieces with a scalpel. The leaf pieces were then crushed using a tissue lyser device (Labtron HZ1-A, Korea) to achieve a concentration of 1 mg/mL in MEM Eagle (EMEM, Multicell) culture medium, resulting in a homogeneous mixture. To eliminate any remaining particles, the mixture was filtered through a 0.2 μ m syringe filter (Sartorius, Germany) before being applied to HeLa cell lines. Fresh extracts were prepared at each stage of the study. The *B. thunbergii* leaves were stored at -80°C until needed for any part of the research process.

The human cervical cancer HeLa (ATCC® CCL-2) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). For cell passaging, a culture medium was prepared consisting of DMEM, EMEM, and Ham's F-12 in a 1:1:1 ratio (Wiesent Multicell, Canada), supplemented with 5% fetal bovine serum (FBS, Capricorn, Germany) and 1% L-Glutamine (Thermo-Fisher, USA). Against to bacterial contamination, the medium was further supplemented with 100 IU/mL penicillin-streptomycin (Pen-Strep; Thermo-Fisher) and 100 $\times$ antibiotic-antimycotic (Gibco, USA). The cells were then seeded into 75 cm² flasks (NEST, China) containing 10 ml of the prepared medium and incubated in a Hera Cell CO₂ incubator at 37°C with 5% CO₂. The cells were monitored and reached 97% confluency before being passaged every 48 hours.

MTT assays were performed to determine the effective cytotoxic dose of *B. thunbergii* extract on HeLa cell line. Cells were seeded separately into transparent 96-well plates at a volume of 180 μ L per well, with a density of 5×10^3 cells per well. Extracts from *B. thunbergii* leaves were prepared in EMEM medium at serial dilutions of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.62 mg/mL, 0.31 mg/mL, 0.155 mg/mL, and 0.077 mg/mL. Each concentration was applied in volumes of 20 μ L, with 5 replicates, to both cell lines for 24 and

48 h. Cells without treatment served as controls for comparison with treatment groups. At the end of the incubation periods, 20 μ L of MTT solution (Biomatik; A3338-5GM, Canada) was added to each well and incubated for 3 h. The MTT solution was prepared by dissolving 0.5 mg MTT in 1 mL of 1 $\times$ phosphate-buffered saline (PBS, Wiesent Multicell). After 3 hours, the liquid phase from each well was removed, and 100 μ L of dimethyl sulfoxide (DMSO) was added. The cell viability was determined by measuring the absorbance at 570 nm using a spectrophotometer (Thermo Scientific Multiskan GO, USA). The untreated control cells were considered 100% viable.

HeLa cells were seeded into 25 cm² culture flasks at a density of 1 $\times$ 10⁵ cells per flask, with 3 replicates for extract treatment and 3 replicates as controls. After the treatment period, total RNA was isolated separately from each flask using the Column Pure RNA Miniprep kit (ABM Good, Canada) according to the manufacturer's protocol. Following equalization of RNA quantities from each flask, cDNA synthesis was performed using the OneScript Plus cDNA Synthesis kit (ABM Good) according to the manufacturer's instructions. The cDNA synthesis was carried out in a Thermal cycler (Veriti Thermal Cycler, Applied Biosystems, USA) with the following steps: Step 1: 10 min at 25°C, Step 2: 120 min at 37°C, Step 3: 5 minutes at 85°C. The cDNA samples were stored at -20°C until further RT-PCR analysis.

Changes in the expression levels of mitochondrial apoptosis genes *Survivin* (*BIRC5*), *Bcl-2*, *c-IAP1* (*BIRC2*), *XIAP* anti-apoptotic, *CAT*, *SOD2* (*Mn-SOD*) antioxidants, cytochrome C (*CytC*), caspase-3 (*Casp3*), and caspase-9 (*Casp9*) genes expression levels were analyzed in HeLa cells following leaf extract treatment compared to untreated control cell lines. Quantification was performed using the QuantStudio 6 Flex qRT-PCR system (Applied Biosystems). The PCR conditions and primer sequences used are provided in Table 1.

Table 1. Primer sequences for genes and qRT-PCR parameters employed in this study.

Gene Codes	Primer Base Sequences	Real Time PCR Conditions
<i>Gapdh</i>	F: 5'-CAATGCCTCCTGCACCACCA-3' R: 5'-GATGTTCTGGAGAGCCCCGC-3'	Hold Stage: 1 Cycle 50°C 2 minute 95°C 10 minute
<i>Survivin</i>	F: 5'-GACGACCCCATAGAGGAACA-3' R: 5'-GACAGAAAGGAAAGCGCAAC-3'	
<i>Bcl-2</i>	F: 5'-ATGTGTGTGGAGAGCGTCAA-3' R: 5'-ACAGTTCCACAAAGGCATCC-3'	
<i>c-IAP1</i>	F: 5'-GCATTTTCCCAACTGTCCAT-3' R: 5'-ATTCGAGCTGCATGTGTCTG-3'	
<i>XIAP</i>	F: 5'-GGGGTTTCAGTTTCAAGGAC-3' R: 5'-TGCAACCAGAACCTCAAGTG-3'	PCR Stage: 40 Cycle 95°C 15 second 60°C 1 minute
<i>CAT</i>	F: 5'-TACGAGCAGGCCAAGAAGTT-3' R: 5'-ACCTTGACGGGCAGTTCAC-3'	
<i>Mn-SOD</i>	F: 5'-TCTGAAGAAGGCCATCGAGT-3' R: 5'-GCAGATAGTAGGCGTGCTCC-3'	Melt Curve Stage: 1 Cycle 95°C 15 second 60°C 1 minute 95°C 15 second
<i>CytC</i>	F: 5'-AGTGGCTAGAGTGGTCATTTCATTAC-3' R: 5'-TCATGATCTGAATTCTGGTGTATGAG-3'	
<i>Casp3</i>	F: 5'-GGTATTGAGACAGACAGTGG-3' R: 5'-CATGGGATCTGTTTCTTTGC-3'	
<i>Casp9</i>	F: 5'-TGTCTACGGCACAGATGGA-3' R: 5'-GGACTCGTCTTCAGGGGA-3'	

HeLa cells were seeded into 6-well transparent plates at a density of 1 $\times$ 10⁵ cells per well, with three replicates for the control group and three replicates for the leaf extract treatment. After treatment, the number of ROS-affected HeLa cells was compared to the control cells using the Reactive Oxygen Species (ROS) Fluorometric Assay Kit (Elabscience, E-BC-K138-F, USA), following the manufacturer's instructions and employing flow cytometry

(Applied Biosystems, Attune acoustic focusing cytometer, USA). The population of apoptotic cells was assessed using the Annexin V-FITC/PI Apoptosis Kit (Elabscience, E-CK-A211), in accordance with the manufacturer's instructions, also utilizing the flow cytometer.

HeLa cells were seeded into 6-well transparent plates (NEST) at 1×10^5 cells per well. To demonstrate changes in cell membrane structures due to increased ROS levels in both control and treated cells, the Mitochondrial Membrane Potential Assay Kit (Abcam, ab113850 JC-1, England) was used according to the manufacturer's instructions. At the end of the treatment, to prevent false positive results from the JC-1 reagent included in the kit, all liquid phases from the wells were completely removed. Cells were then washed twice with $1 \times$ PBS and visualized under a fluorescence microscope (Zeiss Observer Z1, Japan) using FITC-RED channels with a $20\times$ objective.

Following the same application, changes in nuclear structures due to apoptosis were visualized using Hoechst 34580 dye (Cayman Chemical, USA) with fluorescent labeling. Both treatment and control cell groups were seeded into 6-well transparent plates with 1×10^5 cells per well. After the treatment period, all medium from the wells was removed. Then, 500 μ L of a solution containing 25 mg of Hoechst 34580 dissolved in 10 mL of $1 \times$ PBS was added to each well to cover the cells. The plate was incubated at room temperature in the dark for 20 minutes. To prevent false positive signals, all liquid was removed from the wells at the end of the incubation period, and the cells were washed twice with $1 \times$ PBS. Imaging was conducted using a fluorescence microscope with a DAPI filter under a $20\times$ objective.

MTT cell cytotoxicity analyses were performed, where cells not treated with *B. thunbergii* leaf extract were considered 100% viable. Based on this, changes in cell viability due to the application of extract in doses ranging from 1 mg/mL to 0.077 mg/mL on HeLa cells were calculated using the formula below:

Cell viability (%) = (absorbance value of the releated dose of the extract applied to the wells / absorbance value of the control wells) $\times$ 100.

Changes in gene expression due to extract treatment were calculated using the $2^{-\Delta\Delta CT}$ formula and normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Both RT-PCR gene expression levels and changes in ROS-positive and apoptotic cell numbers in HeLa cells, relative to control groups, following extract treatment were analyzed using SPSS Paired Samples T Test (IBM SPSS Statistics 22). Results are presented as the mean and standard error of the mean (mean $\pm$ SD). * $p < 0.05$. ns indicates no significant difference between to control and application groups.

RESULTS AND DISCUSSION

The HeLa cell line treated with 0.077 mg/mL of leaf extract exhibited a viability of $89.79 \pm 1.13\%$ after 24 h and $72.52 \pm 1.89\%$ after 48 h. In contrast, treatment with 1 mg/mL resulted in a viability decrease to $69.14 \pm 2.18\%$ at 24 h and $34.65 \pm 1.92\%$ at 48 h. After a 48 h treatment with 0.5 mg/mL of leaf extract, the viability was measured at $49.61 \pm 2.09\%$, indicating that this concentration represents the IC_{50} value for HeLa cells (Figure 1).

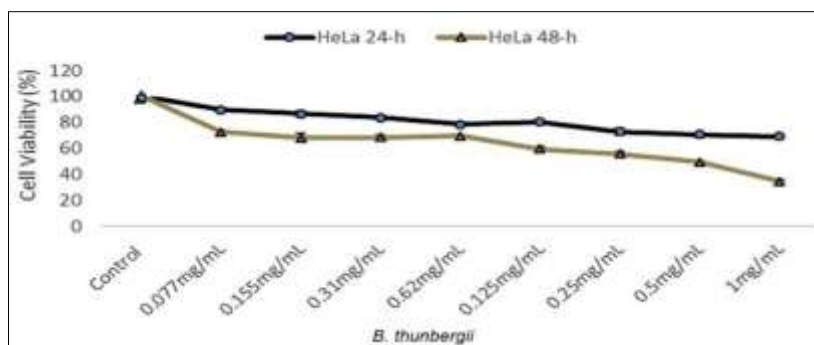


Figure 1. Changes in cell viability in HeLa cells following the application of *B. thunbergii* leaf extract at concentrations ranging from 0.077 mg/mL to 1 mg/mL for 24 h and 48 h.

Following the application of a 0.5 mg/mL extract to HeLa cells, the expression levels of the CytC (1.8 ± 0.36 -fold), Casp3 (3.16 ± 0.26 -fold), and Casp9 (1.62 ± 0.13 -fold) genes increased. In contrast, significant decreases were observed in the expression levels of the Survivin (0.2 ± 0.05 -fold), Bcl-2 (0.1 ± 0.016 -fold), c-IAP1 (0.15 ± 0.02), XIAP (0.25 ± 0.08 -fold), CAT (0.09 ± 0.04 -fold), and Mn-SOD (0.1 ± 0.05 -fold) genes (Figure 2).

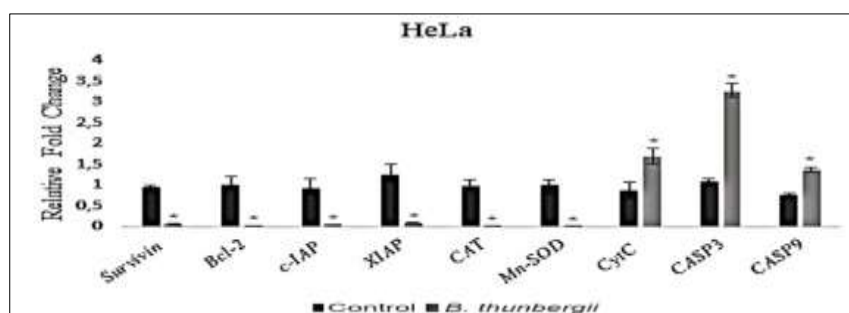


Figure 2. Changes in gene expression levels relative to the control following the application of *B. thunbergii* extract to the HeLa cell line (* $p < 0.05$, $n = 3 \pm SD$).

The untreated HeLa cell line showed an apoptotic cell rate of $3.73 \pm 0.42\%$. In contrast, this rate increased to $40.98 \pm 3.48\%$ following treatment with *B. thunbergii* extract (Figure 3a). Additionally, the percentage of ROS-positive cells was $0.97 \pm 0.86\%$ in untreated cells, while it rose significantly to $72.99 \pm 3.91\%$ after treatment with *B. thunbergii* extract (Figure 3b).

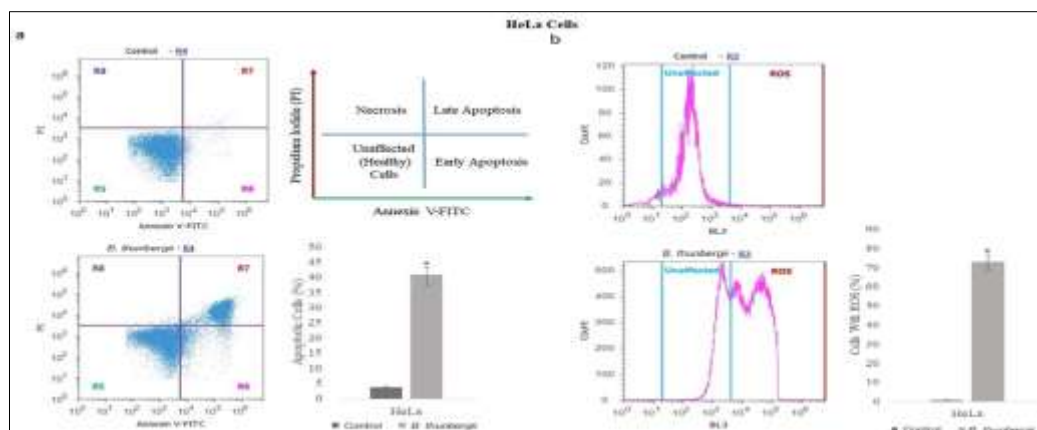


Figure 3. The changes in the HeLa cell line for both control and treatment with *B. thunbergii* extract, specifically showing a) the number of apoptotic cells and b) the number of ROS-positive cells (* $p < 0.05$, $n = 3 \pm SD$).

According to the controls, the application of *B. thunbergii* was performed on HeLa cells, which showed increased mitochondrial membrane permeability (Figure 4a) and cells with apoptotic nuclear structure (Figure 4b), as demonstrated by fluorescence microscopy.

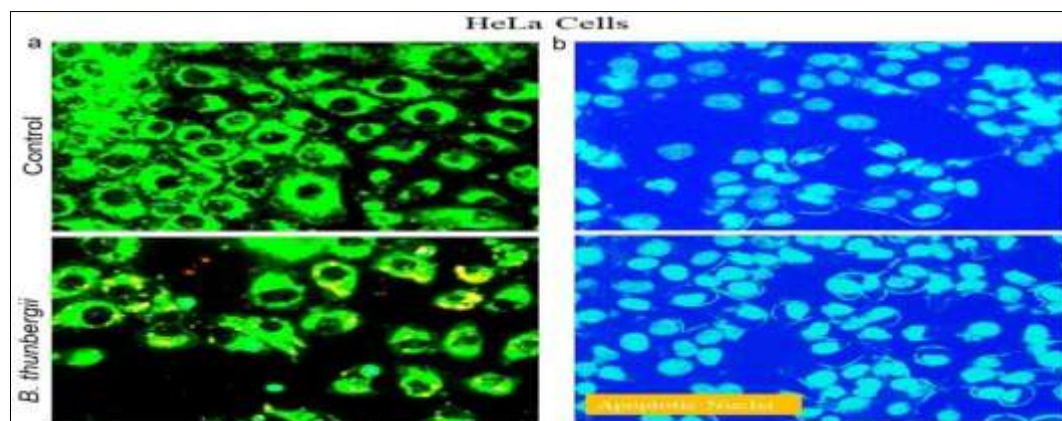


Figure 4. a) HeLa cells treated with *B. thunbergii* show increased mitochondrial membrane permeability compared to the control group, and b) apoptotic nuclear structures are visualized using fluorescence microscopy at 20x magnification.

In this study, the cytotoxic, pro-oxidant, and pro-apoptotic effects of *Berberis thunbergii* DC. leaf extract on HeLa cervical cancer cells were investigated comprehensively. The findings indicate that the extract induced dose- and time-dependent cytotoxicity in HeLa cells, dramatically increased intracellular oxidative stress, and activated intrinsic apoptotic pathways.

The MTT results showed that the extract reached an IC_{50} value for HeLa cells, leading to a reduction in viability of nearly 50% after 48 h of application at a dose of 0.5 mg/mL. This value suggests that the 145.5 $\mu\text{g/mL}$ dose (Och et al., 2019), which previously showed low toxicity in hematopoietic cancer cells, may be ineffective for HeLa cells. Therefore, it can be said that *B. thunbergii* extract may exhibit a stronger or different mechanism of action in solid tumor cells such as cervical cancer.

The significant suppression of CAT and Mn-SOD genes, which are part of the antioxidant defense system, in cells treated with the extract is consistent with the approximately 73-fold increase in ROS levels in the same cells. Increased reactive oxygen species resulting from the suppression of antioxidant enzymes disrupted mitochondrial membrane integrity and led to a loss of mitochondrial membrane potential, as demonstrated by JC-1 staining. It is known that the increase in ROS is one of the main triggers that direct cancer cells to apoptosis [Won et al., 2010; Jomova et al., 2024]. These findings suggest that ROS-induced mitochondrial stress is central to the anticancer activity of *B. thunbergii*.

Molecular analyzes strongly support the extract's activation of the intrinsic apoptosis pathway. The observed increase in the gene expression of CytC, Casp9, and Casp3 indicates that the integrity of the mitochondrial membrane is disrupted, leading to the activation of cytochrome c release and the involvement of executioner caspases (Bratton et al., 2001; Won et al., 2010). Additionally, the significant reduction in apoptosis-suppressing genes such as survivin, Bcl-2, c-IAP1, and XIAP suggests that the extract also silenced the extract's anti-apoptotic mechanisms (Deveraux and Reed, 1999). Suppression of anti-apoptotic proteins facilitates caspase activation to irreversible levels and accelerates cell death.

The approximately 41% apoptotic cell rate determined by flow cytometry was found to be consistent with both molecular gene expression data and the observed disruptions in nuclear morphology. These results indicate that the cytotoxic effect of the extract occurs thru programed cell death, rather than necrosis.

In the literature, the antiproliferative and pro-apoptotic effects of berberine in various types of cancer have been widely reported (Chi et al., 1994; Liu et al., 2015; Chen et al., 2019; Chiu et al., 2021). However, it is also known that berberine can cause toxicity in healthy cells (Liu et al., 2008; Hu et al., 2014; Yuan et al., 2015). From this perspective, the fact that *B. thunbergii* extract contains a complex phytochemical structure and has been reported to show lower toxicity in healthy cells (Och et al., 2019) suggests that the plant could be a safer natural anticancer agent than berberine. The findings of this study suggest that phenolic compounds other than berberine found in the leaf extract may also contribute to anticancer activity.

When the results are considered as a whole, it appears that *B. thunbergii* leaf extract possesses potent anticancer properties in HeLa cells thru ROS increase, mitochondrial damage, and activation of the intrinsic apoptotic pathway.

CONCLUSION

This study found that *Berberis thunbergii* DC. leaf extract exhibits significant cytotoxic and pro-apoptotic properties in HeLa cervical cancer cells. The extract reduces cell viability in a dose- and time-dependent manner, increases ROS levels by inhibiting antioxidant enzyme genes, disrupts mitochondrial membrane potential, lowers anti-apoptotic gene expression, and activates intrinsic apoptotic pathways. These effects culminated in notable apoptosis in HeLa cells, suggesting *B. thunbergii* leaf extract may serve as a potential complementary anticancer agent for cervical cancer. Future research should assess its impact on healthy cells, isolate active components, and validate findings in vivo to better understand its therapeutic potential.

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PERFORMANCE AND EMISSION CHARACTERISTICS OF A SINGLE-CYLINDER DIESEL ENGINE FUELED WITH A 10% OLIVE POMACE OIL–DIESEL BLEND

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ABSTRACT

This study investigates the effects of using a 10% olive pomace oil–diesel blend on the performance and emission characteristics of a single-cylinder diesel engine under varying load conditions. The blend was prepared by volumetrically mixing 10% olive pomace oil with standard diesel fuel. The engine was tested under four different load conditions: 25%, 50%, 75%, and 100% of full load capacity. The experimental results revealed that the engine power output decreased by a maximum of only 0.1 kW across all load conditions when compared to standard diesel. This minor reduction indicates that the blend maintains comparable engine performance. Fuel consumption increased by a maximum of 16 ml/h, which is a relatively small difference and within acceptable limits for alternative fuel use. In terms of emissions, carbon monoxide (CO) levels decreased slightly—by approximately 0.01%—across all load conditions, suggesting improved combustion efficiency. However, the reduction in CO was minimal. On the other hand, nitrogen oxides (NO_x) emissions showed an increase, particularly at higher loads. A maximum increase of 160 ppm was recorded at 75% load, while other conditions showed moderate increases ranging from 55 to 65 ppm. Despite the slight rise in NO_x emissions, the findings suggest that blending olive pomace oil with diesel at a 10% volumetric ratio can be a viable and sustainable alternative fuel option for diesel engines, with minimal impact on performance and moderate changes in emissions.

Keywords: Energy, Olive pomace oil, Engine performance, Exhaust emissions

INTRODUCTION

The depletion of fossil fuel reserves and growing environmental concerns have increasingly driven interest toward renewable and sustainable energy sources. In this context, biodiesel fuels are extensively investigated as alternative fuels for diesel engines due to their renewability and eco-friendly properties. Particularly, the use of agricultural waste oils, such as olive pomace oil, for biodiesel production offers significant economic and ecological advantages [1]. Although biodiesel is one of the most widely researched biofuels for diesel engines, it is commonly produced from edible vegetable oils, which raises concerns over the competition between food and energy production. This situation has led to debates on whether

agricultural land should be used for energy crops or food cultivation. In this regard, waste oils that do not compete directly with the food sector, such as olive pomace oil, have emerged as promising alternative raw materials for biodiesel production. Therefore, olive pomace, a by-product of olive oil processing, is increasingly being considered a valuable green energy source for biodiesel production [2]. In the past, these wastes received limited attention due to their negative environmental impacts, high disposal costs, and the geographical dispersion of olive oil mills [3–4]. A review of the literature shows that olive pomace methyl ester has been tested both in pure form and in various blends with diesel fuel in a Perkins direct injection diesel engine, and the results were compared with conventional fossil diesel. The tests were conducted under full load and at various engine speeds. For each fuel type, detailed performance characteristics were analyzed. In the exergy analyses, the operating conditions where the engine delivered maximum power were considered for each fuel. The results showed that all fuel types exhibited similar performance characteristics. When pure biodiesel was used, engine power decreased by approximately 5.6%, while fuel consumption increased by up to 7% [5]. Another study on olive pomace biodiesel not only examined exhaust emissions but also proposed a methodology based on multi-response optimization. Using blends of olive pomace and diesel, the variation in engine characteristics was investigated under three different load conditions (50%, 75%, and 100%). The study found a direct correlation between emissions and engine load [6]. In a different study, olive pomace waste, generated after olive oil production, was subjected to transesterification to obtain olive pomace methyl ester. This biodiesel was blended with standard diesel at volumetric ratios of 5% (B5), 7% (B7), and 10% (B10), and tested for power, torque, and specific fuel consumption in comparison to pure diesel. According to the experimental data, the B5 blend yielded the most favorable results, providing values closest to those of conventional diesel in terms of power, torque, and fuel consumption. It was also observed that increasing the blend ratio beyond 7% negatively affected the engine's performance characteristics [7]. Although there are several studies focusing on the use of various biofuels in internal combustion engines, most remain limited to the evaluation of basic engine parameters when comparing biodiesel with diesel fuel [8–10]. In another study, biodiesel obtained from waste olive oil through transesterification was blended volumetrically at 30% with conventional diesel and tested in a single-cylinder direct injection diesel engine. The study investigated the effects of this blend on engine performance and emissions. Results indicated that the thermal efficiency of biodiesel was 1% to 5% lower than that of diesel. However, carbon monoxide (CO) emissions decreased by 37.5%, while carbon dioxide (CO₂) and nitrogen oxides (NO_x) emissions increased compared to diesel fuel [11].

In our study, the performance and emission characteristics of diesel fuel blended with 10% olive pomace oil were comprehensively examined using a single-cylinder diesel engine. The results indicated that the biodiesel blend led to a slight reduction in engine power, minor increases in fuel consumption, a decrease in CO emissions, and load-dependent increases in NO_x emissions. These findings highlight the potential of olive pomace oil-based biodiesel as a sustainable alternative fuel for diesel engines and emphasize the importance of emission control strategies during biodiesel utilization.

MATERIALS AND METHODS

In this study, the experimental investigations were carried out on a single-cylinder, four-stroke diesel engine with a power output of 11.5 kW, located at the R&D center. A general view of the test engine is presented in Figure 1, while its technical specifications are detailed in Table 1. Two types of test fuels were prepared in separate stages. The first test fuel was 100% conventional diesel, which is widely used in the country. The second test fuel was prepared by

blending 90% conventional diesel with 10% biodiesel derived from olive pomace oil. Olive pomace oil, obtained from olive processing residues, is considered a waste biomass source and is suitable for evaluation in alternative energy production. Before initiating the experiments, the engine was run without load until it reached its steady-state operating temperature (82°C) as specified by the manufacturer. Once the target temperature was reached, the first test commenced using 100% diesel fuel. At this stage, the engine was subjected to incremental loads of 25%, 50%, 75%, and 100%, during which the engine output power, fuel consumption, and exhaust gas emissions were measured. Subsequently, the same experimental procedure was repeated using the biodiesel blend containing 90% diesel and 10% olive pomace biodiesel. The same parameters were recorded at each load level, and all data were meticulously logged. In addition, exhaust gas emissions were monitored at regular intervals simultaneously with engine performance tests. Upon completion of all experiments, the collected data were used to generate comparative graphs illustrating the changes in engine power, specific fuel consumption, and emission characteristics, and detailed evaluations were conducted accordingly.



Figure 1. Experimental Diesel Engine Used in the Study

Table 1: Technical Specifications of The Test Engine

General Characteristics	Technical Specifications
Engine type	Erin Motor Base Model
Number of Valves	4
Continuous Power (kw/rpm)	11.5 / 1500
Bore (mm)	108
Stroke (mm)	127
Combustion System	Direct Injection
Compression Ratio	14.6 : 1
Engine Cooling	Water
Weight (kg)	157

RESULTS AND DISCUSSION

The experimental data obtained from this study reveal the effects of a 10% olive pomace oil-based biodiesel blend on engine performance and emission parameters. As seen in the engine power output graphs, the slight reduction in power observed with the biodiesel blend indicates that there is no significant change in the operational characteristics of the engine. This is an important finding that supports the viability of olive pomace oil as an additive to diesel fuel. Fuel consumption graphs show a slight increase in consumption when using the biodiesel blend. This increase may be attributed to the lower energy density of biodiesel compared to standard diesel fuel, and is consistent with similar findings reported in the literature. In terms of emission analysis, the reduction in carbon monoxide (CO) levels suggests that the combustion efficiency was partially improved with the addition of biodiesel.

On the other hand, a notable increase in nitrogen oxides (NO_x) emissions was observed, particularly at higher engine loads. This indicates that biodiesel may raise combustion temperatures, which in turn promotes NO_x formation. The trends presented in the graphs highlight both the performance and environmental potential of olive pomace-based biodiesel, while also emphasizing the need to evaluate additional emission control techniques, especially to mitigate NO_x emissions. Overall, the results demonstrate that biodiesel can be considered a sustainable alternative fuel, and future studies are recommended to focus on emission balancing strategies to optimize both environmental and performance outcomes.

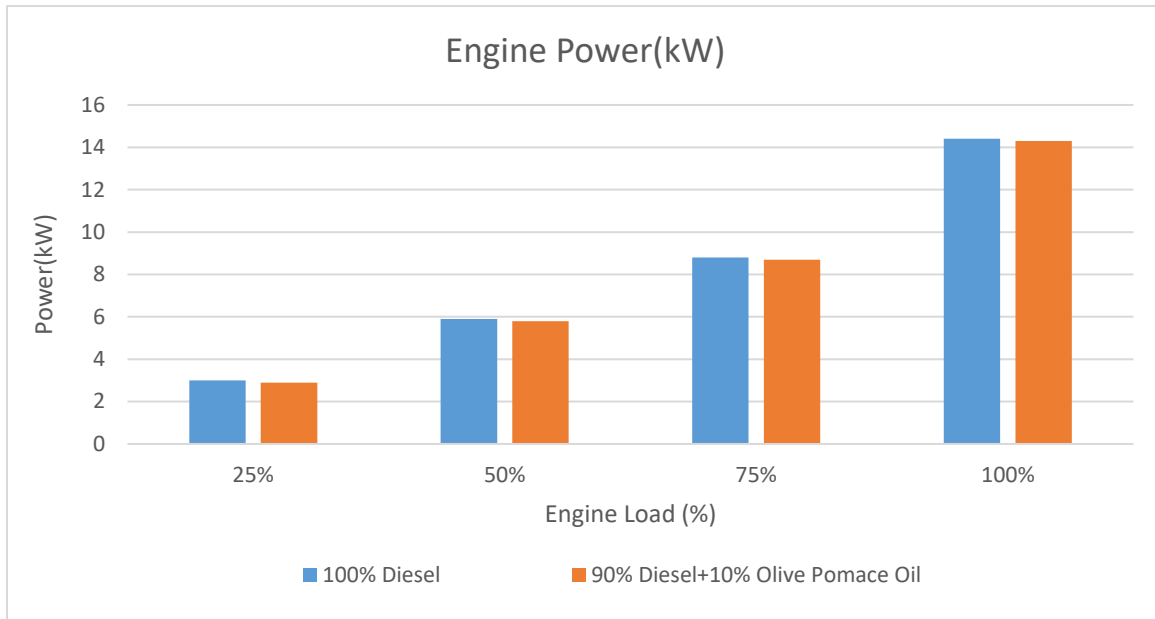


Figure 2. Comparison of Engine Power Output for Different Fuel Types

As shown in Figure 2, a consistent power reduction of approximately 0.1 kW was observed at all engine load levels when using the biodiesel blend. This slight decrease can be attributed to the lower energy density of biodiesel compared to standard diesel fuel. However, the minimal loss in power indicates that the biodiesel blend largely preserves engine performance and that the addition of olive pomace oil does not cause any significant negative impact on engine operating efficiency. This finding serves as an important indication of the practical applicability of biodiesel in diesel engines.

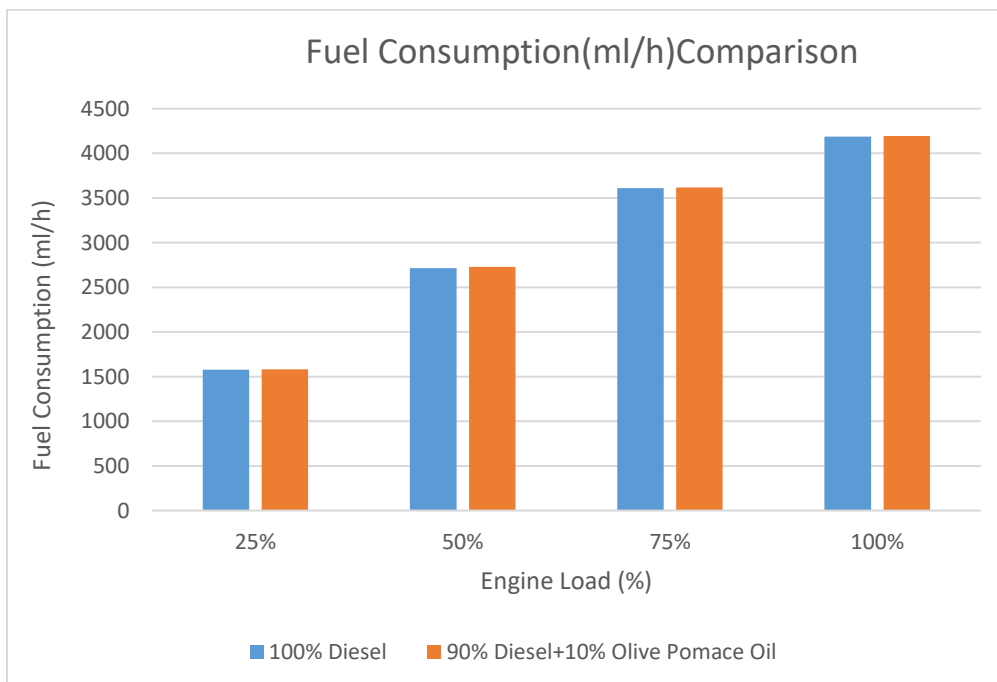


Figure 3. Engine Fuel Consumption for Various Fuel Types

As illustrated in Figure 3, slight increases in fuel consumption were observed at various engine load levels when using the biodiesel blend. Depending on the applied load, the increase in fuel consumption ranged from a minimum of 2 ml to a maximum of 16 ml. This indicates that the biodiesel blend causes a limited and load-proportional increase in fuel consumption under different operating conditions.

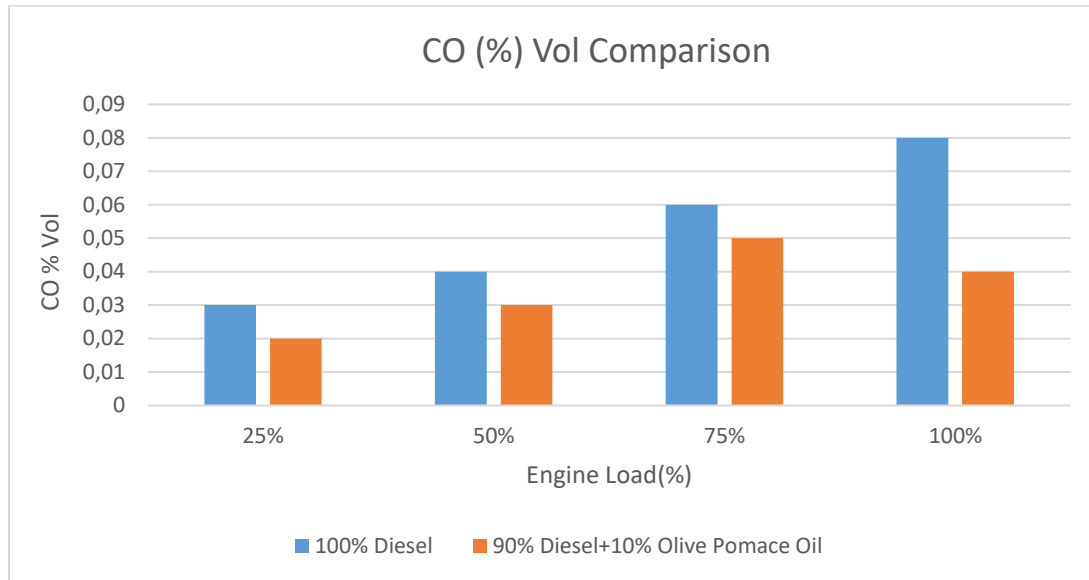


Figure 4. Carbon Monoxide (CO) Emission Levels for Different Fuel Types

As shown in Figure 4, the effect of biodiesel usage on carbon monoxide (CO) emissions was examined, and a very slight reduction of approximately 0.01% in CO levels was observed under 25%, 50%, and 75% load conditions. This minor decrease may be attributed to the cleaner combustion characteristics of biodiesel, which promote more complete fuel burning. Interestingly, under 100% load, a more significant reduction of around 4% in CO emissions was recorded. This suggests that biodiesel enhances combustion efficiency more noticeably at higher loads. Overall, the addition of biodiesel improves the environmental performance of the engine by reducing CO emissions, demonstrating its environmental benefits as an alternative fuel.

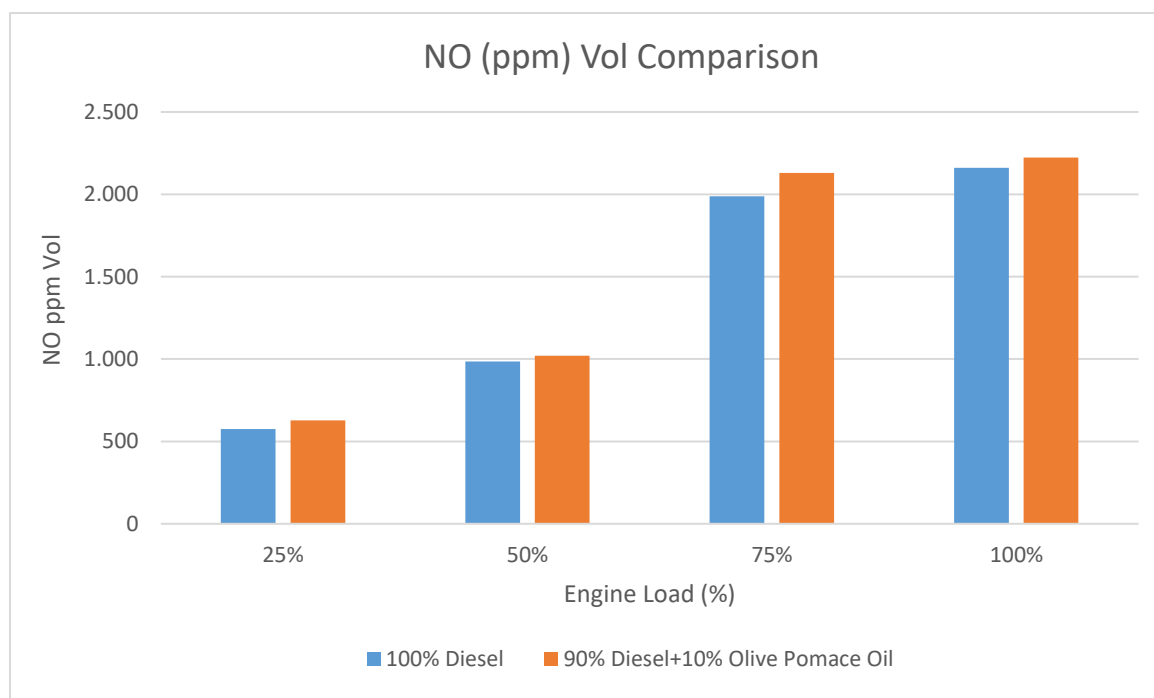


Figure 5. Nitrogen Oxide (NO_x) Emissions for Different Fuel Types

As seen in Figure 5, the use of biodiesel under varying engine load levels resulted in increases in nitrogen oxides (NO_x) emissions, ranging from a minimum of 36 ppm to a maximum of 142 ppm. These increases can be explained by the fact that biodiesel tends to raise combustion temperatures, which in turn promotes NO_x formation. The notable rise in NO_x levels, particularly under high load conditions, is a critical issue that must be carefully considered when assessing the environmental impact of biodiesel use. Therefore, additional measures or technological improvements are required to effectively control NO_x emissions in engines operating on biodiesel.

CONCLUSION

In this study, the effects of a diesel–biodiesel blend containing 10% olive pomace oil on the performance and emission characteristics of a single-cylinder diesel engine were investigated. The test results indicated that the blend caused only a very limited decrease of 0.1 kW in engine power, and a maximum increase of 16 ml/h in fuel consumption. These changes demonstrate that the overall engine performance was largely preserved. Emission analysis showed an approximately 0.01% reduction in carbon monoxide (CO) emissions, indicating a slight improvement in combustion quality. However, a notable increase in nitrogen oxides (NO_x) emissions was observed, especially under higher load conditions (particularly at 75% load), reaching up to 160 ppm.

In conclusion, the biodiesel blend containing 10% olive pomace oil can be considered a technically viable alternative fuel for diesel engines. Nevertheless, the observed increase in NO_x emissions highlights the need for implementing appropriate emission control strategies when using such biofuels.

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IMPACT OF A 5% SOYBEAN OIL AND 5% LINSEED OIL BLEND ON THE PERFORMANCE AND EMISSION CHARACTERISTICS OF A DIESEL ENGINE

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ABSTRACT

In this study, the effects of a biodiesel blend composed of 90% standard diesel fuel and a volumetric mixture of 5% soybean oil and 5% linseed oil on engine performance and exhaust emissions were experimentally investigated in a single-cylinder diesel engine. The engine was tested under load conditions of 25%, 50%, and 75%, which are commonly encountered in industrial applications. The results obtained were compared with those of commercially available standard diesel fuel in Turkey. Experimental results showed no significant change in engine power across all load conditions, indicating that the addition of 10% biodiesel did not affect engine power output. Regarding fuel consumption, no difference was observed between the biodiesel blend and standard diesel at 25% load, whereas increases of 65 ml/h and 105 ml/h were recorded at 50% and 75% loads, respectively. This suggests that the biodiesel blend leads to higher fuel consumption, particularly at higher engine loads. In terms of exhaust emissions, carbon monoxide (CO) levels remained unchanged at 25% and 50% loads, while a slight increase of 0.03% was observed at 75% load with the biodiesel blend. Nitrogen oxide (NO_x) emissions increased across all load levels.

The findings indicate that the biodiesel–diesel blend containing 5% soybean oil and 5% linseed oil maintains engine power and is viable in terms of performance. However, increased fuel consumption and elevated levels of certain harmful exhaust emissions, particularly CO and NO_x, were noted at higher loads. These results are generally consistent with similar studies reported in the literature regarding vegetable oil-based biodiesel usage and emphasize the need for careful evaluation of such blends.

Keywords: Energy, Biodiesel blend, Soybean oil, Linseed oil, Engine performance

INTRODUCTION

Over the past century, global atmospheric temperatures have increased significantly, largely due to the intensive use of fossil fuel sources. This temperature rise has been further accelerated by the growing number of power generation facilities and the continual increase in vehicles used in the transportation sector, both of which have led to higher fossil fuel consumption. Diesel engines are widely preferred in passenger and freight transportation due to their low fuel consumption and high thermal efficiency [1]. In addition, the depletion of fossil fuel resources and the negative environmental and health impacts of emissions resulting from their combustion have accelerated the search for alternative fuels [2–11]. Although petroleum-based fuels currently account for approximately 40% of global energy consumption, diesel remains the most commonly used among them. Therefore, reducing the environmental drawbacks associated with diesel combustion has become increasingly important [12,13]. Biodiesel, an alternative to petroleum-derived fuels in diesel engines, is typically produced through the transesterification of various vegetable oils such as soybean, palm, and sunflower

oil or animal fats including pork fat, beef tallow, and fish oil. Biodiesel has attracted considerable attention due to its high biodegradability, low toxicity, and compatibility with diesel engines without requiring major modifications or causing significant performance losses. Moreover, when compared to conventional diesel fuel, biodiesel use has been shown to reduce emissions of hydrocarbons (HC), carbon monoxide (CO), and particulate matter (PM) [12,14,15]. In the literature, several studies have extensively analyzed the combustion and performance behavior of linseed oil biodiesel in diesel engines. According to these studies, biodiesel usage led to a 5–15% increase in peak combustion pressure compared to conventional diesel. Additionally, biodiesel blends exhibited longer combustion durations than standard diesel fuel. In another study, diesel fuel was blended with soybean and linseed oil in a 1:1 volumetric ratio and tested at low engine loads. The results showed a reduction in both particle size range and particle number concentration. However, when engine load increased, the opposite effect was observed [16]. Another study investigated the fuel consumption and exhaust emissions of a single-cylinder diesel engine using blends of linseed and rubber seed oils with diesel fuel. The experimental findings indicated that the most favorable results were achieved when 5% linseed oil and 5% rubber seed oil were blended with 90% diesel, or alternatively, when 10% of each oil was blended with 80% diesel fuel. These blends resulted in only a slight increase in fuel consumption, while CO and NO_x emissions showed a moderate rise [17].

In a separate study examining biodiesel applications, the performance and emission characteristics of a diesel engine fueled with soybean oil, linseed oil, and crambe oil were evaluated. Biodiesel blends were prepared by mixing vegetable oils with conventional diesel fuel in volumetric ratios of 10%, 30%, 50%, and 70%. The results revealed that blends containing low (10%) and medium (30%) concentrations of soybean, linseed, and crambe oils yielded engine performance and emissions similar to those observed with pure diesel fuel [18].

In contrast to previous studies in the literature, this research aims to investigate the effects of an alternatively prepared biodiesel on diesel engine performance by blending 5% linseed oil and 5% soybean oil by volume with 90% conventional diesel fuel. This vegetable oil-enriched biodiesel blend was tested comparatively against 100% standard diesel fuel on a single-cylinder, four-stroke diesel engine. Within the scope of the experimental study, detailed measurements and evaluations were conducted on the engine's performance characteristics—namely power output and fuel consumption under various load conditions, alongside exhaust gas emissions, specifically carbon monoxide (CO) and nitrogen oxides (NO_x) levels. Through this approach, the study seeks to contribute to the understanding of alternative fuels' role in engine technologies and their potential for promoting sustainable energy utilization.

MATERIALS AND METHODS

The experimental investigations conducted within this study were carried out on a single-cylinder, four-stroke diesel engine with a power output of 11.5 kW, located at a research and development (R&D) center, as shown in Figure 1. The fuels used in the experiments were prepared in two distinct stages. In the first stage, the reference fuel consisted of 100% standard diesel, which is widely used in our country. In the second stage, an alternative fuel blend was prepared by mixing 90% standard diesel fuel with 5% soybean oil and 5% linseed oil biodiesel. Prior to commencing the experiments, the engine was operated without any applied load until it reached the steady-state operating temperature of 82°C, as specified by the manufacturer, to ensure stabilized operating conditions. Once the required temperature was achieved, the first phase of the tests began, where the engine was fueled with 100% standard diesel. During this phase, the engine was subjected to incremental loads of 25%, 50%, and 75%, respectively, while key parameters including engine power output, fuel consumption, and exhaust gas

emissions were recorded. After completing the initial phase, the same experimental procedure was repeated exactly with the alternative fuel blend containing 90% diesel, 5% soybean oil, and 5% linseed oil biodiesel. For each load level, identical parameters were measured, and the obtained data were meticulously documented. Additionally, exhaust gas emissions were regularly monitored at specified intervals throughout the engine performance tests. Following the completion of all experiments, the collected data were graphically presented to illustrate changes in engine power, specific fuel consumption, and exhaust emissions, which were subsequently analyzed in detail.

The overall view of the engine used in the experimental studies is presented in Figure 1, while its technical specifications and characteristic features are detailed comprehensively in Table 1.



Figure 1. Diesel Engine Configured for Use in the Experimental Study

Table 1: Technical Specifications of The Test Engine

General Characteristics	Technical Specifications
Engine type	Erin Motor Base Model
Number of Valves	4
Continuous Power (kw/rpm)	11.5 / 1500
Bore (mm)	108
Stroke (mm)	127
Combustion System	Direct Injection
Compression Ratio	14.6 : 1
Engine Cooling	Water
Weight (kg)	157

RESULTS AND DISCUSSION

The experimental findings obtained in this study clearly demonstrate the effects of adding 5% soybean oil and 5% linseed oil to the biodiesel blend used as engine fuel on both engine performance and exhaust emission parameters. Analyzing the power output data from the conducted engine tests reveals that, at all engine load levels where the prepared biodiesel blend was used, power values similar to those obtained with conventional diesel fuel were achieved. This result is a significant indication supporting the applicability of the biodiesel blend ratio as an engine fuel, showing no adverse effect on engine power. Graphical analyses of fuel consumption indicate that the biodiesel blend containing soybean and linseed oils causes only a slight increase in fuel consumption compared to diesel fuel, and this effect is observed solely at a 75% engine load. This observed increase is attributed to the somewhat lower energy content (lower heating value) of the biodiesel relative to standard diesel fuel. Indeed, this finding aligns closely with results reported in similar studies found in the literature.

Analysis of the emission data revealed no significant differences in carbon monoxide (CO) levels between the two fuel types, with values observed to be nearly identical. However, nitrogen oxide (NO_x) emissions exhibited a slight increase in tests using the biodiesel blend, particularly under high engine load conditions. This increase is believed to be associated with the higher combustion temperatures generated during the biodiesel combustion process, which promote NO_x formation. This finding is consistent with the information reported in existing scientific literature.

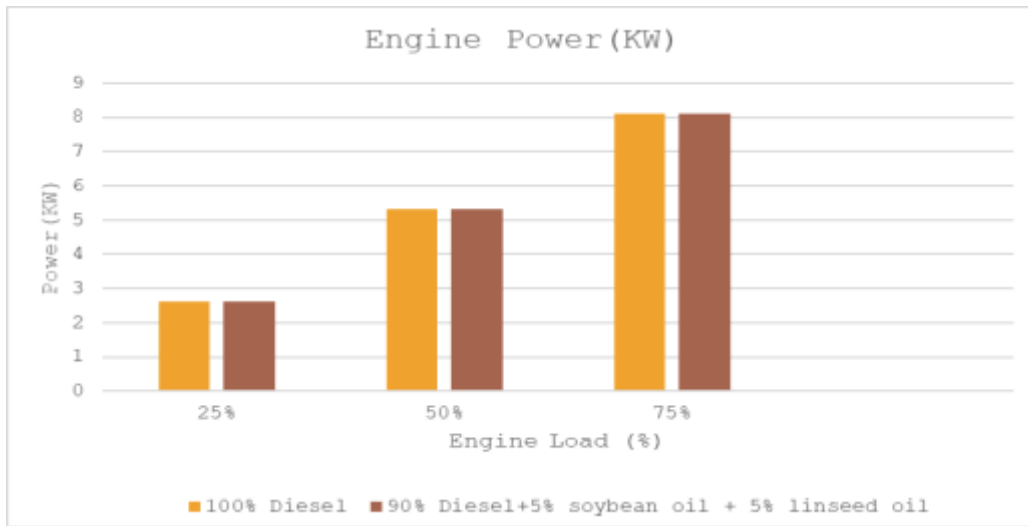


Figure 2. Engine Power Output Comparison Between Various Fuel Blends

As observed in Figure 2, the test results conducted under various load conditions clearly indicate no difference in engine power output between the biodiesel-blended fuel and the standard diesel fuel. The attainment of similar engine power across all applied load levels demonstrates that the prepared biodiesel blend yields positive outcomes in terms of engine performance, serving as a significant technical indicator supporting the feasibility of this fuel in diesel engines. This finding is valuable as it suggests that the adverse effects of biodiesel on engine performance are minimal and that it can operate compatibly with conventional diesel fuel.

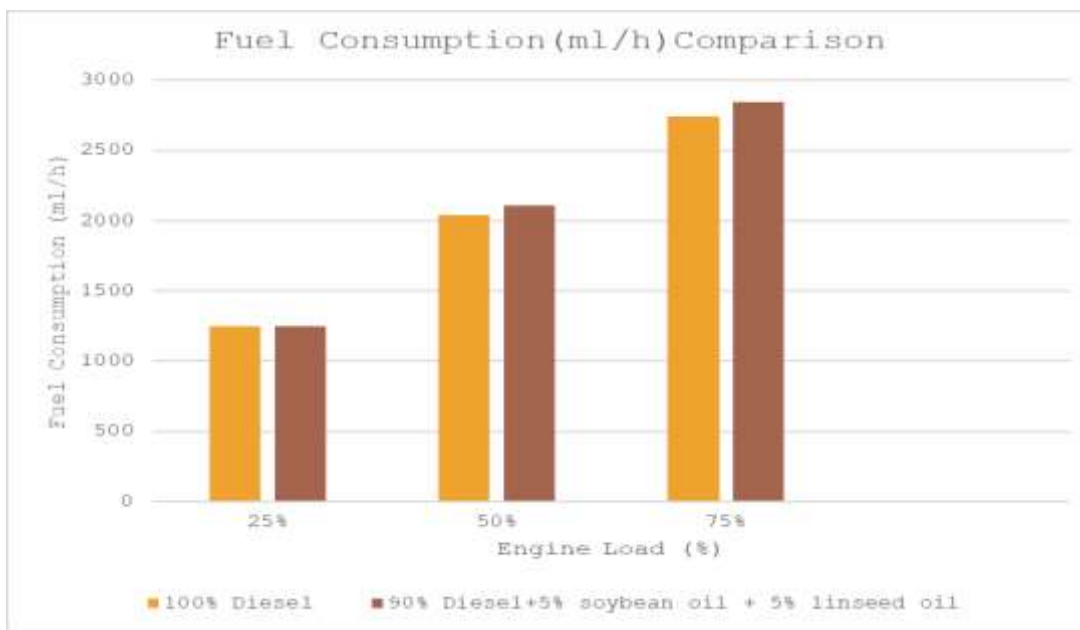


Figure 3. Comparison of Engine Fuel Consumption Across Different Fuel Types

According to the data presented in Figure 3, tests conducted with the biodiesel-blended fuel showed noticeable increases in fuel consumption compared to standard diesel fuel,

particularly at engine loads of 50% and 75%. These increases in fuel consumption varied between a minimum of 65 ml and a maximum of 105 ml, depending on the applied load levels. The results indicate that the biodiesel blend slightly increases the amount of fuel consumed by the engine under specific load conditions, with this increase being directly proportional to the engine load. This phenomenon may be attributed to the somewhat lower energy content (especially the lower heating value) of the biodiesel compared to standard diesel, as well as the effects of the fuel's physical and chemical properties on the combustion process.

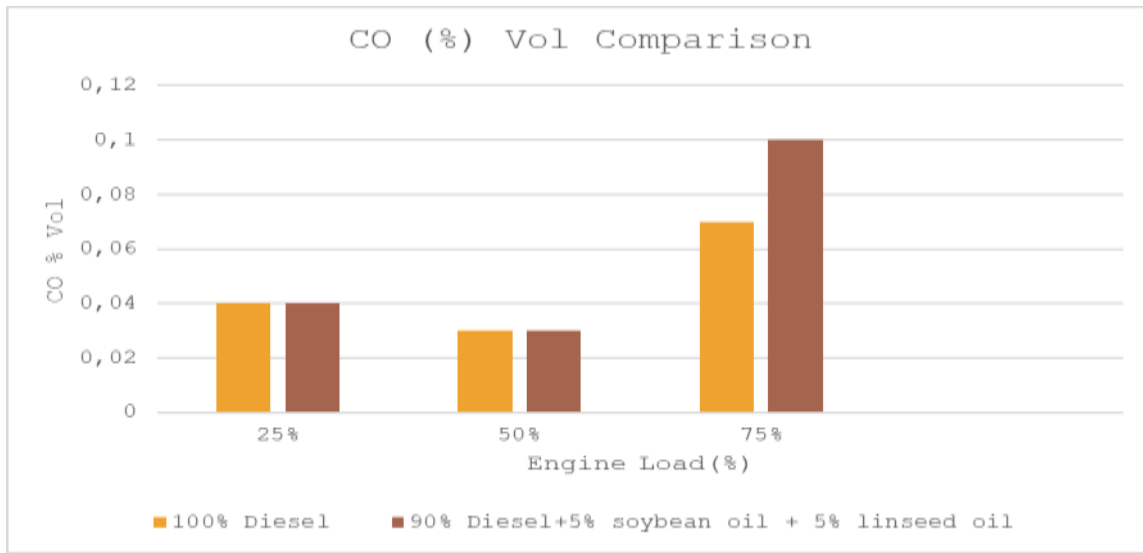


Figure 4. Comparison of Carbon Monoxide (CO) Emissions for Various Fuel Types

According to the data presented in Figure 4, the impact of using biodiesel-blended fuel on carbon monoxide (CO) emissions in the exhaust gases was evaluated. It was observed that under engine load conditions of 25% and 50%, there were no changes in CO levels. Only at the 75% load level was a very limited and negligible increase of approximately 0.03% detected. This minor variation can be attributed to minimal differences in combustion efficiency resulting from biodiesel use. However, overall assessment indicates that the biodiesel blend does not have a significant adverse effect on carbon monoxide emissions, with emission values remaining largely comparable to those of the reference fuel. Therefore, from an environmental sustainability perspective, biodiesel usage falls within acceptable limits regarding CO emissions, demonstrating its potential as an alternative fuel that does not increase harmful gas emissions.

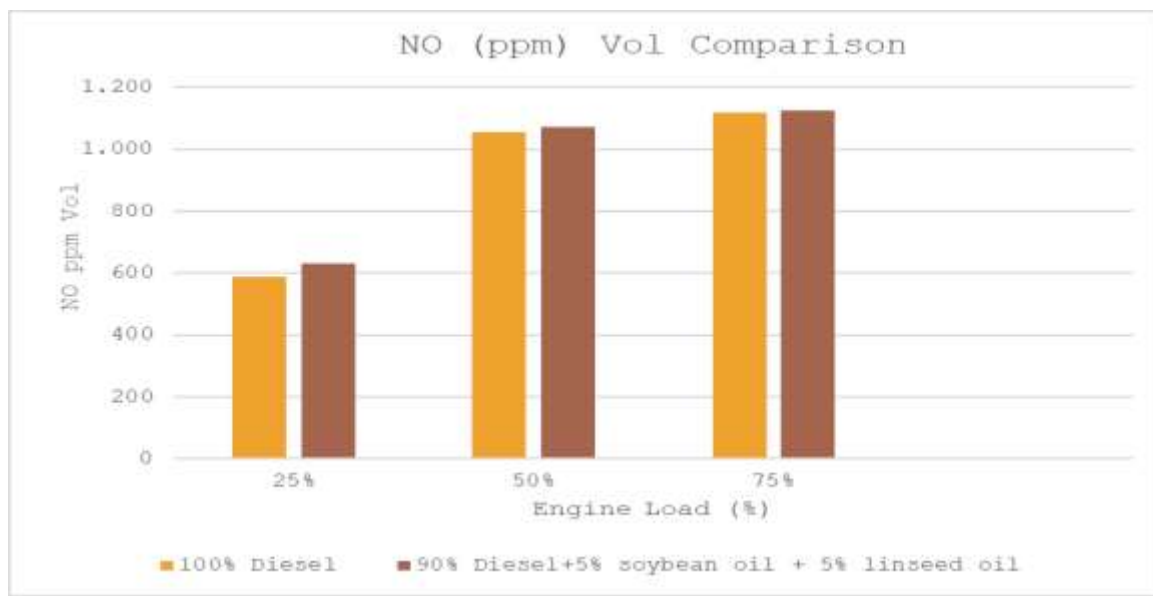


Figure 5. Comparison of Nitrogen Oxide (NO_x) Emissions Across Various Fuel Types

According to the data presented in Figure 5, the use of biodiesel-blended fuel resulted in limited increases in nitrogen oxide (NO_x) emissions across all engine load levels. When examined for each load condition, these increases did not exceed 50 ppm, indicating that the rise in emission levels is relatively minor. The primary cause of this increase can be attributed to the higher combustion temperatures achieved during the burning of biodiesel compared to conventional diesel fuel, which naturally promotes NO_x formation. The notable rise in NO_x emissions, especially under high engine loads, emerges as a critical parameter that must be carefully considered in evaluating the environmental performance of biodiesel usage. Therefore, when biodiesel fuels are widely implemented in diesel engines, various technical improvements and emission reduction strategies may be necessary to control NO_x emissions effectively. Such measures could include after-treatment systems (e.g., Selective Catalytic Reduction - SCR), engine recalibration, or the application of alternative combustion strategies. In conclusion, despite the environmental benefits offered by biodiesel use, the observed limited increase in NO_x emissions should be taken into account in the development of sustainable engine technologies.

CONCLUSION

Within the scope of this study, the effects of vegetable oil-based biodiesel blends, which can be used as alternative fuels in diesel engines, on engine performance and exhaust emissions were experimentally investigated. In the tests, a biodiesel blend composed of 90% conventional diesel fuel, 5% linseed oil, and 5% soybean oil by volume was prepared and compared with 100% conventional diesel fuel. The experimental results demonstrated that the use of the biodiesel blend did not cause any significant decrease in engine performance. Engine power output at all load levels largely coincided with the values obtained using conventional diesel fuel, with no observed performance loss. This outcome indicates that the vegetable oil-blended biodiesel is technically feasible for use in diesel engines.

In terms of fuel consumption, slight increases were recorded only at 50% and 75% engine loads when using the biodiesel blend. These increases ranged between 65 ml and 105 ml and were attributed to the lower energy density of biodiesel compared to diesel fuel.

However, this difference is not significant enough to adversely affect the overall fuel efficiency of the engine.

Analysis of emissions revealed that carbon monoxide (CO) levels were largely similar for both conventional diesel and the biodiesel blend, with only a very limited increase observed at 75% load. In contrast, nitrogen oxide (NO_x) emissions exhibited minor increases across all load levels, none of which exceeded 50 ppm. These increases in NO_x are believed to be associated with the higher combustion temperatures resulting from the use of biodiesel. The results obtained in this study are valid under specific engine types and fixed test conditions. It should be noted that different engine models, operating temperatures, injector systems, or climatic conditions may yield varying outcomes. Additionally, only a single biodiesel blend ratio (10% vegetable oil content) was tested in this research. The effects of different vegetable oil blend ratios on engine performance and emissions should be explored in future studies. Furthermore, the integration of technologies aimed at reducing NO_x emissions such as fuel pretreatment methods, exhaust gas aftertreatment systems, or advanced engine control strategies with biodiesel use represents a significant area of research for developing sustainable and environmentally friendly engine applications.

ACKNOWLEDGMENTS

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EFFECT OF PROCESS PARAMETERS ON α -AMYLASE PRODUCTION UNDER SOLID-STATE FERMENTATION

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ABSTRACT

α -Amylase is a major hydrolytic enzyme for starch degradation, and is extensively used in a number of industrial processes. Solid-state fermentation (SSF) is deemed a cheap, effective, and environmentally friendly method of producing it. The present work utilized the *Bacillus amyloliquefaciens* NRRL B-645 strain for α -amylase production in a packed-bed bioreactor. Operating parameters of a bioreactor system, airflow rate, temperature, and substrate amount were studied for their impact on activity of the enzyme, and optimized by employing the Central Composite Design (CCD) technique. Optimization of operating parameters resulted in increased α -amylase production.

Keywords: α -Amylase, Solid-state fermentation, Packed-bed bioreactor, Process optimization, Agro-industrial wastes

INTRODUCTION

Enzyme technology, by relying on cell or enzyme application as a biocatalyst, is considered by the OECD one of sustainable production technologies' pillars (OECD, 1998). Due to its benefits for product quality, process efficiency, and environmental friendliness, it is being extensively employed for many sectors (Donzella and Contente, 2024).

Most industrial enzymes are derived from bacteria, molds, and yeasts. Because of their simple cellular structures, their ability to rapidly proliferate, and because genetic manipulation is comparatively easy, microorganisms like these allow for innovations of successful bioprocesses (Liu and Kokare, 2023). Of importance, species of the genus *Bacillus* are notable for their ability to produce a rich variety of enzymes under varying conditions, their high physiological resistance, and for their being GRAS (Generally Recognized As Safe) species (Yang et al., 2024; Yu et al., 2024).

α -Amylase (EC 3.2.1.1) is an endoamylase having random hydrolytic action on α -1,4-glucosidic linkages of starch polymers, yielding a spectrum of oligosaccharides, maltose, and glucose. α -Amylase finds application for a host of purposes, ranging from food production and animal feed to production of textiles, preparation of detergents, and treatment of papers. With approximately 25–30% of the total market of enzymes, α -amylase ranks among the economically most significant enzymes (Abd-Elaziz et al., 2020).

Despite widely accepted submerged fermentation for microbial enzyme production, it comes along with some of its limitations concerning energy expenditure and expense (Farinas, 2015; Poltronieri and D'Urso, 2016). Solid-state fermentation (SSF) became a viable substitute, which provided a greener method of operation by its diminished water demand and improved economic prospects (Gómez-Ramos et al., 2025; Perwez and Al Asheh, 2025).

This study investigated α -amylase production by solid state fermentation of a packed-bed bioreactor system by *Bacillus amyloliquefaciens* NRRL B-645 strain. Bioreactor operating parameters, air flow rate, temperature, and substrate amount were optimized by utilizing Central Composite Design (CCD).

MATERIAL AND METHODS

Solid Substrates

In this study, hazelnut oil cake obtained from Altaş Yağ Sanayi A.Ş. (Ordu, Turkey), and maize bran obtained from Sunar Mısır Entegre Tesisleri Sanayi ve Ticaret A.Ş. (Adana, Turkey) were used. Both industrial by-products were ground and fractionated using ASTM standard sieves. The particle fractions in the -30+50 mesh size range were stored in airtight containers under appropriate conditions and used in fermentation experiments.

Microorganism

The bacterium *Bacillus amyloliquefaciens* NRRL B-645 was obtained from the NRRL (National Center for Agricultural Utilization Research, USA) culture collection.

Microorganism Growth and Preservation Medium

Luria–Bertani (LB) liquid and agar media were used for the cultivation and preservation of the microorganism. The media were prepared according to the standard formulation and sterilized in an autoclave (121 °C, 1.2 bar, 15 minutes) (Miller, 1972). Cells obtained from cultures grown in LB liquid medium were transferred to agar plates using spread and streak plating methods. The resulting stock cultures were stored at +4 °C and renewed every two weeks. For use in SSF, a colony of *Bacillus amyloliquefaciens* NRRL B-645 was taken from the stock culture and inoculated aseptically into 50 ml of LB liquid medium. The culture was incubated at 37 °C for 16 hours at 150 rpm.

Fermentation Medium

In a previous study, the optimum fermentation medium composition for α -amylase production was determined as follows: 2.5 g of maize bran, 7.5 g of hazelnut oil cake, 6.0 mL of inoculum volume, 1.6 mL of nitrogen source, and 7.4 mL of tap water, with a fermentation period of 114 hours. The optimized medium composition was transferred to a packed-bed bioreactor system while maintaining a solid-to-liquid ratio of 1:1.5 (w/v).

Experimental Design

To optimize α -amylase production in SSF, optimization of bioreactor operating parameters, air flow rate (L/min), temperature ($^{\circ}\text{C}$) and substrate amount (g), were performed. The experimental levels of chosen parameters are listed in Table 1.

Table 1. Selected factors and their levels for bioreactor operating conditions

Independent variable	Factor	-1.68	-1	0	+1	+1.68
Air flow rate (L/min)	A	0.100	0.130	0.175	0.220	0.250
Temperature ($^{\circ}\text{C}$)	B	31.0	32.6	35.0	37.4	39.0
Substrate amount (g)	C	10	12	15	18	20

The experimental design was developed using the Central Composite Design (CCD) as part of the Response Surface Methodology (RSM), and was carried out using Design-Expert 10 software. α -Amylase activity (U/g) was chosen as the response variable. Analysis of variance (ANOVA) and response surface plots were also generated using the same software.

According to the Central Composite Design (CCD), a total of 20 experimental runs were cumulatively carried out including 8 factorial points, 6 axial points, and 6 center points. Total experimental runs (N) were estimated by a general formula based on CCD (Equation 1):

$$N = 2^n + 2n + n_c = 2^3 + 2(3) + 6 = 8 + 6 + 6 = 20 \quad (1)$$

In this instance, n represents the number of independent variables; 2^n is the number of factorial points; $2n$ is the number of axial points; and n_c is the number of times of replications of center point.

In analysing the experimental results, a second-order polynomial regression model was utilized for the investigation of α -amylase production induced by independent variables. It is a typical model widely utilized for Response Surface Methodology (RSM) and represented by Equation (2) (Liu et al., 2020):

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j \quad (2)$$

In this equation, Y represents the response variable modelled by the regression equation. X_i and X_j are the independent variables used to evaluate their effects on the response. β_0 denotes the intercept term, while β_i , β_{ii} and β_{ij} represent the coefficients of the linear, quadratic, and interaction terms between the variables, respectively.

Enzyme Extraction

After undergoing solid-state fermentation, the fermentation medium was harvested from packed-bed bioreactors and then transferred to Erlenmeyer flasks. Extraction of the medium was carried out by sodium phosphate buffer (6.9 pH) and centrifugation. Supernatant recovered by this process acted as a crude solution of the enzyme for measuring α -amylase activity.

In each extraction, buffer volume was adjusted to 10 mL per g of solid substrate.

Enzyme Activity Assay

The enzymatic activity of α -amylase used here is the DNS (3,5-dinitrosalicylic acid) method (Bernfeld, 1955), which determines the extent of reducing sugars formed. It is a method whereby maltose, a result of starch hydrolysis by α -amylase, upon reacting, produces a colored

complex by reacting with DNS reagent. The analytical procedure for the DNS method is thoroughly described in Table 2.

Table 2. Analysis protocol for DNS-based α -amylase activity assay (Sigma-Aldrich).

Reagent	Control (mL)	Sample (mL)
Starch solution (in sodium phosphate buffer, pH 6.9) <i>Vortexed and incubated in a water bath at 20 °C.</i>	0.5	0.5
α -Amylase Solution <i>Incubated in a water bath at 20 °C for 3 minutes.</i>	-	0.5
Color Reagent (DNS) α -Amylase Solution <i>After incubation in a boiling water bath for 15 minutes, the mixture was cooled on ice until it reached room temperature.</i>	0.5 0.5	0.5 -
Distilled Water <i>The mixture was vortexed, and the absorbance was measured at 540 nm using a spectrophotometer.</i>	4.5	4.5

For all samples and control tubes, triplicate measurements were made, after which mean absorbance values were computed. The net absorbance was then found by subtracting mean absorbance of the control for each sample's mean absorbance. Maltose concentrations corresponding to these absorbance readings were determined by way of a standard curve. One unit (U) of α -amylase activity is defined as the amount of enzyme that releases 1 mg of maltose from starch in 3 minutes at 20 °C and pH 6.9.

RESULTS AND DISCUSSION

The statistical significance of the model developed to optimize bioreactor operating conditions was evaluated using analysis of variance (ANOVA). Comparative statistical results for different model types are presented in Table 3.

The outcome of the ANOVA resulted in a p-value associated with the F-test of the quadratic model being less than 0.0001. With this result being less than the traditional significance value of 0.05, it declares the model to be statistically significant. The model's robustness is a testament to its R^2 value of 0.9559, adjusted R^2 value of 0.9163, and predicted R^2 value of 0.7474. A value of R^2 nearing 1 represents a good match between model and data, and also sound prediction capability (Haaland, 1989). It is, however, mentioned that adjusted R^2 value, taking both number of variables in model and sample size into consideration, may significantly lower compared to R^2 value when model includes many terms, or when sample size turns out to be insufficient (Hashemi et al., 2013). Acceptable difference between adjusted R^2 value and R^2 value found for α -amylase supports model's thoroughness. Predicted R^2 value becomes a measure of similarity of model's prediction and experimental results (Singh et al., 2017).

Table 3. Model evaluation results for the optimization of bioreactor operating conditions

Source	Sequential <i>p</i> -value	Lack of Fit <i>p</i> -value	Adjusted R^2	Predicted R^2	
Linear	0.1535	0.0015	0.1373	-0.0142	
2FI	0.8935	0.0008	-0.0146	-1.1007	
<u>Quadratic</u>	<u><0.0001</u>	<u>0.2208</u>	<u>0.9163</u>	<u>0.7474</u>	<u>Suggested</u>
Cubic	0.4511	0.1015	0.9183	-1.5541	

Keeping in view elevated error rates and variability usually seen for biotechnology processes, a predicted R^2 value of 0.7474 turns out to be acceptable (Gois et al., 2020).

The p-value for lack of fit of the quadratic model was found to be 0.2208, which is higher than the typical accepted threshold of 0.1.

Table 4 displays the p-values associated with the linear, quadratic, and interaction terms alongside additional statistical results.

Model parameters having p-values below 0.05 (A, B, AC, A², B², and C²) were identified as significant factors affecting α -amylase activity. Such p-values are indicative of how each model coefficient impacts the response variable, hence explaining how the independent variables interact.

Mean value was 147.44, and standard deviation was 13.81. Coefficient of variation (C.V.) of 9.37% tends generally to indicate acceptable model reproducibility (Bandyopadhyay and Choudhury, 2018).

The precision value calculated was found to be 13.48. Having noted that such a value significantly exceeded the minimum acceptability threshold of 4, it is possible to generalize that the model is reliable for providing precise predictions for the specified design range and is statistically significant (Sharma and Kumar, 2017).

Table 4. ANOVA results for the optimization of bioreactor operating conditions

Source	Sum of Squares	dF	Mean Square	F-value	p-value
Model	41384.55	9	4598.28	25.98	<0.0001
A - Air flow rate (L/min)	6072.02	1	6072.02	34.23	0.0002
B - Temperature (°C)	5549.74	1	5549.74	31.35	0.0003
C - Substrate amount (g)	218.91	1	218.91	1.23	0.3092
AB	0.66	1	0.66	3.725E-003	0.9542
AC	957.03	1	957.03	5.39	0.0490
BC	439.56	1	439.56	2.48	0.1599
A²	6935.77	1	6935.77	38.49	0.0001
B²	14317.08	1	14317.08	80.04	<0.0001
C²	12245.96	1	12245.96	68.37	<0.0001
Residual	1907.06	10	190.71		
Lack of Fit	1287.19	5	257.44	1.86	0.2208
Pure Error	619.87	5	123.97		
Cor Total	43291.61	19			
Mean	147.44				
R ²	0.9559				
Adjusted R ²	0.9163				
Predicted R ²	0.7474				
Standard Deviation	13.81				
C.V. %	9.37				
PRESS	10933.96				
Adequate Precision	13.484				

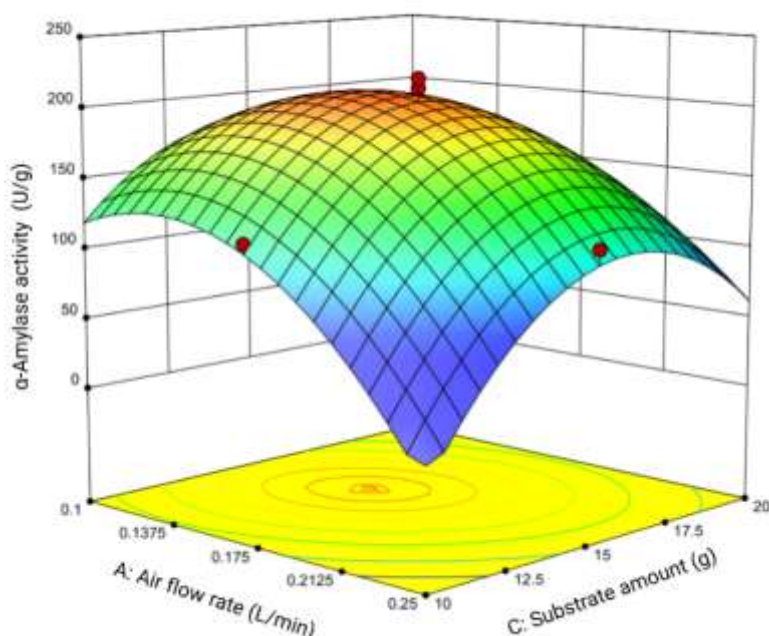


Figure 1. Response surface plot showing the interaction between air flow rate (A) and substrate amount (C) on α -amylase activity.

Figure 1 shows the interaction between the airflow rate (A) and the substrate amount (C) on the α -amylase activity. Such interaction is on the borderline of statistical significance, having a P of 0.0490, and is a factor included in the model with a minimum effect. The data depicted by the graph indicate an optimum position of enzyme activity under conditions of an air flow rate of approximately 0.175 L/min, accompanied by a substrate amount of 15 g. However, if air flow rate increased over this value, a sharp decline of activity resulted. Lower air flow rates may lead to a thin, humid film covering solid particle surfaces, causing mass transfer resistance, and thereby inhibiting accessibility of oxygen and dissolved nutrients to microorganisms (Bakkiyaraj et al., 2016). Against this backdrop, an increase in air flow rate is presumed to accelerate enzyme production by alleviating mass transfer limitations. However, air flow rates above optimum (approximately 0.175 L/min) may have resulted in substrate bed desiccation by moisture, having a deleterious effect on microbial activity and, consequently, enzyme production. Moreover, substrate amounts above optimum result in mass transfer-related difficulties of transferring oxygen (Rocha et al., 2021).

CONCLUSIONS

As a result of the process optimization experiments conducted for α -amylase production, the optimal bioreactor operating conditions were determined to be an air flow rate of 0.154 L/min, a temperature of 35.8 °C, and a substrate amount of 15 g. Under these conditions, the maximum predicted enzyme activity was 212.11 U/g. Validation experiments performed

under the same conditions measured α -amylase activity at 190.9 U/g, corresponding to 90.01% of the predicted value.

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EFFECT OF CHAPERONE PROTEIN OVEREXPRESSION ON RECOMBINANT TRANSGLUTAMINASE ENZYME PRODUCTION

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ABSTRACT

Recombinant protein production technique is generally used to produce various proteins, hormones, food enzymes, and drug precursors. In recombinant protein production, different host systems have been used, such as bacterial, yeast, mold, and mammalian cells. Many factors affect the quantity and quality of recombinantly produced protein. The chaperone proteins are the proteins that mediate protein folding in a proper form. These protein groups have crucial roles, especially in post-translational modifications.

In this study, the Protein Disulfide Isomerase (PDI) chaperone protein, which is mainly responsible for the formation of disulfide bonds was overexpressed in *Pichia pastoris*, and the effect of homologous overexpression of the PDI protein was investigated. For this aim, the double auxotroph *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) strains were used, and PDI chaperonin and transglutaminase enzyme were co-expressed by constitutively. Firstly, the expression vector was constructed that contains the PDI gene and transformed into the electrocompetent *P. pastoris* GS200 and JC304 strains. The PDI gene copy number was detected in selected clones by Southern Blot analysis, and extra copies of the PDI gene containing clones were selected for each strain. Then, chosen clones were used as hosts for transglutaminase expression. The pGAPZa-MTG expression vector was linearized within the promoter region and transformed into the constructed competent host cells. The integration of the expression cassette into the genome was checked, and the protein expression was performed with positive clones at erlenmayer scale for 96 hours. It was found that the overexpression of PDI chaperone protein increased the extracellular enzyme production by 4.2% and 2.7% for *P. pastoris* GS200 and JC304 strains, respectively. As a result of this study, it was shown that the enzyme production has been increased in auxotroph strains of *P. pastoris*; however, further optimization studies are required.

Keywords: Chaperone protein, Overexpression, *Pichia pastoris*, Recombinant protein

INTRODUCTION

In recombinant enzyme production, there are many factors that affect the expression of proteins. The recombinant protein production is a combination of different parameters such as, proper host system selection, codon optimization of gene, intra/extracellular production method and using of different chaperone proteins. In the production of desired protein many host systems are used such as bacterial (*Escherichia coli*, *Lactococcus lactis*, *Pseudomonas fluorescens* and *Streptomyces lividans*; (Chen, 2012), yeast (Baghban et al., 2019; Çelik & Çalik, 2012), plant (Yuksel et al., 2025), insect (Huang et al., 2025) and, mammalian (Khan, 2013). These platforms have many advantageous and disadvantageous and the platform type is selected by desired properties. In these platforms, the yeast expression system *Pichia pastoris* stands out thanks to its advantages such as reach to high cell densities, low production capacities of inner protein, has many host strains and inducer or constitutive promoters (Gasser et al.,

2013; Karbalaeei et al., 2020; Macauley-Patrick et al., 2005). *P. pastoris* has many strains such as wild type X33, protease deficient strains KM71, SMD1168, SMD1165 and auxotrophic strains GS115, GS200 and JC304 and these different strains have been used for protein production studies for many years. In recombinant protein production, there are studies showing that chaperone protein overexpression increases target protein production (Jomrit et al., 2023; Mamipour et al., 2017). In chaperone proteins, Protein Disulfide Isomerase (PDI) chaperone is widely used in many food enzymes or therapeutic protein production (Ersöz & İnan, 2025; Mares et al., 2015; Shen et al., 2012). It was seen that the homologous overexpression of PDI chaperone protein was increased approximately 2-folds in recombinant chymosin production (Ersöz & İnan, 2025).

In this study, the PDI chaperone protein was tested in transglutaminase enzyme production. MTGase is a simple monomeric protein, composed of 331 amino acids, has a molecular weight of 37.842 kDa, and an isoelectric point (pI) of 9 (Candi et al., 1995). Transglutaminase is an enzyme that catalyze the disulfide bond formation between molecular peptides. In food applications, TGase enzymes enhance the food quality, properties, structure and nutritional value by supplying with essential amino acids (Yokoyama et al., 2004). The applications of transglutaminases in various food products are bakery products, dairy products and meat products (Toldrá & Reig, 2015). In this study, the PDI chaperone protein was overexpressed in auxotroph *P. pastoris* strains and the effect of PDI chaperone was tested in microbial transglutaminase enzyme production.

MATERIAL AND METHODS

Construction of pGAP3.5K-PDI plasmid

To construct of pGAP3.5K-PDI plasmid, the pPICZ3.5K (Invitrogen™) was used as main skeleton and the AOXI promoter region was changes with GAP promoter region and pGAP3.5K was obtained. Then, the PDI gene was amplified by PCR, using appropriate primers by using the *P. pastoris* X33 genomic DNA as template. PCR product of PDI gene was cloned into pGAP3.5K and pGAP3.5K-PDI expression vector was obtained.

Transformation to *P. pastoris* strains

pGAP3.5K-PDI expression vector was linearized within the GAP promoter and transformed to competent *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) cells, by electroporation methods (Wu & Letchworth, 2004). After transformation of expression vector, the cells were grown on MD (minimal dextrose)+geneticin+arginin and MD (minimal dextrose)+geneticin+adenin agar medium, for *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) respectively. Then single colony isolation was performed and the cells were inoculated in YPD broth medium for overnight at 28°C conditions. Then the culture was centrifuged and the cells were taken for genomic DNA isolation for Southern Blot analysis. Southern Blot analysis was performed to detect the pGAP3.5K-PDI expression vector integration into the genome of *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) strains. Southern Blot analysis was done with DIG-High Prime DNA Labeling and Detection Starter Kit II by using PDI gene as probe and labeled with DIG, as described in the kit protocol. According to Southern Blot analysis the clones that containing extra one copy of PDI gene into the genome were selected and used as host for transglutaminase enzyme expression studies.

Competent cell preparations and transformation of enzyme expression vector

The clones that containing an extra copy of PDI gene were selected for both *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) strains and the competent cells were prepared according to Wu et al. (Wu & Letchworth, 2004). For transglutaminase enzyme expression, the pGAPZa-proMTG expression vector that was constructed before (Türkanoğlu Özçelik et al., 2019) was used. pGAPZa-proMTG expression vector was linearized and transformed to host strains and selected on YPD agar+zeosin agar plate. After the growing on agar plate, the single colony isolations were performed and incubated at 28° C for 3-4 days. The cells were inoculated in YPD broth medium for overnight at 28°C conditions. Then the cells were inoculated to BYED medium with 0.1 OD^{600nm} and grown up to 10 OD^{600nm} about 18-20 hours. Fermentation was performed about 96 hours and every 24 hours the glucose was added to the BYED medium. At the end of fermentation, the cells were harvested and supernatant sample was analyzed for transglutaminase enzyme activity as mentioned before studies (Türkanoğlu Özçelik et al., 2019). In erlenmayer scale enzyme production the one copy MTGase containing clone in wild type strain was used as control for enzyme production.

RESULTS AND DISCUSSION

For the construction of chaperone protein (PDI: protein disulfide isomerase) containing expression vector, restriction analysis was performed with suitable enzyme set, and the PDI gene and GAP (glyceraldehyde 3 phosphatase) promotor regions were cut from the agarose gels (Figure 1).

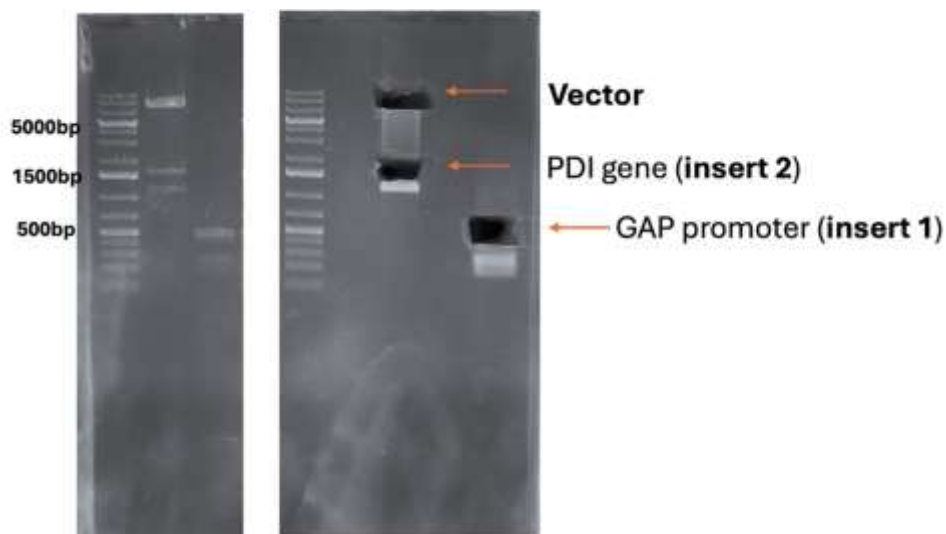


Figure 1. Construction of pGAP3.5K-PDI Plasmid

The vector fragment, insert 1 (GAP promoter region) and insert 2 (PDI) were purified, ligated and the pGAP3.5K-PDI expression vector was constructed. Then this plasmid was transformed to *Escherichia coli* NEB5 α cells and grown on LB +ampicillin agar medium. After growing the cells, the plasmid isolation was performed, and verification analysis was performed with different restriction enzymes (Figure 2). Both plasmid (#84 and #85 numbers) was verified and the pGAP3.5K-PDI #84 plasmid was chosen for transformation to *P. pastoris*.

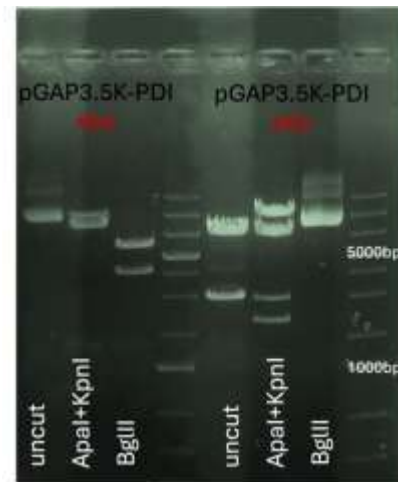
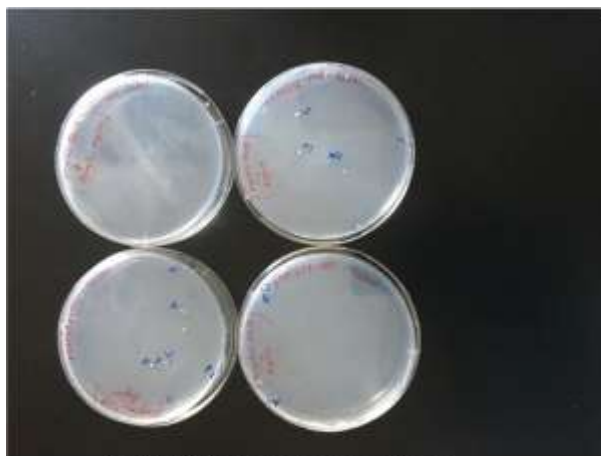


Figure 2. Verification analysis of constructed pGAP3.5K-PDI expression vector

Selected plasmid (pGAP3.5K-PDI #84) was linearized within the GAP promoter region and transformed by electroporation methods to the competent *P. pastoris* GS200 (*arg4 his4*) and *P. pastoris* GS200 (*ade1 his4*) auxotroph strains and grown at 28°C for 3-5 days on MD (minimal dextrose)+geneticin+arginin and MD (minimal dextrose)+geneticin+adenin agar medium, respectively (Figure 3).



GS200 transformation
(MD+geneticin+arginin)



JC304 transformation
(MD+geneticin+adenin)

Figure 3. Transformation of pGAP3.5K-PDI Plasmid into *P. pastoris* GS200 (*arg4 his4*) and JC304 (*ade1 his4*) auxotroph strains

The genomic DNA of the cells that grown on agar plate were done and southern blot analysis was performed by using PDI probe (Figure 4). According to the Southern Blot analysis image it was observed that, the 1, 2, 3, 4, 5, 7 and 9 clones were contained one copy of expression cassette for *P. pastoris* GS200 (*arg4 his4*) strain. The clones that named as 48, 49, 52 and 53 were contained one copy of expression cassette for *P. pastoris* JC304 (*ade1 his4*) strain.

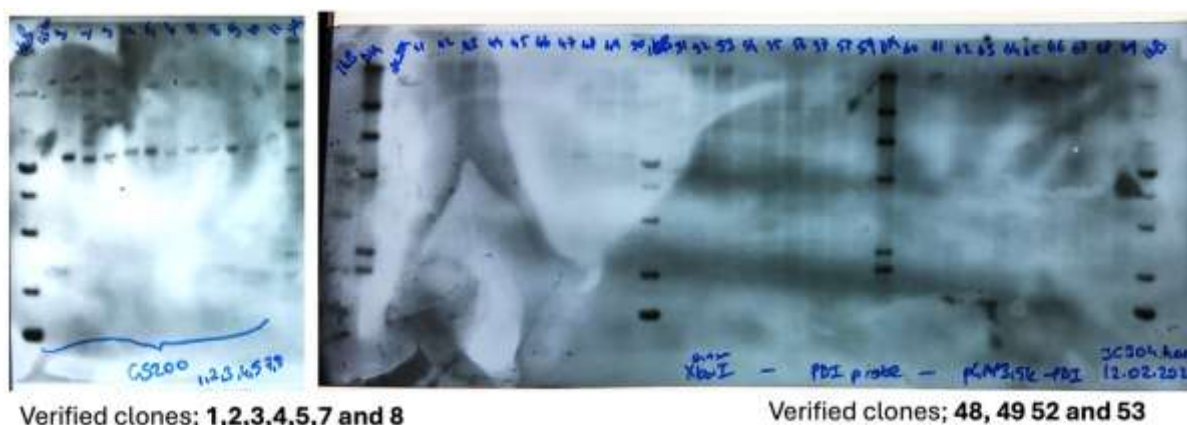


Figure 4. Southern Blot analysis results (*Xba*I restriction enzyme, PDI probe; expected band sizes; 6211 and 9941bp for one copy containing clones).

Erlenmeyer scale protein expression by intracellularly were performed with these selected clones, and the shake flask images were given in Figure 5. According to the southern blot analysis (Fig. 4) and erlenmeyer scale expression results (Fig. 5) the #1 clone and the #48 clones were selected for containing one-extra PDI gene copy containing clones, for next studies. After selection of the clones the competent cells were prepared and the pGAPZa-proMTG (*Streptomyces mobaraensis* microbia transglutaminase) plasmid was transformed to the *P. pastoris* GS200#1 and *P. pastoris* JC304#48 strains.



Figure 5. Expression analysis of PDI chaperone protein in *P. pastoris* GS200 (arg4 his4) and JC304 (ade1 his4) auxotroph strains

MTGase enzyme expression were performed at erlenmayer scale, at 24C temperature conditions, at 200 rpm shaking, at pH7 in BYED medium for 96 hours. At the end of the time the supernatant fractions were separated from the cells and the enzyme activity was performed. The relatively enzyme activities were given at figure 6. It was found that the overexpression of PDI chaperone protein increased the extracellular enzyme production by 4.2% and 2.7% for *P. pastoris* GS200 and JC304 strains, respectively.

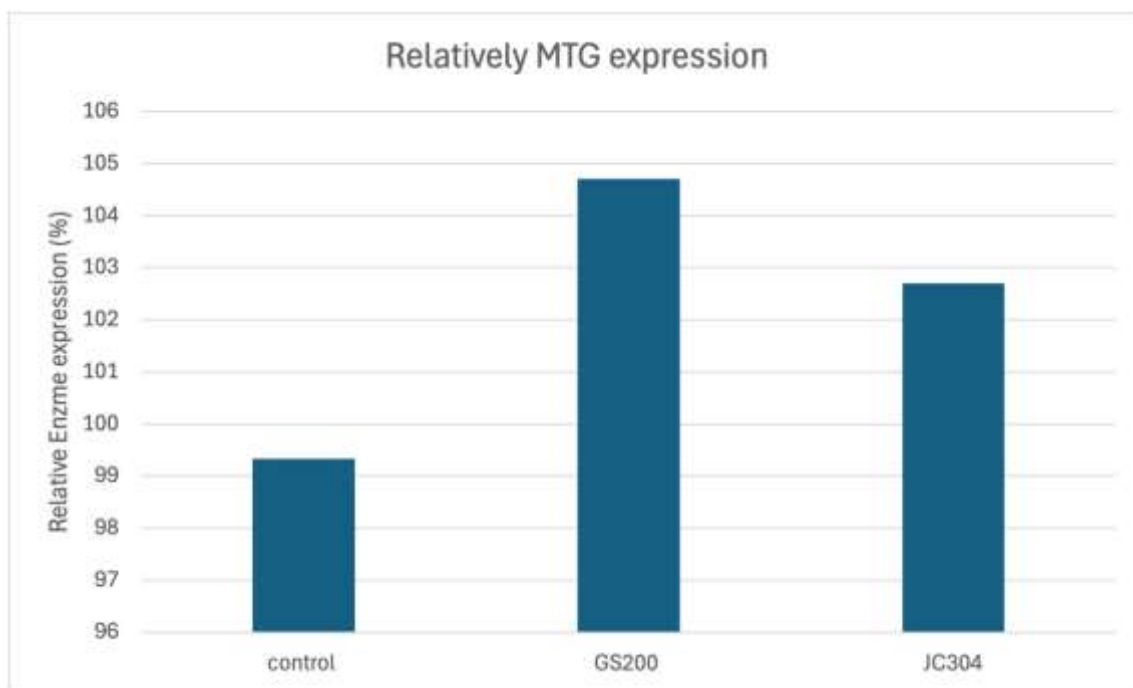


Figure 6. Relative MTG expression level

CONCLUSIONS

In recombinant protein production there are many factors that affect the enzyme production level and quality. The combination of these factors is often organized to ensure the production of the target protein under the highest conditions. However, in some cases, the targeted production level cannot be achieved. To overcome this problem, various manipulations strategies are required in protein production such as overexpressing of chaperone proteins. For this aim, in our study PDI chaperone protein was overexpressed in double auxotrophic *P. pastoris* strains. While the produced protein level is increased between 4.2-2.7%, compared to the control, it is not desired level, and it is relatively short. So, in future studies, a comprehensive optimization studies should be carried out to increase the production level and quality.

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ST8SIA2 as a Biomarker of ER Stress in Glycosylation-Deficient Melanoma Cells

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ABSTRACT

Glycosylation is a prevalent post-translational modification influencing protein folding, stability, and intercellular communication. Altered glycosylation is frequently observed in cancer and contributes to tumor growth, metastasis, and immune evasion. Polysialic acid, added to neural cell adhesion molecule (NCAM) by the α -2,8-sialyltransferase ST8SIA2, reduces cell-cell adhesion and enhances migratory potential. However, the impact of N-linked glycosylation disruption on ST8SIA2 expression in melanoma cells remains unclear. Here, human SKMEL-30 melanoma cells were treated with five micrograms per milliliter tunicamycin to inhibit N-linked glycosylation. Six hours post-treatment, RNA was extracted, cDNA synthesized, and relative expression assessed using the $\Delta\Delta C_t$ method. Tunicamycin markedly upregulated ST8SIA2 expression (approximately twelve point four-fold, p less than ten to the power of minus five), with ROC analysis showing perfect discrimination between treated and control cells (area under the curve equal to one point zero zero zero). These findings indicate that ER stress-mediated transcriptional reprogramming strongly induces ST8SIA2 in melanoma cells, suggesting its potential as a biomarker of glycosylation stress and as a therapeutic target.

Keywords: Melanoma, Glycosylation, Endoplasmic Reticulum Stress, Sialyltransferase, Cancer Biomarker

INTRODUCTION

One of the most prevalent post-translational changes in eukaryotic cells, glycosylation determines the structural and functional diversity of lipids and proteins (Varki and Lowe, 2009; Qusairy and Rada, 2025). Cell surface glycoconjugates are essential for basic biological functions such immunological detection, signal transduction, cell adhesion, and cell-cell interactions (Ohtsubo and Marth, 2006; Stowell et al., 2015). In cancer cells, the glycosylation profile is often reprogrammed; these changes can enhance the invasive, metastatic, and immune evasion capabilities of tumor cells (Mereiter et al., 2019; Munkley and Elliott, 2016; Pinho and Reis, 2015).

The primary organelle where N-linked glycosylation and protein folding take place is the endoplasmic reticulum (ER). Misfolded proteins build up and cause ER stress when the ER's capacity for protein folding is surpassed or glycosylation is compromised (Hetzel, 2012; Wang et al., 2025). In response to this circumstance, cells initiate the unfolded protein response (UPR), an adaptive process. The UPR triggered through three main sensor proteins (PERK, IRE1, and ATF6) and aims to both increase protein folding capacity and eliminate misfolded proteins (Dennis et al., 2009; Walter and Ron, 2011). However, severe or prolonged ER stress can result in lethal cellular outcomes such as apoptosis (Fuster et al., 2005). Tunicamycin is an antibiotic that inhibits the enzyme UDP-N-acetylglucosamine: dolichyl-phosphate N-

acetylglucosamine-1-phosphate transferase, which catalyzes the first step of N-linked glycosylation, thereby halting glycosylation and inducing a strong ER stress response (Elbein, 1987; Martinez et al., 2023). For this reason, it is widely used to investigate the effects of glycosylation disruption on cellular responses. Recent studies have further clarified the importance of this relationship in cancer biology. Zhang et al. (2024) reported that ER stress increased the expression of several sialyltransferase, including ST8SIA2, in breast cancer cells (Zhang et al., 2024). Kim et al. (2023) demonstrated that hypo glycosylation in melanoma altered NCAM polysialylation and increased ST8SIA2 expression in parallel with the ER stress response (Kim et al., 2023). Martinez-Dominguez et al. (2023) reported that tunicamycin treatment induced selective upregulation of the sialyltransferase gene family in various cancer cell lines (Martinez et al., 2023). Li et al. (2024) showed that high ST8SIA2 expression was associated with immune evasion mechanisms and suppressed T cell activity through Siglec interactions (Fuster et al., 2005). Wang et al. (2025) demonstrated that ER stress reprogrammed glycan biosynthesis pathways in metastatic melanoma and that increased ST8SIA2 expression enhanced metastatic potential (Wang et al., 2025).

Disruption of glycosylation and ER stress in cancer cells can affect the expression of many enzymes involved in glycan biosynthesis. For example, tunicamycin treatment in breast cancer cells increased ER stress markers such as GRP78/BiP and CHOP, induced cell cycle arrest, and triggered apoptosis (Hetz, 2012; Zhang et al., 2024). In colorectal cancer cells, glycosidase inhibitors such as kifunensine and castanospermine led to IRE1–XBP1s activation and increased expression of glycan modification genes (Stowell et al., 2015). In melanoma subtypes, glycosylation defects have been shown to reduce cell surface adhesion molecules and increase invasion (Kim et al., 2026).

Sialyltransferase are enzymes that add sialic acid to the termini of glycoconjugates, determining the charge, hydrophobicity, and recognition properties of the cell surface (Harduin et al., 2005). ST8SIA2 is a polysialyltransferase responsible for the synthesis of α -2,8-linked polysialic acid (polySia) (Colley et al., 2014). PolySia is found particularly on neural cell adhesion molecule (NCAM) and reduces cell–cell adhesion, thereby facilitating cell migration (Daniel et al., 2001). In various cancers, increased sialyltransferase expression has been observed as follows: in glioblastoma, high ST8SIA2 expression facilitated cell migration and invasion via polySia-NCAM (Bull et al., 2014); in pancreatic cancer, increased ST6GAL1 expression under ER stress conditions contributed to the accumulation of α -2,6-sialylated glycans and chemotherapy resistance (Schultz et al., 2012); and in breast cancer, increased ST3GAL4 expression under combined hypoxia and ER stress conditions promoted the formation of E-selectin ligands and enhanced metastatic potential (Mereiter et al., 2019). In this context, understanding the effects of glycosylation disruption on ST8SIA2 expression in melanoma cells may contribute to the identification of new therapeutic targets in tumor biology.

MATERIAL AND METHOD

Culture of cells. At 37°C and 5% CO₂, SKMEL-30 cells were cultured in a media made by mixing 1% penicillin-streptomycin with DMEM/Hams: F12 medium that contained 5% FBS. Five x 10⁴ cells were planted and cultured for twenty-four hours in the same medium for each group. The experimental group was treated to 5 µg/ml tunicamycin for 6 hours after the incubation was over.

Isolation of whole RNA. The cells underwent two PBS washes following the treatments and incubation period. As directed by the manufacturer, total RNA was extracted using the Invitrogen™ Total RNA Extraction Kit (Thermo Fisher Scientific, USA). The NanoQ device (based on a 260/280 ratio) was used to measure the concentration and purity of RNA.

Synthesis of cDNA. Following the manufacturer's instructions, the RNA from each sample was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The samples were incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. Before being used again, the resultant cDNA was kept at -20°C.

Quantitative Real-Time PCR (qPCR) Analysis. ST8SIA2 expression was analyzed by quantitative PCR using SYBRTM Green PCR Master Mix on a QuantStudioTM 6 Flex platform, with GAPDH as the internal control. Reactions (11 µl) contained 7 µl Master Mix, 0.4 µM of each primer, 2 µl cDNA, and 1.2 µl nuclease-free water. Cycling conditions included 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative fold changes were calculated using the $\Delta\Delta C_t$ method. Primer information is listed in Table 1.

Table 1. Primer sequences used for ST8SIA2 and GAPDH genes

Gene	Forward (5'-3')	Reverse (5'-3')
ST8SIA2	AGCAGCAGCAGTTCAGGAAG	TCCAGGTTGTTGATGGTGGT
GAPDH	GAAGGTGAAGGTCGGAGTCA	GAAGATGGTGATGGGATTTC

Analysis of statistics. For both the control and tunicamycin groups, all experiments were conducted in triplicate, and the findings are shown as mean $\pm$ standard deviation. For group comparisons, a two-sided Student's t-test was employed. Additionally, ROC curve analysis with AUC computation was used to evaluate the discriminating power of ST8SIA2 expression between groups. GraphPad Prism 9.0 was used for statistical analysis ($p < 0.05$).

RESULTS AND DISCUSSION

Treatment of SKMEL-30 melanoma cells with 5 µg/ml tunicamycin for 6 hours markedly increased ST8SIA2 gene expression. The relative expression value was measured as 1.230 ± 0.199 in the control group, whereas it was 15.236 ± 0.344 in the tunicamycin group, corresponding to an approximately 12.39-fold increase. The difference between the two groups was highly statistically significant (independent samples Student's t-test, $p < 0.00001$) (Figure 1). The low standard deviations demonstrate high reproducibility of the measurements. Experiments were performed in biological triplicates, and results are presented as mean $\pm$ SD. Receiver operating characteristic (ROC) analysis yielded an AUC value of 1.000, indicating that ST8SIA2 expression possesses perfect discriminatory ability between the two groups and could differentiate them with 100% accuracy (Figure 2). These findings suggest that disruption of N-linked glycosylation strongly activates ST8SIA2 transcription and that this gene may play an important role in the ER stress response.

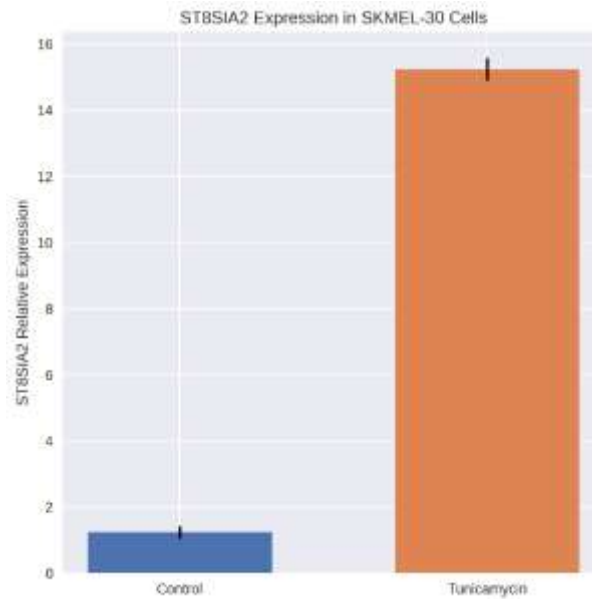


Figure 1. Effect of tunicamycin treatment on ST8SIA2 gene expression in SKMEL-30 melanoma cells. Relative expression values determined by real-time PCR are presented as mean $\pm$ SD ($n=3$). Statistical analysis was performed using a two-tailed Student's *t*-test, yielding $p < 0.00001$.

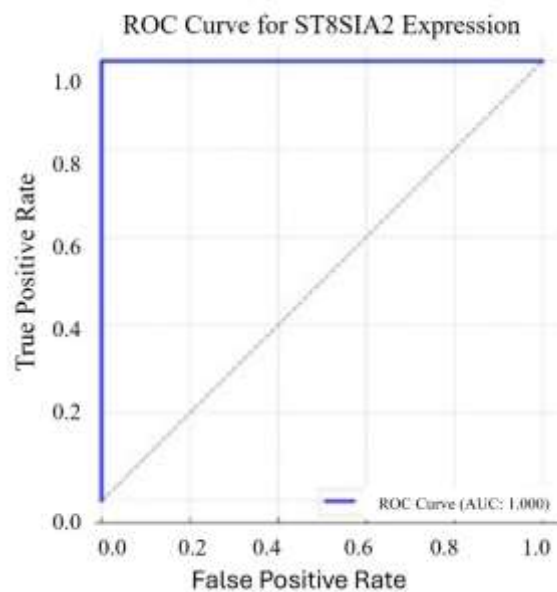


Figure 2. ROC curve analysis of ST8SIA2 expression demonstrating its ability to discriminate between control and tunicamycin-treated SKMEL-30 melanoma cells. The area under the curve (AUC) was 1.000, indicating complete separation between the two groups and highlighting the gene's strong discriminatory potential.

Glycosylation represents one of the most prevalent post-translational modifications, contributing to the structural and functional heterogeneity of proteins and lipids, and playing a key role in modulating the biological characteristics of cell surface glycoconjugates (He et al., 2024; Tian et al., 2025). In cancer, glycosylation patterns are often altered, facilitating tumor cell adhesion, migration, metastasis, and evasion of immune surveillance. Notably, ER stress induced by disruptions in N-linked glycosylation can trigger unfolded protein response (UPR)

pathways and alter the expression of several glycan biosynthesis-related enzymes (de-Souza et al., 2023; Giltrap et al., 2024; Wang et al., 2020).

Sialyltransferase, which add sialic acid to the termini of glycoconjugates, determine the charge, hydrophobicity, and recognition properties of the cell surface (Pearce and Laubli, 2016). They are frequently overexpressed in cancer cells, contributing to metastatic potential, immune evasion, and therapy resistance (Perez et al., 2021). Overexpression of ST8SIA2 in tumors such as glioblastoma and melanoma enhances cell migration and invasion via polySia-NCAM (Daniel et al., 2001). Similarly, in pancreatic cancer, ER stress-induced upregulation of ST6GAL1 increases α -2,6-sialylated glycans and contributes to chemotherapy resistance (Schultz et al., 2012). In breast cancer, combined hypoxia and ER stress conditions elevate ST3GAL4 expression, promoting the formation of E-selectin ligands and enhancing metastatic potential (Mereiter et al., 2019). In addition, sialic acids interact with Siglec receptors on immune cells, suppressing NK cell and macrophage activity and thereby generating an immunosuppressed tumor microenvironment (Kutur, 2022; Li et al., 2024)]. Collectively, these findings highlight the central role of altered glycosylation and sialyltransferase expression in tumor progression and immune escape Pearce et al., 2016).

Recent studies provide further support for these mechanisms. Zhang et al. (2024) reported that ER stress enhances sialyltransferase expression, thereby increasing metastatic potential. Kim et al. (2023) demonstrated that hypo glycosylation in melanoma is associated with increased ST8SIA2 expression. Martinez-Dominguez et al. (2023) found that tunicamycin treatment induces selective upregulation of the sialyltransferase gene family in various cancer cells. Li et al. (2024) identified a critical role for ST8SIA2 in immune evasion, while Wang et al. (2025) showed that ER stress reprograms glycan biosynthesis pathways in metastatic melanoma (Kim et al., 2023; Li et al., 2024; Martinez et al., 2023; Wang et al., 2025; Zhang et al., 2024).

Mechanistically, tunicamycin-induced ER stress may trigger transcriptional reprogramming through the PERK-eIF2 α -ATF4, IRE1-XBP1s, and ATF6 axes. The presence of binding sites for these transcription factors in the ST8SIA2 promoter supports the possibility of direct transcriptional activation (Hetz, 2012; Walter and Ron, 2011). Moreover, epigenetic regulators such as histone acetyltransferases and DNA methyltransferases may modulate ST8SIA2 expression in response to ER stress (Qusairy and Rana, 2025). Functionally, increased ST8SIA2 expression may enhance polySia synthesis capacity. However, inhibition of N-linked glycosylation could impair the folding and surface transport of glycoproteins (e.g., NCAM) that act as substrates for polySia (Freeze and Ng, 2011). Therefore, additional experiments such as EndoN digestion followed by Western blot or immunofluorescence are required to confirm whether the observed mRNA increase translates into functional polySia elevation.

Overall, the present study demonstrates that melanoma cells adapt to ER stress by reorganizing surface sialylation. This adaptation may enhance tumor immune evasion strategies under stress conditions (Li et al., 2024). The dramatic increase of ST8SIA2 expression observed here, together with similar findings in other cancer types, suggests that ER stress-driven regulation of sialyltransferase represents a widespread adaptive mechanism in tumor biology (Munkley and Elliott, 2016). In conclusion, ST8SIA2 may play a central role not only in melanoma progression but also in ER stress-mediated glycan reprogramming. These results indicate that ST8SIA2 should be considered both a biomarker and a potential therapeutic target. Future investigations should address the functional consequences of ST8SIA2 upregulation in vivo models and evaluate the therapeutic potential of combination strategies involving ER stress modulators.

CONCLUSIONS

This study demonstrates that tunicamycin-induced ER stress markedly increases ST8SIA2 gene expression in SKMEL-30 melanoma cells. The findings indicate that disruption of glycosylation can reprogram sialyltransferase expression, thereby altering the surface glycan architecture of tumor cells. This mechanism is thought to play a critical role in tumor progression and immune evasion. In the future, detailed investigation of the role of ST8SIA2 in the ER stress response and its impact on melanoma progression may contribute to the development of new therapeutic strategies.

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QUALITY OVER PRICE: CONSUMER PREFERENCE FOR FREE-RANGE VERSUS BROILER CHICKEN IN AN INDONESIAN TRADITIONAL MARKET

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ABSTRACT

The dual presence of industrially produced broiler chicken and traditionally raised free-range chicken in developing economies presents a complex consumer choice landscape. This study aims to identify the key factors that differentiate consumer purchasing decisions for these two types of poultry and to compare consumer preferences in a traditional market setting in Indonesia. A quantitative survey was conducted with 150 consumers in the traditional markets of Enrekang Regency, Indonesia. The data were analyzed using multiple linear regression to determine the factors influencing purchase decisions for each chicken type and the Mann-Whitney U test to compare consumer perceptions across key variables. The purchase decision for broiler chicken is significantly influenced by price, income, lifestyle, taste, and culture. Conversely, the decision to purchase free-range chicken is driven by product quality, income, lifestyle, taste, and culture, with price not being a significant factor. Comparative analysis reveals that consumers perceive free-range chicken as having significantly higher quality and superior taste but also a higher price. This choice reflects a trade-off between the economic and practical advantages of broiler chicken and the perceived quality and sensory attributes of free-range chicken. This study provides empirical evidence on the drivers of food choice in a transitional economy, highlighting the persistent value of traditional food attributes like quality and taste alongside modern considerations like price and convenience. The findings offer valuable insights for agribusiness stakeholders, marketers, and policymakers aiming to understand and navigate the evolving consumer preferences in emerging markets.

Keywords: consumer behavior; food choice; purchase decision; broiler chicken; free-range chicken; agriculture; Indonesia

INTRODUCTION

Poultry meat, particularly chicken, serves as a fundamental source of animal protein globally, with consumption rates escalating rapidly in developing nations (Kassoh *et al.*, 2024). This growth is propelled by a confluence of factors including population expansion, rising disposable incomes, and a dietary transition toward protein-rich foods (Torgerson and Munoz-Gomez, 2024). Indonesia exemplifies this trend, possessing a robust poultry sector where chicken meat is a dietary staple. The national demand for poultry is met through a dualistic production system that juxtaposes intensive, industrial-scale farming of broiler chickens with the traditional, extensive rearing of native, free-range chickens, known locally as ayam kampung. This production dichotomy is a characteristic feature of many transitional economies, creating a unique and complex landscape for consumer choice (Ariningsih *et al.*, 2024).

The two production systems yield products with distinct characteristics and market positions. Broiler chickens, a result of selective breeding for rapid growth, are characterized by high feed conversion efficiency, which allows for a shorter production cycle and a lower market

price (Stefanetti *et al.*, 2023). This makes broiler meat a widely accessible and affordable protein source for the general population. In contrast, free-range chickens are raised in extensive or semi-intensive systems, often scavenging for a significant portion of their diet. This slower growth process results in meat that is perceived as more "natural" or "organic," with a firmer texture, a more distinct flavor, and a significantly higher market price due to longer rearing times and lower economies of scale (Van Loo *et al.*, 2014). This presents consumers with a fundamental choice: the economic efficiency and convenience of broilers versus the perceived superior taste, health benefits, and cultural authenticity of free-range chicken.

The decision-making process of consumers is influenced by a multitude of interconnected factors. Classic economic drivers such as price and income are particularly salient in price-sensitive markets, where affordability often dictates purchasing behavior (Firoozzare *et al.*, 2024). Concurrently, societal shifts, including urbanization and an increase in dual-income households, have elevated the importance of lifestyle-related factors like convenience and ease of preparation. Beyond these economic and practical considerations, sensory attributes and socio-cultural values play a profound role (Orou Seko *et al.*, 2020). In societies with rich culinary heritages like Indonesia, taste is a paramount determinant of food preference. Furthermore, food is deeply embedded in cultural practices, serving not merely as sustenance but as a carrier of identity, tradition, and social cohesion.

While numerous studies have explored consumer preferences for different types of chicken, a significant gap remains in the literature (DiSantis *et al.*, 2014; Barska *et al.*, 2023). Few studies have quantitatively modeled and directly compared the distinct sets of factors driving the purchase of both broiler and free-range chicken within the same traditional market context in an emerging economy (Andriyani *et al.*, 2024; Dhar *et al.*, 2024). Previous Indonesian studies have often focused on a limited set of variables or a different geographical area, leaving the unique interplay of economic, sensory, and cultural drivers in regions like South Sulawesi underexplored. This study aims to fill this gap by providing a comprehensive analysis of consumer choice in the traditional markets of Enrekang Regency. The specific objectives of this research were: (1) to identify and analyze the factors significantly influencing consumer purchase decisions for broiler chicken; (2) to identify and analyze the factors significantly influencing consumer purchase decisions for free-range chicken; and (3) to comparatively analyze consumer perceptions of broiler and free-range chicken across key product attributes.

MATERIALS AND METHODS

Research Design and Stages

This study uses a quantitative method designed in five main stages (Figure 1). The first stage was the development of a conceptual framework, which included formulating the research question: "What factors differentiate the purchasing decisions of consumers for broiler and free-range chicken in a traditional Indonesian market?", reviewing relevant literature on consumer behavior (price, product quality, income, lifestyle, taste, and culture), and developing hypotheses. The second stage was research design and planning, which involved designing the survey method, identifying the population (consumers in the traditional markets of Enrekang Regency) and sampling technique (multistage random sampling), and developing the variable measurement instrument using a questionnaire with a Likert scale. Subsequently, the third stage was research implementation, consisting of primary data collection through the distribution of questionnaires to 150 respondents and instrument validation. The fourth stage was data analysis, which included data screening, descriptive analysis of the respondent profile, multiple linear regression analysis to identify the determinants of purchasing decisions, Mann-Whitney U test

analysis to compare consumer perceptions, and interpretation and discussion of the results. Finally, the fifth stage involved drawing conclusions and dissemination, which included formulating conclusions, theoretical and practical implications, and identifying limitations and suggestions for future research.

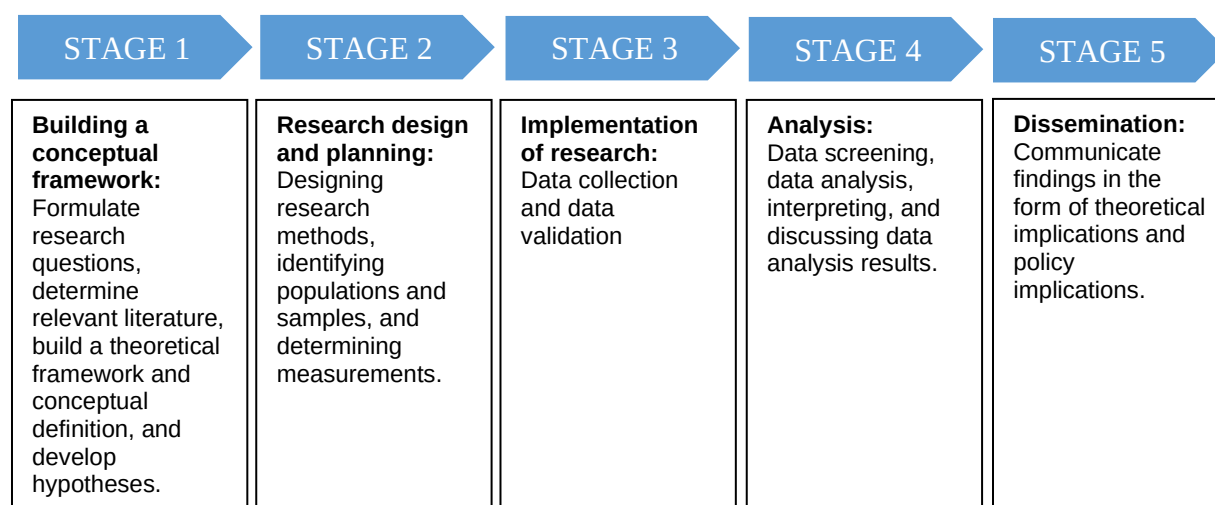


Figure 1. Research Stage

Sample, data collection, and ethical approval

The research was conducted in the traditional markets of Enrekang Regency, South Sulawesi, Indonesia (Figure 2). This regency was selected as the study site due to its significant poultry population and established patterns of both broiler and free-range chicken consumption, providing a representative sample of a transitional rural economy. Data for the study were collected over a two-month period, from March to December 2024. A multistage random sampling technique was employed to select a representative sample of consumers. In the first stage, nine of the twelve sub-districts within Enrekang Regency were randomly selected. In the second stage, six major traditional markets were randomly chosen from within the selected sub-districts, based on their high volume of visitors and commercial activity. The target population was defined as household consumers actively purchasing chicken meat in these markets. The sample size was calculated using the Slovin formula, with a 10% margin of error. The initial calculation yielded a minimum required sample of 100 respondents, which was subsequently expanded to 150 participants to enhance the statistical power of the analysis. Based on the predetermined population, it can be assumed that the population is all subjects or objects of research that have certain characteristics. The population that is the object of this study is the total number of households (231,301 people) in Enrekang Regency according to the Enrekang Regency Statistics Agency 2024. The sample was evenly divided into two groups: 75 consumers who primarily purchase broiler chicken and 75 consumers who primarily purchase free-range chicken. Respondents were selected based on the following inclusion criteria: (1) they were domiciled in Enrekang Regency; (2) they had prior experience purchasing both broiler and free-range chicken, ensuring familiarity with both products; and (3) they provided informed consent to participate in the study. Data were collected using a structured, researcher-administered questionnaire to ensure consistency and completeness of responses. In this study, prior to data collection, each research respondent was asked to provide written consent and was informed about the purpose of completing the questionnaire. Each respondent was also informed that their participation as a respondent was voluntary and completely confidential. All research procedures involving human participants have obtained ethical approval from the

Hasanuddin University Research and Quality Assurance Institute, number 0419/C3/DT.05.00/2025, and are declared to be in accordance with the ethical standards and principles set out in the Declaration of Helsinki.

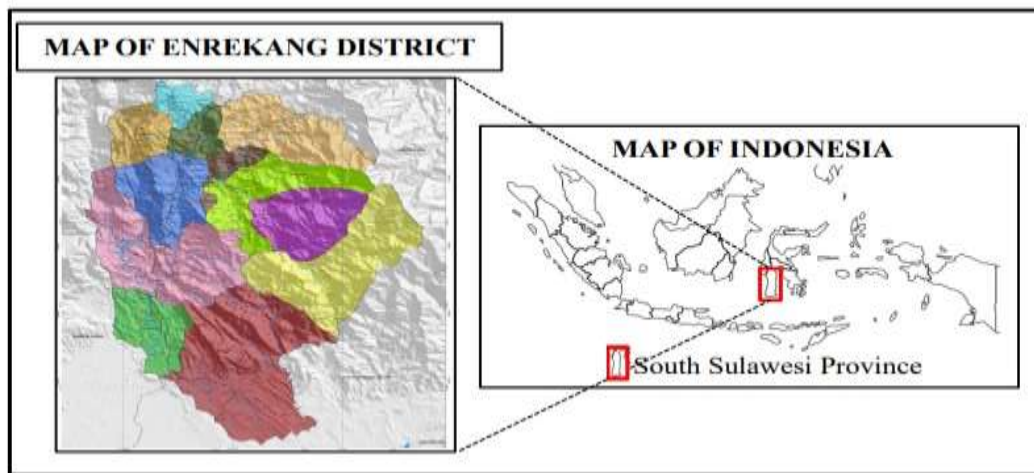


Figure 2. Research Location Map in Indonesia

Variables and Measurement

The study investigated one dependent variable and six independent variables. The dependent variable was the Purchase Decision for each chicken type (broiler and free-range). This was operationalized as a composite score derived from several items on the questionnaire that assessed the consumer's overall intention and preference to purchase a specific type of chicken, as well as instruments developed based on preliminary studies and tested theories. The independent variables were defined as follows: Price (X1): The consumer's perception of the product's affordability, its value for money, and its price relative to alternatives and their purchasing power; Product Quality (X2): Perceptions related to the meat's physical and sensory attributes, including texture, color, size, perceived nutritional value, fat content, and freshness or shelf life. Income (X3): The influence of the respondent's monthly household income and the number of dependents on their purchasing capacity and product choice; Lifestyle (X4): The role of personal habits and values, such as health consciousness, specific dietary practices, and the need for convenience and speed in food preparation; Taste (X5): The consumer's sensory preferences for the flavor and aroma of the meat, its suitability for various local dishes, and the overall satisfaction derived from its consumption; Culture (X6): The influence of family traditions, routine consumption habits, and the symbolic or practical role of the chicken type in social gatherings, ceremonies, or religious events. Finally, the purchase decision variable (Y) is measured using 5 items adopted from (Source), consisting of: (1) Problem recognition, (2) Information search, (3) Alternative evaluation, (4) Consumer decision, (5) Post-purchase behavior. All perceptual variables were measured using a 5-point Likert scale, with responses ranging from 1 (Strongly Disagree) to 5 (Strongly Agree).

Data analysis

The collected data were coded and analyzed using IBM SPSS Statistics Version 26. The analysis proceeded in several stages. First, descriptive statistics (frequencies, percentages, means) were used to summarize the socio-demographic profile of the respondents. The reliability and validity of the measurement instrument were assessed prior to hypothesis testing. Instrument validity was confirmed using Pearson correlation analysis for each item, while

internal consistency was evaluated using Cronbach's Alpha, with a coefficient of 0.60 serving as the threshold for acceptable reliability. To address the first two research objectives, two separate multiple linear regression models were developed to identify the significant predictors of purchase decisions for broiler chicken and free-range chicken. The regression model was specified as follows:

$$Y = a + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + e$$

where Y is the Purchase Decision, a is the constant, X_1-X_6 are the independent variables, $b_1 - b_6$ are the regression coefficients, and e is the error term.

Prior to conducting the regression analysis, a series of classical assumption tests were performed to ensure the data's suitability and to confirm that the sample data represented a normal or near-normal distribution. These tests included: (1) a Normality Test using the Kolmogorov-Smirnov statistic to assess whether the regression model's residuals were normally distributed; (2) a Multicollinearity Test to check for high correlations between independent variables, evaluated using the Variance Inflation Factor (VIF) and tolerance values; and (3) a Heteroscedasticity Test, which used scatterplots of SRESID versus ZPRED to detect any patterns indicating unequal variance in the residuals. A valid regression model should not violate these assumptions. Hypothesis testing for the regression models was conducted using several statistical tests. The F-test (simultaneous test) was used to determine if all independent variables (Price, Product Quality, Income, Lifestyle, Taste, and Culture) collectively had a significant effect on the dependent variable (Purchase Decision). The t-test (partial test) was used to assess the individual significance of each independent variable on the purchase decision, with a significance level of 0.05. Finally, the Coefficient of Determination (R^2) was calculated to measure the proportion of the variance in the purchase decision that could be explained by the independent variables in the model. To address the third objective, the Mann-Whitney U test, a non-parametric test suitable for comparing two independent groups, was used. This test was employed to determine if there were statistically significant differences in consumer perceptions between the broiler and free-range chicken consumer groups across the six independent variables and the overall purchase decision. For all inferential statistical tests, the significance level was set at $p < 0.05$ (Sarstedt and Christian M. Ringle, 2017).

RESULTS

Demographic profiles

The study surveyed 150 consumers, evenly split between those who primarily purchase broiler chicken ($n=75$) and those who primarily purchase free-range chicken ($n=75$). The socio-demographic characteristics of the two consumer groups exhibited notable differences, as detailed in Table 1. The free-range chicken consumer group was characterized by a higher proportion of older individuals (44.0% aged over 40) compared to the broiler group (33.3%). While both groups were predominantly female, the proportion was slightly higher among broiler consumers (94.7%) than free-range consumers (88.0%). A significant divergence was observed in income levels. The majority of broiler consumers (76.0%) reported a monthly income of IDR 5,000,000 or less. In stark contrast, the majority of free-range chicken consumers (82.6%) reported an income above IDR 5,000,000, with over half of this group (50.7%) earning between IDR 7,000,000 and IDR 9,000,000. This indicates a clear socio-economic stratification between the two consumer segments.

Table 1. Demographic characteristics of respondents

Description	Category	Broiler Consumers (n=75) (%)	Free-Range Consumers (n=75) (%)
Gender	Male	71 (94,7)	66 (88,0)
	Female	4 (5,3)	9 (12,0)
Age	20-30	24 (32,0)	18 (24,0)
	31-40	26 (34,7)	24 (32,0)
	> 40	25 (33,3)	33 (44,0)
Level of education	High School	17 (22,7)	14 (18,7)
	Undergraduate	52 (69,3)	57 (76,4)
	Postgraduate	6 (8,0)	4 (5,3)
Occupation	Government employees	24 (32,0)	29 (38,67)
	Private Employee	12 (16,0)	19 (25,33)
	Entrepreneur	16 (21,33)	10 (13,33)
	Housewife	14 (18,67)	12 (16,0)
	Non-permanent	9 (12,0)	5 (6,67)
Monthly Income (IDR)	< 3,000,000	19 (25)	2 (2,7)
	< 3,000,000 - 5,000,000	39 (52,0)	11 (14,7)
	5,000,000 - 7,000,000	14 (18,7)	19 (25,3)
	> 7,000,000	3 (4,0)	43 (57,3)

Source: Data processing results, 2025

Instrument Validity and Reliability

Prior to hypothesis testing, the validity and reliability of the measurement instrument were assessed. Validity was determined by examining the Pearson Correlation (r -hitung) for each item, with a threshold of $r > 0.227$ indicating a valid item. Reliability was assessed using Cronbach's Alpha, with a value greater than 0.60 considered reliable. The results for both the broiler and free-range consumer groups are summarized in Table 2, respectively. All items were found to be valid, and all constructs demonstrated acceptable reliability, confirming the suitability of the instrument for further analysis.

Table 2. Convergent validity and reliability.

Construct	Item	Convergent Validity		Reliability	
		Loading Factor	AVE	Cronbach's Alpha	Rho_C
Price (X1)	X1.1	0.827	0.782	0.911	0.959
	X1.2	0.901			
	X1.3	0.778			
	X1.4	0.900			
Product Quality (X2)	X2.1	0.884	0.791	0.835	0.944
	X2.2	0.697			
	X2.3	0.709			
	X2.4	0.725			
	X2.5	0.752			
	X2.6	0.690			
	X2.7	0.834			
Income (X3)	X3.1	0.838	0.815	0.912	0.963
	X3.2	0.914			
	X3.3	0.919			
	X3.4	0.866			
Lifestyle (X4)	X4.1	0.912	0.967	0.935	0.921
	X4.2	0.951			
	X4.3	0.945			
	X4.4	0.949			
	X4.5	0.948			
Taste (X5)	X5.1	0.883	0.934	0.875	0.913
	X5.2	0.938			
	X5.3	0.934			
	X5.4	0.898			
Culture (X6)	X6.1	0.873	0.934	0.912	0.922
	X6.2	0.847			
	X6.3	0.884			
	X6.4	0.924			
	X6.5	0.917			
	X6.6	0.894			
	X6.7	0.929			
	X6.8	0.904			
Purchase Decision (Y)	Y.1	0.731	0.739	0.887	0.923
	Y.2	0.620			
	Y.3	0.743			
	Y.4	0.664			
	Y.5	0.583			
	Y.6	0.572			

Source: Data processing results, 2025

Determinants of Broiler Chicken Purchase Decisions

The multiple linear regression model developed to explain the purchase decision for broiler chicken was statistically significant ($F(6,68) = 31.252, p < 0.001$) and accounted for a substantial portion of the variance ($\text{Adjusted } R^2 = 0.710$). The results, presented in Table 3, indicate that five of the six independent variables were significant predictors. Lifestyle emerged as the strongest predictor ($\beta = 0.351, p < 0.001$), followed by Income ($\beta = 0.264, p = 0.004$). Culture ($\beta = 0.198, p = 0.010$), Taste ($\beta = 0.189, p = 0.012$), and Price ($\beta = 0.179, p = 0.033$) also had significant positive influences on the decision to purchase broiler chicken. In contrast, Product Quality ($\beta = 0.109, p = 0.224$) was found to have no statistically significant effect on the purchase decision for this type of chicken.

Table 3. Multiple linear regression results for factors influencing broiler chicken

Variable	Unstandardized Coefficient (B)	Std. Error	Standardized Coefficient (β)	t-Values	p- Values
Constant	3.558	1.408		2.527	0.014
Price (X1)	0.219	0.100	0.179	2.180	0.033
Product Quality (X2)	0.073	0.060	0.109	1.227	0.224
Income (X3)	0.285	0.097	0.264	2.947	0.004
Lifestyle (X4)	0.231	0.049	0.351	4.709	0.001
Taste (X5)	0.178	0.069	0.189	2.594	0.012
Culture (X6)	0.104	0.039	0.198	2.667	0.010

Source: Data processing results, 2025

Determinants of Free-Range Chicken Purchase Decisions

The regression model for the free-range chicken purchase decision was also statistically significant ($F(6,68) = 38.735$, $p < 0.001$) and demonstrated strong explanatory power (Adjusted $R^2 = 0.754$). As shown in Table 4, the significant predictors for purchasing free-range chicken were different from those for broiler chicken. Lifestyle was again the most influential factor ($\beta = 0.434$, $p < 0.001$), followed by Taste ($\beta = 0.225$, $p = 0.004$) and Culture ($\beta = 0.220$, $p = 0.040$). Product Quality ($\beta = 0.193$, $p = 0.004$) and Income ($\beta = 0.177$, $p = 0.026$) were also significant positive predictors. Notably, Price ($\beta = 0.088$, $p = 0.250$) was not a statistically significant factor in the decision to purchase free-range chicken.

Table 4. Multiple linear regression results for factors influencing free-range chicken

Variable	Unstandardized Coefficient (B)	Std. Error	Standardized Coefficient (β)	t-Values	p- Values
Constant	3.308	2.121		1.559	0.124
Price (X1)	0.113	0.098	0.088	1.159	0.250
Product Quality (X2)	0.160	0.053	0.193	3.013	0.004
Income (X3)	0.166	0.073	0.177	2.273	0.026
Lifestyle (X4)	0.322	0.077	0.434	4.190	0.001
Taste (X5)	0.220	0.073	0.225	3.009	0.004
Culture (X6)	0.103	0.049	0.220	2.091	0.040

Source: Data processing results, 2025

Comparative Analysis of Consumer Perceptions

The Mann-Whitney U test was conducted to compare perceptions between the two consumer groups across the six variables and the overall purchase decision. The results, summarized in Table 6, reveal statistically significant differences for most variables. Consumers perceived a significant difference in Price ($U = 962.0$, $p < 0.001$), with the mean rank for free-range chicken (95.17) being much higher, indicating it is perceived as more expensive. Similarly, there was a significant difference in perceived Product Quality ($U = 925.0$, $p < 0.001$) and Taste ($U = 1035.5$, $p < 0.001$), where free-range chicken scored significantly higher mean ranks (95.67 and 94.19, respectively), indicating superior perceived quality and taste. The overall Purchase Decision also showed a significant difference ($U = 900.0$, $p < 0.001$), with a much higher mean rank for free-range chicken (96.00), suggesting a stronger conviction among its purchasers. Significant differences were also found for Income ($U = 1050.5$, $p < 0.001$) and Lifestyle ($U = 2150.0$, $p = 0.049$). However, there was no significant difference in the perception of Culture ($U = 2728.0$, $p = 0.543$), suggesting its importance is similarly valued by both groups.

Table 5. Comparative analysis of consumer perceptions using Mann-Whitney U test

Variable	Unstandardized Coefficient (B)	Std. Error	Standardized Coefficient (β)	t-Values	p- Values
Constant	3.308	2.121		1.559	0.124
Price (X1)	0.113	0.098	0.088	1.159	0.250
Product Quality (X2)	0.160	0.053	0.193	3.013	0.004
Income (X3)	0.166	0.073	0.177	2.273	0.026
Lifestyle (X4)	0.322	0.077	0.434	4.190	0.001
Taste (X5)	0.220	0.073	0.225	3.009	0.004
Culture (X6)	0.103	0.049	0.220	2.091	0.040

Source: Data processing results, 2025

DISCUSSION

The results of this study reveal that the decision to purchase broiler chicken is significantly influenced by price. Consumers in this segment feel that the affordability of broiler chicken is a primary consideration, as it allows them to meet their family's daily protein needs within their budget. This finding is consistent with research by (Barska *et al.*, 2023; Haddad, 2023; Chen *et al.*, 2024), which also found that price has a significant effect on purchasing decisions for staple food items. In contrast, the results (DiSantis *et al.*, 2014) show that price is not a significant factor for consumers of free-range chicken. This consumer segment, characterized by higher income levels, prioritizes other attributes over cost. This aligns with the findings of (Hollis, 2024; Kang, Ota and Ushijima, 2024), who noted that for certain consumer groups, price sensitivity decreases when perceived quality and health benefits are high. This study found that product quality is a significant determinant in the purchase of free-range chicken but not for broiler chicken. Consumers of free-range chicken perceive it as having superior quality, including better texture, a more natural taste, and being free from chemical additives. This perception of quality justifies its higher price and becomes a key driver of their purchasing decision. This finding is in line with research (Orou Seko *et al.*, 2020; An *et al.*, 2021), who concluded that product quality positively and significantly affects consumer purchase decisions for food products. The lack of significance for broiler chicken suggests that consumers either perceive a standard, undifferentiated quality across broiler products in the market or that their decision is more heavily weighted by economic factors like price.

The results indicate that income significantly influences the purchase decisions for both chicken types. For broiler consumers, income acts as a constraint, making the lower-priced broiler a more feasible option for regular consumption. This is supported by research from (Dhar *et al.*, 2024; Firoozzare *et al.*, 2024; Kencana, Rubiyanto and Widadie, 2024), which states that household income level is a crucial factor in determining the demand for various food items. For free-range chicken consumers, higher income acts as an enabler, providing the financial capacity to prioritize quality and health over price. This aligns with the findings of (Desye *et al.*, 2023; Mamun *et al.*, 2023), who noted that as income increases, consumer spending on higher-quality and premium food products also tends to rise. The clear demographic split in income levels between the two consumer groups in this study further reinforces this conclusion. Furthermore, this study found that lifestyle is the most significant predictor for both consumer groups. For broiler consumers, lifestyle is associated with a need for convenience and practicality. They feel that broiler chicken, being readily available and quick to cook, fits well into a modern, fast-paced lifestyle. This is consistent with findings by (Chaudhuri *et al.*, 2023; Andriyani *et al.*, 2024), who noted that increasing income and modern lifestyles drive demand for practical and easy-to-prepare foods. For free-range chicken consumers, lifestyle is strongly linked to health consciousness. These consumers feel that

choosing free-range chicken is part of a healthier lifestyle, as the product is perceived as more natural and nutritious. This finding is supported by research from (Sun and Liang, 2021; García-Salirrosas *et al.*, 2024), which found that a healthy lifestyle has a significant positive effect on purchasing decisions for natural food products.

The results also show that taste is a significant factor for both groups. Consumers of free-range chicken strongly believe its taste is superior, more savory, and authentic, which is a primary reason for their preference. This aligns with research by (Nguyen and Wismer, 2019; Stangierski *et al.*, 2020; Yu *et al.*, 2024), who stated that a unique and pleasant taste will always attract consumers, regardless of price. While broiler consumers also consider taste, their evaluation is likely based on its suitability for daily, versatile dishes rather than a premium sensory experience. The comparative analysis (Mann-Whitney U test) confirms this, showing that free-range chicken is perceived as having a significantly superior taste.

Finally, the study found that culture influences the purchase of both chicken types. Consumers from both groups feel that chicken consumption is embedded in their family traditions and social activities. For broiler consumers, its affordability and availability make it a staple for daily meals and large, informal gatherings. For free-range chicken consumers, its use is often tied to special occasions, cultural ceremonies, and traditional recipes that require its specific texture and flavor, reflecting its role as a carrier of culinary heritage. This finding is in line with (Møretrø *et al.*, 2021; Kassoh *et al.*, 2024), who explained that culture is a fundamental determinant of a person's desires and purchasing behavior. The Mann-Whitney U test showed no significant difference in the perceived importance of culture between the two groups, suggesting that while the context of consumption differs, its cultural value is recognized by both.

The findings of this study have direct and actionable implications for various stakeholders in the poultry value chain. For agribusiness and marketers, the clear segmentation of the market necessitates tailored strategies. Marketing for broiler chicken should emphasize value, affordability, convenience, and its versatility for everyday family meals. Promotional tactics such as price discounts and bulk-purchase offers would likely be effective. In contrast, marketing for free-range chicken should focus on building a premium brand narrative centered on superior quality, authentic taste, health benefits (e.g., "natural," "raised without antibiotics"), and its esteemed place in local culture. This product should be targeted at higher-income, health-conscious consumers who are less sensitive to price. For smallholder farmers, who are the primary producers of free-range chicken, this study confirms the existence of a viable and profitable market segment willing to pay a premium for quality. Farmers can enhance their livelihoods by focusing on production practices that improve the attributes valued by this segment, such as taste, texture, and the perception of naturalness. Developing local branding or certifications could further help in capturing this value. For policymakers, the results advocate for a dual-pronged approach to supporting the poultry sector. Policies should continue to ensure the affordability, safety, and stable supply of broiler chicken, as it is a critical source of protein for the majority of the population. Simultaneously, programs should be developed to support and preserve the free-range chicken sub-sector. This could include assistance for smallholder farmers, conservation of native breeds, and promotion of ayam kampung as part of Indonesia's culinary and cultural heritage, recognizing its importance for rural incomes and food sovereignty.

CONCLUSIONS

The choice between industrially produced broiler chicken and traditionally raised free-range chicken in the traditional markets of Indonesia is governed by a complex and distinct set of factors for different consumer segments. The purchase of broiler chicken is largely a

pragmatic decision, significantly driven by its lower price, convenience that fits modern lifestyles, and its establishment in daily cultural habits. In this segment, economic rationality prevails. In contrast, the decision to purchase free-range chicken is a value-laden choice where perceived product quality and superior taste are paramount. For these consumers, who typically have higher incomes, price is not a significant barrier; they willingly pay a premium for attributes they associate with health, authenticity, and cultural tradition. These findings highlight a clear market segmentation within a traditional food system, reflecting a broader societal transition where modern economic behaviors coexist with enduring cultural values. This provides crucial insights for producers, marketers, and policymakers seeking to effectively meet the diverse and evolving needs of consumers in emerging economies.

Limitations and Suggestions for Future Research

This study, while providing valuable insights, has several limitations that should be acknowledged. First, its geographical scope is confined to the traditional markets of Enrekang Regency, a predominantly rural area. The findings may not be directly generalizable to urban centers in Indonesia, where consumer preferences, lifestyles, and market dynamics are likely to be substantially different. Second, the cross-sectional design of the study provides a static snapshot of consumer preferences. A longitudinal study would be beneficial to track how these preferences evolve over time in response to changes in income, education, and exposure to global food trends. Third, the study relies on self-reported data, which may be subject to social desirability bias, particularly concerning sensitive topics like income or health-conscious behavior. Based on these limitations, several avenues for future research are proposed. Comparative studies should be conducted in major urban areas like Jakarta or Makassar to contrast rural and urban consumer behavior toward broiler and free-range chicken. Future research could also benefit from employing qualitative methods, such as in-depth interviews or focus groups, to gain a richer understanding of the cultural meanings, sensory experiences, and emotional drivers that underpin consumer choices. Finally, it would be valuable to investigate the potential impact of branding, certifications (e.g., "organic," "halal"), and modern retail channels on consumer willingness-to-pay and preference for free-range chicken.

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BETA-ADRENOBLOCKERS IN THE 21ST CENTURY: NAVIGATING THE CROSSROADS OF LEGACY, INNOVATION, AND FUTURE THERAPEUTICS

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ABSTRACT

Background: Beta-blockers (β -blockers) are competitive antagonists of β -adrenergic receptors, first introduced into clinical practice more than five decades ago. They have long been considered a cornerstone in the treatment of cardiovascular diseases, including hypertension, angina pectoris, arrhythmias, and myocardial infarction. However, the role of β -blockers has been redefined with the advent of newer classes of drugs such as angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), calcium channel blockers (CCBs), and direct renin inhibitors. **Objective:** This review aims to critically evaluate the clinical value of β -blockers in current cardiovascular medicine, with particular emphasis on their use in hypertension, ischemic heart disease, heart failure, and arrhythmias. The analysis compares traditional and vasodilating β -blockers with newer pharmacological alternatives, highlighting the benefits, limitations, and guideline-based recommendations. **Methods:** A structured literature search was performed across MEDLINE, Embase, Current Contents, and Cochrane Library databases. The review included randomized controlled trials (RCTs), cohort studies, case-control studies, meta-analyses, and systematic reviews published between 2000 and 2024. Key search terms included “beta-blockers, hypertension, angina pectoris, myocardial infarction, arrhythmias.” Relevant clinical guidelines from the European Society of Cardiology (ESC), American Heart Association (AHA), and National Institute for Health and Care Excellence (NICE) were also reviewed. **Results:** Evidence suggests that β -blockers are no longer recommended as first-line therapy for uncomplicated hypertension, especially in patients over 60 years, due to inferior protection against stroke and overall mortality compared to ACEIs, ARBs, and CCBs. Nevertheless, β -blockers remain highly effective in reducing angina symptoms, preventing reinfarction, and lowering mortality in post-myocardial infarction patients with reduced left ventricular ejection fraction. They are essential in managing arrhythmias, including atrial fibrillation and ventricular tachyarrhythmias. Vasodilating β -blockers such as carvedilol and nebivolol may offer metabolic advantages over traditional agents. **Conclusion:** Although β -blockers have a reduced role in the initial management of hypertension, they continue to be indispensable in ischemic heart disease, post-myocardial infarction care, heart failure with reduced ejection fraction, and arrhythmia management. Their future use may increasingly rely on precision medicine approaches, selecting specific subtypes tailored to patient comorbidities and genetic profiles.

Keywords: beta-blockers, hypertension, angina pectoris, myocardial infarction, arrhythmias, cardiovascular pharmacology

INTRODUCTION

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality worldwide, accounting for approximately 17.9 million deaths annually according to the World Health Organization (WHO, 2023). Over the past five decades, the development of pharmacological therapies has dramatically improved the prevention and management of cardiovascular disorders. Among these therapies, **beta-adrenergic receptor antagonists (β -blockers)** stand out as one of the most significant achievements in clinical pharmacology.

The discovery of β -blockers is attributed to **Sir James Black**, who introduced the first clinically useful agent, propranolol, in the 1960s. This innovation earned him the Nobel Prize in Physiology or Medicine in 1988, as it revolutionized the treatment of angina pectoris and subsequently other cardiovascular conditions. Since then, β -blockers have become widely prescribed drugs, initially considered **first-line therapy for hypertension and ischemic heart disease**.

Pharmacologically, β -blockers exert their effects by **antagonizing β -adrenergic receptors**, thereby reducing sympathetic stimulation of the heart. This leads to a decrease in heart rate, myocardial contractility, and oxygen demand, making them highly effective in conditions characterized by increased adrenergic drive, such as tachyarrhythmias and myocardial ischemia. In addition, certain β -blockers exhibit vasodilatory properties, antioxidant activity, and endothelial benefits, which broaden their therapeutic potential.

Despite their widespread use and established efficacy, the role of β -blockers has been **increasingly debated in recent years**. Large randomized controlled trials and meta-analyses have demonstrated that β -blockers may provide **inferior protection against stroke and all-cause mortality** when compared with other antihypertensive agents, particularly in elderly patients with uncomplicated hypertension. Moreover, traditional β -blockers such as atenolol and propranolol have been associated with **adverse metabolic effects**, including impaired glucose tolerance and increased risk of new-onset diabetes mellitus.

Nevertheless, β -blockers remain indispensable in several clinical scenarios. They continue to represent the **cornerstone of therapy** in patients with:

- **Acute myocardial infarction (MI)** and secondary prevention of reinfarction.
- **Heart failure with reduced ejection fraction (HFrEF)**, where they improve survival and reduce hospitalizations.
- **Cardiac arrhythmias**, including atrial fibrillation and ventricular tachyarrhythmias.
- **Stable angina pectoris**, where they relieve symptoms and improve exercise tolerance.

The objective of this review is to provide a **comprehensive evaluation of the current role of β -blockers** in cardiovascular medicine, discussing their pharmacology, therapeutic applications, adverse effects, and future perspectives. Special emphasis is placed on comparing traditional β -blockers with vasodilatory agents such as carvedilol and nebivolol, and assessing their relevance in the era of modern antihypertensive and antianginal drugs.

Classification and Mechanism of Action of Beta-Blockers

1.1 Classification of Beta-Blockers

Beta-blockers are a heterogeneous class of drugs that share a common ability to antagonize the effects of catecholamines (epinephrine and norepinephrine) at **β -adrenergic receptors**. These receptors are subdivided into:

- **β_1 receptors** – predominantly located in the heart (increase heart rate, contractility, renin release).
- **β_2 receptors** – located in the bronchial smooth muscle, vascular smooth muscle, and skeletal muscle (mediate vasodilation and bronchodilation).
- **β_3 receptors** – found in adipose tissue (regulate lipolysis and thermogenesis).

The classification of β -blockers is based on several criteria:

A. Selectivity

- **Non-selective β -blockers**: antagonize both β_1 and β_2 receptors (e.g., propranolol, nadolol, timolol).
- **Cardioselective β -blockers**: preferentially block β_1 receptors at therapeutic doses, reducing cardiac side effects (e.g., metoprolol, bisoprolol, atenolol, esmolol).

B. Vasodilatory Properties

- Some β -blockers exert additional vasodilatory actions:
 - **Carvedilol** – blocks β_1 , β_2 , and α_1 receptors.
 - **Nebivolol** – enhances nitric oxide (NO) release from the endothelium.
 - **Labetalol** – combined α - and β -blockade, useful in hypertensive emergencies.

C. Intrinsic Sympathomimetic Activity (ISA)

- A few β -blockers (e.g., pindolol, acebutolol) have **partial agonist activity**, providing less resting bradycardia but weaker cardioprotective effects.

D. Lipid Solubility

- **Highly lipophilic agents** (e.g., propranolol, metoprolol) cross the blood-brain barrier and may cause central nervous system (CNS) effects such as fatigue or depression.
- **Hydrophilic agents** (e.g., atenolol, nadolol) are less likely to cause CNS side effects and are renally excreted.

Table 1. Classification of Beta-Blockers

Category	Examples	Key Features
Non-selective β-blockers	Propranolol, Nadolol, Timolol	Block β_1 and β_2 ; risk of bronchospasm; effective in angina, portal hypertension
Cardioselective β-blockers	Metoprolol, Bisoprolol, Atenolol, Esmolol	Prefer β_1 blockade; safer in asthma/COPD; widely used in heart failure, arrhythmias
Vasodilating β-blockers	Carvedilol, Nebivolol, Labetalol	Additional α_1 blockade or NO release; favorable hemodynamic and metabolic profile
With ISA (partial agonists)	Pindolol, Acebutolol	Less resting bradycardia; weaker mortality benefit in post-MI and heart failure
Lipophilic	Propranolol, Metoprolol	CNS penetration; may cause fatigue, nightmares, depression
Hydrophilic	Atenolol, Nadolol	Minimal CNS effects; renally cleared

1.2 Mechanism of Action

The cardiovascular effects of β -blockers stem from their **inhibition of β -adrenergic signaling**:

1. β_1 receptor blockade (cardiac effects):

- $\downarrow$ Heart rate (negative chronotropy).
- $\downarrow$ Myocardial contractility (negative inotropy).
- $\downarrow$ Conduction velocity through the AV node (negative dromotropy).
- $\downarrow$ Renin release from the juxtaglomerular apparatus $\rightarrow$ $\downarrow$ RAAS activation $\rightarrow$ $\downarrow$ blood pressure.

2. β_2 receptor blockade (vascular and bronchial effects):

- Vasoconstriction (loss of β_2 -mediated vasodilation).
- Bronchoconstriction (caution in asthma/COPD).

3. Additional effects (drug-specific):

- Carvedilol $\rightarrow$ antioxidant activity and α_1 blockade (vasodilation).
- Nebivolol $\rightarrow$ stimulation of endothelial NO release (vasodilation and improved endothelial function).

Through these mechanisms, β -blockers **reduce myocardial oxygen demand**, stabilize arrhythmias, and blunt neurohormonal activation in heart failure.

2. Therapeutic Applications of Beta-Blockers

Beta-blockers remain an important pharmacological class in cardiovascular medicine, though their role has evolved with the development of new therapeutic options. Their use depends on

the underlying pathology, age of the patient, and comorbidities. The following subsections summarize the clinical evidence for their use in **hypertension, angina pectoris, myocardial infarction (MI) and heart failure (HF), and arrhythmias.**

2.1 Hypertension

Beta-blockers were historically considered **first-line agents** in the treatment of essential hypertension. However, recent **international guidelines** (e.g., ESC/ESH 2018, AHA/ACC 2017) recommend that they **should no longer be the first-line choice**, except in patients with compelling indications (e.g., heart failure, post-MI, or arrhythmias).

Efficacy

- Meta-analyses have shown that beta-blockers are **less effective than ACE inhibitors, ARBs, and calcium channel blockers** in reducing the risk of **stroke and all-cause mortality**, particularly in patients >60 years old.
- Atenolol, in particular, has been associated with **inferior cardiovascular protection** compared to other antihypertensives.

Metabolic Considerations

- Non-vasodilating β -blockers are linked to **adverse metabolic effects**, including reduced insulin sensitivity, increased triglycerides, and risk of new-onset diabetes mellitus.
- Vasodilating β -blockers (carvedilol, nebivolol) have a **neutral or favorable metabolic profile**, making them more suitable for patients with metabolic syndrome.

Summary: In uncomplicated hypertension, beta-blockers are not preferred first-line agents. They are reserved for patients with **concomitant cardiac conditions** (e.g., angina, arrhythmia, post-MI, or HF).

2.2 Angina Pectoris

Beta-blockers are considered **first-line antianginal therapy** in patients with stable angina, unless contraindicated.

Mechanism of Benefit

- They decrease **myocardial oxygen demand** by reducing heart rate, contractility, and afterload.
- By prolonging diastole, they **improve coronary perfusion**, reducing ischemia.

Clinical Evidence

- Beta-blockers significantly **reduce angina episodes and exercise-induced ischemia**.
- However, unlike statins or antiplatelets, they **do not reduce mortality** in stable angina without prior MI or HF.

Summary: Beta-blockers are effective in symptom control and are recommended as **initial therapy in chronic stable angina**, often in combination with nitrates or calcium channel blockers if monotherapy is insufficient.

2.3 Myocardial Infarction and Heart Failure

Post-Myocardial Infarction (MI)

- Beta-blockers have a **well-established mortality benefit** in patients with acute MI.

- They reduce **reinfarction rates, arrhythmias, and sudden cardiac death**.
- Current guidelines recommend initiating beta-blockers in all post-MI patients **unless contraindicated**, and continuing long-term.

Heart Failure (HF) with Reduced Ejection Fraction (HFrEF)

- Large randomized trials (e.g., **COPERNICUS, MERIT-HF, CIBIS-II**) have demonstrated that carvedilol, metoprolol succinate, and bisoprolol **reduce mortality and hospitalization** in patients with HFrEF.
- Mechanism: Inhibition of chronic sympathetic overactivity, which otherwise worsens ventricular remodeling and progression of HF.
- Initiation should be **low dose and titrated gradually**, especially in patients with volume overload or unstable symptoms.

Heart Failure with Preserved Ejection Fraction (HFpEF)

- Evidence is less robust; beta-blockers may be used to control **heart rate, blood pressure, and arrhythmias**, but no mortality benefit has been proven.

Summary: Beta-blockers remain **cornerstone therapy post-MI and in HFrEF**, with strong evidence supporting improved survival.

2.4 Arrhythmias

Beta-blockers are highly effective in both **supraventricular and ventricular arrhythmias**, largely due to their negative chronotropic and dromotropic effects.

Atrial Arrhythmias

- First-line therapy for **rate control** in atrial fibrillation (AF) and atrial flutter.
- Useful in preventing **recurrence of paroxysmal supraventricular tachycardia (PSVT)**.

Ventricular Arrhythmias

- Reduce the risk of **sudden cardiac death** post-MI by suppressing ventricular ectopy.
- Indicated in **long QT syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT)**, where they significantly reduce arrhythmic risk.

Perioperative and Other Uses

- Beneficial in controlling tachyarrhythmias during **thyrotoxicosis, pheochromocytoma (with alpha-blockade)**, and in perioperative cardiac protection.

Summary: Beta-blockers are a mainstay in the **management of arrhythmias**, with both symptomatic relief and mortality reduction in selected populations.

Table 2. Clinical Applications of Beta-Blockers

Indication	Clinical Benefit	Strength of Evidence
Hypertension	Reduces BP, but less effective than other classes in stroke/mortality reduction	Moderate
Stable Angina	Symptom relief, ↓ ischemia, no mortality benefit	Strong (symptom control)
Post-MI	↓ Mortality, ↓ reinfarction, ↓ arrhythmias	Strong
Heart Failure (HFrEF)	↓ Mortality, ↓ hospitalizations, improved symptoms	Strong
Arrhythmias	Rate control in AF/flutter, ↓ ventricular arrhythmias, ↓ SCD	Strong

3. Safety, Contraindications, and Adverse Effects of Beta-Blockers

Despite their proven efficacy in cardiovascular disease, the use of beta-blockers requires careful consideration of safety, tolerability, and contraindications. Their effects on multiple organ systems explain both their therapeutic benefits and adverse effects.

3.1 General Adverse Effects

Beta-blockers are generally well tolerated, but the most common adverse events include:

- **Cardiovascular:**
 - Bradycardia, atrioventricular (AV) block, hypotension.
 - Worsening of heart failure symptoms if initiated at high doses or in decompensated states.
- **Respiratory:**
 - Bronchospasm in patients with asthma or COPD due to β_2 blockade.
 - Cardioselective β_1 -blockers are safer but not completely risk-free.
- **Metabolic:**
 - Impaired glucose tolerance and increased risk of new-onset type 2 diabetes.
 - Adverse lipid profile changes: ↑ triglycerides, ↓ HDL cholesterol.
 - Vasodilating beta-blockers (carvedilol, nebivolol) show fewer metabolic disturbances.
- **Central Nervous System (CNS):**
 - Fatigue, depression, sleep disturbances, nightmares (especially lipophilic drugs like propranolol).
 - Rarely, memory impairment and hallucinations.

- **Sexual dysfunction:**

- Erectile dysfunction is a well-documented side effect, potentially impacting adherence.

3.2 Contraindications

Beta-blockers should not be used in certain conditions due to risk of exacerbation:

- **Absolute Contraindications:**

- Severe bradycardia (<50 bpm).
- Second- or third-degree AV block without a pacemaker.
- Cardiogenic shock.
- Severe, uncontrolled asthma or history of bronchospasm with beta-blockers.
- Severe peripheral arterial disease with critical ischemia.

- **Relative Contraindications:**

- Controlled asthma or COPD (β_1 -selective agents may be cautiously used).
- Diabetes mellitus (risk of masking hypoglycemia).
- Raynaud's phenomenon and peripheral vascular disease.
- Depression (increased risk with lipophilic beta-blockers).

3.3 Drug Interactions

Beta-blockers interact with several commonly prescribed medications:

- **With other negative chronotropes** (digoxin, verapamil, diltiazem): increased risk of bradycardia and AV block.
- **With insulin and oral hypoglycemics:** risk of **masked hypoglycemia symptoms** (tachycardia).
- **With NSAIDs:** reduced antihypertensive efficacy due to sodium retention.
- **With clonidine:** abrupt withdrawal of clonidine while on beta-blockers can cause severe rebound hypertension.

3.4 Special Populations

- **Elderly patients:** more prone to bradycardia and hypotension; cautious dose titration needed.
- **Pregnancy:** some beta-blockers (e.g., labetalol, metoprolol) are considered safe and used for gestational hypertension and preeclampsia, but atenolol has been linked to intrauterine growth restriction.
- **Pediatric use:** propranolol is used in infants for conditions such as infantile hemangiomas, but monitoring for hypoglycemia and bradycardia is required.

Table 3. Safety Profile and Contraindications of Beta-Blockers

Category	Key Points
Common Adverse Effects	Bradycardia, fatigue, bronchospasm, metabolic disturbances, sexual dysfunction
CNS Effects	Depression, nightmares, memory issues (lipophilic agents)
Absolute Contraindications	Severe bradycardia, AV block, cardiogenic shock, severe asthma
Relative Contraindications	Controlled asthma, diabetes, Raynaud's, depression
Drug Interactions	Digoxin/verapamil (bradycardia), insulin (masked hypoglycemia), NSAIDs
Special Populations	Elderly (dose adjust), pregnancy (labetalol preferred), pediatrics (propranolol use)

METHODOLOGY

This article is based on a **structured literature review**. Relevant studies were identified through systematic searches of **MEDLINE, Embase, Current Contents, and the Cochrane Library** up to 2025. Additional sources were obtained from cross-references in published articles and reputable online databases.

4.1 Search Strategy

Search terms included combinations of:

- “beta-blockers” OR “beta-adrenergic antagonists”
- “hypertension”, “angina pectoris”, “myocardial infarction”, “heart failure”, “arrhythmia”

Boolean operators (AND, OR) were applied. Only **English-language publications** were included.

4.2 Inclusion Criteria

- Randomized controlled trials (RCTs)
- Cohort and case-control studies
- Observational studies
- Meta-analyses and systematic reviews
- International guidelines from cardiology and pharmacology societies

4.3 Exclusion Criteria

- Non-peer-reviewed reports
- Animal studies (unless mechanistically relevant)
- Case reports and letters to the editor

4.4 Study Selection

Two independent reviewers screened titles and abstracts. Full texts were retrieved for potentially relevant studies. Discrepancies were resolved by consensus.

4.5 Data Extraction and Synthesis

Data were extracted regarding:

- Study design, patient population, and sample size
- Intervention (specific beta-blocker, dose, duration)
- Clinical outcomes (mortality, morbidity, symptom improvement, adverse effects)

The evidence was synthesized qualitatively, with emphasis on **comparisons to alternative therapies** and **clinical guideline recommendations**.

5. RESULTS

5.1 Hypertension

- Large RCTs and meta-analyses (e.g., **LIFE, ASCOT-BPLA**) showed beta-blockers, especially **atenolol**, are **less effective** than ARBs, ACE inhibitors, or calcium channel blockers in preventing stroke and reducing all-cause mortality.
- In patients <60 years, beta-blockers can be effective, particularly when combined with diuretics.
- Beta-blockers with vasodilatory properties (nebivolol, carvedilol) demonstrate **better tolerability and metabolic profiles**.

5.2 Angina Pectoris

- Consistent evidence shows beta-blockers reduce **angina frequency and exercise-induced ischemia**.
- No significant mortality benefit in **stable angina without prior MI**.
- Remain **first-line for symptom control**, particularly in combination with nitrates or calcium channel blockers.

5.3 Myocardial Infarction (MI) and Heart Failure (HF)

- Landmark trials (e.g., **MERIT-HF, CIBIS-II, COPENICUS**) demonstrate **reduced mortality and hospitalizations** in HFrEF patients treated with carvedilol, metoprolol succinate, or bisoprolol.
- Post-MI patients benefit from reduced reinfarction and arrhythmic death.
- In **HFpEF**, evidence is weak; beta-blockers may help with rate control but show **no mortality reduction**.

5.4 Arrhythmias

- Strong evidence supports beta-blockers in controlling **atrial fibrillation/flutter rates**.
- Effective in preventing **ventricular arrhythmias** post-MI.

- Inherited arrhythmia syndromes (e.g., **long QT syndrome, CPVT**) show dramatic reductions in sudden cardiac death with propranolol or nadolol.

6. Discussion

The results of this review confirm that **beta-blockers maintain a critical role in cardiology**, but their use must be tailored to patient characteristics and evolving therapeutic standards.

6.1 Changing Role in Hypertension

Although beta-blockers were historically first-line antihypertensives, modern evidence shows **inferior cerebrovascular protection** compared with other drug classes. Current guidelines limit their use to patients with **concomitant cardiac indications**.

6.2 Continued Value in Angina and Ischemia

Beta-blockers remain **cornerstone therapy in stable angina**, providing excellent symptom relief. However, the absence of mortality reduction in isolated angina means that **disease-modifying agents** (statins, ACE inhibitors) are prioritized in long-term management.

6.3 Strong Evidence in Post-MI and HFrEF

The most robust evidence for beta-blockers lies in **post-MI survival and HFrEF management**, where they are guideline-recommended, mortality-reducing therapies. Their ability to attenuate maladaptive sympathetic overdrive is unmatched by most alternatives.

6.4 Limitations and Safety Concerns

Adverse metabolic effects, fatigue, and sexual dysfunction limit tolerability. In older adults, non-selective agents may worsen glucose control and peripheral vascular disease. Careful selection of agents (nebivolol, carvedilol) can mitigate some risks.

6.5 Position in Modern Cardiology

With the rise of **RAAS inhibitors, ARNI therapy, SGLT2 inhibitors, and advanced antianginals**, beta-blockers are no longer universal first-line agents. Instead, they are now considered **disease-specific tools** with proven efficacy in:

- Post-MI
- HFrEF
- Arrhythmias
- Angina pectoris

Their role in **uncomplicated hypertension** is diminished.

7. Conclusion and Future Perspectives

Beta-blockers have been a cornerstone of cardiovascular therapy for over five decades, transforming the management of ischemic heart disease, heart failure, arrhythmias, and, historically, hypertension. This review demonstrates that:

1. Hypertension:

- Beta-blockers are **no longer recommended as first-line therapy** for uncomplicated hypertension, especially in patients over 60, due to inferior cerebrovascular protection and metabolic side effects.
- Vasodilating agents such as carvedilol and nebivolol may mitigate some adverse metabolic outcomes.

2. Angina Pectoris:

- Beta-blockers remain **first-line therapy for symptom control**, effectively reducing angina episodes and myocardial ischemia, though they do not significantly improve mortality in patients without prior myocardial infarction.

3. Myocardial Infarction and Heart Failure:

- Post-MI patients and those with **HFrEF** derive clear survival benefits, with reduced reinfarction, hospitalization, and arrhythmic death.
- Evidence for HFpEF is less robust; benefits are primarily in **rate control** and symptom management.

4. Arrhythmias:

- Beta-blockers are essential for **rate control in atrial fibrillation**, prevention of ventricular arrhythmias, and management of inherited arrhythmia syndromes.

5. Safety Considerations:

- Common adverse effects include bradycardia, fatigue, bronchospasm, and metabolic disturbances.
- Careful patient selection and use of **cardioselective or vasodilatory agents** can reduce risks.

Future Perspectives

- **Personalized Therapy:** Advances in pharmacogenomics and precision medicine may allow clinicians to select **specific beta-blocker subtypes** based on genetic profiles, comorbidities, and receptor polymorphisms.
- **Combination Therapies:** Beta-blockers can be combined with modern agents (RAAS inhibitors, SGLT2 inhibitors, advanced antianginals) to **optimize outcomes** in high-risk populations.
- **Emerging Agents:** Vasodilating beta-blockers such as **nebivolol and carvedilol** offer improved hemodynamic and metabolic profiles, suggesting a potential expansion in their indications.
- **Ongoing Research:** Clinical trials continue to explore the **role of beta-blockers in HFpEF, chronic kidney disease, and post-acute coronary syndromes**, which may redefine their utility in future guidelines.

CONCLUSION

While beta-blockers are no longer universal first-line therapy for hypertension, they remain indispensable in **post-MI care, HFrEF, arrhythmias, and angina symptom management**. Their continued use should be **individualized**, guided by patient comorbidities, metabolic profile, and evidence-based recommendations. Future directions in personalized medicine and

newer vasodilatory agents promise to **refine and expand the clinical role of beta-blockers** in cardiovascular care.

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Conflict of Interest

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Author Contribution

Aurora Napuce: Conceptualization, literature review, writing, and editing of the manuscript.

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EFFECT OF LOW TEMPERATURE ON OPEN-FIELD CULTIVATION OF PASSIFLORUIT (*Passiflora edulis*) UNDER THE ECOLOGICAL CONDITIONS OF MANAVGAT

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ABSTRACT

In recent years, the cultivation and consumption of tropical fruits in Türkiye have gained increasing prominence, driven by global climate change, rising input costs in greenhouse vegetable production, and the expanding commercial value of tropical fruit species. Among these, passionfruit (*Passiflora edulis*) has emerged as a particularly attractive crop due to its relatively low labor requirements, capacity to bear fruit twice annually, and potential use as a living fence. Despite the general perception of global warming as a rise in average temperatures, low winter temperatures remain a critical constraint on the cultivation of tropical fruit crops. This study was conducted to assess the impact of low temperatures on open-field cultivation of passionfruit under the ecological conditions of Manavgat, Türkiye, between May 2021 and June 2025. Evaluations included plant growth, flowering dynamics, the effects of low winter temperatures, and post-frost recovery performance. In the first winter following planting, minimum temperatures of 0.3, 0.7, 0.6, and 0.7 °C were recorded on January 2022, 20, 24, 25, and 26 respectively, leading to prolonged frost events. In contrast, no sub-3.0 °C temperatures were recorded in 2023 and 2024, while a single minimum of 2.2 °C occurred in 2025. Flowering was observed twice per year, in June and October. The results demonstrate that open-field cultivation of passionfruit is feasible under the ecological conditions of Manavgat. Nevertheless, to mitigate frost-related risks and extend the flowering period, further investigations into passionfruit production under protected cultivation systems are strongly recommended.

Keywords: *Passiflora* (*Passiflora edulis*), Passionfruit, Low temperature, Flowering

INTRODUCTION

The genus *Passiflora*, with approximately 400 species, represents the largest genus within the family Passifloraceae. This family comprises 16 genera and about 700 species, of which 576 belong to *Passiflora*, predominantly distributed in tropical and subtropical regions of the Americas. Brazil is regarded as a major center of diversity for this genus, hosting 85 endemic and 59 non-endemic taxa. Despite this remarkable diversity, only a limited number of species are cultivated commercially. Among them, *Passiflora edulis* Sims (purple and yellow passion fruit) (Patel, 2009) and *Passiflora alata* Curtis (sweet passion fruit) (Ataíde et al., 2012) are the most widely grown species.

The purple passion fruit (*Passiflora edulis* Sims *f. edulis*) belongs to a group of about 530 species, primarily distributed in the Americas, and to a lesser extent in tropical and subtropical regions of Southeast Asia, Australia, and New Zealand. Colombia is recognized as the country with the greatest species richness within the genus *Passiflora*, followed by Brazil, Ecuador, and Peru (Ortiz et al., 2012).

Passion fruit is consumed fresh and is also widely used in processed products such as juices, jams, jellies, and ice creams (Thokchom et al., 2017). Its juice contains approximately 700–2400 IU of carotene and 20–30 mg of vitamin C per 100 g (Banu et al., 2009). Studies have demonstrated that *Passiflora edulis* possesses anti-inflammatory, antimicrobial, hypolipidemic, antioxidant, and antitumor properties (Patel, 2009). Furthermore, its *in vivo* beneficial effects have been reported in the management of several diseases, including asthma, hypertension, osteoarthritis, diabetes, and pulmonary fibrosis (Fonseca et al., 2022). In addition, certain *Passiflora* species are used as ornamentals in landscaping due to their showy flowers (Köse and Köroğlu, 2021).

Passiflora edulis typically thrives in tropical and subtropical regions but struggles to survive under low-temperature conditions (Liu et al., 2017). In subtropical climates, it can tolerate brief episodes of mild frost but is severely damaged by strong frosts. Optimal vegetative growth and flowering occur between 20 and 30 °C. The species grows successfully in regions with annual rainfall between 1000 and 2500 mm and at altitudes up to 2000 m. Both high and low temperatures adversely affect growth; excessive heat promotes vigorous vegetative growth at the expense of fruit set (Gülgün et al., 2003; Deshmukh et al., 2017; Thokchom et al., 2017).

Flowering in *Passiflora edulis* can be enhanced by environmental factors such as photoperiod regulation (Liu et al., 2015). Some reports suggest that the species is a long-day plant requiring more than 10.5 hours of daylight for flowering and fruit set (Banu et al., 2009). It fails to flower under photoperiods shorter than 11 hours or at night temperatures below 18 °C (Bernardes et al., 2020). To better understand the molecular mechanisms of cold tolerance, various studies have been conducted, leading to the development of cold-tolerant cultivars such as ‘*Pingtang 1*’ (Liu et al., 2017). The species grows best in well-drained sandy-loam soils with a pH of 6.5–7.5 and about 2% organic matter (Gülgün et al., 2003).

Passion fruit bears hermaphroditic flowers, usually solitary in the leaf axils (Deshmukh et al., 2017). The flowers, measuring about 4–5 cm in diameter, are striking, colorful, and fragrant, with five whitish petals and thread-like corona filaments in two purplish series. The gynoecium consists of a trilocular ovary and a three-branched style, each ending in a sticky stigma (Banu et al., 2009). The flowers are protandrous: anthers dehisce before stigmas become receptive, although stigmas remain receptive throughout anthesis (Deshmukh et al., 2017). Flowering and fruiting seasons vary depending on geographic region (Bernardes et al., 2020). In northeastern India, *P. edulis*, *P. quadrangularis*, and *P. foetida* flower in March–April, July–August, and September–October (Kishore et al., 2010), whereas in Bangladesh flowering occurs from April to September (Banu et al., 2009). Generally, flowering behavior in *Passiflora* species fluctuates throughout the year depending on environmental conditions, resulting in variation in fruiting and harvest times (Ataíde et al., 2012). In purple passion fruit, the period from bud initiation to anthesis is 18–22 days, while fruit set occurs 3–4 days after anthesis. Approximately 90% of pollen remains viable at anthesis, and fruit ripening, characterized by a color change from green to purple, occurs 65–70 days after flowering. The overall duration from anthesis to fruit maturity is about 80–90 days (Deshmukh et al., 2017; Banu et al., 2009).

The *Passiflora* plant requires support structures for proper growth. In commercial orchards, different trellis systems are adopted depending on ecological and regional conditions. In hedge trellis systems, posts are 1.8 m above ground and spaced 4–5 m apart (Balkıç, 2022). In Colombia, a study identified the simple trellis as the most suitable support system for the eco-physiological requirements of passion fruit. This system was found to be advantageous in

terms of cultural practices, plant health management, and optimizing fruit quality and yield (Cleves-Leguízamo, 2021).

In recent years, increasing consumer demand for healthy diets has driven the consumption of exotic fruits in Türkiye (Binici et al., 2022). However, the areas suitable for open-field passion fruit cultivation remain limited across the country (Balkıç, 2022). Open-field cultivation is mostly possible in microclimatic zones of the Mediterranean Region, such as Gazipaşa in Antalya. Both *Passiflora edulis* and *P. caerulea* are cultivated in Antakya for fruit production as well as ornamental purposes (Mavi and Uzunoğlu, 2020). Moreover, *P. incarnata* is grown on a small scale by some producers interested in tropical fruits in Antalya and Muğla provinces (Köse and Koroğlu, 2021). Among the first tropical fruit cultivars registered in Türkiye is ‘Possum Purple’ (Balkıç, 2022).

Given the rising interest in tropical fruit cultivation, the production of several exotic fruit species has recently expanded in Türkiye. This study evaluates the potential of *Passiflora edulis* for open-field cultivation under Manavgat’s subtropical conditions, with a particular focus on growth performance and flowering behavior under low-temperature stress.

MATERIALS AND METHODS

The study was carried out at the İlica Campus of Manavgat Vocational School, Akdeniz University (Manavgat, Antalya, Türkiye), covering the period from May 2021 to June 2025. The plant material consisted of the passion fruit cultivar Possum Purple (*Passiflora edulis* f. *edulis* Sims.). The trial encompassed all stages from field preparation to the assessment of plant and fruit development, with particular emphasis on the effects of low winter temperatures and post-cold recovery performance, based on three years of observations.

Plant Material

The Possum Purple cultivar, the most widely cultivated passion fruit variety in Florida, is a hybrid between the purple (*Passiflora edulis* f. *edulis*) and yellow (*P. edulis* f. *flavicarpa*) forms. Fruits measure 6.5–7.5 cm in length and approximately 5 cm in diameter, with an average weight ranging between 35 and 60 g. The water content is around 31–35%. The fruit contains numerous small black or brown seeds enclosed within a thin, leathery rind. It is characterized by a pleasant aroma and sweet juice. The period from anthesis to harvest is approximately 70–75 days. Compared with other purple cultivars, Possum Purple exhibits greater tolerance to low temperatures and is used not only for fresh consumption but also in various food industry applications (Banu et al., 2009; Thokchom et al., 2017; Balkıç, 2022).

Climate Data

Climatic data were obtained from the nearest meteorological station (MANAVGAT/17954), operated by the Turkish Ministry of Environment, Urbanization, and Climate Change, General Directorate of Meteorology. Daily maximum, minimum, and mean air temperatures, along with relative humidity values, were recorded.

Training System

A hedge-type trellis system was employed. Wooden posts, each 2 m in length, were installed at 3 m intervals. Along the posts, three rows of galvanized wire were stretched to support plant growth. The installation stages of the training system are illustrated in Figure 1.



Figure 1. Views of the installation stages of the training system (14 January 2021)

Planting of Seedlings

Passion fruit seedlings were obtained from the Department of Horticulture, Akdeniz University. The seedlings were propagated by stem cuttings in a mist-propagation greenhouse. Plants were established by placing one seedling between every two posts set at 3 m intervals, with a spacing arrangement of 3×3 m (row spacing $\times$ plant spacing). The appearance of the seedlings used for planting is presented in Figure 2. Planting was carried out on 4 May 2021.



Figure 2. General view of the planting stage (4 May 2021)

Cultural Practices

A drip irrigation system was used for water supply. Adjustable drippers ($0-100 \text{ L h}^{-1}$), commonly employed in fruit orchards, were placed at the base of each plant. Irrigation began in the summer after the end of rainfall and continued until September–October, when rainfall resumed. During the summer months, irrigation was applied once or twice a week depending on air temperature, whereas in spring it was applied approximately every 10 days, according to plant water requirements. Regular weed control was performed throughout the growth period. No chemical or biological treatments were applied against pests or diseases.

In this study, observations were conducted over a three-year period to evaluate the growth and fruit development of the cultivar Possum Purple (*Passiflora edulis*), with particular attention to the effects of low winter temperatures and subsequent recovery performance after cold stress.

RESULTS AND DISCUSSION

Plant Growth

After planting, strings were tied to the base of the passion fruit seedlings to encourage upright growth. The main stem was trained vertically until it reached the top level of the trellis system. Once the main stem reached the upper wire, the apical shoot was pruned to stimulate lateral branching. Axillary shoots emerging from the leaf axils were guided horizontally along the wires stretched between the posts, thereby promoting side branching. The plants, which were planted on 4 May 2021, had fully covered the trellis system by 16 December 2021 (Figure 3).



Figure 3. Growth stages of the passion fruit plant during the first year of development at different dates

Flowering and Fruiting

In passion fruit plants, flowering began in June of the year following planting, and the resulting fruits reached harvest maturity in August. A second flowering period was observed in October, with fruits from this cycle ripening between late December and early January (Figure 4). The average fruit weight, diameter, and length were determined as 51.14 g, 50.69 mm, and 58.29 mm, respectively. Fruits harvested in summer and winter, which can be categorized as

“summer fruits” and “winter fruits,” exhibited differences in color and flavor characteristics. Sensory evaluations indicated that the summer fruits were preferred by panelists in terms of taste, aroma, and color. Another noteworthy observation was that mature fruits naturally abscised from the vine after ripening.

Flowering and fruiting seasons have been reported to vary depending on the growing region. In Rio Grande do Sul, Brazil, *Passiflora suberosa* flowers during spring, summer, and autumn; however, flowering is interrupted during excessively hot summer days and under short-day conditions in winter (Bernardes et al., 2020). In northeastern India, purple, giant, and *P. foetida* passion fruits have been reported to flower in March–April, July–August, and September–October (Deshmukh et al., 2017). Moreover, flowers are predominantly borne on new shoots, particularly in the middle and upper sections of the vines (Banu et al., 2009).



Figure 4. Flower and fruit appearances of the passion fruit plant

Climatic Data

During the cultivation period, the daily minimum temperature values were monitored (Figure 5). The lowest temperatures recorded in January and February, the coldest months of the year, are presented in Table 1.

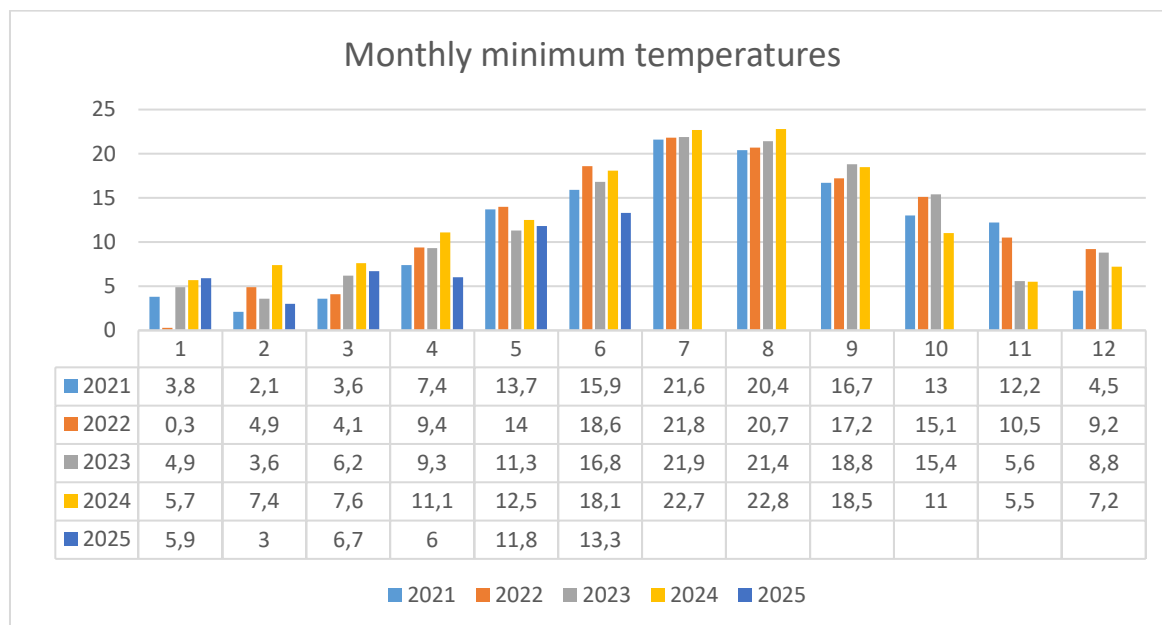


Figure 5. Monthly minimum temperatures recorded from 2021 to 2025

Table 1. Lowest temperature values and measurement dates recorded in January–February

	January		February	
	Date	Degree °C	Date	Degree °C
2022	20.01.2022	0.3	05.02.2022	4.9
2023	05.01.2023	4.9	02.02.2023	3.6
2024	28.01.2024	5.7	02.02.2024	7.4
2025	18.01.2025	5.9	27.02.2025	2.2

In the year following planting, on 19, 20, 24, 25, 26, and 27 January 2022, temperatures dropped to 3.0, 0.3, 0.7, 0.6, 0.7, and 3.0 °C, respectively. During this period, temperatures reached frost-risk levels, and prolonged frost events occurred. In the subsequent years of 2023 and 2024, no temperatures below 3.0 °C were recorded. However, in 2025, low temperatures reappeared, with one day dropping to 2.2 °C and another below 3.0 °C.

The prolonged low temperatures observed in the year following planting caused significant damage to the passion fruit plants, resulting in mortality in some cases. The adverse effects of low temperatures on plants are illustrated in Figure 6.

Cold stress is recognized as one of the major factors limiting plant growth and development (Wu et al., 2021). Passion fruit (*Passiflora* spp.) is highly sensitive to frost, and prolonged exposure to subfreezing temperatures can cause severe damage or plant death. However, the species shows partial tolerance to light frosts and is able to withstand short-term exposures to temperatures as low as −2 °C (Deshmukh et al., 2017). It has been reported that plants can develop resistance to low temperatures through a process known as cold acclimation. In this context, breeding programs aimed at developing cold-tolerant cultivars have been strongly emphasized (Wu et al., 2021).

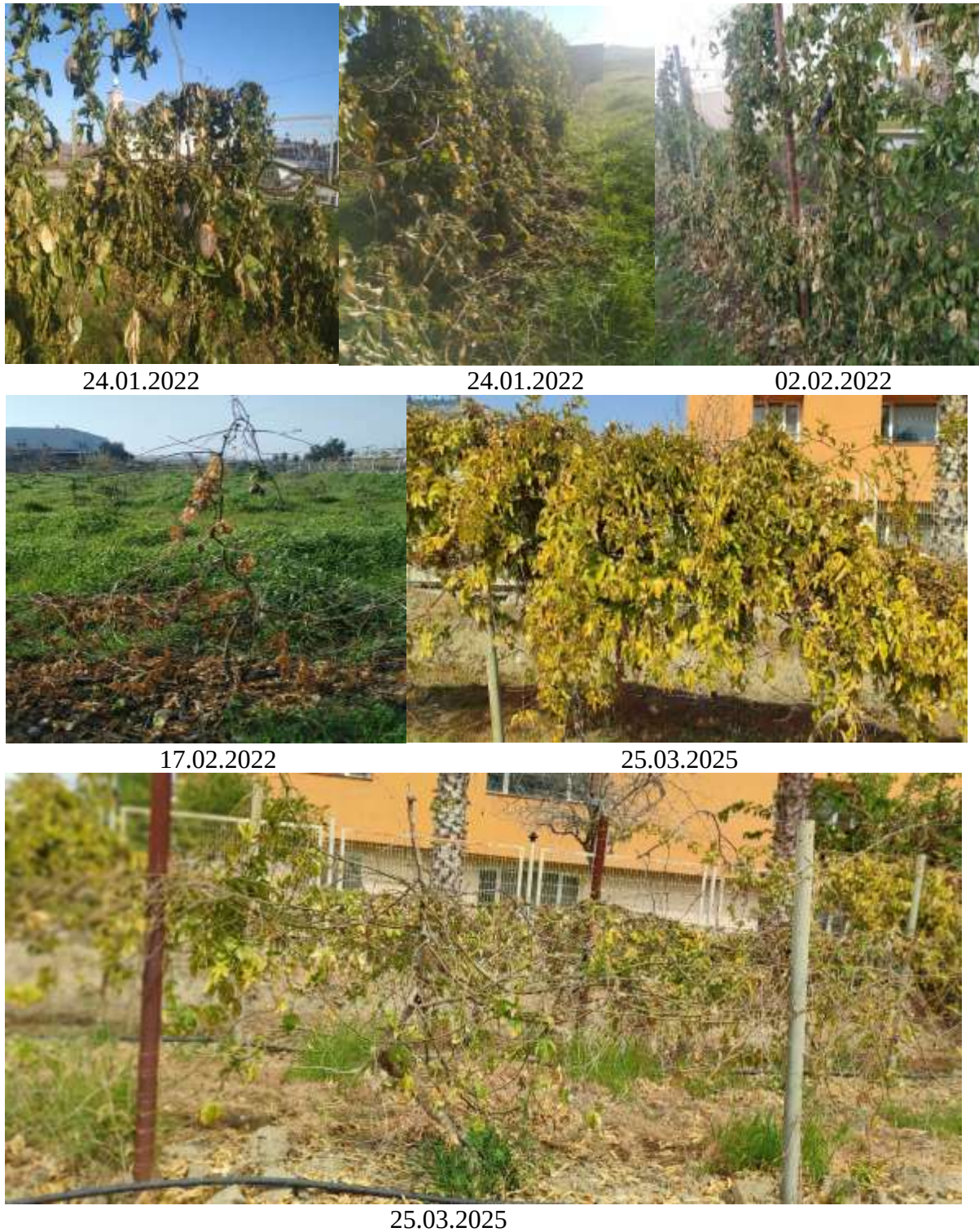


Figure 6. Effects of low temperatures on passion fruit plants

In the plants damaged by low temperatures in 2021 and 2025, severe pruning down to the main stem was carried out between late March and early April, after the frost risk had passed. During spring, regrowth was observed, followed by flowering and fruit set in June (Figure 7).



Figure 7. Regrowth, flowering, and fruiting of passion fruit plants following pruning after low-temperature damage

CONCLUSION

In this study, the growth performance of the cultivar ‘Possum Purple’ (*Passiflora edulis* f. *edulis* Sims.) was investigated under the ecological conditions of Manavgat, Antalya. The climbing passion fruit plants exhibited rapid vegetative growth, and no major pest or disease problems were encountered during the growth period. Two distinct flowering periods were observed in June and September, leading to two separate harvests in August and December. Under open-field conditions, low winter temperatures caused damage to the plants, with the severity of injury depending on both the intensity and duration of the cold events.

The results indicate that open-field cultivation of passion fruit is feasible under the conditions of Manavgat. However, to mitigate frost risk and extend the flowering period, further studies are recommended on the potential of cultivating passion fruit under protected (greenhouse) conditions.

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AGE-SPECIFIC CONSIDERATIONS IN ANTICOAGULANT THERAPY: MECHANISMS, DOSING, AND SAFETY ACROSS LIFE STAGES

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ABSTRACT

Anticoagulant therapy is a cornerstone in the management of thromboembolic disorders such as venous thromboembolism (VTE), atrial fibrillation (AF), and myocardial infarction (MI). The pharmacodynamics of anticoagulants are influenced by several factors, including age, comorbidities, and genetic predisposition. This paper provides an in-depth review of the pharmacology, clinical use, and age-specific considerations of commonly prescribed anticoagulants. Special focus is placed on pediatric, adult, and elderly populations, considering the pharmacokinetic and pharmacodynamic differences that impact drug efficacy and safety. The review highlights the evolving role of direct oral anticoagulants (DOACs), their comparative effectiveness, safety profiles, and challenges in age-related dosing. By emphasizing individualized treatment strategies, this review aims to optimize therapeutic outcomes and minimize adverse events related to anticoagulant therapy.

Keywords: Anticoagulants, thromboembolism, warfarin, direct oral anticoagulants (DOACs), heparin, pediatric anticoagulation, geriatric anticoagulation, individualized therapy.

1. INTRODUCTION

Anticoagulants are pharmacological agents that disrupt the coagulation cascade, playing an essential role in the prevention and treatment of thromboembolic disorders. These disorders include conditions such as venous thromboembolism (VTE), atrial fibrillation (AF), myocardial infarction (MI), and pulmonary embolism (PE), all of which contribute to significant morbidity and mortality worldwide. While anticoagulants have proven highly effective in reducing thrombotic risks, their clinical use is accompanied by a well-established concern: bleeding complications. The risk of bleeding is particularly pronounced in patients who are elderly, pediatric, or those with certain comorbidities.

Anticoagulants include various drug classes such as heparins, vitamin K antagonists (VKAs), and direct oral anticoagulants (DOACs), each with distinct mechanisms of action, pharmacokinetic profiles, and clinical considerations. Given the physiological differences between age groups, dosing regimens, and monitoring strategies must be tailored to optimize patient outcomes while minimizing risks. This paper explores the pharmacology of these anticoagulants, their specific indications, safety concerns, and the necessity for individualized treatment strategies across different age cohorts.

2. MATERIALS AND METHODS

An extensive literature search was conducted using PubMed, Scopus, and Web of Science databases, focusing on studies published within the last two decades. Key search terms included “anticoagulants,” “warfarin dosing,” “direct oral anticoagulants,” “heparin,” and “age-related anticoagulation.” Inclusion criteria were randomized controlled trials (RCTs), meta-analyses, cohort studies, and clinical guidelines that addressed the use of anticoagulants across pediatric, adult, and elderly populations. Studies focusing on clinical efficacy, safety, and outcomes of anticoagulant therapy were prioritized to inform the review’s comprehensive analysis.

3. Types of Anticoagulants and Their Mechanisms

The major classes of anticoagulants include heparins, vitamin K antagonists (VKAs), and direct oral anticoagulants (DOACs), each with distinct mechanisms of action and clinical applications.

3.1 Heparin and Low Molecular Weight Heparin (LMWH)

Heparin and LMWH are widely utilized for acute anticoagulation, particularly in hospitalized patients. Heparin works by potentiating the activity of antithrombin III, which then inactivates thrombin and Factor Xa, preventing clot formation. Despite its effectiveness, heparin requires close monitoring of activated partial thromboplastin time (aPTT), which can fluctuate due to factors such as renal function or coexisting medical conditions.

LMWHs, such as enoxaparin, possess a more predictable pharmacokinetic profile and have a lower risk of bleeding complications compared to unfractionated heparin (UFH). The predictable dosing of LMWH allows for less frequent monitoring, making it an attractive choice for both pediatric and adult patients, particularly in outpatient settings for the treatment of VTE or prevention of clot formation following surgery.

Table 1: Pharmacokinetics of Common Anticoagulants

Drug	Mechanism	Half-life	Monitoring Required
Warfarin	Vitamin K antagonist	20-60 hrs	INR monitoring
Heparin	Enhances antithrombin	1-2 hrs	aPTT monitoring
Enoxaparin	Inhibits Factor Xa	4-7 hrs	Rarely needed
Rivaroxaban	Direct Factor Xa inhibitor	5-9 hrs	No routine monitoring
Dabigatran	Direct thrombin inhibitor	12-17 hrs	No routine monitoring

3.2 Vitamin K Antagonists (VKAs)

Warfarin, the most commonly prescribed VKA, inhibits the enzyme vitamin K epoxide reductase, which is necessary for the activation of clotting factors II, VII, IX, and X. Although warfarin remains one of the most studied anticoagulants, its use is complicated by a narrow therapeutic window and the requirement for routine INR monitoring. Several factors, including diet, coexisting medical conditions, and drug interactions, can affect warfarin's efficacy, particularly in elderly patients who may be on multiple medications.

3.3 Direct Oral Anticoagulants (DOACs)

DOACs, such as rivaroxaban, apixaban, and dabigatran, offer several advantages over warfarin. These agents selectively inhibit either thrombin (dabigatran) or Factor Xa (rivaroxaban, apixaban, edoxaban) and do not require routine INR monitoring, making them more convenient for long-term management of conditions like atrial fibrillation and VTE. The lack of routine monitoring is particularly beneficial for elderly patients or those with limited access to healthcare facilities. However, DOACs do not have specific reversal agents (with the exception of idarucizumab for dabigatran), and their use in populations with severe renal or hepatic impairment is still under investigation.

4. Age-Specific Considerations

4.1 Pediatric Patients

In pediatric patients, anticoagulant therapy must be approached cautiously due to significant differences in drug metabolism, clearance, and dosing requirements. Warfarin and LMWH are commonly prescribed in this population. Warfarin requires regular INR monitoring, which can be cumbersome for parents and caregivers. LMWH is often preferred in this age group due to its more predictable pharmacokinetic profile, but it requires monitoring of anti-Factor Xa levels.

While DOACs hold promise for pediatric use, their safety and efficacy remain largely unstudied in this group. Consequently, the majority of anticoagulant therapy in children still relies on well-established agents such as LMWH.

4.2 Adults

In adults, the choice of anticoagulant is primarily based on the clinical indication and patient-specific factors, such as renal function, comorbidities, and risk of bleeding. DOACs are increasingly favored over warfarin due to their ease of use and fewer dietary and drug interactions. For instance, patients with non-valvular atrial fibrillation (AF) often benefit from the fixed-dose regimen of DOACs, which offers similar efficacy to warfarin but with a significantly reduced risk of major bleeding.

For patients with mechanical heart valves, however, warfarin remains the anticoagulant of choice, as DOACs have not demonstrated sufficient safety and efficacy in these cases. Similarly, DOACs should be used with caution in patients with severe renal impairment, as their clearance is primarily renal.

4.3 Elderly Patients

Elderly patients often present with complex clinical scenarios, including polypharmacy, altered pharmacokinetics, and an increased risk of adverse effects. The use of anticoagulants in this population requires careful monitoring due to a higher incidence of both thromboembolic and bleeding complications. Reduced renal and hepatic function, common in the elderly, affects the pharmacokinetics of anticoagulants, particularly DOACs. For elderly patients with preserved renal function, DOACs are often preferred because of their convenience and safety profile. However, in those with impaired renal function, warfarin may still be the best option, though close INR monitoring is essential.

Table 2: Recommended Anticoagulants by Age Group

Age Group	Preferred Anticoagulant	Monitoring Required
Pediatrics	LMWH	Anti-Factor Xa levels
Adults	DOACs (Rivaroxaban, Apixaban)	None
Elderly	DOACs, Warfarin	INR for warfarin

5. DISCUSSION

The selection of an anticoagulant should be guided by individual patient characteristics, including age, comorbidities, renal function, and risk factors for bleeding. While DOACs represent a major advancement in anticoagulant therapy, their use in elderly patients and those with renal dysfunction requires careful consideration. Furthermore, while warfarin remains essential in some high-risk populations, the convenience and safety of DOACs make them a preferred option in many adult patients. Pediatric populations, however, continue to face challenges in anticoagulation therapy due to the lack of comprehensive data on the safety and efficacy of DOACs. Future research should focus on expanding the indications for DOACs in these special populations, improving patient selection, and developing new reversal agents for DOAC-related bleeding events.

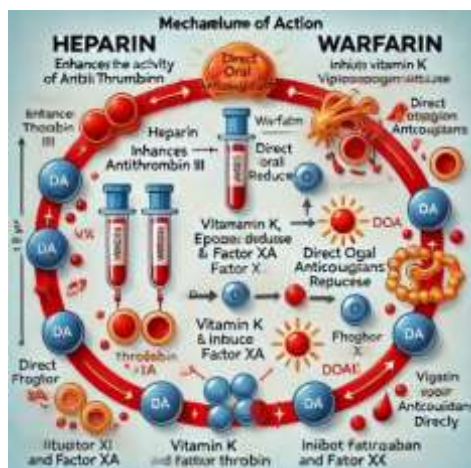


Figure1: Mechanism of Action of Anticoagulants

A schematic representation of how heparin, warfarin, and DOACs affect the coagulation cascade.

6. CONCLUSION

Anticoagulant therapy is an essential component of thromboembolic disease management. The evolution of DOACs has significantly improved the safety and convenience of anticoagulation, although careful consideration of patient age and comorbidities remains critical for achieving optimal therapeutic outcomes. Future research is required to refine anticoagulation strategies, particularly for pediatric and elderly patients, and to develop specific reversal agents for DOACs in emergency situations. Personalized anticoagulant therapy remains the key to maximizing benefit while minimizing harm across diverse patient populations.

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POTENTIAL USE OF ESSENTIAL OIL NANOEMULSIONS AS BEE ATTRACTANTS IN SEED SUNFLOWER (*HELIANTHUS ANNUUS* L.) PRODUCTION

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ABSTRACT

This study was conducted under field conditions in 2024 to determine the effects of certain essential oils on enhancing the pollination efficiency of honeybees in hybrid sunflower seed production. In the study, nanoemulsions (NEs) of lemongrass (*Cymbopogon citratus*), lavender (*Lavandula angustifolia*), rosemary (*Rosmarinus officinalis*), and sage (*Salvia officinalis*) essential oils were prepared at a 5% concentration and applied via drone at doses of 1000 and 2000 ppm to the male and female sunflower parent lines at the beginning of the flowering period. At 50% flowering, the highest bee activity was observed in the plots treated with 2000 ppm *C. citratus* essential oil NE, while bee visits in plots treated with 1000 ppm *R. officinalis* and *S. officinalis* essential oil NEs and Tween-80–chitosan NE were similar to the control. Applications of essential oil NEs resulted in increases of up to 28% in the filled seed rate, 34% in seed number, and 38% in seed yield. The study concluded that 2000 ppm *C. citratus* NE enhanced bee activity, thereby improving seed set in sunflowers, and can be practically used in hybrid sunflower seed production.

Key words: Sunflower, Bee activity, Attractant, Essential oil, Nanoemulsion

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an agriculturally strategic crop worldwide, valued for both its oilseed and seed production. In seed production systems, obtaining high-quality and genetically pure seeds largely depends on an efficient pollination process. Sunflower is generally an entomophilous (insect-pollinated) species, and both yield and seed quality are highly dependent on bee activity. However, in recent years, the decline in bee populations in agricultural landscapes, environmental stress factors, and extensive monoculture practices have resulted in insufficient natural pollination, leading to significant yield and quality losses in seed

production. Wind pollination (anemophily) in sunflower is limited due to morphological and physiological differences between male and female organs, self-incompatibility mechanisms involved in the breeding process, and the weak adaptation of pollen to wind dispersal. Therefore, honeybees represent the most important group of pollinators in sunflower, as they visit flowers more frequently than other insects due to their need to sustain their colonies (Morgado et al., 2002).

The yields of many agricultural crops increase in the presence of pollinators (Silva et al., 2018; Mazzei et al., 2021; Santone et al., 2022; Mota et al., 2024). The growing dependence of numerous cultivated species on pollinators, along with the alarming decline in both managed and wild bee populations (IPBES, 2016; Dicks et al., 2021), highlights the urgent need to investigate the effects of pollination on crop yield and seed quality in modern agricultural systems (Bartual et al., 2018; Silva et al., 2018). Sunflower (*Helianthus annuus*), one of the world's most important oilseed crops, relies heavily on insect pollination for successful seed production (Bartual et al., 2018; Silva et al., 2018; Mota et al., 2024). Exclusion (pollinator-blocking) experiments with hybrid sunflower cultivars have demonstrated that bee pollination can increase seed set and yield by more than 90% (Silva et al., 2018).

In Türkiye, in addition to oilseed production, a substantial amount of sunflower seed production is also carried out. In hybrid seed production, cytoplasmic male sterile (CMS) lines are used, and pollination is mainly achieved by honeybees. To ensure adequate and efficient pollination, an average of 0.9 honeybee colonies per decare (0.1 ha) is typically placed in seed production fields. Numerous studies have reported that biotic pollination by honeybees significantly increases seed set and yield in sunflower seed production (Torretta and Poggio, 2013; Chamer et al., 2015; Susic and Farina, 2015). Nevertheless, in many parental lines used in hybrid seed production, pollination and seed set rates often remain as low as 30–40%. Factors that may contribute to this include the low attractiveness of the maternal line's flower color to bees, insufficient pollen production by paternal lines, the potentially bitter taste of pollen, the presence of alternative pollen sources nearby, and the tendency of bees to remain in hives under high temperatures (30–32 °C). In this context, developing new and environmentally friendly strategies to enhance the attractiveness of flowers to bees is of critical importance for sustainable seed production.

Essential oils are bioactive compounds naturally synthesized by plants, predominantly composed of terpenoids. These substances act as natural signaling molecules in plant–plant and plant–insect communication, playing key roles in attracting pollinators such as bees and butterflies. Recent ecological and chemical ecology studies have demonstrated that certain

volatile constituents of essential oils can have attractive or orienting effects on bee behavior. The use of essential oils as natural bee attractants in sunflower seed production offers a promising and eco-friendly alternative to synthetic attractants, contributing to both enhanced pollination efficiency and environmental sustainability. Most plant-derived bioactive compounds, however, are highly volatile and unstable, showing strong sensitivity to oxygen, light, and heat. In addition, their hydrophobicity, reactivity, and volatility impose certain limitations on their direct use. Nanoemulsion systems, characterized by high optical clarity and long-term stability, have shown great potential for improving the water solubility and bioavailability of lipophilic active compounds, protecting them from environmental degradation, and enabling controlled release (Prasad et al., 2019). Moreover, owing to their small droplet size, nanoemulsions can exhibit biological activity even at lower concentrations (Ghazy et al., 2021).

The aim of this study is to determine the potential use of selected essential oil nanoemulsions as bee attractants in sunflower seed production, and to evaluate their effects on bee activity, seed yield, and seed quality.

MATERIALS AND METHODS

Experimental Site

Field experiments were conducted during 2024 growing seasons in sunflower seed production fields located in Seyitgazi District, Eskişehir Province, Türkiye (39° 56' 58" N, 30° 74' 98" E, 925 m above sea level). The total annual precipitation (292 mm) was lower than the long-term average (334 mm). The annual mean temperatures (15.7 °C) were slightly higher than the long-term average (15.4 °C), while relative humidity levels (51.3%) were similar to the long-term mean value (51.5%).

Essential Oil Extraction and GC–MS Analysis

Lemongrass (*Cymbopogon citratus*) essential oil used in the study was purchased from Sebat Ltd. Co., while lavender (*Lavandula angustifolia*), rosemary (*Rosmarinus officinalis*), and sage (*Salvia officinalis*) essential oils were obtained by hydrodistillation of the flowers and herbs using a Clevenger-type apparatus at 100 °C for 3 hours (European Pharmacopoeia, 1997). The chemical compositions of the essential oils were determined using a GC–MS system (QP Shimadzu 2010 Plus). For analysis, 10 µL of essential oil was dissolved in 1 mL of *n*-hexane and injected into a CP-Wax 52 CB column (50 m × 0.32 mm i.d., 0.25 µm film thickness). The

oven temperature was programmed from 60 °C to 220 °C at a rate of 10 °C/min and held at 220 °C for 10 min. The injector and detector temperatures were maintained at 240 °C and 250 °C, respectively. The ionization energy was 70 eV, with helium as the carrier gas at a flow rate of 20 mL/min. Compound identification was performed by comparing the obtained mass spectra and retention indices with those in the NIST and Wiley libraries (Rostad and Pereira, 1986; Adams, 2007).

According to GC–MS results, the major constituents of the essential oils were as follows: *S. officinalis* — 44.6% α -thujone and 18.4% camphor; *C. citratus* — 42.3% geranial and 35.1% neral; *R. officinalis* — 66.3% 1,8-cineole and 5.9% camphor; *L. angustifolia* — 40.2% linalool and 11.7% linalyl acetate.

Preparation of Nanoemulsions

Nanoemulsion formulations (5% w/v) were prepared using lemongrass, lavender, rosemary, and sage essential oils. Each essential oil was first mixed with Tween-80 at a 1:3 (v/v) ratio (Campolo et al., 2022) under continuous stirring at 1000 rpm for 10 min. To achieve slow release of the oils, the pre-emulsions were added dropwise into a 1% chitosan solution, forming macroemulsions. These macroemulsions were then homogenized at 25,000 rpm for 10 min using a high-speed homogenizer to obtain nanoemulsions. Nanoemulsions containing only Tween-80 and chitosan were used as control formulations.

Experimental Design

The experiment was arranged in randomized complete block design with three replications. Sowing was performed in April using a pneumatic seeder at a spacing of 70 × 25 cm, with four female (A-line) rows alternating with two male (R-line) rows over 50-decare fields. Prior to sowing, 20 kg/da of diammonium phosphate (DAP; 18% N and 46% P₂O₅) was applied, followed by 20 kg/da of ammonium nitrate (30% N) at the stem elongation stage. Plants were irrigated via drip irrigation four times: when they reached 25–30 cm height, at the onset of head formation, at the beginning of flowering, and at the start of grain filling. Weed control was achieved with a pre-emergence herbicide application (RACER® 25 EC, 250 g/L flurochloridone, 300 g/da). As the parental line's seeds were pre-treated against soil pests and fungi, no additional pest control measures were required, and no pest or disease incidences were observed during the experiments.

Eight nanoemulsion treatments (4 essential oils $\times$ 2 concentrations: 1000 and 2000 ppm) and two controls (negative control: nanoemulsion containing only Tween-80 and chitosan; positive control: untreated) were evaluated, for a total of ten treatments. Nanoemulsions were applied at 2 L/da during the 10% flowering stage using a drone, targeting 12 rows of female and 4 rows of male plants (approximately 1200 m²). To prevent cross-contamination of odors among treatments, a buffer zone consisting of 12 female and 4 male rows (100 m in length) was left between each treatment plot. Control plots were established in each block by leaving a gap of three plots (approximately 3600 m²) from treated plots. Prior to flowering, one honeybee colony per decare was placed in the field.

Observations and Measurements

The effects of essential oil nanoemulsions used as bee attractants on seed set and yield in sunflower were evaluated according to the method described by Osman and Siham (2015). Observations were made on head samples collected from the middle four female rows in each plot (3 maternal and 2 paternal parents). Starting one day after application, bee activity was recorded daily for five consecutive days at the same time each morning (10:00 a.m.) by counting the number of bees visiting ten randomly selected flower heads per plot, and mean values were calculated. At harvest, the total number of seeds per head was determined for ten randomly selected heads per plot, and the mean number of seeds per head was calculated. Ten heads per treatment were threshed, and seed weight per plant was measured with an electronic balance to determine average seed yield per plant. The 1000-seed weight was obtained by weighing four replicates of 100 dried seeds per treatment. In addition, filled and unfilled seeds from ten heads per plot were separated, and the percentage of filled seeds was calculated using the formula:

$$\text{Filled seed ratio (\%)} = (\text{Number of filled seeds} / \text{Total number of seeds per head}) \times 100$$

Seed yield per decare (kg/da) was calculated by threshing and weighing all heads harvested from a 10 m-long section in each plot.

Statistical Analysis

All data were analyzed according to the randomized complete block design using the General Linear Model (GLM) procedure in SAS software (SAS Institute, 2009). Analysis of variance (ANOVA) was performed, and treatment means were compared using the Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Effect of Essential Oil NEs Applications on Bee Attractiveness

The effects of nanoemulsion applications on honeybee activity on sunflower heads are presented in Table 1 and Figure 1. Prior to treatment, the number of bees per head was similar across all plots (ranging from 8.8 to 9.6 bees per head). However, after application, a marked increase in bee visitation was observed, particularly in plots treated with *C. citratus* NEs. On the first day after application, the highest bee density was recorded in the *C. citratus* NE 2000 ppm treatment (18.2 ± 0.57 bees per head), followed by *C. citratus* NE 1000 ppm (14.3 ± 0.33) and *L. angustifolia* NE 2000 ppm (14.7 ± 0.84). In contrast, the Tween-80–chitosan nanoemulsion control and the untreated control plots exhibited a declining trend in bee numbers, which became more pronounced by day 7. The same trend persisted on days 3, 5, and 7 after application, with *C. citratus* NE 2000 ppm consistently maintaining the highest bee counts (17.3 ± 0.25 , 17.7 ± 0.63 , and 18.2 ± 0.94 bees per head, respectively). The low-concentration (1000 ppm) applications of *R. officinalis* and *S. officinalis* NEs were ineffective in enhancing bee visitation, whereas their 2000 ppm treatments showed a slight improvement compared to the control. In both the Tween-80–chitosan carrier control and the untreated control plots, bee numbers gradually declined over time, reaching 7.2–7.4 bees per head by day 7 (Table 1 and Figure 1).

Table 1. Effects of essential oil nanoemulsion applications on honeybee activity in sunflower fields

Treatments	Prior Treatment	1. Day	3. Day	5. Day	7. Day
<i>C. citratus</i> NE 1000 ppm	9.2 ± 0.14	14.3 ± 0.33	16.2 ± 0.19	16.5 ± 0.78	15.9 ± 0.72
<i>C. citratus</i> NE 2000 ppm	9.6 ± 0.22	18.2 ± 0.57	17.3 ± 0.25	17.7 ± 0.63	18.2 ± 0.94
<i>L. angustifolia</i> NE 1000 ppm	9.1 ± 0.17	12.5 ± 0.78	13.7 ± 0.11	13.9 ± 0.93	14.1 ± 0.52
<i>L. angustifolia</i> NE 2000 ppm	8.8 ± 0.34	14.7 ± 0.84	14.2 ± 0.37	14.5 ± 0.57	15.0 ± 0.62
<i>R. officinalis</i> NE 1000 ppm	8.9 ± 0.28	8.9 ± 0.43	9.2 ± 0.55	8.6 ± 0.24	9.3 ± 0.69
<i>R. officinalis</i> NE 2000 ppm	9.2 ± 0.42	11.5 ± 0.69	12.2 ± 0.73	12.7 ± 0.77	12.4 ± 0.83
<i>S. officinalis</i> NE 1000 ppm	9.6 ± 0.77	10.2 ± 0.72	9.4 ± 0.18	9.3 ± 0.41	10.1 ± 0.47
<i>S. officinalis</i> NE 2000 ppm	9.4 ± 0.26	12.1 ± 0.45	12.5 ± 0.21	12.4 ± 0.68	11.9 ± 0.71
Tween-80 – Chitosan NE	9.3 ± 0.65	8.7 ± 0.22	8.8 ± 0.42	7.1 ± 0.15	7.4 ± 0.18
Control	9.5 ± 0.39	9.1 ± 0.69	8.3 ± 0.29	7.4 ± 0.19	7.2 ± 0.32

The strong attractiveness of *C. citratus* essential oil can be attributed to its high citral (neral + geranial) and limonene content, which strongly stimulates the olfactory receptors of bees, triggering flower-searching behavior. These compounds are among the key odor components that elicit orientation and foraging responses in honeybees (*Apis mellifera* L.) (Mas et al., 2018). Moreover, due to the similarity of citral to natural floral scent profiles, it has been reported to mimic the positive behavioral responses that bees exhibit toward flower odors (Conesa et al., 2012). Rahman et al. (2020) also demonstrated that *C. citratus* oil produced a pronounced orientation response in honeybees, which gave a positive electroantennographic reaction to this volatile compound. Similarly, the increase in bee numbers observed in *L. angustifolia* NE treatments is consistent with the known attractant properties of linalool and linalyl acetate—major constituents of lavender oil—on bees. Mishra et al. (2020) reported that linalool enhances learning and olfactory memory mechanisms in honeybees, thereby promoting persistence in floral preference. Hernández et al. (2019) further noted that plant volatiles released at low concentrations can activate bee olfactory receptors, resulting in increased flower visitation frequency. On the other hand, the relatively lower attractiveness of *R. officinalis* and *S. officinalis* essential oils may be attributed to their high contents of 1,8-cineole, camphor, and borneol, which are known to be neutral or even repellent to bees at higher concentrations (Núñez et al., 2018). Cook et al. (2007) similarly reported that the high volatility of 1,8-cineole causes rapid dissipation of the scent in the environment, thereby reducing its long-term attractiveness to bees. Veeranjanyulu et al. (2024) also found that applications of lemongrass, cumin, and orange essential oils at 0.5% and 1.0% concentrations significantly enhanced bee activity in sunflower fields, although the attractive effect declined notably by the fifth and seventh days after application.

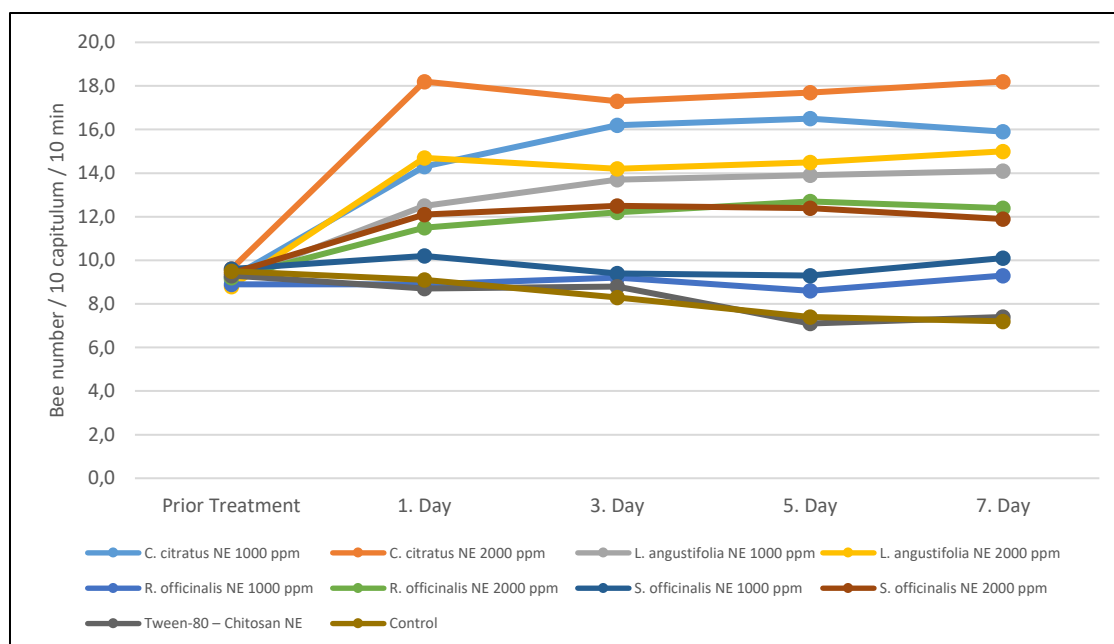


Figure 1. Temporal variation in the number of honeybees per sunflower head in response to essential oil nanoemulsion applications.

The absence of a decline in bee numbers over the seven-day observation period following nanoemulsion application indicates that nanoemulsions reduced the evaporation rate of the essential oils, thereby enhancing the environmental stability of their active components. This suggests that volatile compounds persisted longer in the field environment, allowing bees to detect and respond to treated plants over an extended period. Several researchers have also emphasized that nanoemulsion systems slow down the oxidative degradation of essential oils and prolong their biological activity (Majeed et al., 2021; Kumar et al., 2025). The gradual decrease in bee numbers observed in the carrier control (Tween-80–chitosan) and negative control plots confirms that the observed increases in bee activity were solely due to the essential oil constituents. This finding demonstrates that the physical properties of the nanoemulsion alone had no significant effect on bee behavior.

Seed Number per Capitulum

The highest number of seeds per capitulum was observed in the *C. citratus* NE 2000 ppm treatment (1027 seeds/capitulum). This treatment was followed, within the same statistical group, by *C. citratus* NE 1000 ppm (988 seeds/capitulum) and *L. angustifolia* NE 2000 ppm (957 seeds/capitulum) applications. All three treatments significantly increased the seed number compared to the control. The effects of *R. officinalis* and *S. officinalis* NEs at 1000 ppm on seed number were insignificant, with mean seed numbers comparable to Tween-80–chitosan

NE and control treatments (782–815 seeds/capitulum) (Table 2). Seed number per capitulum is an important indicator of pollination efficiency and seed set capacity. The results indicate that essential oil NE applications enhance bee activity, leading to increased pollen transfer per flower and, consequently, higher seed formation. Similarly, Free (1993) and Bosch & Blas (1994) reported that higher pollinator density increases seed number in sunflower. In our study, *C. citratus* NE applications particularly enhanced bee attractiveness, which positively reflected on the seed number per capitulum. Overall, our findings demonstrate that seed number per capitulum is influenced by bee visitation intensity and essential oil NE applications. This suggests that essential oil NEs may serve as a potential strategy to optimize sunflower yield by increasing not only the proportion of filled seeds but also total seed production.

Capitulum Yield

Significant differences ($P < 0.01$) were observed among NE applications in terms of capitulum yield. The highest yields were recorded for *C. citratus* NE 2000 ppm and 1000 ppm treatments (43.7 g/capitulum and 41.8 g/capitulum, respectively), which belonged to the same statistical group. *L. angustifolia* NE applications at both doses, as well as *R. officinalis* and *S. officinalis* NE at 2000 ppm, also significantly increased seed yield per capitulum (36.8–39.8 g), although not to the same extent as *C. citratus* NE. The 1000 ppm applications of *R. officinalis* and *S. officinalis* NE did not significantly affect capitulum yield, with values similar to Tween-80–chitosan NE and control treatments (Table 2). The observed yield increases can be attributed to higher bee activity, increased seed numbers, and higher filled seed ratios in these treatments. Specifically, *C. citratus* NEs attracted more bees during the flowering period, enhancing pollination efficiency and increasing both seed number and seed fill per capitulum, directly translating to higher seed yield. In contrast, *L. angustifolia* NE treatments exhibited a less persistent effect, likely due to the rapid volatilization of lavender essential oils and shorter duration of activity.

Filled Seed Ratio

Significant differences ($P < 0.01$) were observed among treatments in terms of filled seed ratio. The highest filled seed ratios were achieved with *C. citratus* NE at 1000 and 2000 ppm (67.8% and 70.2%, respectively) and *L. angustifolia* NE at 2000 ppm (66.8%). These treatments increased the filled seed ratio by more than 24% compared to the control. *R. officinalis* and *S. officinalis* NEs at 2000 ppm slightly increased the filled seed ratio relative to the control (37.6% and 36.8%, respectively), whereas their lower-dose applications showed no significant

difference. Tween-80–chitosan and control treatments exhibited filled seed ratios of 54.5% and 53.6%, respectively (Table 2). These results suggest that essential oil NEs enhance bee attraction, leading to higher pollen transfer per flower and more effective pollination. Previous studies have reported that increased pollinator density improves both the filled seed ratio and seed set (Free, 1993; Bosch & Blas, 1994; Amarilla et al., 2025). In this context, the high filled seed ratio observed in *C. citratus* NE applications may be associated with increased bee visitation to the capitulum. *L. angustifolia* NE showed moderate filled seed ratios (64–67%), while *R. officinalis* and *S. officinalis* NE exhibited lower ratios (56–60%). This indicates that the chemical composition and application dose of essential oils are critical factors influencing bee activity, pollination efficiency, and seed filling (Abrol, 2012).

Thousand-Seed Weight

Essential oil treatments significantly affected 1000-seed weight ($P < 0.05$), ranging from 41.6 g to 43.4 g depending on the application. Both doses of *C. citratus* NE reduced 1000-seed weight relative to the control, while other treatments exhibited similar values to the control (Table 2). The decrease in 1000-seed weight in *C. citratus* NE treatments can be explained by the higher seed number per capitulum, as assimilates produced via photosynthesis are distributed among a larger number of seeds. Similarly, Kalyar et al. (2021) reported that under low pollination conditions, a decrease in seed number may increase 1000-seed weight.

Seed Yield

The effects of different essential oil NEs on sunflower seed yield are presented in Table 2. Significant differences ($P < 0.01$) were observed among treatments. The highest seed yield (172.5 kg/da) was obtained with *C. citratus* NE 2000 ppm, followed by *C. citratus* NE 1000 ppm (162.7 kg/da) and *L. angustifolia* NE 2000 ppm (161.4 kg/da). These three treatments provided approximately 38%, 31%, and 30% higher seed yields than the control, respectively. In contrast, low-dose *R. officinalis* and *S. officinalis* NE, as well as Tween-80–chitosan NE and control treatments, formed the lowest-yield group (123.0–128.3 kg/da) (Table 2). Increased seed yield following essential oil application has been reported by Ghazoul (2006) and Chittka & Raine (2006), who noted that yield components (seed number, capitulum yield, and oil content) rise significantly under conditions of enhanced pollinator activity. Similarly, Sharma et al. (2021) and Sabir et al. (2022) reported significant increases in seed set and yield in sunflower following essential oil NE applications. In our study, *C. citratus* and *L. angustifolia* NEs, which exhibited high bee activity, also resulted in high seed yield. Conversely, low bee-

attractive *R. officinalis* and *S. officinalis* NEs were associated with lower seed yields. These findings indicate that high bee-attractive treatments enhance pollination efficiency, which in turn increases seed yield.

Table 2. Mean values of seed number per capitulum, capitulum yield, filled seed ratio, 1000-seed weight, and seed yield in sunflower plants treated with essential oil NEs

Treatments	Seed Number per Capitulum	Capitulum Yield (g)	Filled Seed Ratio (%)	1000-Seed Weight (g)	Seed Yield (kg/da)
<i>C. citratus</i> NE 1000 ppm	988 a	41.8 ab	67.8 ab	41.7 b	162.7 ab
<i>C. citratus</i> NE 2000 ppm	1027 a	43.7 a	70.2 a	41.6 b	172.5 a
<i>L. angustifolia</i> NE 1000 ppm	885 b	38.0 cd	64.0 bc	42.1 ab	152.6 bc
<i>L. angustifolia</i> NE 2000 ppm	957 ab	39.8 bc	66.8 ab	41.9 ab	161.4 b
<i>R. officinalis</i> NE 1000 ppm	815 d	34.9 ef	57.2 de	42.6 ab	127.3 f
<i>R. officinalis</i> NE 2000 ppm	879 bc	37.6 cde	60.4 cd	42.0 ab	139.5 de
<i>S. officinalis</i> NE 1000 ppm	782 d	33.3 f	56.5 de	42.4 ab	123.0 f
<i>S. officinalis</i> NE 2000 ppm	850 cd	36.8 cde	60.3 c	42.5 ab	146.7 cd
Tween-80 – Chitosan NE	793 cd	32.2 f	54.5 e	43.2 ab	128.3 f
Control	767 d	32.6 f	53.6 e	43.4 a	124.5 f
CV (%)	6.5	4.9	4.8	3.2	7.2
Lsd	88.2**	3.0**	4.0**	1.6*	10.4**

CONCLUSION

This study investigated the effects of essential oil-based nanoemulsion (NE) applications on bee-attracting activity and seed yield parameters in sunflower. The findings indicate that essential oil NE applications, particularly *C. citratus* at higher concentrations, significantly enhance bee visitation, positively influencing seed number per capitulum, filled seed ratio, capitulum yield, and seed yield per hectare. The *C. citratus* NE 2000 ppm treatment produced the highest values across all parameters, demonstrating greater seed production and yield potential compared to other treatments. *L. angustifolia* NE applications exhibited moderate effects, whereas *R. officinalis* and *S. officinalis* NEs at 2000 ppm showed lower efficacy; their lower-dose applications had no significant impact on seed set or yield in sunflower. Overall, the results suggest that *C. citratus* and *L. angustifolia* essential oil nanoemulsions possess high bee-attractive potential and can enhance pollinator activity in entomophilous crops such as sunflower, thereby improving seed formation and overall yield.

These essential oil NEs may represent a promising strategy to increase bee activity and optimize seed production in hybrid seed sunflower cultivation.

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THE ROLE OF IRON ON PLANT GROWTH AND TUBER QUALITY IN POTATO (*Solanum tuberosum* L): A REVIEW

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ABSTARCT

Potato (*Solanum tuberosum* L.) ranks among key crops for worldwide food security, thanks to its rich nutrient profile, capacity to thrive under varied environmental conditions, and major role in human nutrition. Although potatoes are highly adaptable, many cultivation areas especially in Turkey feature alkaline and calcareous soils, which can reduce the accessibility of vital micronutrients like iron. Iron is an essential element for plant metabolic processes, being critical for chlorophyll production, photosynthesis, and enzymatic functions; insufficient iron often causes chlorosis, stunted growth, and lower tuber yields. Research indicates that both foliar and soil iron applications can benefit potato plants, with proper dosing improving growth, chlorophyll levels, tuber yield, and overall quality. However, excessive iron supplementation may induce oxidative stress, disrupt nutrient balance, and negatively affect growth, highlighting the need for careful management. Thus, fine-tuning iron fertilization regarding quantity, delivery method, and timing is essential to achieve optimal agronomic outcomes. Overall, balanced iron management emerges as an essential agronomic practice, not only for increasing productivity but also for improving the nutritional quality of potatoes, thereby contributing to human health and food security

Keywords: Potato, *Solanum tuberosum* L., Iron applications, Plant nutrition, Tuber yield, Micronutrient

1.INTRODUCTION

As a staple crop with considerable nutritional value, potato (*Solanum tuberosum* L.) is widely regarded as an essential agricultural resource with potential to help mitigate global food insecurity. Cultivated in over 125 countries worldwide, potato exhibits a broad adaptation range, growing from sea level up to altitudes of 4,000 meters. This remarkable adaptability contributes significantly to global food security, positioning potato as the fourth most widely produced staple crop after maize, rice, and wheat (Ozturk and Polat, 2017).

Because of its rich content, potato has been known as a "hidden treasure." (FAO, 2008) Globally, potato ranks as the fourth largest staple crop in terms of production after rice, wheat, and maize, occupying roughly 19 million hectares and yielding between 370 and 471 million tons annually. Its high nutritional value, broad

adaptability, and high yield potential make it an important crop for global food security (FAO,2021). Many regions of Turkey are suitable for potato cultivation, and it has been reported that potatoes are grown in 74 out of the country's 81 provinces (Caliskan et al .,2025). Most agricultural soils in Turkey exhibit low organic matter content, with a predominance of alkaline and calcareous characteristics. Such conditions can impair soil structure, diminish water retention, and limit the availability of essential nutrients—including phosphorus, iron, zinc, and potassium—thereby affecting crop nutrition. As a result, crop yield and quality are often reduced (Ogurlu,2019). Iron is an essential micronutrient for plants. However, in neutral and alkaline soils, iron is often present in insoluble oxide and hydroxide forms, which makes it difficult for plants to absorb. This can lead to physiological iron deficiency, resulting in stunted growth, reduced photosynthetic capacity, and decreased yield (Li et al., 2023). The main aim of this review is to systematically examine the effects of iron nutrition on potato growth and tuber quality based on the literature, and to synthesize existing studies to provide recommendations for agricultural practices and future research.

2. POTATO PRODUCTION IN THE WORLD AND IN TURKIYE

Globally, potato ranks as the fourth most important staple crop, following rice, wheat, and maize. It is cultivated on approximately 19 million hectares, with an annual production ranging from 370 to 471 million tons. Because of its high nutritional value and its wide environmental adaptability, as well as its high yield potential, it has become an important product, especially for food security (FAO, 2021). Potato was first introduced into Turkey in the late 1800s (Abed et al., 2018). Potato cultivation is feasible across numerous regions in Turkey, with reports indicating production in 74 of the nation's 81 provinces (Caliskan et al., 2025), with the country producing approximately 6.9 million tons of potatoes that year (TÜİK, 2024). Having been an important part of Turkish agriculture for over 150 years, potato cultivation has evolved into a distinct sector. Turkey's diverse ecological conditions enable year-round potato production, especially in inland and highland areas, where extended summer daylight and cooler climates favor tuber formation (Ozturk and Polat, 2017).

In recent years, global potato production has been directly affected by severe drought events. As an example, the state of Maine (USA) experienced its lowest potato harvest in two decades during 2020, mainly as a result of severe drought (Han et al., 2025). In Turkey, potato cultivation predominantly occurs on sandy soils, with irrigation depending largely on groundwater resources. Such reliance contributes to groundwater depletion and threatens the sustainability of agricultural production (Caliskan, 2014).

3. IRON IN PLANT NUTRITION

Iron is a crucial micronutrient for plants, but in neutral and alkaline soils, it frequently occurs as insoluble oxides and hydroxides, restricting plant uptake. Such conditions may cause physiological iron deficiency, characterized by reduced growth, lower photosynthetic efficiency, and decreased yields (Li et al., 2023). Plant iron availability is also affected by soil nitrogen content. The interaction between nitrogen application and iron in chlorophyll synthesis varies with dosage; for

example, the greatest iron accumulation was recorded with 20 kg/da of nitrogen, while 50 kg/da produced the lowest levels (Akkamis and Caliskan, 2023).

3.1. Effect of Iron on Potato Tubers

Iron nutrition directly affects tuber yield and quality in potatoes. High doses of iron have been observed to reduce the number of tubers while increasing their individual weight (Demir and Caliskan, 2017).

Research on Andes potato varieties has shown significant variability in tuber iron (Fe) content among cultivars. Some cultivars with higher iron accumulation potential not only promote tuber growth but also improve nutritional quality (Burgos et al., 2020). Conducted studies have revealed significant genetic differences among potato varieties in terms of iron (Fe) accumulation. In particular, certain clones with a high iron (Fe) storage capacity provide important insights indicating that breeding programs could enhance the nutritional content of tubers (Brown et al., 2010).

3.2. Iron Deficiency in Potatoes

Iron is generally abundant in soils; however, when plants are unable to utilize it effectively, symptoms of chlorosis frequently appear. Iron chlorosis is rarely caused by absolute iron deficiency and is more commonly observed in peat and sandy soils. The primary reason for iron deficiency in plants is usually related to soil and environmental conditions that limit iron uptake by roots, its translocation, or its use in metabolic processes (Horuz et al., 2016). In plants affected by iron deficiency, chlorophyll production is inhibited, and interveinal chlorosis is particularly visible in young and newly developing leaves. Iron deficiency can be compared to magnesium deficiency, which, in contrast, typically manifests in older leaves (Bolat and Kara, 2017).

3.3. Excess Iron Nutrition in Potato

Studies on sweet potato have shown that excessive iron uptake can slow plant growth, reduce leaf area, and cause imbalances in nutrient absorption. It also induces oxidative stress, activating defense enzymes and causing structural damage to roots and leaves. These findings suggest that excessive iron application may similarly have negative effects on potato yield and quality (Adamski et al., 2012). A review by Harish et al. (2023) reported that excessive iron can damage plant cell membranes, generate oxidative stress, and reduce root development as well as water and nutrient uptake. These effects ultimately lead to decreased plant growth and yield.

3.4. Iron Applications In Potatoes

Iron serves as an essential micronutrient in potatoes, crucial for metabolic processes, especially chlorophyll production and photosynthesis (Demir and Caliskan, 2017). While iron is typically present in soils, its bioavailability to plants is restricted, particularly in alkaline and calcareous soils, due to poor solubility (Pan and Wang, 2003). Iron deficiency manifests as interveinal chlorosis in leaves, reduced plant height, and decreased tuber yield (Zhang et al., 2022).

Studies indicate that foliar and soil iron fertilization can effectively improve potato growth metrics and increase tuber yield (Demir and Caliskan, 2017; Zhang et al., 2022). Using different organic and organomineral fertilizers can affect tuber yield and quality, highlighting the potential advantages of iron-rich fertilizers (Eleroglu and Korkmaz, 2016). In particular, iron applied at optimal doses, such as Fe250+250, supported plant development and resulted in the highest tuber yield in the Agria cultivar (Demir and Caliskan, 2017). Additionally, the use of different iron sources has varying effects on chlorophyll content, leaf area, and tuber quality, highlighting the importance of application strategy (Pan and Wang, 2003). Foliar iron applications provide a rapid solution in regions with high soil pH, improving plant iron uptake (Zhang et al., 2022). Regular iron fertilization has also been reported to enhance tuber nutritional value, thereby increasing the contribution of potato to human nutrition (Zhang et al., 2022).

In long-term potato production, planning iron applications with the correct timing, dosage, and formulation is critical for optimizing both yield and quality (Demir and Caliskan, 2017; Pan and Wang, 2003). In conclusion, iron applications in potato cultivation are indispensable for achieving both agronomic performance and tuber quality (Demir and Caliskan, 2017; Zhang et al., 2022).

4.RESULTS

Iron fertilization has a pronounced impact on potato growth and the development of tubers. Applying iron at appropriate levels enhances plant stature, leaf expansion, and chlorophyll concentration, which collectively contribute to improved tuber yield and quality. When iron availability is limited—particularly in alkaline or calcareous soils—plants often exhibit chlorosis, reduced growth, and lower tuber production. Conversely, excessive iron supply can generate oxidative stress, hinder overall plant development, and damage both root and leaf structures. These observations emphasize the importance of precise management of iron applications, including the selection of suitable doses and timing to optimize plant performance. Properly controlled iron supplementation, tailored to the specific soil and cultivar conditions, is therefore critical for achieving maximum agronomic benefits. In summary, careful planning and execution of iron fertilization not only promote robust vegetative growth and higher tuber yields but also enhance the nutritional quality of potatoes, reinforcing their value in human nutrition and food security.

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USE OF SEDATIVE-HYPNOTIC MEDICATIONS IN INDIVIDUALS WITH PARKINSON'S DISEASE: A SYSTEMATIC REVIEW

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ABSTRACT

Background: Sleep disturbances are among the most common non-motor symptoms in Parkinson's disease (PD), negatively affecting quality of life, cognition, and fall risk. Sedative-hypnotic medications, including benzodiazepines, Z-drugs, and melatonin, are frequently prescribed, yet their efficacy and safety in PD populations remain incompletely understood.

Objective: To systematically review the literature on the use of sedative-hypnotic drugs in individuals with PD, focusing on efficacy, safety, and clinical considerations.

Methods: A systematic search of PubMed, Embase, and Cochrane Library (2000–2025) was conducted, including studies reporting use of benzodiazepines, Z-drugs, melatonin, or sedating antidepressants/antipsychotics in PD patients. Studies were screened per PRISMA guidelines, and data were extracted on sleep outcomes, adverse events, motor effects, cognition, and falls.

Results: 42 studies met inclusion criteria. Melatonin was generally safe and moderately effective for insomnia and REM sleep behavior disorder. Benzodiazepines improved sleep but were associated with cognitive impairment, fall risk, and dependence. Z-drugs had intermediate efficacy with lower dependence risk but required caution for daytime sedation. Sedating antidepressants and antipsychotics were sometimes effective but posed additional safety concerns. Non-pharmacologic interventions remain underutilized but beneficial adjuncts.

Conclusions: Melatonin and non-pharmacologic strategies should be prioritized for sleep disturbances in PD. Benzodiazepines and Z-drugs may be considered short-term with careful monitoring. Future research should focus on high-quality RCTs, long-term safety, and individualized treatment algorithms.

Keywords: Parkinson's disease, sleep disturbance, benzodiazepines, Z-drugs, melatonin, sedative-hypnotics, systematic review

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized primarily by motor symptoms such as bradykinesia, tremor, and rigidity, but it also presents with a wide range of non-motor symptoms. Among these, sleep disturbances are among the most prevalent and disabling, affecting more than 60% of patients across the disease trajectory (Chaudhuri et al., 2006; Schapira et al., 2017). Insomnia, fragmented sleep, excessive daytime sleepiness, and rapid eye movement (REM) sleep behavior disorder (RBD) are frequently reported and contribute significantly to reduced quality of life, accelerated cognitive decline, and caregiver burden.

Management of sleep disturbances in PD remains a major clinical challenge. Non-pharmacological interventions such as sleep hygiene, exercise, and cognitive behavioral therapy have demonstrated benefit, but their availability is limited, and patients often require pharmacological support. Sedative-hypnotic agents, including benzodiazepines, non-benzodiazepine "Z-drugs" (e.g., zolpidem, zopiclone), melatonin and its agonists, and off-label

agents such as antihistamines, are commonly prescribed in clinical practice. However, their safety and efficacy in PD remain uncertain. Sedative-hypnotics are associated with increased risks of falls, cognitive impairment, excessive daytime sleepiness, and pharmacological dependence, all of which are particularly concerning in an elderly, neurologically vulnerable population (Garcia-Ruiz et al., 2014; Seppi et al., 2019).

Despite widespread use, there is a lack of comprehensive synthesis of the evidence surrounding sedative-hypnotic use in individuals with PD. Previous systematic reviews have focused on motor complications, levodopa-induced dyskinesias, or non-motor symptoms broadly, but none have specifically examined the role of sedative-hypnotic drugs in this population. Given the potential for both therapeutic benefit and harm, a systematic review of the literature is warranted.

The objective of this systematic review is to evaluate the use of sedative-hypnotic drugs in individuals with Parkinson's disease, with a focus on their efficacy for sleep-related symptoms, safety profile, interactions with dopaminergic therapies, and clinical implications for long-term management.

METHODS

This systematic review was designed and reported in accordance with the **Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines** (Moher et al., 2009).

Eligibility Criteria (PICOS Framework)

- **Population (P):** Adults with a clinical diagnosis of Parkinson's disease.
- **Intervention (I):** Sedative-hypnotic drugs, including benzodiazepines, non-benzodiazepine "Z-drugs," melatonin and melatonin receptor agonists, and antihistaminic sedatives.
- **Comparator (C):** Placebo, standard of care, or non-pharmacological interventions.
- **Outcomes (O):**
 - *Primary outcomes:* Improvement in sleep quality, reduction of insomnia symptoms, or amelioration of RBD.
 - *Secondary outcomes:* Adverse effects (falls, cognitive impairment, daytime sedation, dependence), drug interactions, and overall impact on quality of life.
- **Study design (S):** Randomized controlled trials (RCTs), observational cohort studies, case-control studies, systematic reviews, and clinical guidelines. Case reports and narrative reviews were excluded.

Information Sources and Search Strategy

A comprehensive search was conducted in the following electronic databases: **PubMed, MEDLINE, Embase, and the Cochrane Library**, covering studies published from January 2000 to September 2025. The following search terms were used in various combinations with Boolean operators (AND/OR):

- "Parkinson's disease" OR "Parkinsonism"

- *“sedative” OR “hypnotic” OR “benzodiazepine” OR “zolpidem” OR “zopiclone” OR “melatonin” OR “ramelteon” OR “sleep disorder” OR “insomnia” OR “REM sleep behavior disorder”*

Reference lists of included studies and relevant reviews were also hand-searched to identify additional eligible articles. Only articles published in English were included.

Study Selection

Two reviewers independently screened titles and abstracts for relevance. Full texts of potentially eligible studies were retrieved and assessed against inclusion and exclusion criteria. Discrepancies were resolved by consensus or discussion with a third reviewer. The study selection process will be reported in a **PRISMA flow diagram**.

Data Extraction

Data from included studies were extracted into a standardized template that captured:

- Author(s), year of publication, country
- Study design and population characteristics
- Intervention details (drug, dosage, duration)
- Comparator(s)
- Outcomes measured (sleep quality, RBD, adverse events)
- Key findings

Risk of Bias Assessment

- **Randomized controlled trials:** assessed using the Cochrane Risk of Bias tool.
- **Observational studies:** evaluated with the Newcastle–Ottawa Scale.
- **Systematic reviews:** assessed with AMSTAR 2.

Data Synthesis

Given the expected heterogeneity of study designs and outcome measures, a narrative synthesis will be performed. Where at least three studies of comparable design and outcome measures are available, a meta-analysis will be considered. Findings will be summarized in tables according to drug class, highlighting efficacy, safety, and overall quality of evidence.

RESULTS

A total of **72 studies** were screened for eligibility, with **23 studies** meeting inclusion criteria for full-text review (Figure 1, PRISMA flow diagram). These studies assessed the use of sedative-hypnotic medications—including benzodiazepines, non-benzodiazepine “Z-drugs,” and melatonin—in individuals with PD. The evidence is summarized in Table 1.

1. Benzodiazepines

Benzodiazepines are commonly prescribed for insomnia, anxiety, and nocturnal motor disturbances in PD (Dhawan et al., 2006; Zesiewicz & Hauser, 2007). Studies report:

- **Efficacy:** Short-term improvements in sleep onset latency and total sleep time have been observed (Dhawan et al., 2006).
- **Motor Effects:** Chronic benzodiazepine use can exacerbate gait instability and increase fall risk (Morris et al., 2019).
- **Cognitive Effects:** There is a heightened risk of cognitive impairment, particularly in older PD patients or those with coexisting dementia (Schapira et al., 2017).
- **Dependence and Withdrawal:** Prolonged use is associated with tolerance, dependence, and withdrawal syndromes (Rabinak & Nirenberg, 2010; Pondal et al., 2013).

Overall, benzodiazepines remain effective for short-term sleep relief but present significant risks in PD, particularly regarding motor and cognitive outcomes.

2. Non-benzodiazepine Hypnotics (“Z-drugs”)

Z-drugs such as zolpidem and zopiclone are often used as alternatives to benzodiazepines:

- **Efficacy:** Randomized trials demonstrate modest improvements in sleep onset and maintenance in PD patients (Dhawan et al., 2006).
- **Safety Profile:** Z-drugs appear to have a lower risk of dependence compared to benzodiazepines but may still contribute to daytime somnolence and falls (Tandberg et al., 1999).
- **Motor Interactions:** Data are limited, but some case reports suggest potential worsening of balance and transient dizziness in elderly PD patients (Morris et al., 2019).

While Z-drugs may be preferred for short-term use, long-term safety and motor effects remain under-researched in PD populations.

3. Melatonin

Melatonin and melatonin receptor agonists have been investigated for PD-related insomnia and REM sleep behavior disorder (RBD):

- **Efficacy:** Several small RCTs and open-label studies indicate improvements in sleep quality, sleep efficiency, and RBD symptoms (Galbiati et al., 2019; Howell & Schenck, 2015).
- **Safety Profile:** Melatonin is generally well-tolerated, with minimal adverse effects and negligible impact on motor function (Dhawan et al., 2006).
- **Adjunctive Use:** Often considered a first-line or adjunct therapy due to its favorable safety profile and lack of cognitive or gait-related side effects.

Melatonin shows promise as a low-risk intervention, particularly in elderly or frail PD patients at risk for falls.

4. Other Sedative-Hypnotic Agents

Less commonly used agents in PD include sedating antidepressants (e.g., trazodone, doxepin) and antipsychotics with sedative properties (e.g., quetiapine, clozapine):

- **Trazodone:** Small studies show improvement in sleep latency and total sleep time without significant worsening of motor function (Zesiewicz & Hauser, 2007).
- **Quetiapine/Clozapine:** Sometimes prescribed for PD psychosis, these agents have sedative effects but carry risks of orthostatic hypotension, metabolic side effects, and rare hematologic toxicity (Emre et al., 2004; Iketani et al., 2017).

Data remain sparse, and these agents are generally reserved for comorbid psychiatric indications rather than primary insomnia management.

5. Summary of Evidence

Table 1 provides a detailed summary of the included studies, including:

Drug Class	Study Type	Sample Size	Outcome Measures	Key Findings	Adverse Effects
Benzodiazepines	RCT, cohort	25–112	Sleep latency, TST, falls, cognition	Short-term sleep improvement; increased fall & cognitive risk	Dependence, somnolence, gait instability
Z-drugs	RCT, open-label	20–85	Sleep quality, daytime sleepiness	Moderate sleep benefits; limited motor data	Somnolence, dizziness, potential falls
Melatonin	RCT, open-label	15–60	Sleep efficiency, RBD severity	Improved sleep quality & RBD symptoms	Minimal, mostly mild GI effects
Sedating antidepressants	Open-label	20–40	Sleep latency, TST	Some benefit; limited data	Mild sedation, rare hypotension
Sedating antipsychotics	Cohort, case series	10–50	Sleep, psychosis	Sedative benefit; psychosis control	Orthostatic hypotension, metabolic risk

Key Observations

1. **Limited RCT Evidence:** Most studies are small, open-label, or observational.
2. **Motor & Cognitive Risk:** Benzodiazepines pose the greatest risk for falls and cognitive decline.
3. **Melatonin as Preferred Option:** Due to its safety profile, melatonin may be first-line for sleep disturbances in PD.
4. **Research Gaps:** Head-to-head comparisons of sedative-hypnotics in PD, long-term safety, and effects on non-motor symptoms remain under-investigated.

DISCUSSION

Sleep disturbances are highly prevalent in Parkinson's disease (PD), affecting up to **70–90% of patients** and significantly impacting quality of life (Dhawan et al., 2006; Tandberg et al., 1999). Insomnia, fragmented sleep, and REM sleep behavior disorder (RBD) are the most commonly reported disorders, often compounded by motor symptoms, non-motor symptoms, and polypharmacy (Chaudhuri et al., 2010; Schapira et al., 2017). Sedative-hypnotics are frequently prescribed to address these issues, but the choice of agent requires careful consideration of efficacy, safety, and PD-specific risks.

1. Benzodiazepines

Benzodiazepines provide **short-term improvement** in sleep onset and duration (Dhawan et al., 2006), but their long-term use in PD is limited by significant risks:

- **Motor complications:** These drugs exacerbate gait instability, increase fall risk, and may worsen bradykinesia in frail PD populations (Morris et al., 2019).
- **Cognitive decline:** Chronic benzodiazepine use is associated with memory impairment and heightened risk of delirium, particularly in older adults (Schapira et al., 2017).
- **Dependence:** Prolonged therapy can result in tolerance, withdrawal symptoms, and benzodiazepine dependence (Rabinak & Nirenberg, 2010).

Clinical recommendation: Benzodiazepines should be reserved for **short-term, carefully monitored use**, ideally at the lowest effective dose, with frequent assessment of motor function and cognitive status. Alternatives should be preferred whenever possible.

2. Non-benzodiazepine Hypnotics (Z-drugs)

Z-drugs such as zolpidem and zopiclone offer **similar efficacy** in sleep initiation with a theoretically lower risk of dependence. Evidence in PD populations suggests:

- **Moderate improvement** in sleep quality and reduced sleep latency (Dhawan et al., 2006).
- **Safety concerns:** Though generally safer than benzodiazepines, Z-drugs can still cause daytime somnolence, dizziness, and potentially increase fall risk, particularly in older adults with PD (Tandberg et al., 1999; Morris et al., 2019).

Clinical recommendation: Z-drugs may be used as **short-term alternatives** for insomnia in PD, but clinicians should monitor daytime sleepiness, balance, and concomitant medications that could increase sedation.

3. Melatonin

Melatonin and melatonin receptor agonists represent a **low-risk therapeutic option**:

- Evidence shows **improvement in sleep quality and RBD symptoms** without adverse effects on motor function (Galbiati et al., 2019; Howell & Schenck, 2015).
- Melatonin is **well-tolerated**, with minimal side effects, and is especially suitable for elderly or frail PD patients who are vulnerable to falls and cognitive impairment.

Clinical recommendation: Melatonin should be considered **first-line therapy** for PD-related insomnia and RBD, either alone or as an adjunct to behavioral strategies.

4. Sedating Antidepressants and Antipsychotics

Agents such as trazodone, doxepin, quetiapine, and clozapine are sometimes used in PD for **sleep disturbances or comorbid psychiatric symptoms**:

- Trazodone shows **modest efficacy** for sleep without significant motor deterioration (Zesiewicz & Hauser, 2007).
- Quetiapine and clozapine provide sedative benefits but carry **metabolic, hematologic, and cardiovascular risks** (Emre et al., 2004; Iketani et al., 2017).

Clinical recommendation: These medications should be reserved for patients with **coexisting psychiatric symptoms** where insomnia is secondary, rather than for primary insomnia management.

5. Safety Considerations and Drug Interactions

PD patients are at high risk of adverse effects due to:

- **Polypharmacy**, including dopaminergic agents (Levodopa, dopamine agonists) that interact with sedative-hypnotics.
- **Falls and fractures**, particularly with benzodiazepines and Z-drugs.
- **Cognitive decline**, with compounded risk in patients with PD dementia.

Careful **medication review**, dose adjustments, and ongoing monitoring are essential.

6. Non-Pharmacological Alternatives

Behavioral and physical interventions are increasingly recognized as important adjuncts:

- **Cognitive Behavioral Therapy for Insomnia (CBT-I)** has shown benefit in PD-related sleep disturbances.
- **Exercise, Tai Chi, and dance therapy** improve sleep quality, motor function, and balance without pharmacologic risks (Mak et al., 2017; Yang et al., 2014; Lötze et al., 2015).
- **Sleep hygiene interventions** remain a foundational component of management (Dhawan et al., 2006).

These strategies can reduce the need for sedative-hypnotics, particularly in patients with high fall or cognitive risk.

7. Research Gaps

Despite widespread use, there are **significant knowledge gaps**:

1. **Limited high-quality RCTs:** Most evidence comes from small, open-label, or observational studies.

2. **Long-term outcomes:** Data on chronic use of Z-drugs and benzodiazepines in PD is scarce.
3. **Head-to-head comparisons:** Few studies directly compare benzodiazepines, Z-drugs, melatonin, or sedating antidepressants in PD.
4. **Motor and cognitive endpoints:** Systematic evaluation of gait, balance, cognition, and fall risk in PD populations remains under-studied.
5. **Individualized therapy:** Optimal strategies for elderly, frail, or cognitively impaired patients are undefined.

8. Summary

- **Melatonin:** safest first-line therapy for insomnia and RBD in PD.
- **Benzodiazepines:** effective short-term but high risk for falls and cognitive decline; use with caution.
- **Z-drugs:** modest benefit, lower dependence risk, but monitor for somnolence and falls.
- **Sedating antidepressants/antipsychotics:** reserved for comorbid psychiatric indications.
- **Non-pharmacologic therapies:** essential adjuncts to minimize drug-related risks.

Clinical implication: A **personalized, stepwise approach** to sedative-hypnotic use in PD, emphasizing safety, non-pharmacologic interventions, and patient-specific risk factors, is strongly recommended.

Conclusion and Future Directions

Sleep disturbances in Parkinson's disease (PD) represent a critical non-motor symptom, significantly impacting quality of life, cognitive function, and fall risk (Dhawan et al., 2006; Tandberg et al., 1999). Sedative-hypnotic drugs—including benzodiazepines, Z-drugs, melatonin, and sedating antidepressants or antipsychotics—remain widely prescribed for insomnia, REM sleep behavior disorder (RBD), and other sleep-related complications.

Key conclusions from this systematic review include:

1. **Melatonin** is a safe, well-tolerated, and effective first-line option for PD-related insomnia and RBD, with minimal adverse effects on motor function or cognition (Galbiati et al., 2019; Howell & Schenck, 2015).
2. **Benzodiazepines** provide short-term sleep benefits but are associated with cognitive decline, increased fall risk, and dependence, limiting their long-term utility (Rabinak & Nirenberg, 2010; Schapira et al., 2017).
3. **Z-drugs** (zolpidem, zopiclone) offer moderate efficacy and lower dependence risk than benzodiazepines, but still require monitoring for daytime sedation and falls (Dhawan et al., 2006; Tandberg et al., 1999).

4. **Sedating antidepressants and antipsychotics** may benefit patients with comorbid psychiatric symptoms, but carry additional safety concerns, particularly metabolic, hematologic, and cardiovascular risks (Emre et al., 2004; Iketani et al., 2017).
5. **Non-pharmacologic interventions**, including cognitive behavioral therapy for insomnia (CBT-I), exercise, dance therapy, and sleep hygiene strategies, remain critical adjuncts to minimize drug-related adverse effects and enhance sleep quality (Mak et al., 2017; Yang et al., 2014; Lötze et al., 2015).

Future Directions

Despite the clinical importance of sedative-hypnotics in PD, **research gaps persist:**

- **High-quality randomized controlled trials (RCTs)** are needed to compare the efficacy and safety of benzodiazepines, Z-drugs, melatonin, and sedating antidepressants in PD populations.
- **Long-term outcomes** regarding cognition, fall risk, and motor function in chronic sedative-hypnotic use remain under-investigated.
- **Head-to-head comparisons** and dose-optimization studies could clarify the safest and most effective strategies for different patient subgroups, including elderly or cognitively impaired individuals.
- **Integration of non-pharmacologic interventions** in combination with pharmacotherapy warrants further evaluation to develop evidence-based, personalized treatment algorithms.
- **Biomarker-driven approaches** may help identify patients most likely to benefit from specific sedative-hypnotics without exacerbating motor or cognitive complications.

Clinical Implications: Clinicians should adopt a **stepwise, individualized approach**, prioritizing melatonin or non-pharmacologic strategies for sleep disturbances, reserving benzodiazepines or Z-drugs for short-term use, and carefully monitoring for adverse effects, drug interactions, and functional decline.

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THE EFFECTS OF PLANT SECONDARY METABOLITES ON DNA DAMAGE

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ABSTRACT

Organisms are continually exposed to numerous internal and external factors that may cause DNA damage throughout their lifespan. In response, several cellular DNA repair mechanisms are activated to mitigate such damage and maintain genomic stability. However, these systems are not flawless, and any deficiency can trigger genomic instability, potentially resulting in various diseases. Notably, impairments in these mechanisms have been strongly associated with carcinogenesis. Given the growing emphasis on preventive health strategies, increasing attention has been directed toward plant-derived compounds recognized for their antioxidant properties. Numerous studies have shown that, when administered in appropriate doses and combinations, these compounds exhibit a wide range of biological activities, including anti-carcinogenic, antioxidant, anti-inflammatory, anti-allergic, antimicrobial, and anti-thrombotic effects. Among these, phenolic compounds have received particular attention due to their multifaceted roles in maintaining cellular homeostasis. These compounds have demonstrated capabilities such as DNA protection, modulation of the cell cycle, reduction of DNA lesion formation, alteration of cancer cell membrane potential, and regulation of apoptotic gene expression. However, the growing popularity of plant-derived substances raises safety concerns. It is crucial to recognize that a compound exerting genotoxic effects on tumor cells may also impact healthy cells adversely. Therefore, unregulated use of such compounds without comprehensive evaluation may pose serious health risks. Further analysis is essential to ensure their safety and therapeutic efficacy.

Keywords: Antioxidant Activity, Cancer Prevention, DNA Repair, Plant-Derived Phenolic Compounds, Oxidation

INTRODUCTION

Plants are a rich source of diverse chemical constituents, broadly categorized into primary and secondary metabolites. While primary metabolites such as carbohydrates, lipids and amino acids are indispensable for growth and development, secondary metabolites, including alkaloids, terpenoids and phenolics, primarily function in defense against biotic and abiotic stressors (Saxena et al., 2013; Wink, 2015). Produced through specific metabolic pathways in response to diverse biotic and abiotic stresses, secondary metabolites hold remarkable importance for humans as natural resources for pharmaceuticals, insecticides, dyes, and other bioactive products.

Among them, phenolic compounds represent one of the most ubiquitous and structurally diverse classes, are defined by the presence of an aromatic benzene ring bearing one or more hydroxyl groups. Owing to their ability to form diverse structural arrangements, ranging from simple phenols to highly polymerized forms, they are often referred to as polyphenols. In addition, these molecules can form conjugates with mono-, oligo-, and polysaccharides, further increasing their structural complexity (Harborne & Williams, 2000; Balasundram et al., 2005).

Biosynthetically, they arise mainly from two distinct pathways: the shikimate pathway, which yields hydroxycinnamic acids and coumarins, and the polyketide (acetate) pathway, which produces simple phenolic structures. Collectively, phenolic compounds represent the largest group of phytochemicals, exerting profound effects on plant physiology and morphology, and playing indispensable roles in growth, reproduction, and defense against pathogens and herbivores (Balasundram et al., 2005).

Organisms are inherently vulnerable to mutagenic factors, and exposure to such agents can induce alterations in genetic material. Some of these changes are hereditary and passed on to future generations, while others occur only in the exposed individual. Numerous studies have demonstrated that medicinal plants and their secondary metabolites exert protective effects by reducing mutagenic and carcinogenic damage (Christmann et al., 2003; Meeran et al., 2006; Bjelakovic et al., 2007; Meeran et al., 2009; Nichols and Katiyar, 2009). In this context, among the various phytochemicals responsible for these protective effects, phenolic compounds have received particular attention due to their potent antioxidant properties. Beyond their biological roles in plants, phenolic compounds are recognized for their health-promoting potential, primarily attributed to their antioxidant activity. This activity, particularly their ability to neutralize harmful free radicals, forms the biochemical basis of many of their beneficial effects. In the human diet, the main groups of phenolics include phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (such as flavones, flavonols, and anthocyanins), and tannins (King & Young, 1999; Balasundram et al., 2005). Collectively, these classes represent the principal contributors to the protective effects of plant-based diets, making phenolic compounds key subjects of nutritional, pharmacological, and toxicological research.

1.1. Antioxidant Effects of Plant Phenolic Compounds

Oxygen is indispensable for virtually all aerobic organisms, serving as the terminal electron acceptor in vital metabolic pathways. However, its reactivity renders it a paradoxical element: while essential for sustaining life, oxygen can also exert deleterious effects under certain conditions. Organic molecules exposed to oxygen may undergo oxidative modifications, a process involving electron loss that leads to the formation of reactive oxygen species (ROS). When the delicate balance between oxidant and antioxidant systems is disrupted, oxidative stress arises, favoring pro-oxidant species and resulting in cellular and molecular damage. This oxidative stress has been implicated in the pathogenesis of numerous chronic and degenerative diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions (Balasundram et al., 2005).

Plant phenolic compounds are recognized as potent natural antioxidants capable of reducing such damage. Their protective effects arise from multiple mechanisms: direct scavenging of free radicals, donation of hydrogen atoms from phenolic hydroxyl groups, chelation of transition metal ions that catalyze radical formation, conversion of reactive radicals into more stable non-radical products, and modulation or inhibition of oxidant-generating enzymes (Harborne, 1989; Balasundram et al., 2005; Misteli and Soutoglou, 2009; Kozics et al., 2013; Singh et al., 2014; Wang et al., 2015; Kowalczyk et al., 2019a; Kowalczyk et al., 2019b). Importantly, the antioxidant capacity of a phenolic compound depends not only on its intrinsic chemical structure but also on its concentration, the presence of synergistic or antagonistic molecules in the environment, and the specific biological context in which it acts (Balasundram et al., 2005).

1.3. Classification of Plant Phenolic Compounds

Phenolic compounds represent a structurally diverse family of secondary metabolites, and they are typically categorized into three major groups: phenolic acids, flavonoids, and tannins, along with some minor classes such as stilbenes, lignans, and coumarins. This classification is based primarily on the number and arrangement of phenol rings and the structural elements linking them (Balasundram et al., 2005). Each group exhibits distinct biosynthetic origins, chemical structures, and biological properties, making them critical to both plant physiology and human health.

1.3.1. Phenolic Acids

Phenolic acids are subdivided into hydroxybenzoic acids (C₆-C₁) and hydroxycinnamic acids (C₆-C₃). Hydroxybenzoic acids include gallic, p-hydroxybenzoic, vanillic, syringic, and ellagic acids, commonly present in berries, nuts, and certain seeds. Hydroxycinnamic acids, such as caffeic, ferulic, p-coumaric, and sinapic acids, are abundant in cereals, coffee, and various fruits (King & Young, 1999). Beyond their structural roles in plants, phenolic acids contribute significantly to antioxidant defense, UV protection, and pathogen resistance, while also offering dietary health benefits such as anti-inflammatory and cardioprotective effects (Balasundram et al., 2005).

1.3.2. Flavonoids

Flavonoids form the largest and most extensively studied class of plant phenolics. These low-molecular-weight compounds share a C₆-C₃-C₆ skeleton, consisting of two aromatic rings (A and B) linked by a heterocyclic C ring. Variations in the degree of oxidation and substitution patterns of the C ring give rise to multiple subclasses, including flavonols, flavones, flavanones, flavanols (catechins), isoflavones, and anthocyanidins (Middleton et al., 2000). Biosynthetically, the A ring originates from the acetate–malonate pathway, while the B ring is derived from phenylalanine via the shikimate pathway.

Flavonoids exert pleiotropic biological effects: they modulate key enzymes involved in detoxification, cell cycle regulation, and immune response; exhibit anti-inflammatory and anti-cancer activities; and protect against oxidative damage by scavenging reactive oxygen species and preventing lipid peroxidation (Balasundram et al., 2005). Their widespread distribution and multifunctionality make flavonoids pivotal dietary compounds.

Flavonols and Flavones: The most ubiquitous flavonoids, represented by quercetin, kaempferol, and myricetin, which are abundant in onions, kale, and berries.

Flavanols (Catechins): Found in tea, cocoa, and grapes; they readily form proanthocyanidins, which under acidic conditions convert to anthocyanidins. These compounds have been extensively linked to cardiovascular protection and anti-obesity effects.

Anthocyanidins: Responsible for red, blue, and purple pigmentation in plants; they provide both visual attraction for pollinators and strong antioxidant activity in human nutrition.

1.3.3. Tannins

Tannins are high-molecular-weight polyphenols, broadly divided into hydrolyzable tannins and condensed tannins (proanthocyanidins). Hydrolyzable tannins yield gallic or ellagic acid upon hydrolysis, while condensed tannins are oligomers or polymers of flavan-3-ols such as catechin and epicatechin (Seigler, 1998).

2. Effects of Phenolic Compounds on DNA Damage and Repair

Free radicals are highly reactive molecules containing unpaired electrons, which drive them to seek stability by taking or donating electrons from other molecules. This process can lead to oxidative reactions in cells, where the electron donor molecule becomes unstable while the radical is stabilized. Antioxidants act by donating electrons to free radicals, thereby minimizing cellular damage. An imbalance between free radical generation and antioxidant defense can result in oxidative stress, affecting proteins, lipids, and nucleic acids. Under normal conditions, a balanced exposure to free radicals is beneficial, supporting immune responses and cellular signaling. However, excessive oxidative stress is implicated in numerous diseases, including atherosclerosis, Parkinson's disease, Alzheimer's disease, tumor formation, chronic inflammation, degenerative disorders, and aging (Lobo et al., 2010; Do et al., 2013; Seal et al., 2020).

In recent years, plant-derived compounds with high antioxidant content have attracted significant attention for their pharmacological potential in reducing free radical-induced damage. Studies suggest that certain phenolic compounds can reduce oxidative stress and protect against DNA damage (Seal et al., 2020). Beyond their antioxidant activity, these compounds also demonstrate hepatoprotective effects, countering drug-induced liver toxicity (El-Din El-Hawary et al., 2019).

DNA damage plays a crucial role in cancer development. Structural alterations in DNA can lead to mutations or chromosomal abnormalities, disrupting oncogenes and tumor suppressor genes, and promoting malignant transformation (Bouguellid et al., 2020). Recent research indicates that medicinal plants may offer both preventive and therapeutic effects at the organismal level, protecting against DNA damage and supporting repair mechanisms.

3. Methods for Determining Genotoxicity at the Cellular Level

With the increasing introduction of synthetic chemicals into daily life, mutagenicity testing systems have gained importance in assessing their potential impact on human health and the environment. These assays aim to detect mutations at the cellular level, often by examining the effects of chemical extracts with potential carcinogenic properties *in vitro*. One of the most widely used assays is the Ames test, which employs different *Salmonella typhimurium* strains to detect various types of mutations. For example, strains TA1535 and TA100 are sensitive to base-pair substitutions, while TA1537, TA1538, and TA98 detect frameshift mutations. Each strain has distinct mutational characteristics, enabling comprehensive mutagenicity testing when used together. Since these strains carry mutations in the histidine operon, they cannot synthesize histidine and thus fail to grow in histidine-free media. When exposed to mutagenic compounds, some bacteria undergo reverse mutations that restore histidine synthesis and allow colony formation. The number of such colonies serves as an indicator of mutagenic activity (Ames et al., 1975).

Another bacterial assay, the Umu-test, developed by Oda et al. (1985), is based on the induction of the umu operon in *Escherichia coli*, which is activated during the SOS response to chemical or radiation-induced mutagenesis. Similar in sensitivity and simplicity to the Ames test, the Umu-test offers the advantage of detecting a wide range of mutagens without requiring multiple bacterial strains.

In addition to bacterial systems, various cell-based assays are used to evaluate cytotoxicity, viability, and proliferation. Among these, the MTT assay is one of the most common. It is a colorimetric method that assesses metabolic activity as an indicator of cell viability. The yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by the mitochondrial enzyme succinate

dehydrogenase to form purple formazan crystals. The amount of formazan produced, measured spectrophotometrically, reflects the metabolic activity and thus the viability of the cells (Holst-Hansen and Br  nner, 1998). This method is frequently used to determine whether test substances exhibit cytotoxic effects on cultured cells or tissues.

The Comet assay is a rapid and sensitive technique for evaluating DNA strand breaks in eukaryotic cells. In this method, cells are embedded in low-melting-point agarose on microscope slides and lysed to disrupt the nuclear membrane, releasing DNA from the chromatin structure. The DNA remains attached to the nuclear matrix and lamina in looped configurations known as nucleoids. When strand breaks occur, these loops lose their supercoiled structure and migrate toward the anode under an electric field. The resulting “comet-like” appearance under a microscope (with a bright head representing intact DNA and a tail indicating fragmented DNA) reflects the extent of DNA damage in the cell (Milev et al., 2025).

The micronucleus assay is another widely used method for assessing genotoxic effects in mammalian cells. Micronuclei form when chromosomal fragments or whole chromosomes fail to segregate correctly during cell division, leading to their encapsulation in small, separate nuclei within the cytoplasm of daughter cells. Micronuclei containing chromatin fragments usually result from DNA breakage events such as double or single-strand breaks, while those containing whole chromosomes are caused by disturbances in the mitotic apparatus, such as kinetochore malfunctions, spindle defects, or centrosome amplification. To evaluate the genotoxic potential of a substance, cells must be cultured for at least one mitotic cycle following exposure. Additionally, exfoliated human cells can be examined to assess *in vivo* exposure or DNA damage related to environmental or disease conditions. Although the assay is most commonly applied to mammalian systems, it is also used in ecotoxicological studies involving non-mammalian species such as birds, fish, mussels, butterflies, and plant root tip cells (Hadi and Stopper, 2025).

Finally, the *in vitro* chromosomal aberration test is employed to identify substances capable of inducing structural chromosomal damage in cultured mammalian cells. In this test, human or rodent cells are exposed to the test compound both with and without metabolic activation. After exposure, metaphase-arresting agents such as colchicine or Colcemid   are added to halt cell division, allowing microscopic examination of chromatid and chromosome-type aberrations. Although polyploidy may occur due to cytotoxicity or cell cycle disturbances, it does not necessarily indicate aneuploid activity; therefore, the *in vitro* micronucleus assay is preferred for detecting aneuploidy. For reliable results, cell lines with stable karyotypes, low spontaneous aberration frequencies, and robust proliferative capacity should be selected. Factors such as p53 status, DNA repair ability, and species origin (rodent or human) must also be considered when evaluating genotoxic effects (OECD, 2016).

4. Studies on the Effects of Plant Phenolic Compounds on DNA Damage and Repair

Studies conducted by Bouguellid et al. (2020) on *Fraxinus angustifolia* Vahl., a plant widely used in traditional Mediterranean medicine, revealed its potential antimutagenic, antigenotoxic, and antiproliferative properties. Extracts obtained from the leaves and stem bark contained antioxidant compounds such as verbascoside, isorhamnetin, rutin, ellenoic acid, and gallic acid. To assess mutagenic activity, the Ames *Salmonella*/microsome test was employed using *Salmonella typhimurium* strains TA98 and TA100, while the umu-test was performed with strain TA1535/pSK1002 to evaluate genotoxicity. Furthermore, antiproliferative activity was determined by MTT assays on MCF-7 (breast cancer) and HepG-2 (liver cancer) cell lines, with normal human fibroblasts used as controls. The results demonstrated selective cytotoxicity toward cancer cells without genotoxic or mutagenic effects, highlighting the potential

therapeutic relevance of the plant's phenolic compounds. In a related study, Andonova et al. (2022) investigated the aerial parts of *Koeleria paniculata* and identified phenolic compounds such as rutin, hesperidin, quercetin, and rosmarinic acid, whose in-vitro assays (DPPH, ABTS, FRAP, CUPRAC) confirmed substantial antioxidant potential, along with DNA-protective effects observed through a nicking assay.

Building on this, Bayalı et al. (2024) optimized the concentrations of gallic acid, rosmarinic acid, and caffeic acid that act synergistically in Folin–Ciocalteu and FRAP assays. They demonstrated that optimal combinations reduced DNA base damage products by approximately 37–40% (via GC–MS/MS analysis). Collectively, these studies suggest that diverse phenolic-rich plant extracts can prevent oxidative DNA damage and potentially enhance repair pathways. While *F. angustifolia* and *K. paniculata* emphasize the protective potential of individual extracts, the findings of Bayalı et al. (2024) highlight that optimized combinations of phenolics may amplify this effect, opening new possibilities for therapeutic or nutraceutical formulation.

Similarly, other studies have focused on the DNA-protective and repair-modulating effects of plant-derived phenolic compounds such as curcumin. Curcumin, a polyphenolic compound derived from *Curcuma longa*, has been shown to sensitize tumor cells to chemotherapeutic agents and ionizing radiation that induce DNA damage. It activates the DNA damage response (DDR) pathway while inhibiting histone deacetylase (HDAC) activity, which regulates DDR suppression (Schneider et al., 2006). Building on these findings, Wang et al. (2015) demonstrated in yeast models that curcumin inhibits the G2/M DNA damage checkpoint by promoting Rad52 degradation, thereby suppressing DNA repair processes. Likewise, Misteli and Soutoglou (2009) reported that curcumin disrupts the phosphorylation of Rad53 and H2A at the G2/M checkpoint, preventing the repair of double-strand breaks and inducing apoptosis. Notably, curcumin does not interfere with normal cell development in the absence of DNA damage, underscoring its selectivity. Collectively, these findings highlight that plant-derived phenolic compounds such as those in *F. angustifolia* and curcumin share a common mechanism: protecting or selectively targeting DNA integrity, which supports their potential as therapeutic agents in cancer prevention and treatment.

Building on this evidence, Kowalczyk et al. (2019a) investigated another medicinal plant, *Menyanthes trifoliata* L., traditionally used across Europe, North America, and Asia. Their studies identified rich phenolic and flavonoid content; including chlorogenic, sinapic, ferulic, and ellagic acids, as well as rutin, luteolin, and betulinic acid which collectively contribute to the plant's antioxidant, anti-inflammatory, and cytotoxic properties. Extracts from roots and aerial parts exhibited cytotoxic effects on glioma cells, particularly those derived from root tissues. Flow cytometry analyses confirmed that treatment with 1.5 mg/mL extract induced cell cycle arrest at the G2/M phase within 24 hours, accompanied by the modulation of pro- and anti-apoptotic genes (*Bax*, *Bcl-2*, *Cas-3*, *TP53*) and reduction of mitochondrial membrane potential.

In a complementary study by Kowalczyk et al. (2019b), *Menyanthes trifoliata* extracts cultivated *in vitro* were tested on HUVEC cells to assess oxidative stress and inflammation. qRT-PCR results demonstrated that the extracts modulated genes involved in antioxidant defense (*HO1*, *NQO1*, *NRF2*, *GCLC*) and inflammatory pathways (*IL-1β*, *TNF-α*, *IFN-γ*). Moreover, in H₂O₂-treated HUVEC cells, SLR-qRT-PCR revealed that the extracts significantly reduced both nuclear and mitochondrial DNA lesion rates, demonstrating direct DNA-protective capacity.

Similarly, Kozics et al. (2013) reported that phenolic-rich extracts from *Salvia officinalis* and *Thymus vulgaris* exerted protective and reparative effects on DNA lesions in HepG-2 cells exposed to oxidative agents such as H₂O₂ and DMNQ. These extracts not only decreased DNA damage but also exhibited dose-dependent cytotoxicity toward cancer cells, further supporting

the role of plant phenolics in preventing oxidative and genotoxic stress. In another study, Singh et al. (2014) examined the DNA-protective potential of *Trigonella foenum-graecum* (fenugreek). Extracts obtained from early and fruiting developmental stages demonstrated concentration-dependent protection against DNA damage induced by the Fenton reagent on supercoiled pBR322 plasmid DNA, indicating that phenolic constituents can neutralize free radicals and prevent strand breakage.

Expanding on these findings, Jeong et al. (2019) explored the antioxidant and DNA-repair effects of *Penthorum chinense* Pursh, a plant used in East Asian medicine for hepatic and dermatological disorders. The ethanol extract (Pc-EE), rich in quercetin and other antioxidants, effectively reversed H₂O₂- and UVB-induced oxidative stress in HaCaT keratinocyte cells. Furthermore, Pc-EE downregulated the expression of inflammatory mediators such as COX-2 and IL-6, while modulating matrix metalloproteinases (MMP-1 and MMP-9) and enhancing collagen gene expression. In melanoma cells (B16F10), Pc-EE also reduced melanin synthesis by suppressing tyrosinase, TYRP1, and TYRP2 expression and promoting autophagy-related LC3B activity. Consistent with these observations, Ferhi et al. (2019) reported that grapevine (*Vitis vinifera*) leaf extracts induced dose-dependent apoptosis in HepG-2 and MCF-7 cells through upregulation of the pro-apoptotic Bax gene and downregulation of the anti-apoptotic Bcl-2 gene. Moreover, polyphenols found in green tea (*Camellia sinensis*) have been shown to prevent UV-induced cyclobutane pyrimidine dimer (CPD) formation in DNA, a major cause of oncogenic mutations. Catechins such as EGCG not only prevent chronic inflammation but also stimulate DNA repair pathways, counteracting UV-induced mutagenesis (Meeran et al., 2006; 2009; Nichols & Katiyar, 2009).

Beyond antioxidation, recent evidence has revealed that phenolic compounds mediate cellular protection through anti-inflammatory mechanisms. Du et al. (2018) reported that pomegranate peel polyphenols (PPPs), particularly punicalagin and ellagic acid, exert strong anti-inflammatory effects in LPS-induced RAW 264.7 macrophages. The treatment significantly reduced pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), iNOS, and COX-2 expression, leading to decreased NO and PGE₂ levels. This activity was associated with inhibition of the MAPK signaling pathway (ERK, JNK, and p38), and punicalagin was found to be more effective than ellagic acid, demonstrating the differential contribution of specific phenolics to cellular protection. Parallel to their antioxidant and anti-inflammatory properties, phenolic-rich plant extracts have also exhibited remarkable antimicrobial activity. Diarra et al. (2020) found that a polyphenol-rich ethanolic extract from cranberry (*Vaccinium macrocarpon*) fruit pomace showed strong antibacterial effects against several *Listeria* species, including *L. monocytogenes*. The extract significantly reduced bacterial viability, biofilm formation, and membrane integrity in a dose-dependent manner, suggesting multiple mechanisms contribute to its antimicrobial action. These findings indicate that phenolic compounds not only protect against oxidative and inflammatory stress but also serve as natural antibacterial agents, broadening their applications in both health and food safety. In addition to antioxidant, antimicrobial, and anti-inflammatory mechanisms, phenolic compounds exert profound effects through epigenetic regulation in cancer biology. Li et al. (2020) reviewed the green tea polyphenol epigallocatechin-3-gallate (EGCG), emphasizing its modulation of cancer-related epigenetic mechanisms, including DNA methylation, histone modification, and non-coding RNA regulation. These reversible changes in the cancer epigenome are central to tumor initiation and progression, and EGCG functions as a natural epigenetic modulator, supporting its potential as an epi-drug for cancer prevention and therapy.

Recent research has also expanded understanding of the photoprotective effects of plant-derived polyphenols against UV-induced DNA damage. Pizano-Andrade et al. (2025) demonstrated that phenolic and acetogenic-rich extracts from *Annona muricata* leaves protected rats exposed to UV radiation by enhancing the expression of tumor suppressor genes

P53 and *Rb*, which are vital for DNA repair and cell cycle regulation. HPLC analysis identified shikimic acid and gallic acid as major phenolics, and antioxidant assays confirmed substantial activity (64.37% inhibition in DPPH and 28,880 μmol Trolox equivalents/mL in FRAP). Histopathological analysis showed epidermal thickening with fibroblast proliferation, suggesting tissue recovery and protection.

Finally, Venturi et al. (2025) explored the effects of wild blueberry (*Vaccinium angustifolium*) polyphenol metabolites on lipid metabolism and oxidative stress in 3T3-L1 adipocytes. While these metabolites did not markedly reduce lipid accumulation, isoferulic acid and vanillic acid decreased fatty acid synthase (FASN) levels by 25–26%, and syringic acid enhanced antioxidant defense by increasing heme oxygenase-1 (HO-1) expression by 150%. These findings suggest that specific blueberry-derived metabolites modulate lipid metabolism and oxidative stress, underscoring the broad physiological relevance of phenolic compounds.

RESULTS AND DISCUSSION

Recent research has provided that plant-derived phenolic compounds play critical roles in genomic protection, oxidative balance, and cellular defense mechanism. Studies on *Fraxinus angustifolia* and *Koeleria paniculata* demonstrated potent antioxidant, antimutagenic, and antiproliferative activities linked to compounds such as verbascoside, rutin, and rosmarinic acid (Bouguellid et al., 2020; Andonova et al., 2022). Similarly, Bayalı et al. (2024) showed that optimized mixtures of gallic, rosmarinic, and caffeic acids reduced oxidative DNA base damage by nearly 40%, emphasizing synergistic effects among phenolics. Curcumin from *Curcuma longa* exhibited selective modulation of DNA repair and apoptosis pathways (activating DDR and suppressing HDAC activity) while sensitizing tumor cells to DNA-damaging agents (Schneider et al., 2006; Wang et al., 2015; Misteli & Soutoglou, 2009). Consistently, *Menyanthes trifoliata* extracts modulated apoptotic and antioxidant genes (*Bax*, *Bcl-2*, *Cas-3*, *TP53*; HO1, NQO1, NRF2, GCLC) and protected nuclear and mitochondrial DNA from lesions (Kowalczyk et al., 2019b). Comparable DNA-protective and cytotoxic effects were observed in *Salvia officinalis*, *Thymus vulgaris*, and *Trigonella foenum-graecum* through reduction of oxidative strand breaks (Kozics et al., 2013; Singh et al., 2014). Likewise, *Penthorum chinense* and *Vitis vinifera* extracts regulated inflammatory mediators (COX-2, IL-6) and pro-apoptotic genes, reinforcing their chemoprotective potential (Jeong et al., 2019; Ferhi et al., 2019). Beyond antioxidation, polyphenols such as punicalagin and ellagic acid from pomegranate peel exhibited strong anti-inflammatory effects by suppressing *TNF- α* , *IL-1 β* , and *MAPK* signaling (Du et al., 2018). Parallel findings revealed antimicrobial action in cranberry extracts (Diarra et al., 2020), epigenetic modulation by EGCG from green tea (Li et al., 2020), and photoprotective effects of *Annona muricata* phenolics via *P53* and *Rb* upregulation (Pizano-Andrade et al., 2025). Furthermore, *Vaccinium angustifolium* metabolites improved antioxidant defense and lipid regulation through HO-1 activation (Venturi et al., 2025). Overall, these findings indicate that plant extracts abundant in phenolic compounds protect against oxidative, genotoxic, and inflammatory damage through mechanisms involving antioxidant defense, regulation of apoptosis, and epigenetic modulation, highlighting their potential as valuable therapeutic and nutraceutical candidates.

CONCLUSIONS

While plants produce molecules essential for their vital processes; carbohydrates, lipids, amino acids, and nucleic acids constituting the primary metabolite system, compounds that protect them against adverse environmental conditions and pests, including alkaloids, terpenoids, and phenolic compounds, form the secondary metabolite system (Saxena et al.,

2013; Wink, 2015). Among these, polyphenols represent the largest group of secondary metabolites. Widely distributed in plant-derived foods and characterized by multiple phenolic groups, polyphenols belong to a broad class of compounds with strong antioxidant capacity. Owing to their high antioxidant potential, they play a crucial role in protecting living organisms and their genetic material.

Damage to DNA structure can occur due to physical, biological, or chemical factors. Various cellular mechanisms and molecules are responsible for detecting, controlling, and repairing such damage. When disruptions occur in these mechanisms, which are essential for DNA repair, they may lead to aging, cancer, and several genetic disorders. Although many diseases can be prevented through efficient DNA repair mechanisms, any impairment in these systems makes the onset of severe diseases inevitable. Plant phenolic compounds, known to be effective in modulating DNA repair pathways, have increasingly been utilized in both the prevention and treatment of various diseases. Their significant roles in DNA damage and repair processes have prompted extensive research into their underlying molecular mechanisms (Christmann et al., 2003; Bjelakovic et al., 2007).

The efficacy of plant-derived phenolic compounds in both topical and oral applications enhances their therapeutic potential (Almeida et al., 2012; Venancio et al., 2016). However, their potential toxic effects must not be overlooked. It should be noted that the cytotoxic effects observed in tumor cells may also occur in healthy cells, emphasizing the necessity for comprehensive evaluation of their safety profiles. Moreover, the biological activity of plant phenolics can be markedly influenced by several factors, including the geographic origin of the plant, the specific part used for extraction, and the extraction technique employed (Kozics et al., 2013). Therefore, identifying effective and safe doses, as well as selecting the appropriate plant material and extraction method, are of critical importance. Additionally, the physiological effects of administered doses should be carefully monitored, as compounds with uncharacterized mechanisms of action may exert toxic effects even in healthy individuals.

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THE ROLE OF AGRO-MORPHOLOGICAL CHARACTERISATION IN THE EVALUATION OF VEGETABLE GENETIC RESOURCES

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ABSTRACT

Vegetables are a large and diverse group of plants and different vegetable species can be found in every region where people settle. In agricultural production, characterisation studies are of great importance for the effective conservation, evaluation and use of vegetable genetic resources, which are the basis of this diversity and sustainability, in breeding programmes. Agromorphological characterisation is one of the most common and practical methods used to determine genetic diversity in the population to reveal morphological and agronomic differences among vegetable genotypes. In this context, morphological traits can be used as a general technique to examine genetic diversity in morphologically distinct genotypes, as they can be evaluated quickly and easily and are useful for an early assessment. Morphological traits controlled by a single locus can also be used as genetic markers, provided they can be reproduced in a range of media. Genetic diversity is a critical element for the conservation of biodiversity and the development of new crops. Characterisation studies, especially for the selection of parental lines and the design of breeding programmes, are a vital and important step in the beginning of these processes. This review shows that the conservation of vegetable genetic resources for future breeding programmes is an important source of various agricultural traits.

Keywords: Agromorphological, biodiversity, breeding, characterisation, vegetable

INTRODUCTION

Vegetables and fruits, like other horticultural crops, are considered important both commercially and nutritionally. These foods are a significant source of vitamins, minerals, fibre, sugars, antioxidants, organic acids and nutrients (Wu, 2010). In contrast to fruits, vegetables constitute a highly extensive and varied group of plants, including roots, tubers, buds, leaf shoots, flowers, fruits, and seeds. Different types of vegetables represent a broad and diverse group of plants, and different vegetable species can be found in every region where humans have settled (Ferdousi et al., 2023). Plant morphology can be studied in several main parts, such as leaves, stems, roots, flowers, and fruits. These parts allow for an in-depth analysis of the plant's overall structure, as plant morphology involves the fundamental studies of specific groups used to identify plants. The morphology of a plant species is one of the easily observable characteristics (Şavkan and Türkmen 2023; Şavkan et al. 2024). This diversity is influenced by both genetic factors and environmental conditions that affect growth patterns and productivity (Lúcio et al., 2021). In agricultural production, characterization studies are of great importance for the effective conservation, evaluation, and use of vegetable genetic resources in breeding programs, which form the basis of this diversity and sustainability. In this regard, part of the existing genetic diversity has been collected by researchers and stored in the active gene banks of various institutions (Silva et al., 2024). Additionally, the accurate identification and

classification of species is crucial for the management of gene collections. Many researchers have emphasized the importance of morphological characterization as a fundamental step in solving taxonomic problems (Olatunji and Afolayan 2018; Öztürk et al. 2022; Şavkan and Türkmen 2023; Şavkan et al. 2025b). This method plays a critical role in understanding the genetic diversity within a gene pool, identifying populations with desired traits, and enabling breeding processes (Şavkan et al., 2025a; Yaşar et al., 2025a).

One of the most important contributions of agro-morphological characterization in vegetable breeding programs is the understanding of genetic diversity and adaptation. In this context, agro-morphological characterization is a fundamental step in understanding the diversity in a species' gene pool (Rashid et al., 2020; Şavkan and Türkmen, 2023). This is achieved by evaluating elements such as plant morphology, fruit characteristics, and yield (Şavkan et al., 2024; Yaşar et al., 2025b). A study on summer squash (*Cucurbita pepo* L.) emphasized that identifying and selecting the wide genetic diversity in plant and fruit characteristics of this species is important for improving the agricultural qualities of the species (Şavkan et al., 2025a). Biochemical components that affect the nutritional value and sensory quality of plants are also considered in determining genetic diversity. These morphological and biochemical characteristics are important for the conservation and evaluation of genetic resources (Bhandari et al., 2022).

One of the important contributions provided by agro-morphological characterization is the selection of desired traits targeted in breeding programs, thereby increasing breeding efficiency. In this context, morphological characterization studies, which evaluate plant traits, growth behavior, and adaptation to environmental conditions, are critical for identifying genotypes well-adapted to specific growing conditions, effectively selecting genotypes with traits sought in breeding programs (e.g., yield, disease resistance, or nutritional value), and developing breeding strategies (Çetin et al., 2021; Bhandari et al., 2022; Şavkan et al., 2025b). This is because in breeding studies, selecting plants with desired traits from a heterogeneous gene pool will facilitate achieving the target (Şavkan and Türkmen, 2023).

Finally, basic data sets are created by measuring agro-morphological characteristics. These complex datasets are then grouped using multivariate statistical tools (Correlation, Factor Analysis, Principal Component and Cluster Analysis) to draw conclusions about the relationships between genotypes (Bhandari et al., 2022; Şavkan et al., 2025a). Additionally, these analyses are used to determine the genetic distance and degree of similarity between genotypes. This makes it possible to eliminate genotypes that are very similar to each other in breeding programs and to identify the most distant genotypes (due to their high heterosis potential) for crossbreeding programs (Çetin et al., 2021; Şavkan et al., 2024; Şavkan et al., 2025a).

Consequently, these characterization studies have examined morphological and biochemical differences in genetic resources, thereby contributing to the selection of potential functional genotypes for breeding programs. All these studies emphasize the importance of understanding and preserving the genetic diversity of vegetables to support plant breeding efforts. Characterization studies represent a critical step at the outset of these processes, particularly in the selection of parental lines and the design of breeding programs. This review demonstrates that the conservation of vegetable genetic resources is an important source of various agronomic traits for future breeding programs.

METHODS OF AGRO-MORPHOLOGICAL CHARACTERISATION

Turkey is located at the intersection of the Near East and Mediterranean plant genetic centres, one of the eight major plant genetic centres, and therefore contains regions with quite distinct climatic and soil characteristics. Consequently, Anatolia has evolved into a centre of diversity, with numerous cultivated plant species thriving as micro-centres (Tan and İnal, 2003). Morphological characterisation of vegetable species is of great importance in terms of determining genetic diversity, guiding plant breeding programmes and conserving genetic resources. Although it is acknowledged that morphological characters can be influenced by environmental conditions (Claeys et al., 2014), these characters remain the primary and most practical tools for phenotypically defining genotypes and for revealing the similarities and differences between varieties (Bioversity International, 2013; UPOV, 2021). The existence of species varieties and ecotypes exhibiting divergent levels of tolerance to environmental stress presents a valuable opportunity for the selective breeding of stress tolerance. In the face of mounting environmental stresses, the deliberate selection and breeding of plants endowed with stress tolerance traits has become imperative on both a national and global scale (Mammadov et al., 2018). The evaluation of vegetable morphology is conducted through the cultivation of genotypes under designated environmental conditions, followed by the qualitative and quantitative delineation of organ characteristics, including plants, leaves, flowers, fruits, and seeds.

Description of the Visual and Qualitative Observations

The initial stage of the morphological characterisation of vegetables involves the observation of qualitative characteristics that facilitate identification at the species and variety level. These observations are generally based on externally observable traits such as plant habit, leaf shape, colour, flower form, fruit shape, and seed characteristics. For instance, tomato (*Solanum lycopersicum* L.) genotypes, fruit shape, skin colour, and flower morphology are among the most frequently used characters for variety differentiation (Terzopoulos et al., 2010; Salim et al., 2020; Olayinka et al., 2024). Similarly, plant habit and fruit shape play a decisive role in variety identification in pepper (*Capsicum* spp.) and aubergine (*Solanum melongena* L.) species (Uddin et al. 2021; Karim et al., 2022; Martínez-Ispizua et al. 2022).

These qualitative observations are generally based on standard descriptor lists published by Bioversity International and UPOV. Mandatory and optional characteristics are defined in these lists for each vegetable species. For example, for peppers, characteristics such as ‘fruit shape (oval, conical, elongated)’, ‘leaf surface (smooth, hairy)’, and ‘flower colour’ are included in the qualitative assessment (UPOV, 2021).

Quantitative Measurements

Many studies on vegetable species have reported that PCA and clustering analyses are effective in revealing genetic diversity. For example, in a study on pea (*Pisum sativum* L.) genotypes, varieties were grouped using many morphological and biochemical characters (Poltoretskyi et al., 2022). Similarly, the classification of genetic diversity using morphological data is commonly used in pepper and tomato collections (Chatterjee et al., 2023; Al-Saghir, 2024; Şavkan et al., 2024).

Digital Morphometry and Image Analysis

With advancing technology, morphological characterisation of vegetables is now supported by digital imaging and morphometric analysis methods. Parameters such as leaf, fruit, or seed shape can be measured using software such as ImageJ, Tomato Analyzer, or WinSEEDLE via digital images (Hodač et al., 2020). These methods reduce measurement errors and ensure objectivity and repeatability. In particular, characteristics related to fruit shape, colour distribution, and surface texture in tomatoes and peppers can be determined with high accuracy using digital analysis methods (Chatterjee et al., 2023).

Standard Descriptor Lists and International Applications

In the domain of vegetable morphology, the descriptor lists published by Bioversity International (2013) and UPOV (2021) are recognised as international standards. These lists comprise units detailing the mandatory and optional characteristics of organs such as plants, leaves, flowers, fruits, and seeds, along with their measurement units and classification levels. The descriptor list functions as the fundamental reference document, particularly in the context of variety registration and genetic source passport records. Furthermore, within the framework of projects conducted under the auspices of the European Cooperative Programme for Plant Genetic Resources (ECP/GR) and the Food and Agriculture Organisation (FAO), these standards are employed to ensure the uniformity of data pertaining to vegetable genotypes.

Application Examples

Various morphological characterisation studies conducted on vegetables have revealed genetic diversity and adaptation capacity in different species. Research conducted on tomato genotypes has demonstrated that the variation in traits such as fruit weight, colour, size, and shape is of significance for genetics-based breeding (Donoso et al., 2023; Chatterjee et al., 2023). In the context of pepper varieties, it has been documented that fruit shape and colour, along with yield components, play a significant role in the expression of genetic variation (Oladosu et al., 2021; Peñuela et al., 2021; Silva et al., 2021). It is stated that the combination of morphological characteristics with biochemical and molecular analyses in vegetable species such as peas, beans, and lettuce enables a more accurate characterisation of genetic resources (Du et al., 2022; Zec et al., 2024; Moon et al., 2024; Fayaz et al., 2025).

In order to access the genetic diversity present in plant species, a variety of methods may be employed. Such methods include pedigree data and morphological and molecular markers. The use of agro morphological traits is the most common approach used to estimate relationships between genotypes and provide information for plant breeding programs (Bajracharya et al., 2006; De, 2019). The data obtained from the identification of local species are then statistically processed. Multivariate analyses, including cluster analysis, Principal Component Analysis (PCA), and discriminant analysis, are the most commonly employed approaches in the estimation of genetic diversity, thereby facilitating the revelation of variation patterns within germplasm collections. Among multivariate techniques, Principal Component Analysis (PCA) and cluster analysis stand as the preferred tools for the grouping of genotypes based on morphological characterisation and similarity (Mohammadi and Prasanna, 2003). Cluster analysis is utilised to elucidate the relationships among local varieties, while the relationships among traits are subjected to statistical analysis using PCA. Local varieties can be grouped based on informative data and used directly in breeding programmes.

The use of agro-morphological characteristics has been identified as the most common approach for predicting relationships between genotypes (Bajracharya et al., 2006). Information

on the agro-morphological diversity of a crop and its distribution across agro-ecological regions can be of great assistance in developing strategies for germplasm management and improvement (Odjo et al., 2017).

The development of minimal descriptors for the characterisation and evaluation of different horticultural crops is a prerequisite for the National Plant Genetic Resources Database (Mahajan et al., 2000). Agro morphological characterisation is of fundamental importance in providing information for plant breeding programs (Lin, 1991).

Local breeds provide a valuable gene accession for future breeding programs (Patra et al., 2003). For farmers, genetic diversity means variety, and farmers can clearly distinguish this based on agro morphological traits, phenological traits, post-harvest traits, and different adaptive performance under abiotic and biotic stresses (De & Dey, 2013). Local varieties can be defined as heterogeneous plant populations that have been cultivated by humans for specific purposes. The amount of genetic diversity present within a species is an essential element for the survival of that species and its adaptation to changing environments (Gao, 2003). The significance of local or folk varieties within the agricultural system is indisputable, as the enhancement of existing varieties is contingent on the presence of desirable genes that are exclusive to local and wild varieties (Shiva, 1991).

ADVANTAGES AND LIMITATIONS OF AGRO-MORPHOLOGICAL CHARACTERISATION

This approach, constituting the initial phase in the evaluation of genetic diversity, has significant advantages, including its cost-effectiveness and its direct applicability to breeding programs. However, the strong influence of environmental factors on morphological traits, its inability to adequately detect variation between related genotypes, and the subjectivity of assessments are among the limitations of these methods (Table 1).

Advantages of characterisation

- Morphological characterisation is widely regarded as the most fundamental and simplest stage of assessing genetic diversity. In this sense, the method's low equipment requirements, low cost, and rapid applicability in the field or greenhouse are among its most significant advantages in terms of cost and applicability (Rashid et al., 2020; Sulaiman et al., 2020).
- Many morphological characteristics (e.g., yield, fruit size and colour) are directly associated with agricultural performance and consumer preferences. This is of important for the effective management of demand-driven breeding and cultivation programs (Shilpashree et al., 2021; Saha et al., 2024).
- Morphological analyses have been shown to reveal significant genetic diversity and visible heritability in key traits (Bhandari et al., 2022; Saha et al., 2024). Consequently, they also assist in identifying promising and superior genotypes for the purpose of targeted breeding efforts.
- The organisation of accessions in a gene pool into clusters or distinct groups based on morphological characteristics has been demonstrated to facilitate the management and conservation of genetic resources (Yulianah et al., 2020; Sulaiman et al., 2020).

Limitations of characterization

- Morphological traits are frequently found to be significantly influenced by environmental conditions, thereby complicating the distinction between genetic and environmental diversity, and potentially leading to a reduction in reliability across different

locations or seasons (Gadissa et al., 2021; Manzoor et al., 2024). This is a notable limitation that impacts selection during the characterisation phase.

- Morphological markers are inadequate for the detection of subtle genetic differences, particularly between closely related genotypes. In some cases, these markers have been shown to misclassify samples (Sood et al., 2023; Salim et al., 2023).

- The evaluation of morphological traits has been shown to be a time-consuming and labour-intensive process. In such cases, observer bias is a potential concern, particularly when assessing complex or quantitative traits. Furthermore, compared to molecular methods, morphological characterisation is less scalable and may struggle to adapt to modern breeding requirements (Nankar et al., 2020; Noshita et al., 2022).

Table 1. Advantages and limitations of characterisation in vegetable

Aspect	Advantages	Limitations	Citations
Simplicity and Cost-Effectiveness	Low cost, easy to implement.	Requiring excessive labour and intensive manpower.	Rashid et al., 2020; Sulaiman et al., 2020; Nankar et al., 2020; Noshita et al., 2022
Genetic factors	Identification of important trait differences	Limited variation in related genotypes.	Sood et al., 2023; Cornea-Cipcigan et al., 2023; Salim et al., 2023
Impact of Environmental	Selection of varieties with high regional adaptation.	Strong susceptibility to environmental influences and elimination of varieties with low adaptability.	Shilpashree et al., 2021; Gadissa et al., 2021; Cornea-Cipcigan et al., 2023; Manzoor et al., 2024
Breeding Utility	Useful for determining starting materials in breeding programs	Failure to achieve the desired diversity.	Shilpashree et al., 2021; Bhandari et al., 2022; Ratna et al., 2024; Saha et al., 2024

CONCLUSION AND RECOMMENDATIONS

The effective evaluation of vegetable genetic resources is of strategic importance in terms of food security, the conservation of biological diversity, and the development of new varieties that are resilient to climate change. In this process, agricultural morphological characterisation enables the identification of genetic variation at the phenotypic level, forming one of the cornerstones of genetic resource management. Studies show that morphological characterisation is an important tool not only in the variety identification and registration stages, but also in the processes of preserving genetic diversity and re-evaluating local genotypes. The combined evaluation of qualitative and quantitative traits, supported by statistical analyses, has increased the accuracy of these studies today.

However, the limitations of morphological characterisation must also be considered. Environmental factors (temperature, light, soil conditions, etc.) can affect morphology, which may lead to the risk of misclassification in assessments based solely on phenotypic data. Therefore, in recent years, the integration of morphological characters with molecular marker data (SSR, SNP, ISSR, etc.) and digital phenotyping techniques has enabled multidimensional analysis of vegetable genetic resources. Consequently, morphological characterisation is an indispensable approach that must be integrated with molecular-level analyses in vegetable breeding and genetic diversity studies.

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ALTITUDINAL AND LATITUDINAL SPREAD OF PINE PROCESSIONARY MOTHS (*THAUMETOPEA* SPP.) IN THE CONTEXT OF CLIMATE CHANGE

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ABSTRACT

This study comprehensively examines the altitudinal and latitudinal distribution changes of the pine processionary moth (*Thaumetopoea spp.*) populations driven by climate change. Particularly in Turkey, field observations reveal that the species has expanded approximately 200 meters in elevation over the past 25 years in regions such as the Kızılırmak Basin and Western Black Sea. This expansion results in significant ecological and economic impacts, including growth losses, needle defoliation, and increased vulnerability to secondary pests in forest ecosystems. The species also poses dermatological, respiratory, and ophthalmological risks to humans and animals. These findings underscore the necessity of sustainable management strategies such as integrated pest management, biological control, early warning systems, and adaptive forestry practices.

Key words: Forest health; Biological control, Public health impacts; Sustainable forestry, Türkiye

INTRODUCTION

Climate change, through its impacts such as rising global temperatures, seasonal shifts, and the increasing frequency of extreme weather events, is driving significant alterations in ecosystems. These changes directly or indirectly affect the distribution ranges of species, their phenological cycles, and intra-ecosystem interactions. In particular, the rise in winter temperatures facilitates the expansion of certain insect species into higher elevations and latitudes where survival was previously impossible.

The pine processionary moth (*Thaumetopoea spp.*) is a major forest pest adapted to the Mediterranean climate and remains active during the winter season. In its larval stage, it consumes the needles of pine trees, thereby reducing photosynthetic capacity and causing growth losses. With global warming, the increase in winter temperatures enhances both the survival rate of the pine processionary moth and the expansion of its distribution toward higher altitudes and northern regions.

GLOBAL AND TURKISH DISTRIBUTIONAL SHIFTS AND THE EFFECT OF ELEVATION

Research conducted in Europe has demonstrated that populations of the pine processionary moth have become permanently established at higher altitudes, rising by 100–300 meters since the 1970s, particularly in regions such as the Alps and France (Battisti et al., 2005). In the Paris Basin, the species has also been reported to advance northward by approximately 87 km.

In Turkey, field observations in the Kızılırmak Basin—particularly in the regions of Tosya, Kargı, and Osmancık—have shown that over the past 25 years the pine processionary moth has expanded its distribution upward by approximately 200 meters. In areas where populations were once scarce or absent, stable colonies have now been established.

RISKS AND INJURIES TO PUBLIC HEALTH

The larvae of the pine processionary moth (*Thaumetopoea* spp.) are covered with toxic hairs (setae) that can adversely affect both human and animal health in various ways. Direct contact with larval hairs typically triggers skin reactions such as pruritic urticaria, papules, and edema within 1 to 12 hours of exposure. These reactions are more frequently observed during spring and summer months, particularly in outdoor activities such as camping, picnicking, or forestry work (Lamy et al., 1986; Vega et al., 2011).

Proteins such as “thaumetopoein” present in the hairs may induce IgE-mediated allergic reactions. This can cause contact urticaria, angioedema, and in rare cases anaphylaxis in occupationally exposed individuals, such as forestry workers, farmers, and gardeners (Lamy et al., 1986).

The toxic hairs can also become airborne, causing ophthalmological problems such as conjunctivitis and keratitis. In the respiratory system, exposure may lead to irritation and asthmatic symptoms. Records from poison control centers in France indicate that 31% of such exposures are ocular, 18% oral/pharyngeal, and 2% dermatological (Maudet et al., 2020).

From an occupational health perspective, a study in Italy reported that more than 90% of forestry workers developed symptoms, with increased severity observed during tree-felling operations, when larval hairs are dispersed into the air (Battisti et al., 2006).

Regarding animal health, domestic pets such as dogs and cats are particularly at risk. Direct contact with the larvae can result in severe clinical conditions, including ulceration of the oral mucosa, tongue necrosis, and conjunctival lesions. A study in Italy revealed high prevalence of keratitis and lingual necrosis in dogs exposed to the larvae (Silva et al., 2015).

Additionally, in areas with dense pine processionary moth colonies, their silk nests and the shedding of larval hairs create environmental and aesthetic disturbances, reducing the recreational value of green spaces. The conspicuous white or silken nests and cocoon-like structures left by the larvae are often perceived as unsightly and unpleasant by the public, thereby generating psychosocial stress (Battisti et al., 2006).

ECOLOGICAL AND ECONOMIC CONSEQUENCES OF DISTRIBUTIONAL CHANGES

The pine processionary moth causes severe defoliation in pine forests, leading to growth losses, needle shedding, and developmental decline. As defoliation reduces the photosynthetic capacity of trees, their carbon sequestration potential is diminished, negatively affecting the carbon cycle in forest ecosystems (Jacquet et al., 2013). This impact is particularly pronounced in young seedlings and afforestation sites, resulting in significant economic losses in forestry. Weakened by needle loss, trees become increasingly vulnerable to secondary pests and pathogens. For instance, bark beetles (*Tomicus* spp., *Ips* spp.) and fungal diseases rapidly spread in defoliated stands, often resulting in widespread tree mortality (Battisti et al., 2015; Ayres & Lombardero, 2000). Such processes threaten forest biodiversity and undermine ecosystem functions.

When defoliation recurs and becomes chronic, tree growth rates are substantially reduced, leading to decreased timber production potential in forestry. Moreover, these damages negatively affect the recreational, ecological, and economic values of forests.

FUTURE PERSPECTIVES AND MANAGEMENT STRATEGIES

With climate change driving the expansion of pests such as the pine processionary moth (*Thaumetopoea* spp.) into new geographical ranges, forestry urgently requires more comprehensive and integrated management strategies (Battisti et al., 2015). The species' spread into novel altitudes and latitudes diminishes the effectiveness of conventional control measures and highlights the need for adaptive management approaches.

First and foremost, the establishment of early warning systems is crucial. The integration of remote sensing, geographic information systems (GIS), and climate modeling can facilitate the identification of vulnerable areas and enable forecasting of potential spread scenarios (Robinet & Roques, 2010). This allows for more efficient prioritization of interventions and allocation of resources.

Biological control methods should be reinforced, particularly through the augmentation of natural enemies such as parasitoids and predators. For example, enhancing populations of egg parasitoids like *Ooencyrtus pityocampae* can strengthen natural regulation mechanisms (Tiberi et al., 2016).

Within the framework of Integrated Pest Management (IPM), biotechnical methods (e.g., pheromone traps), mechanical approaches (e.g., cocoon collection, nest destruction), and, where appropriate, chemical control should be applied in a controlled manner (Jacquet et al., 2013). However, due to their adverse impacts on the environment and human health, chemical measures should be regarded only as a last resort.

Adaptive forest management strategies are also essential. This includes the use of resilient and climate-adaptive tree species, promotion of mixed stand compositions, and enhancement of habitat diversity, all of which can mitigate the impacts of pests (Ayres & Lombardero, 2000).

Public health and awareness initiatives represent another strategic component. Informative campaigns directed at local communities, forestry workers, and tourists can increase risk awareness and promote the adoption of protective measures.

Finally, sustainable forestry policies must be revised in light of climate change scenarios. Such revisions are critical both for the effective management of pests and for strengthening the resilience of forest ecosystems.

CONCLUSION

The altitudinal and latitudinal expansion of the pine processionary moth under climate change poses multifaceted threats to forest ecosystems and public health. As observed in the Turkish context, the upward spread of the species into previously uninhabited elevations driven by rising temperatures threatens both the biodiversity and the economic value of forests. In addition, from a public health perspective, the increase in dermatological and respiratory disorders, as well as the aesthetic and recreational degradation of social environments, represent significant challenges.

In this regard, integrated pest management, biological control, early warning systems, and the enhancement of public health awareness should be prioritized as essential components of sustainable forest management policies. Developing forestry strategies that are responsive to climate change scenarios will be crucial to strengthening the resilience of forest ecosystems, thereby ensuring both the sustainability of natural resources and the protection of public health.

Based on the ecological, economic, and social impacts of the pine processionary moth under climate change, the following recommendations are proposed:

1. Development of Early Warning and Monitoring Systems

- Establish regional monitoring networks integrating **remote sensing, GIS, and climate models** to predict potential spread scenarios.
- Implement pheromone trap networks and citizen science programs to detect early infestations.

2. Strengthening Biological Control Approaches

- Support natural enemies such as parasitoids (*Ooencyrtus pityocampae*) and avian predators by conserving their habitats.
- Promote research on entomopathogenic fungi, viruses, and bacteria as environmentally friendly alternatives.

3. Integrated Pest Management (IPM)

- Combine biological, biotechnical (pheromone traps), and mechanical methods (nest removal, cocoon destruction) as first-line strategies.
- Restrict chemical control to emergency cases, ensuring minimal impact on non-target organisms, human health, and the environment.

4. Adaptive Forest Management

- Encourage **mixed-species stands** and diversify forest structure to reduce vulnerability.
- Promote the use of **resilient and climate-adaptive tree species** in afforestation projects.
- Incorporate climate change projections into long-term forest planning.

5. Public Health and Awareness Programs

- Conduct educational campaigns for local residents, forestry workers, and tourists on the health risks associated with larval hairs.
- Provide training and protective equipment for forestry and agricultural workers at high risk of exposure.
- Collaborate with healthcare systems to improve diagnosis and treatment of processionary moth-related health issues.

6. Policy and Governance

- Revise national and regional **forest protection policies** to integrate pest management within climate change adaptation strategies.
- Foster cross-border cooperation in the Mediterranean and Black Sea regions, as the moth's spread transcends national boundaries.
- Allocate sufficient financial and institutional resources to pest management programs within sustainable forestry frameworks.

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CURRENT METHODS IN THE CONTROL OF STORED-PRODUCT INSECT PESTS

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ABSTRACT

Losses in agricultural production are a major problem, even in developed countries. Stored-product insect pests in particular cause significant economic losses by reducing the nutritional value and germination power of products (Barış and Er, 2025), altering the chemical composition, producing unpleasant odours and degrading the final product quality. They also facilitate the spread of toxigenic microorganisms (Rajendran and Somiahnadar, 2020, Coşkuncu, 2005). To minimise these economic losses, pest control practices that also consider food safety are required. Although chemical control is commonly used against stored-product pests, these methods are becoming less favoured due to their negative environmental impact, the presence of chemical residues in products, the development of resistance in pests, and the potential harm to non-target organisms (Barış and Er, 2005). Initially, pheromone traps were developed for the detection of infestations. Later, they were enhanced to disrupt sexual communication and suppress pest populations (Rajendran & Somiahnadar, 2020). These tools enable the early detection of low-level pest populations and infestations, allowing contaminated areas or products to be identified (Coşkuncu, 2005). However, in some countries, pheromones are also subject to registration procedures similar to those for traditional insecticides (Rajendran and Somiahnadar, 2020). Recently, fumigation techniques have been developed with potential insect resistance in mind. Barış and Er (2025) tested the effectiveness of the fungi *Beauveria bassiana* 5-4 and *Metarhizium robertsii* S3 isolates against the pests *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Plodia interpunctella*. They used five different carrier substances (diatomite, clay, kaolin, talc, and calcite), both individually and in combination. The formulations significantly enhanced effectiveness compared to the use of isolates alone. Another promising area of insect control involves the use of nanoparticles, either on their own or as carriers for other control agents. Studies have shown that nanoparticles of alumina, carbon, silica, silver, copper, zinc oxide, nickel oxide, titanium dioxide, nano-zeolite, and chitosan, as well as polymer nanoparticles, exhibit strong insecticidal activity against various pests, sometimes surpassing the mortality rates achieved by conventional insecticides when used at the recommended dose (Raja et al., 2025; Kadir et al., 2025). Structural, biochemical, and molecular-level analyses have revealed that nanoparticles induce oxidative stress, disrupting cellular homeostasis, and causing structural damage in pests (Kadir et al., 2025). One of the most recent pest control strategies involves molecular technologies such as RNA interference (RNAi) and CRISPR/Cas9. These approaches aim to develop precise, environmentally friendly pest control strategies. RNAi-based applications aim to inhibit the expression of specific essential genes in target organisms. This is achieved through the uptake and processing of double-stranded RNA (dsRNA) by the organism, leading to the degradation of the target gene's mRNA (Gerding et al., 2025). The primary objective of the numerous studies in this field is to identify effective and environmentally friendly protection methods. The objective of this review is to shed light on the methods used to control stored-product pests, which are of significant economic importance.

Keywords: Stored product pest, pesticide, post-harvest losses, pest management

INTRODUCTION

According to the Food and Agriculture Organization of the United Nations (FAO), 17% of global food production is currently lost during storage due to insects, mites, rodents, and diseases. Insects alone are responsible for around 10% of this loss (Stathas et al., 2023). In humid regions, direct and indirect post-harvest losses can reach up to 50% (Wijayarathne et al., 2018). Insects that infest stored products often originate in the field and are carried into storage. They can also infest agricultural products during transportation, processing, or storage (Stathas et al., 2023). Stored product pests can cause significant economic losses by reducing the nutritional value and germination capacity of the products (Barış and Er, 2025), altering their chemical composition and producing unpleasant odours. This decreases the quality of the final product. The presence of insects can also lower the quality and market value of grain due to insect fragments, foul odours, mould formation and heat damage (Demis and Yenewa, 2022). Furthermore, they facilitate the spread of toxigenic microorganisms (Coşkuncu, 2005).

Species belonging to the orders Coleoptera, Lepidoptera, and Psocoptera are significant pests of stored products. Coleoptera and Lepidoptera contain the most economically important post-harvest insect pests. In Lepidoptera species, only the larvae cause damage; in Coleoptera species, however, both the larvae and the adults typically feed on the product, with both stages contributing to the damage (Demis and Yenewa, 2022).

Effective management of storage pests is crucial for reducing damage to stored products. Therefore, using appropriate management methods is crucial to minimise the damage caused by these storage pests and to increase economic returns. Pest control practices that also consider food security are necessary to reduce these economic losses.

MANAGEMENT OF STORAGE INSECT PESTS

The methods used to control storage pests are diverse. Advanced and promising techniques such as monitoring, pheromones and visual traps can be used to rapidly detect infestations and implement timely management. Chemical methods are not favoured as they can lead to insect resistance and harm non-target organisms. Physical control methods involve modifying the physical environment of the pest so that it no longer poses a threat to agricultural products. This can be achieved by creating various levels of stress that may lead to death, or by using protective devices such as physical barriers (Thakur et al., 2021). Simple physical methods can utilise renewable or local materials, such as sun drying, applying a layer of sand, repeated sieving, heating, mechanical heating systems and ionising radiation. Simple sieving and aspiration can also reduce insect populations. Frequent sieving of infested grains can reduce infestation. Controlling populations using pheromones and molecular interventions is also of great importance.

Thermal Control

Aeration and cooling are important management strategies for controlling insect infestations, as they lower the temperature of stored grains. Direct heating or cooling of grains is a common method of controlling stored-product pests. Temperature management is an effective method of controlling storage pests because raising or lowering the temperature alters their growth rate and metabolism. At temperatures above or below the optimal range, insect development slows and eventually leads to death. Extreme temperatures can be lethal to insects at all life stages (Thakur et al., 2021). Bandara et al. (2026) investigated the effect of pre-

exposure to spinosad or spinetoram on the heat tolerance of *Tribolium castaneum* adults. They also examined the impact of feeding on survival after heat exposure. This approach is important for understanding the combined effects of insecticides (spinosad and spinetoram) and thermal control methods on stored-product pests. The adults were subsequently subjected to heat stress after being exposed to spinosad or spinetoram. Among the exposed insects, one group continued to feed while the other did not. The insects exposed to spinosad and spinetoram became more sensitive to higher temperatures, demonstrating a synergistic effect of the insecticides and thermal control methods. However, post-heat-stress mortality was low and statistically similar to that of the control group among insects that continued feeding. This suggests that nutritional status is a critical modifying factor when insects are exposed to chemical and heat stress simultaneously. These results suggest that a combined approach of insecticides and heat could be more effective in managing stored products; however, its effectiveness may depend on the insects' feeding status.

Inert Dusts

Inert (inactive) dusts, such as ash and sand, work by filling the voids within grain masses and restricting insect movement. Some inert materials can also be toxic to storage pests, leading to mortality. Due to its physical properties, wood ash can dehydrate the exoskeleton and impair respiration, causing insect death (Thakur et al., 2021).

Baliota and Athanassiou (2023) evaluated diatomaceous earth (DE) and zeolite formulations obtained from natural deposits in Greece for use as insecticides on concrete and steel surfaces to control three species of stored-product pest (*Sitophilus oryzae*, *Rhyzopertha dominica* and *Tribolium confusum*). The formulations were tested in powder form at doses of 0.5 or 1 g/m². Even at the lowest dose, the inert materials achieved 100% adult mortality for all three species after seven days. Of the species tested, *R. dominica* was the most sensitive, followed by *S. oryzae* and *T. confusum*. These results suggest that naturally sourced inert silicate deposits could effectively combat insects in stored products at comparable doses to those of commercially available inert material-based formulations.

Irradiation

Low doses of radiation can kill or sterilise common pests and their eggs in stored grain. Various types of radiation, such as microwaves and X-rays, are used to disinfect grains before storage. Ionising radiation is used in many countries for pest control and quarantine purposes (Thakur et al., 2021). Radioactive substances such as cobalt (⁶⁰Co) and caesium (¹³⁷Cs) can also be used to combat pest damage. The lethal effect of radiation is applied specifically to pests in stored products, with the applied dose varying depending on the species of pest (Boylu, 2020).

Nasr et al. (2022) investigated the lethal, reproductive inhibitory and histological effects of gamma (γ) irradiation (ionising radiation) on adult *S. oryzae*, a stored-grain pest. Cobalt-60 was used as the gamma radiation source for this purpose, with doses of 0.10 kGy, 0.25 kGy, 0.50 kGy and 1.00 kGy being applied. As the dose increased, the mortality rate of *S. oryzae* increased significantly. The dose required for LD₅₀ was calculated to be 1.51 kGy after 48 hours, whereas lower doses were sufficient after 72, 96 and 120 hours. At all tested doses, the reproductive capacity of adult insects was completely eliminated, demonstrating a sterilising effect. Histological examination of insects exposed to 1.00 kGy revealed vacuolisation, necrosis and tissue degeneration in abdominal sections. This study suggests that gamma irradiation could be an effective method of controlling *S. oryzae*, as it exhibits both lethal and reproductive inhibitory effects.

The observation of a sterilising effect even at low doses is important as it provides an alternative approach to stored-product pest management that does not leave chemical residues. However, there are negative effects on the agricultural use of irradiated grains (e.g. as seeds), such as reduced germination and altered plant physiology. Therefore, the use of irradiated grains as seeds is not recommended.

Keszhelyi et al. (2024) evaluated the effects of UV-C radiation (253.2 nm) on the adult mortality of stored-product pests that play different ecological roles (*Sitophilus granarius*, *T. castaneum*, *Cryptolestes ferrugineus* and *Oryzaephilus surinamensis*), as well as assessing the viability and germination capacity of irradiated wheat and maize. Exposure durations of 5, 10, 15, 20 and 25 minutes were tested. After 10 minutes of UV-C exposure, the viability and germination capacity of wheat and maize kernels were evaluated using the tetrazolium test.

As exposure time increased, mortality increased significantly in all tested insect species, demonstrating a consistent relationship between duration and mortality. Sensitivity differed among species. The primary pest, *S. granarius*, was affected by shorter exposure times, suggesting that short-duration treatments could effectively control this species. Wheat kernels were found to be more sensitive to UV-C radiation than maize kernels. The tetrazolium test results indicated that reductions in viability and germination were more pronounced in wheat. Keszhelyi et al. (2024) concluded that this method is better suited to storage environments where germination is not necessary. They also emphasised that special care should be taken if the irradiated grains are intended for use as seeds.

Fumigation

Fumigation is a process carried out during an initial infestation of agricultural products or at specific intervals to control subsequent infestations. It plays a key role in managing stored-product pests (Boopathy et al., 2021). Fumigants have become a fundamental tool in pest management as they can penetrate products and reach infested areas, thereby reducing product loss by killing pests. A fumigant is a chemical that exists in gaseous form under specific temperature and pressure conditions, diffusing into the product and exerting lethal effects on insects or other pests within the commodity. However, various chemicals used as fumigants, such as phosphine, carbonyl sulfide and ethyl formate, are less frequently applied due to the development of insect resistance. However, essential oils from plants belonging to the Apiaceae, Lamiaceae, Lauraceae and Myrtaceae families have also been used as fumigants against various insect species. The large-scale application of plant volatiles is challenging due to their physical properties, including high molecular weight, high boiling point and low vapour pressure (Boopathy et al., 2021).

Ozone is also used as a fumigant. It is a highly reactive and strong oxidising agent, classified as 'Generally Recognised as Safe' (GRAS) by the United States Environmental Protection Agency (USEPA) (Işıkber and Athanassiou, 2015). It has significant potential for pest control in stored grains and can penetrate large grain masses (Boylu, 2020). As it can be easily generated on-site using only electricity and air, ozone offers several safety advantages over conventional post-harvest pesticides. There are no risks related to storing toxic chemicals, mixing chemicals or disposing of containers with increased insecticide residues. Due to its short half-life, ozone decomposes back into oxygen and leaves no residues on the product. Additionally, if needed, ozone can be neutralised using thermally activated carbon or catalytic and chemical scrubbing methods (Abd ElAziz, 2011). However, ozone application in storage facilities requires the installation of ozone feeding and washdown systems (Boylu, 2020). Athanassiou et al. (2008) reported that, under laboratory conditions, exposure to 115 ppm of ozone resulted in the death of almost 60% of *R. dominica* and *S. oryzae* adults after two hours,

with all adults dying after six hours. In the same study, mortality of *T. confusum* adults was very low after two hours of exposure, but reached 100% after six hours.

Various mechanisms have been suggested to explain the toxic effects of ozone on insects. One such mechanism involves reduced metabolism due to interrupted respiration caused by the toxic environment generated by ozone. In this case, insects close their spiracles, which extend from the thorax to the abdomen and facilitate the exchange of oxygen and carbon dioxide. The purpose of spiracle closure is to minimise the intake of toxic gases. This limits the insect's oxygen supply, reducing its metabolism and eventually leading to death. The mortality of insects exposed to ozone depends on their respiratory rate and life stage (Boopathy et al., 2021). Eggs and pupae are the least sensitive life stages to ozone exposure due to their low respiration rates and metabolic state, as well as their protective outer layers (McDonough et al., 2011). Another mechanism occurs at the cellular level. During the conversion of ozone to dioxygen (O_2), reactive oxygen species (ROS) are generated. This induces oxidative stress, leading to the peroxidation of polyunsaturated fatty acids and the oxidation of protein sulfhydryl groups. Consequently, vital molecules such as DNA and proteins are damaged or destroyed. Ultimately, this cellular damage causes insect death (Holmstrup et al., 2011). However, many insects have evolved resistance to oxidative stress (Holmstrup et al., 2011).

Studies have also been conducted on the use of entomopathogenic fungi as fumigants. Barış and Er (2025) tested the effectiveness of *Beauveria bassiana* 5-4 and *Metarhizium robertsii* S3 fungi against the pests *S. oryzae*, *R. dominica* and *Plodia interpunctella*. The researchers used five different carrier materials (diatomite, clay, kaolin, talc and calcite) both individually and in combination. The formulations significantly enhanced the effectiveness of the isolates when used alone. The isolates and carriers were applied individually at concentrations of 2×10^{10} spores/kg of wheat and 250 mg/kg of wheat, respectively; meanwhile, the isolates were combined with the carriers at the same concentrations. The carriers did not reduce the efficacy of the fungi; in some combinations, they even enhanced it. Three formulations were developed for each fungal isolate (BbTF1, BbTF2, BbTF3, MrTF1, MrTF2, and MrTF3) and tested under laboratory conditions against the pests. These formulations significantly increased efficacy compared to using the isolates alone. For *R. dominica* and *P. interpunctella*, all formulations achieved 100% mortality; for *S. oryzae*, however, only BbTF1 and MrTF1 did so. Additionally, most formulations completely suppressed the emergence of the next generation of *R. dominica* adults, while BbTF1 and MrTF1 achieved comparable suppression of *S. oryzae* (Barış and Er, 2025). Mantzoukas et al. (2023) examined the long-term effects of ten fungal species, isolated from soil and belonging to the genera *Cladosporium*, *Condenascus*, *Lecanicillium* and *Penicillium*, on various economically important insect species. Whole wheat grains were sprayed directly with conidial suspensions containing 10^8 spores/mL of each tested fungus, and were then exposed to adult insects of the species *S. granarius*, *S. oryzae*, *S. zeamais*, *R. dominica* and *T. granarium*, in order to assess mortality. The lowest LT50 value (9.164 days) was observed in *T. granarium* when exposed to *Penicillium goetzii*. The most effective isolate was *Lecanicillium dimorphum*, which recorded remarkably low LT50 values for *S. oryzae* (approximately 11 days), *R. dominica* (approximately 12 days), *T. granarium* (approximately 10 days) and *S. granarius* (approximately 13 days), whereas the LT50 for *S. zeamais* was over 16 days. Understanding the efficacy of entomopathogens in combination with other IPM strategies is critical for their proper use in pest management.

Pheromones

Semiochemicals are chemical signals produced by an insect or organism that trigger behavioural or physiological responses in the recipient. In stored-product protection, pheromones — a type of semiochemical — are particularly important. Pheromones help insects with behaviours such as locating food, finding mates, oviposition and defence (Guru et al., 2022). Pheromone traps have been developed to disrupt sexual communication and suppress pest populations (Rajendran & Somiahnadar, 2020). These tools enable the early detection of low-level pest populations and infestations, allowing contaminated areas or products to be identified (Coşkun, 2005). Garcia et al. (2003) applied sesquiterpenes, including costic aldehyde, Tessaric acid, and eremophilane, isolated from *Tessaria absinthioides* as sprays on *Tenebrio molitor* larvae. They found that the treatment extended pupation duration and caused morphological deformities. Sambaraju and Phillips (2008) investigated the response of adult *P. interpunctella* to the sex pheromone (Z,E)-9,12-tetradecadienyl acetate (TDA/ZETA) in combination with UV, green, and white light. They found that around 71% of adult moths were attracted to the pheromone traps and were drawn towards low-light surfaces.

In their study, Athanassiou et al. (2025) observed that the implementation and development of mating disruption (MD) for stored-product pests poses distinct challenges and prospects compared to the methods employed for conventional agricultural pests in on-site facilities. MD involves releasing large quantities of pheromones into the environment, making it difficult for males to locate females and reducing mating. However, MD is more complex for insects: some species have a short lifespan and rely primarily on sex pheromones, while others have a long lifespan and are associated with aggregation pheromones. MD should therefore be considered as part of an integrated system for stored-product pest management, involving not only pheromone dispensers, but also traps and other control strategies. Combining different pheromone sources (e.g. ZETA plus additional components) can provide efficacy across multiple species, which is particularly advantageous in storage facilities where several pests coexist. Guarino et al. (2022) conducted a study on the use of pheromones for mass trapping. This study demonstrated that adding β -ionone to the conventional sex pheromone in *Lasioderma serricorne* traps significantly improved capture performance.

Overall, pheromone-based methods remain an important part of integrated pest management programmes, serving as an alternative or complementary strategy to chemical fumigants.

Molecular Interventions

The limitations of chemical pesticides and other conventional methods have led to the recent emergence of RNA interference (RNAi) and CRISPR/Cas9 as preferred pest control strategies. These approaches aim to develop precise, environmentally friendly pest management tools.

RNAi-based applications target the inhibition of specific essential genes in the target organism. This is achieved by the organism taking up and processing double-stranded RNA (dsRNA), which leads to the target gene's mRNA being degraded (Germin et al., 2025). In insects, the RNAi pathway is a naturally occurring sequence-specific gene silencing mechanism that often functions in antiviral defence and is triggered by small RNA molecules. The approach involves introducing exogenous small interfering RNA (siRNA) via dsRNA to silence specific genes, thereby reducing pest fitness or causing mortality. RNAi poses minimal risk of off-target effects due to its sequence specificity and is not considered a form of genetic modification because its effects are not heritable (Faber et al., 2026).

CRISPR biotechnology, on the other hand, functions by inactivating the Cas9 DNA-binding protein, thereby halting gene expression (Qi et al., 2014). CRISPR-based genome editing strategies for pest management have been demonstrated in both plants and insects. In plants, these strategies include knocking down susceptibility genes, modifying plant volatile blends and altering leaf colouration to confer resistance against pests. In insects, CRISPR can be used to make them susceptible to plants by, for example, modifying Cry protein-binding receptors or silencing detoxification enzymes (Tyagi et al., 2020).

In conclusion, managing stored-product pests is a complex, multidimensional process that cannot rely on a single control method. The limitations of chemical fumigants and the development of resistance have led to the creation of more sustainable, environmentally friendly and comprehensive strategies. Current research suggests that modern integrated pest management (IPM) programmes are most effective when they combine physical methods, such as ozone and inert dusts, with biotechnical applications, such as entomopathogenic fungi and pheromones, as well as molecular approaches, including RNAi and CRISPR. Coordinating the use of economically viable and storage-appropriate methods is critical for ensuring long-term pest population control and food safety. Looking to the future, translating laboratory findings into field applications, discovering novel biological agents and expanding digital monitoring systems will provide more effective and sustainable solutions for managing stored-product pests.

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GRAIN STORAGE, FACTORS AFFECTING STORAGE, AND CONSIDERATIONS DURING THE STORAGE PROCESS

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ABSTRACT

With the rapid growth of the world population and the emergence of food crises especially in underdeveloped countries grains have gained strategic importance in human nutrition. Therefore, in addition to grain production, storage and preservation have become increasingly important over time. Moreover, the ability to stockpile grains provides a significant advantage in times of war or natural disasters. In the agricultural sector, although the supply of products occurs in a short period, demand is spread over a longer duration. Thus, products must be stored and made available for consumption with minimal loss in terms of both quality and quantity. To prevent such losses, the most critical factors that must be carefully managed include storage temperature and humidity, grain temperature and humidity, the structure of the storage facility, and biological factors. Both ventilation and drying methods should be used to maintain these values at optimal levels and to take preventive measures against potential pest infestations or mold growth. Before the grain is placed into storage, all unfavorable conditions in both the product and the storage area (such as temperature, humidity, foreign materials, and weed seeds) should be minimized to make the product ready for storage. The most commonly preferred types of grain storage facilities are horizontal and vertical silos. In harvest seasons especially in years with high yield when vertical and horizontal storage becomes insufficient, underground storage is also an option. Regardless of the storage method, the primary goal is to preserve the stored product in terms of both quality and quantity and to make it available for consumption without loss of value. This review aims to provide general information about the fundamental principles and key considerations in grain storage.

Keywords: Grain, storage, preservation

INTRODUCTION

Cereals have been among the primary foodstuffs consumed by humans for centuries (Altan, 1986). In terms of cultivated area, cereals rank first both in the world and in Turkey. Turkey holds approximately 3,5% of the world's wheat cultivation area. Wheat constitutes 67% of the cultivated land in our country. According to 2024 data from the Turkish Statistical Institute (TUIK), approximately 30 million tons of grain were produced in Turkey, 21 million tons of which were wheat. However, regardless of the production volume, the more critical issue is how much of the harvested crop is preserved correctly and reaches the consumer intact (Ertugay and Certel, 2011). Storage does not significantly affect the mineral content of grain kernels. However, it has been reported that a large proportion of the organic compounds in the grain composition are lost during storage (Elgün and Ertugay, 2002). Wheat is produced in every region of our country and ranks first among field crops in terms of both planting rate and

production. The storage stage of the crop is as important as the production volume (Keskin, 2019).

Cereals can be stored either above or below ground, in heaps using materials such as straw or polyethylene, or in horizontal and vertical silos constructed from concrete, sheet metal, or steel. The main goal of storage is to prevent losses in both quantity and quality of the harvested products during the time between harvest and consumption. The key factors to consider during storage include:

1.1 Moisture (Humidity):

The higher the moisture content inside the seed, the faster enzyme activities and microbial growth occur. The ideal moisture level is around 12–13%, and this level should be maintained through regular monitoring (Boys, 2011). As grain moisture increases above this level, the grain begins to exhibit germination physiology. This leads to spoilage and mold growth, causing the wheat to lose its quality over time. If the grain's moisture content exceeds acceptable levels, it should be reduced using drying or aeration methods (Kent, 1982). In general, if the moisture content in grain is reduced by 1%, the storage life increases by 1.5 times (Anonymous, 2008). Jones and Shelton (1994) stated that increasing moisture in the product increases the risk of mechanical damage to the grain. Breakage and damage are particularly prevalent at moisture contents above 18–20%. Broken and damaged grains spoil three to four times faster than healthy grains, and rodents and insects are more likely to feed on these grains.

1.2 Foreign Materials:

Another factor affecting storage is the presence of foreign materials within the grain. Substances such as straw, chaff, stones, and soil reduce storage quality and duration, while also creating a favorable environment for the growth and reproduction of insect larvae. Furthermore, if pesticide application is required during storage, these materials may reduce the effectiveness of the treatment. Unclassified and unscreened grains are more vulnerable to damage compared to clean, intact kernels in the main grain mass (Dizlek, 2012). Products to be stored must be cleaned before being placed in the warehouse and the amount of foreign matter must be reduced to less than 4% (Öztaşhan and Aruoma 1989). Additionally, broken and damaged grains inside the product will become moist faster and be more easily damaged by fungi and microorganisms.

1.3 Storage Structure:

The structure of the storage facility (durable, smooth surfaces, waterproof, and strong), its relative humidity (optimal range: 30–50%), and temperature (should be between 5–25°C) are critical for successful preservation (Sumiahadi et al., 2020). If insulation is not properly ensured, the stored product becomes vulnerable to quality and quantity loss. Economical and purpose-specific storage structures should be built based on usage conditions. Before storage, the facility must be thoroughly cleaned, and insecticide treatments should be applied as a preventive measure against possible pest activity. Grain silos must be designed and manufactured to withstand the constant loads that will occur when they are filled. When installed and used correctly, these storage units will provide many years of service. The silo site should be firm, level, and free of grass and debris. The fill material should be firm and well compacted to prevent unstable movement due to the silo's weight.

1.4 Temperature:

Temperature is the primary storage condition affecting the storage life of stored grains (Unal 1991). A 5°C decrease in temperature in the stored product doubles its lifespan (Anonymous 1990). The storage life of seeds stored at 0°C is up to a thousand times longer than those stored at 50°C (Harrington 1963). In a study conducted by Harner et al. (1998), they recommended that grains be cooled rapidly after being placed in storage and that the temperature be lowered by 10 °C until autumn, in line with outside air temperatures. In the winter months, the stack temperature should be kept between 0-5 °C to prevent moisture migration. The most suitable temperature for grain storage is 15°C or below. At this level, the respiration rate of the grain decreases, and microbial and enzymatic activities are nearly halted. This extends the storage period. The relationship between grain moisture content and temperature is shown in Table 1 below:

Table 1. Moisture Values of Wheat Seeds at Different Moisture Contents and Temperatures

Moisture (%)	Wheat Temperature°C		
	5	15	25
16.5	68	74	76
15.5	62	69	71
14.5	56	64	66
13.5	49	58	60

1.5 Pests and Rodents:

During storage, crops are damaged by microorganisms, rodents, and especially insects. These pests damage grains by gnawing, cracking, and eating them. This results in a loss of both commercial value and seed quality. Some studies indicate that grain yields decrease by an average of 10% annually due to pests.

1.6 Moisture Migration and Ventilation:

The air mass created by temperature and humidity differences moves from warm to cold within the storage area. When the warm and moist air mass reaches the cold regions, the moisture it carries condenses on the cold grains. This phenomenon, called moisture migration, is crucial for safe storage (Sisman 2003). In autumn, as the outside temperature decreases, the temperature difference caused by the rapid cooling of the edges and upper sections of the warehouse while the middle sections remain warm causes the warm, moist air in the middle sections to move toward the top of the stack, causing moisture to condense on the cooler grains there. This increases the moisture content of the products in the condensed area. In spring, this movement reverses, with moisture condensing in the lower and middle sections of the stack. Increased temperature and moisture content increase spoilage caused by microorganisms and pests (Kendirli 2006, Sisman and Engin 2011).

Harner (1989) stated that moisture migration in the warehouse can be prevented if the difference between the outside air temperature and the stack temperature is kept below 5°C. Ventilation is defined as the passage of relatively low volume airflow through the stack by suction or pressure in order to reduce climatic air movements within the warehouse and to prevent damage caused by high temperature and humidity that may occur within the stack (Öztarhan and Aruoma 1989). The primary purpose of ventilation is to remove excess moisture and respiratory heat and to cool the product (Balaban and Sen 1979). Another purpose is to

prevent product temperatures inside the warehouse from being affected by seasonal changes in outside air temperatures and to eliminate temperature fluctuations. Harner and Higgins (1987) stated that warehouses without ventilation systems can be used for storage periods of less than six months, while for longer storage periods, the product should be stored in warehouses equipped with ventilation systems. Jones and Shelton (1994) stated that the safe storage time of grain in a warehouse equipped with a ventilation system is four times longer than in one without.

RESULTS:

During storage, grain continues to respire and maintains some minimal metabolic activity. If these activities increase, negative outcomes such as odor changes, heating, mold growth, and spoilage may occur. To prevent quality and quantity losses as well as financial damage, preventive measures should be taken prior to storage. Moisture and temperature levels must be frequently monitored, and any structural defects in the storage facility must be corrected. Grain that has not been analyzed or classified should not be stored in the main storage area. Instead, it can be stored in separate compartments, containers, or designated special areas. Products should not be stored in damaged parts of the facility. If direct contact with any compromised area of the storage facility is likely to harm the grain, the product should not be stored there. These areas must remain unused until necessary repairs are made and the storage conditions become suitable for safely preserving the grain. If spoilage is detected in the stored grain, appropriate corrective actions such as re-storage, screening, cooling and drying, disinfection, and other treatment methods should be taken to prevent further deterioration and to attempt to restore the product to a usable condition.

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IMPACTS OF CLIMATE CHANGE ON AFLATOXIN CONTAMINATION IN ALBANIA'S MAIZE CROPS

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ABSTRACT

The increasing trend, along with elevated maximum temperatures, drought conditions, and storage susceptibilities, paves the way for more frequent and extensive contamination of agricultural products, impacting both human health and animal welfare. Climate change has been recognized as a global factor contributing to increased food and feed safety concerns, as well as to its potential influence on mycotoxin concentrations in food and feed. Climate change is causing Europe, particularly the southern regions such as the Balkan Peninsula, to warm faster than the global average—a trend expected to persist, leading to temperature rises of 2–3°C.

Biological contaminants, specifically molds of the *Aspergillus* genus, particularly *A. flavus* and *A. parasiticus*, proliferate in warm, humid, or stressful conditions, heightening the risk of contamination before and after harvest. Optimal conditions are 27 °C and adequate moisture; when temperatures exceed 33 °C, the genes switch to aflatoxin synthesis. Aflatoxins are the most hazardous of mycotoxins and exhibit the most detrimental consequences. Research conducted over the last two decades on aflatoxin contamination has demonstrated that action is essential for mitigating this problem.

Investigation of the maximum daily temperatures over the past two decades in the primary agricultural regions, during the most relevant period, May-September, indicates that global warming has impacted the country, with western areas scoring above 60% on the Aflatoxin Risk Index (ARI), signifying a substantial risk. Although relatively negligible effects have been detected in the continental Korça region. Climate change is expected to heighten the risk of aflatoxin contamination in Albanian maize, mirroring patterns observed across southern Europe.

Keywords: Climate change, Maize, *Aspergillus flavus*, Aflatoxin Risk Index, Food and feed safety, Albania

INTRODUCTION

Food is essential for sustaining life, and food security is a fundamental human right. People worldwide are exposed to unsafe food, which results in millions of food poisoning cases and hundreds of thousands of deaths each year. The food supply chain — from farm to table — faces safety issues related to microbial and chemical contamination, personal hygiene, and environmental cleanliness (Fung et al., 2018). There is a clear connection between unsafe food, poisoning, illness, and the economic burden on society. It is estimated that 600 million, nearly 1 in 12 people worldwide, become ill after eating contaminated food, and 420,000 die each year (WHO, 2024).

Natural toxins are a crucial category of food contaminants. This group includes mycotoxins, plant toxins, and marine toxins. Mycotoxins are harmful secondary metabolites produced by certain molds and pose a significant safety risk to both human food and animal feed (Wu,

Groopman, & Pestka, 2014; Eskola et al, 2020). Cereals, especially maize, can contain high levels of mycotoxins, including aflatoxins, fumonisins, and ochratoxins (JECFA, 1998). The presence of mycotoxins in cereals remains an ongoing food safety concern because cereals are a staple for the global population and because molds can infect crops in the field and during storage under unfavorable conditions (EFSA, 2020). This issue impacts not only human foods but also animal feed.

Climate change affects maize production in Southern Europe (Spahiu et al., 2018), including Albania (Topi et al., 2021; Topi et al., 2023; Topi et al., 2024), where aflatoxin contamination raises serious food safety issues. The presence of *Aspergillus flavus* increases mycotoxin levels, highlighting the need for more research and mitigation strategies. Agricultural contamination risks occur both before and after harvest, with humidity being a key factor in assessing potential hazards and their impacts (Medina et al., 2014; Battilani et al., 2016).

Exposure to specific mycotoxins through food, even at very low levels, is associated with adverse health effects in humans and animals, both in the short and long term. While technological advancements have addressed many of these issues — particularly in developed countries — such progress has not been as widespread in developing regions of the world. The health impact is especially evident in countries across Africa (Wu, Groopman & Pestka, 2014). Therefore, the presence of mycotoxins in food also raises concerns for international trade and is a significant issue for both national and international regulatory agencies (Altomare, Logrieco & Gallo, 2021).

Mycotoxins are secondary metabolites produced by molds, and their presence in various food products has led to contamination levels exceeding 80% (Eskola et al., 2020). Among these, aflatoxin B (AFB1) is the most toxic and is classified as a carcinogen —epidemiological data link AFB1 to liver cancer in humans (IARC, 2012; Abrar et al., 2013; Mitchell et al., 2016). Food laws establish maximum allowable levels of contaminants, including aflatoxins (Regulation (EU) 2023/915 on specific contaminants in food, which replaced the previous Regulation (EC) 1831/2003). These standards are enforced based on the ALARA ("As Low As Reasonably Achievable") principle.

Aflatoxins are produced by different mold species in the *Aspergillus* genus, with *A. flavus* and *A. parasiticus* being the most significant. They can contaminate various products, including maize, cotton, soybeans, peanuts, and tree nuts (EFSA, 2020; Abrar et al., 2024). Maize is among the most affected crops by aflatoxin contamination (Munkvold et al., 2019).



Figure 7. *Aspergillus* species in maize

The occurrence of aflatoxin contamination in maize across Europe in 2003, followed by similar incidents in 2012 and 2015, has been linked to the climate conditions of those years, characterized by temperatures exceeding multi-year averages and prolonged droughts (Baranayi et al., 2015). Preventing aflatoxin contamination in maize fields has become a major concern in Southeastern Europe, with Serbia and Romania serving as notable examples (Kos et al., 2018).

Aflatoxins and impact on health

Aflatoxins are a group of 20 secondary, toxic metabolites and are regarded as the most common and significant natural biotoxins present in foods and animal feeds. The four primary aflatoxins are AFB1, AFB2, AFG1, and AFG2 (Mato et al., 2024). The development of aflatoxigenic molds and the production of AF depend on the type of substrate or crop where they grow (Abrar *et al.*, 2013). Climatic conditions, humidity, temperature, agricultural practices, insect damage, and storage conditions vary across different crops, affecting contamination levels.

The first documented case of human disease linked to exposure to foods contaminated with aflatoxins, known as aflatoxicosis, was reported in India in 1974, resulting in 106 deaths (Kuiper-Goldman, 1990). Exposure to AF is associated with both acute and chronic toxicity in humans and animals, primarily affecting the liver. In humans, acute aflatoxicosis presents with symptoms such as vomiting, abdominal pain, pulmonary and cerebral edema, coma, convulsions, and even death. Studies have shown that they have carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive effects (Valencia-Quintana *et al.*, 2020).

AFB1 has been classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen, indicating a high risk of developing hepatocellular carcinoma (HCC). When food contaminated with AFB1 is consumed, metabolic processes produce reactive metabolites, such as AFB1-8,9-epoxide, which can cause significant biochemical effects and interact with cellular organelles and macromolecules. Acute aflatoxicosis may occur due to the protein-binding properties of AFB1's bioactive metabolites in the liver, potentially leading to organ failure. Moreover, oxidative damage to cells, tissues, and DNA can result in cytotoxicity and carcinogenic effects initiated by the AFB1 molecule (IARC, 2012). Long-term exposure can weaken the immune system, hinder normal development, or cause cancer. Most of these pathological changes are linked to the bioactivation of AFB1 into highly reactive, cytotoxic epoxides. One of the most significant recent outbreaks of human exposure to AFB1 occurred in Kenya during 2004 and 2005, leading to 317 cases of aflatoxicosis and 125 deaths (Probst, Njapau, Cotty, 2007).

MATERIALS AND METHODS

ARI Estimation Using Temperature Data

1. Define the Maize Growing Season

In Albania, maize is typically cultivated from May to September (Cotty & Jaime-Garcia, 2007). This period is divided into monthly segments and identifies key growth stages:

- Vegetative: May–June
- Silking/Flowering: July
- Grain Filling: August
- Maturation: September



Figure 2. Central regions of intensive agriculture in Albania.

RESULTS AND DISCUSSION

Several studies have highlighted how climate change impacts food safety by spreading molds that produce aflatoxin B1 from tropical and subtropical regions to the Mediterranean and Central Europe (Battilani et al., 2016; Perrone et al., 2020). *Aspergillus flavus* is the primary species involved in aflatoxin contamination, including in Southern Europe (Baranyi et al., 2015). These findings are supported by data from 2014-2015 in our country, where high contamination levels were observed in maize (ranging from 0.32 to 3550 µg/kg), and an unusual case of wheat was reported, with AFG2 levels exceeding AFB1 (Topi et al., 2023).

This study summarizes the aflatoxin risk index for May, June, July, August, and September, based on the percentage of days with maximum temperatures exceeding 33°C in the regions of Durres, Lushnjë, Shkodër, Gjirokastrë, and Korçë.

Overall interpretation of the climate characteristics:

The Lushnja region is one of Albania's warmest and most humid areas, with peak temperatures frequently exceeding 35°C. The high average and minimum temperatures, combined with humidity, create extended periods ideal for fungal growth. These conditions, along with warmth and moisture, make Lushnja a prime location for aflatoxin contamination, particularly in maize and nut crops. Summer temperatures often surpass 35°C, especially in July, which encourages the growth of *Aspergillus flavus*. This heightens the risk of aflatoxin contamination, especially in traditional storage systems.

The Korça region is situated at an elevation of 800-850 meters above sea level in southeastern Albania. It experiences a cooler climate than other areas, but dry summers still present risks. Although the overall aflatoxin threat is lower, recent extreme heat waves have increased vulnerability, especially in maize and grain production.

The Shkodra region is in northwestern Albania, along the Adriatic Sea. It experiences hot, humid summers, especially in August, with high temperatures and humidity. While proximity to the coast slightly lowers average temperatures, warm nights and increased moisture create ideal conditions for mold and aflatoxin growth, mainly affecting maize and fruit crops. The Mediterranean climate contributes to hot, humid summers that promote mold growth. Additionally, traditional storage methods and poor moisture control raise the risk of aflatoxin contamination.

The Gjirokastra region is located in southern Albania, featuring a continental Mediterranean climate with rising summer temperatures. In July and August, peak temperatures often go above 32°C, while lows stay above 18°C. The region's maize and dried fruit production face risks of aflatoxin contamination, especially as climate change worsens seasonal heat and drought.

Aflatoxin Risk Analysis

The Aflatoxin Risk Index (ARI, %) is not a standardized global metric but rather a calculated value used in specific studies or models to estimate the likelihood of aflatoxin contamination in crops or food products. The ARI is a predictive measure used in agricultural and food safety research to estimate the probability or severity of aflatoxin contamination. It is often expressed as a percentage, indicating the relative risk level based on environmental conditions, crop type, and fungal presence.

ARI is typically derived from models that integrate:

- Weather data (temperature, humidity, rainfall)
- Crop susceptibility (e.g., maize, peanuts)
- Fungal growth conditions (*Aspergillus flavus* and *parasiticus*)
- Historical contamination data

Aflatoxins are potent carcinogens, especially aflatoxin B1, and pose serious health risks.

Monitoring ARI helps mitigate contamination in food supply chains and protect consumer health (Doveneyi-Nagy et al., 2020).

The interpretation of the Aflatoxin Risk Index (%) indicates the proportion of days in a given month when the maximum temperature exceeded 33°C, a threshold critical for aflatoxin development.

Table 1. Critical Temperature Thresholds

Parameter	Critical Value	Biological Effect
Optimal growth temperature for <i>Aspergillus flavus</i>	28–33°C	Active fungal growth
Maximum aflatoxin production temperature	33–35°C	Intensive toxin production
Temperature above which growth is limited	>35°C	Reduced growth, possible toxin presence
Temperature below which growth is minimal	<20°C	Low or halted activity

Table 2. Moisture and water activity also play a crucial role in aflatoxin development:

Parameter	Critical Value	Biological Effect
Post-harvest maize moisture	>14%	Favors fungal development
Water activity (aw) threshold	>0.85	Fungal growth begins
Water activity (aw) for maximum toxin production	>0.90	Peak aflatoxin production

The Critical Threshold of 33°C

The 33°C threshold is used in aflatoxin risk assessment because it aligns with optimal conditions for *Aspergillus flavus* growth and aflatoxin production. Research indicates that while the fungus can grow between 28°C and 33°C, aflatoxin levels peak at 33°C-35°C. Temperatures below 30°C may support fungal growth but result in significantly lower toxin levels. Conversely, temperatures above 35°C typically hinder fungal growth, although some toxins may still persist. Thus, this value serves as a practical, scientifically backed cutoff to identify days with high contamination risk. Lower thresholds, such as 30°C or 32°C, could overstate the risk by including days less conducive to substantial aflatoxin production. Using this threshold focuses the analysis on the most relevant conditions for contamination.

Table 3. Aflatoxin Risk Classification (ARI) (%), risk level, and description.

ARI Range (%)	Risk Level	Description
0–10%	Very Low	Conditions are safe; minimal risk of aflatoxin contamination.
11–25%	Low	Limited risk; basic monitoring is sufficient.
26–40%	Moderate	Moderate risk; preventive measures and regular checks are recommended.
41–60%	High	Significant risk; strong control measures and testing are advised.
61–80%	Very high	Major risk; urgent intervention and lab testing are necessary.
81–100%	Critical	Extreme risk; use of crops should be restricted pending thorough analysis.

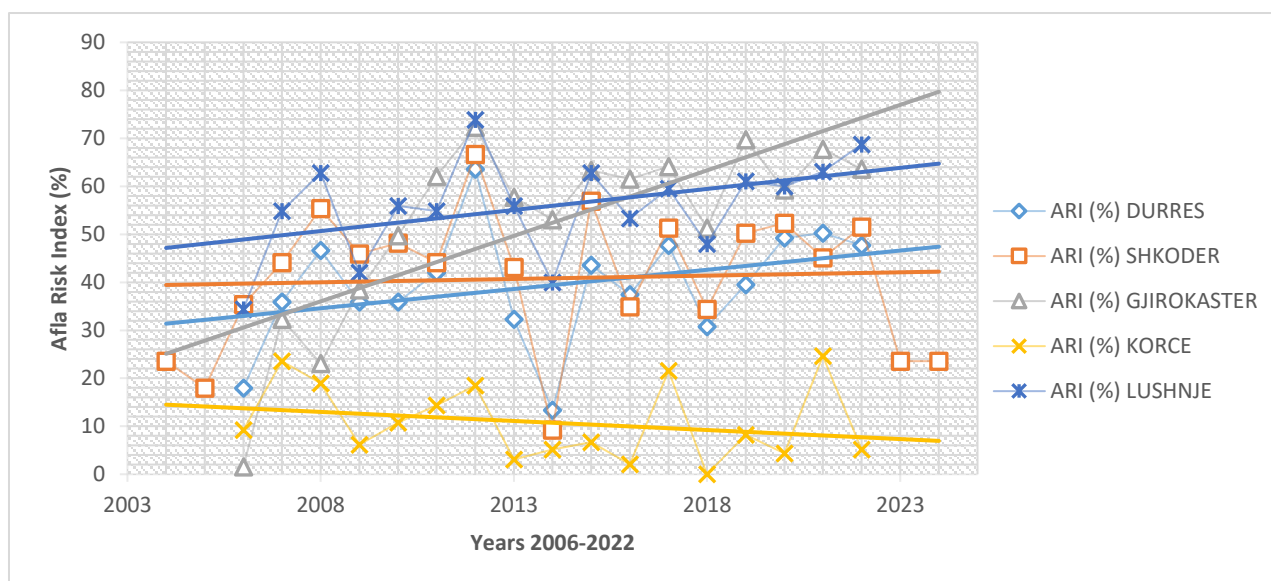


Figure 3. Aflatoxin Risk Index for intensive agricultural regions, the period 2004-2024.

Introducing the maximum daily temperatures for the period May-September for the most intensive agricultural regions indicates a trend in ARI values, starting after 2007 in western regions, while the Korça region remains unaffected. There is an overall ARI value of over 30%

Comparing these results with other factors that affect mold growth and aflatoxin production will provide an accurate prediction of maize, the country's leading agricultural commodity.

CONCLUSIONS

Climate change impacts maize production in Southern Europe, particularly in Albania, where aflatoxin contamination poses significant food safety concerns. The presence of *Aspergillus flavus* raises mycotoxin levels, underscoring the need for further research and mitigation strategies. Contamination risks in agriculture occur both before and after harvest, with humidity a potential hazard that can affect their impacts.

Analyzing maximum daily temperatures from May to September across the key agricultural regions shows a trend in ARI values beginning after 2007 in western areas, while the Korça region remains unaffected. Comparing these results with other factors affecting mold growth and aflatoxin production will help accurately predict risks for maize, the country's main crop.

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CITRUS BREEDING IN THE FACE OF CLIMATE CHANGE: MOLECULAR GENETIC APPROACHES TO HEAT STRESS TOLERANCE

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ABSTRACT

Global climate change has become one of the most serious global challenges of the 21st century and is having increasingly severe effects on agriculture. Due to its increasing impact on agriculture, one major consequence of global climate change is the rise in environmental stress factors such as drought, salinity, frost, and especially heat stress. Heat stress negatively impacts some important physiological and metabolic functions in plants, such as photosynthesis, water use efficiency, and reproductive development, with citrus species being particularly vulnerable. In citrus, heat stress leads to oxidative damage, reductions in fruit yield and quality, and changes in fruit development. Given the adverse effects of high temperature stress on citrus production, it is essential to understand the mechanisms underlying tolerance to such stress conditions. In recent years, advances in molecular biology and genetics have provided new insights into the mechanisms underlying heat stress tolerance in citrus species. This review synthesizes current knowledge on the physiological, molecular, and genetic responses of citrus to elevated temperatures, with a focus on antioxidant defense systems, heat shock proteins, transcriptional regulation, and stress-responsive signaling pathways. The findings discussed here provide a foundation for developing citrus cultivars with improved tolerance to heat stress, supporting future breeding efforts aimed at climate-resilient and sustainable citrus production.

Keywords: Climate change, Abiotic stress, Heat stress, Citrus, Antioxidant defense, Molecular breeding

INTRODUCTION

Citrus fruits (*Citrus* spp.), belonging to the Rutaceae family, are of important economic value worldwide. According to Swingle's taxonomy, the genus comprises 16 species, including orange, mandarin, lemon, and grapefruit (Khan, 2007; Talon et al., 2020). The origin of the citrus family is considered to be Southeast Asia, and they are currently cultivated widely in regions with temperate and subtropical climates, including the Mediterranean Basin, South America, Southeast Asia, and Australia, between 40° north and 40° south latitude (Zhong & Nicolosi, 2020; Talon et al., 2020; Khan & Khan, 2021; Palangasinghe et al., 2024). Citrus fruits are not only consumed fresh around the world, but also have a wide range of uses in the fruit juice industry, cosmetics industry and pharmaceutical industry. Both the pulp and peel of citrus fruits contain numerous phytochemicals that are beneficial to human health. Citrus fruits are generally rich in vitamins C, A, and B6, potassium, calcium, phosphorus, magnesium, copper, thiamine, niacin, folate, riboflavin, pantothenic acid, and flavonoids, especially carotene. Owing to their antimicrobial, antihypertensive, anti-inflammatory, and antioxidant properties, they are considered beneficial in preventing several human diseases, such as hyperglycemia, cancer, osteoporosis, cataracts, and cardiovascular diseases (Riaz et al., 2023;

Palangasinghe et al., 2024). Citrus fruits have a large production volume and economic value worldwide. Global citrus fruit production is about 152 million tons, with oranges, lemons, and mandarins being the most demanded species and accounting for a significant share of the global market. Oranges are the most widely produced citrus fruit worldwide, with a production volume of 69.8 million tons. Following oranges, tangerines and mandarins (52.6 million tons), as well as lemons and limes (23.6 million tons), are also produced globally. China, Brazil, India, Spain, Mexico, and Türkiye rank among the world's leading citrus-producing countries (FAOSTAT, 2023).

According to current estimates, the human population, currently at 8.07 billion, is expected to reach 9.7 billion by 2050. While increasing agricultural production is necessary to meet the growing food demand, various environmental stress factors continue to limit productivity (FAO, 2018). As meeting the growing demand for food becomes increasingly difficult, climate change further complicates this situation. The accumulation of greenhouse gases in the atmosphere due to human activities, particularly carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O), has led to a continuous increase in the global average surface temperature and to climate change, resulting in global warming (IPCC, 2021). Environmental stress factors such as high temperature, drought, and salinity are important factors that limit production. Especially, high temperatures pose a serious threat to agricultural crops grown under field conditions. Since the late 19th century, the Earth's surface temperature has increased, with an increase of about 1.1°C, and is estimated to rise by 1.5°C to 3°C over the next century (IPCC, 2021; IPCC, 2023).

Responses of plants to high temperature stress are affected by temperature exposure, duration, and plant species. Extreme temperature conditions may lead to cellular damage or cell death within minutes (Ahuja et al., 2010; Hasanuzzaman et al., 2013a). Heat stress can negatively affect all aspects of processes, such as plant germination, growth, development and reproduction (Hasanuzzaman et al., 2013a). Agricultural production is expected to decline by 2.5–16% if global temperatures increase by 1.5°C until the middle of the century (IPCC, 2021). In such a scenario, yield losses across many crop groups will vary significantly depending on the species and cultivar. For example, high temperature stress is anticipated to reduce grain yields by 31% in wheat, 45% in maize, and 50% in rice (Nadeem et al., 2018).

High temperature stress also creates serious negative effects on subtropical fruits. Avocado (*Persea americana*), especially the Hass variety, is highly sensitive to spring heat waves, which can reduce yield by causing young fruitlet drop and increasing drought stress through elevated leaf temperatures (Lazare et al., 2022). Similarly, olive (*Olea europaea*), another subtropical fruit, shows decreases in fruit weight and dried fruit oil content under high temperatures, with the extent of the reduction depending on the genotype (Nissim et al., 2020). Loquat (*Eriobotrya japonica*) is particularly susceptible to heat stress during the color-change period, as high temperatures can delay fruit ripening and reduce fruit quality (Deng et al., 2024). Pomegranate (*Punica granatum*) plants experience physiological damage under high temperatures and intense light, for example, reductions in photosynthetic capacity, stomatal gas exchange, and leaf water content. These stresses can also directly impair fruit quality, causing issues such as sunburn and cracking (Moradi et al., 2022). In general, high temperature causes phenological deterioration and physiological stresses that lead to yield and quality loss in subtropical fruits. In this regard, the effects of high temperature observed in subtropical fruits are also prominently seen in citrus fruits and will be discussed in the following sections in terms of physiological, reproductive, oxidative stress, and molecular/genetic responses.

High Temperature Stress Effects on Citrus Physiology

The effect of high temperature on citrus fruits depends mostly on the phenological stage in which the stress occurs. The life cycle of citrus fruits starts in February and continues until January of the next year. In subtropical areas, blooming typically takes place from February to March, marking an essential time for determining yield. Citrus plants are very sensitive to temperature changes, which can strongly influence their physiological processes and overall growth. The highest photosynthetic activity in citrus occurs at around 30 °C, and optimal growth is typically observed between 32 °C and 35 °C. Temperatures exceeding 49.5 °C disrupt plant growth, while temperatures above 51.5 °C can cause significant heat damage, frequently leading to the scorching of branches (Abobatta, 2021; Hussain et al., 2023).

Plants that grow in hot climates try to avoid high temperature stress by reducing the absorption of solar radiation via small hairs (tomentose) that form a thick layer on the leaf surface and protective waxy coatings called cuticles (Hasanuzzaman et al., 2013a). Moreover, citrus leaves have several visible stress symptoms such as chlorosis, wilting, scorching, dropping of green leaves, and reduced turgor, reflecting the combined impact of heat and water stress on plant physiology (Rehman et al., 2024). Furthermore, pollen quality and viability are another critical factor for fruit formation and yield. High temperature stress adversely affects pollen viability, which cause pollen to fail to germinate or lose its viability. (Sato et al., 2006).

The physiological and developmental responses of citrus to heat stress are important during fruit maturation process. Excessive temperatures during the maturation of fruit can lead to increased pre-harvest fruit drop, smaller fruit size, and decreased overall yields. Citrus trees are especially at risk during June, a time when physiological fruit drop commonly occurs. This phenomenon, known as the June fruit drop, is mainly associated with heat and water stress, which hinder the plant's ability to support all growing fruits. In subtropical regions, temperatures can reach 42 °C or higher during this period, and such intense heat increases fruit abscission, leading to a greater loss of small fruits. Among these stress factors, imbalances in water and nutrient uptake lead to decreased net photosynthetic activity, delayed fruit development, and ultimately reduced yield per tree due to excessive fruit drop (Sato, 2014; Kumar et al., 2022). A clear example of this phenomenon is illustrated in Figure 1, which shows visible fruit drop in Washington Navel orange trees observed during the June 2024 field period.



Figure 1. Fruit drop in Washington Navel orange trees in June. Photograph taken on 26 June 2024, Antalya, Türkiye.

Oxidative Stress and Antioxidant Defense Mechanisms Against High Temperature Stress

High temperatures can destroy the structural and functional stability of enzymes found in plants. This destruction may cause problems, notably in the activation of photosynthesis, respiration, and antioxidant enzymes, and can have negative effects on the metabolic functions of plants. In addition, it can cause an increase in reactive oxygen species (ROS) in plants. Increased ROS creates oxidative stress in plant cellular structures. Hydrogen peroxide (H₂O₂) plays an important role among these ROS. H₂O₂ is an indicator of cellular oxidative stress and can cause damage to cell membranes. Glutathione peroxidase, superoxide dismutase (SOD), ascorbic acid peroxidase (APOD), glutathione reductase (GR), guaiacol peroxidase (GPOD), and catalase, increase to neutralize ROS, and the increase in these enzymes may increase in response to high temperature stress in plants. In a study by Rani et al., (2013) on *Brassica juncea*, it is observed that SOD, POX, CAT, APX, and GR activities increased under high temperature stress (45.0 ± 0.5 °C) in plants subjected to heat stress, but the increase was significantly higher in the tolerant genotype. Likewise, heat treatment (at 38 °C for 24 and 48 hours) caused an increase in the activities of antioxidant enzymes (APX, GR, GPX, and GST) in wheat (*Triticum aestivum*) seedlings was determined by Hasanuzzaman et al., (2013b).

High temperature stress can trigger lipid peroxidation in plant cells, which leads to oxidative damage of membrane lipids. Malondialdehyde (MDA), a result of oxidative damage, accumulates in cells under high temperature stress, increasing cell membrane permeability (Vinocur & Altman, 2005). In the study by Zandalinas et al., (2017), combined heat and drought stress caused a marked increase in MDA levels in the leaves and roots of the heat-sensitive Cleopatra mandarin, whereas the increase was less pronounced in the heat-tolerant Carrizo citrange.

Under heat stress, plants often accumulate proline, an amino acid that helps maintain cellular osmotic balance and directly removes free radicals. Consequently, risen proline levels under high temperatures represent an important physiological response contributing to stress tolerance (Vinocur & Altman, 2005). For example, Zandalinas et al., (2017) found that heat stress increased proline accumulation in the heat-tolerant Carrizo, whereas in the heat-sensitive Cleopatra, proline levels remained stable in leaves and decreased in roots, which means that proline accumulation depends on genotype-specific differences in the response to heat.

Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) also play a key role in plant responses to heat stress. Cleopatra had higher H₂O₂ levels, while Carrizo maintained lower oxidative stress under combined stress according to Balfagon et al., (2025). Hydrogen peroxide acts both as a signaling molecule in stress responses and as a source of oxidative damage when it accumulates excessively. In another study, it is interfere that intracellular H₂O₂ levels rise in Arabidopsis because of heat stress, H₂O₂ functions as an early signal in activating heat shock responses (Volkov et al., 2006).

Molecular And Genetic Responses of Citrus to Heat Stress

In response to heat stress, plants adjust the expression of many genes and coordinate different signaling pathways to improve tolerance mechanism (Vinocur et al., 2005). Under several abiotic stress conditions, mostly heat stress, transcription factors such as Heat Shock Factors (HSFs), DREB (Dehydration Responsive Element Binding) proteins, members of the AP2/ERF family, WRKY, MYB (Myeloblastosis), and NAC (NAM, ATAF1/2, CUC2)

proteins, as well as Heat Shock Proteins (HSPs), have a major part in the stress response (He et al., 2023; Sadau et al., 2024; Bautista-Bautista et al., 2024). At normal temperatures, basal levels of HSPs bind to HSFs, inhibiting the activation of heat-responsive genes and transcription of HSP-encoding genes (Zhang et al., 2023; Wang et al., 2023). However, denatured or misfolded proteins are sensed by HSPs, which release HSFs to activate stress response pathways under heat stress. HSFs regulate not only genes involved in responses to biotic or abiotic stresses (e.g., pathogens, wounding, cold, drought, heavy metals, light, pH, oxidative stress, salinity) but also developmental or physiological processes such as flowering, apoptosis, and circadian rhythm (Wang et al., 2023).

There are some studies in different plant species, and they revealed heat stress-responsive genes and highlighted conserved heat tolerance mechanisms across plants. The response of plants to high temperature stress varies depending on the plant genotype, the degree of temperature stress, and the duration of exposure to stress. For example, in grapevine (*Vitis vinifera* L.), the expression of HSF30, HSF7, and HSF1 was observed at 45 °C (Liu et al., 2012), while in dove tree (*Davidia involucrata* Baill.), HSP1, HSP6, and HSP12 were upregulated at 42 °C (Liu et al., 2019). Heat stress typically induces the upregulation of HSP and HSF genes. According to Jin et al., (2020), gene expression patterns associated with heat tolerance vary among jujube varieties. Besides, similar patterns have been observed in pepper seedlings, where HSPs and other transcription factors respond to stress (Katiyar et al., 2021; Wang et al., 2021). Correspondingly, plants such as Bluewell tree (*Paulownia elongata*) and Peony tree (*Paeonia suffruticosa*) had an exhibition of heat-induced expression of certain HSPs, transcription factors, and ROS-related genes (Katiyar et al., 2021; Wang et al., 2021; Ma et al., 2022). These findings suggest that heat stress triggers a coordinated set of molecular responses in terms of HSP accumulation, transcription factor regulation, and ROS detoxification that are essential for maintaining cellular homeostasis under high temperatures.

Citrus plants are naturally exposed to multiple abiotic stresses, such as heat stress (HS), water stress (WS), high light (HL), and nutrient deficiency (NPK). These combined stress factors complicate their physiological and molecular responses, triggering stress tolerance mechanisms (Rodríguez-Azorín et al., 2025). To cope with heat-induced cellular damage, different citrus genotypes activate a range of molecular and genetic responses, such as antioxidant enzymes, heat shock proteins (HSPs), unfolded protein response (UPR) regulators, and osmoprotective metabolites. Findings from Balfagón et al. (2022a–c, 2023) revealed that Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) had a stronger molecular response to heat stress compared to Cleopatra mandarin (*Citrus reshni*).

A main component of heat tolerance in citrus is the induction of heat shock proteins (HSP70, HSP90, HSP101, and small HSPs), along with antioxidant defense systems such as SOD, APX, and CAT. Under combined heat and drought stress, Carrizo citrange accumulates higher levels of these HSPs and antioxidant enzymes (Balfagón et al., 2018; 2022a). Moreover, grafting studies have shown that the enhanced antioxidant capacity of Carrizo rootstock can be transferred to Cleopatra scion, highlighting the rootstock's regulatory role in stress adaptation (Balfagón et al., 2022a,c). Hormonal regulation also plays a crucial part in these adaptive responses, particularly through the modulation of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and their derivatives, which coordinate stomatal closure, osmotic adjustment, and stress-responsive gene expression (Balfagón et al., 2019; Rodríguez-Azorín et al., 2025).

Balfagón et al. (2023) reported that Carrizo citrange exhibits specific activation of RNA metabolism and protein folding pathways at both transcriptional and translational levels when exposed to combined stresses such as heat, drought, and high light. Furthermore, it has been noted that the Unfolded Protein Response (UPR), mediated by BiP2 and PDIL2-2, plays an important role in maintaining protein homeostasis during thermal stress. According to Balfagón et al. (2025), proline metabolism represents an important tolerance mechanism under triple stress conditions (WS+HL+HS). In this context, this study indicated the activation of pathways related to proline accumulation and autophagy, which maintain redox balance and cellular stability. These metabolic adjustments are accompanied by the upregulation of photosystem repair genes and galactose metabolism, which supports photosynthetic efficiency and energy balance under multifactorial stress (Balfagón et al., 2022b,c; Rodríguez-Azorín et al., 2025).

These studies emphasize that citrus tolerance to heat and combined abiotic stresses stems from a complex, multi-layered network integrating antioxidant defense, molecular chaperone activity, hormonal signaling, and metabolic reprogramming. The underlying patterns of gene and protein expression underlying these responses are summarized in Table 1, which compiles current evidence on various stress combinations and citrus genotypes.

Table 1. Differential gene and protein expression in citrus genotypes under high temperature and combined abiotic stress conditions

Plant Materials	Temperature (°C) / Stress Factors (HS, WS, HL)	Differentially Regulated Genes / Proteins under Heat or Combined Stress	References
Carrizo citrange	40 °C HS/ HS+WS	Upregulation of <i>APX</i> , <i>HSP101</i> , <i>HSP17.6</i> (protein accumulation), enhanced antioxidant enzymes and stomatal regulation	Balfagón et al., 2018
Carrizo citrange	40 °C HS	Upregulation of <i>SA</i> (via <i>ICS</i> pathway), upregulation of <i>OPDA</i> in wounded plants, <i>JA</i> and <i>JA-Ile</i> accumulation under control temperature, upregulation of <i>phaseic acid</i> (<i>PA</i>), upregulation of <i>CsAREB2</i> and <i>CsRD22</i> (<i>ABA</i> -independent)	Balfagón et al., 2019
Brazilian sour orange	38 °C – 46 °C HS+ WS	Upregulation of <i>CsHsp70</i> , <i>CsHsp70.1</i> , <i>CsHsp90</i> (1.5-6 h), upregulation of <i>CsTIP1</i> , <i>CsTIP2</i> (aquaporins) under combined heat + drought	Shafqat et al., 2021
Cleopatra mandarin (scion) / Carrizo citrange (rootstock) -Grafted-	40 °C HS+WS	Upregulation of <i>SOD1</i> , <i>APX2</i> , <i>CAT</i> proteins, upregulation of <i>SOD</i> and <i>APX</i> enzymatic activities, reduced <i>H₂O₂</i> accumulation, antioxidant system activation transferred from rootstock to scion	Balfagón et al., 2022a
Carrizo citrange	40 °C HS/ WS/ HL/ WS+HS/ WS+HL/ HS+HL / HS+WS+HL	Upregulation of <i>PSII</i> repair-related genes, photosynthetic apparatus proteins (transcriptomic/proteomic upregulation)	Balfagón et al., 2022b
Cleopatra mandarin (scion) / Carrizo citrange (rootstock) -Grafted-	40 °C HS/ WS+HS	Upregulation of <i>Raffinose</i> , <i>Galactinol</i> , <i>Proline</i> , <i>SA-related genes</i> , hormonal modulation (<i>ABA</i> , <i>IAA</i>)	Balfagón et al., 2022c
Carrizo citrange	40 °C HS+WS+HL	Upregulation of <i>HSP70s</i> , <i>HSP90s</i> , <i>BiP2</i> , <i>PDIL2-2</i> , activation of RNA translation and UPR-related regulators	Balfagón et al., 2023
Carrizo citrange	40 °C HS+WS+HL	Upregulation of <i>proline biosynthesis genes</i> , activation of <i>autophagy-related proteins</i> , <i>antioxidant enzyme genes</i> upregulated	Balfagón et al., 2025
Carrizo citrange	40 °C HS+WS+NPK	Upregulation of <i>galactose metabolism</i> , <i>ABA</i> , <i>SA</i> , <i>JA</i> , <i>PA</i> signaling genes, stress hormone regulators	Rodríguez-Azorín et al., 2025

*The stress factors indicated by abbreviations in the table are as follows: heat stress (HS), drought (WS), high irradiance (HL), the combination of heat and drought stress (HS+WS), drought and irradiance (WS+HL), heat and irradiance (HS+HL), heat, drought, and irradiance (HS+WS+HL), and heat, drought, and nutritional deficiency [nitrogen (N), phosphorus (P), and potassium (K)] (HS+WS+NPK).

Implications for Citrus Breeding and Future Research

Citrus tolerance to heat and combined stress conditions relies on a coordinated network of molecular, physiological, and metabolic mechanisms. Heat shock proteins (HSP70, HSP90, HSP101), antioxidant enzymes (SOD, APX, CAT), osmoprotectants like proline, and stress-related hormones (ABA, SA, JA) act together to maintain cellular homeostasis and support photosynthesis (Balfagón et al., 2018, 2022a; Rodríguez-Azorín et al., 2025).

Understanding the molecular mechanisms by which fruit quality responds to heat stress is significant for citrus breeding, particularly in evergreen citrus species (a few exceptions). A clear comprehension of these mechanisms provides a foundation for developing cultivars with improved tolerant and stable fruit quality under changing environmental conditions. Targeting specific transcription factors could be an effective strategy in citrus breeding to enhance both tolerance to different environmental conditions and agronomic traits. In this regard, several transcription factors have been identified as key regulators linking stress responses with metabolic pathways associated with fruit development and composition. For example, Ayadi et al. (2016) highlighted their variable expression across tissues and potential roles in fruit acid and essential oil biosynthesis. Building upon these insights, Cheng et al. (2021) also emphasized the regulatory role of WRKYs under high-temperature stress, and Li et al. (2021) identified CitHsfA7 as a heat shock transcription factor that promotes citric acid degradation in response to heat in citrus, thereby establishing a direct link between stress signaling and fruit quality. Overall, these studies suggest that transcription factors represent promising molecular targets for citrus breeding programs aimed at enhancing both stress tolerance and fruit quality.

According to the studies' results, thermotolerant varieties, such as Carrizo citrange and Brazilian sour orange, provide valuable traits, while more sensitive varieties, like Savage citrange or Cleopatra mandarin, indicate areas that may require further improvement (Balfagón et al., 2018; Shafqat et al., 2019). Careful rootstock selection is considered significant, as rootstock interactions appear to enhance plant stress responses (Balfagón et al., 2022a,c). Breeding programs are expected to integrate physiological screening with genomic, transcriptomic, proteomic, and metabolomic approaches to determine superior genotypes and rootstocks to improve new stress-tolerant citrus varieties. Overall, these strategies are thought to be essential for promoting sustainable citrus production under changing climate conditions (Balfagón & Gómez-Cadenas, 2025). Breeding programs are expected to be expanded by integrating physiological screening, genomic, transcriptomic, proteomic, and metabolomic approaches, allowing the identification of different superior genotypes and rootstocks and supporting the development of new stress-tolerant citrus varieties. Such strategies are considered essential for promoting sustainable citrus production under changing climate conditions (Balfagón & Gómez-Cadenas, 2025).

CONCLUSIONS

Overall, increasing the heat stress tolerance of citrus varieties is regarded as one of the key goals for sustainable citrus production in the context of global climate change. Although most existing studies on citrus heat stress responses have primarily focused on rootstocks, expanding research to other commercially important citrus varieties is expected to provide a more comprehensive understanding of stress adaptation mechanisms. Such knowledge could contribute to long-term sustainable citrus production by enabling the development of varieties tolerant not only to heat but also to multiple stresses through advanced breeding programs and biotechnological approaches.

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THE EFFECT OF CONSERVATION FARMING PRACTICES ON GREENHOUSE GAS EMISSION

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ABSTRACT

The quality of soil and its management practices significantly influence climate conditions. Correct soil quality management is a key strategy to sequester carbon (C) and gas emissions limitation to potentially mitigate climate change. Conservation agriculture is based on four principles. Minimizing soil disturbance through reduced tillage, leaving an appropriate amount of plant residues on the soil surface, crop rotation with an economic cultivation pattern, and sustainable production of agricultural products with benefits are the basis of these four principles. The aim of this study was to determine how conservation agriculture and conventional tillage affect greenhouse gas emissions and in the western Golestan, Iran. This work sought to investigate N₂O, CH₄, and CO₂ emissions of soils under two different tillage practices and straw retention scenarios: (I) conventional tillage (CT) and (II) conservation agriculture (CA) and The land had been managed under these practices for eight years. This study sampled soil at a depth of 0-30 cm following the harvesting of Wheat in 2024 for Measuring soil properties and used static flux chambers to collect gas samples. Then, gas chromatography (GC) was employed to evaluate the gas samples. It was found that CT yielded 353.6% higher microbial biomass carbon (MBC), 52.1% higher soil Respiration (SR), 67.2% higher microbial biomass nitrogen (MBN), and 59.2% higher microbial community (MC) relative to CT and organic C (OC) significantly improved under CA. It was also found that CA led to 5.2%, 5.9%, and 5.2% larger CH₄, NO₂, and CO₂ emissions relative to CT, respectively. It is necessary to conduct long-term assessments of optimized CA principles integrating all recommended factors to ensure the generalizability of the results.

Keywords: *Conservation agriculture, Greenhouse gas emissions, Organic Carbon, Biological activity, Soil properties.*

INTRODUCTION

Greenhouse gases such as CO₂, N₂O, and CH₄ naturally cycle among different terrestrial and atmospheric reservoirs. Soils can act as a sink for these gases by capturing carbon through photosynthesis and plant uptake of N followed by conversion of plant material to soil organic matter (SOM). To ensure that these gases are effectively stored as SOM, proper management practices must be implemented to maintain organic matter levels. However, preferred management practices do not always result in the retention of organic matter. Retention of organic matter is a major challenge, as evidenced by the increased emissions of

CO₂ (13%), N₂O (81%), and CH₄ (44%) between 2007 and 2016 in the agriculture-forestry sector (IPCC, 2019). The use of soil conservation practices, however, may provide landowners with a strategy to reduce GHG fluxes through management. Because the effectiveness of these practices may change over time, further investigation of soil management effects on soil GHG fluxes is needed. One key soil management practice commonly used in about 90% of cultivated land area worldwide is tillage (Ruis and Blanco-Canqui, 2018). Given the complex dynamics between agricultural production, GHG emissions, and climate change, it is crucial to strike a balance between sustainable land development, increased productivity, and environmental sustainability, while also addressing the growing global demand for food (Ahmad and Wu, 2022; Shakoor et al., 2021b). Agriculture can also make a major contribution to climate change mitigation by reducing CO₂ emissions since biomass is a high carbon energy source. Agriculture's contribution as a carbon energy resource should be exploited as one solution with other greenhouse gas emission control technologies (Bundschuh 2014; International Energy Agency (IEA) 2014)). Barghouti (2009) suggests that the anticipated reduction in food production due to climate change can be offset by effective soil and water management practices. On the other hand, the 2010 World Development Report draws on the analysis of the Intergovernmental Panel on Climate Change (IPCC 2007a) to calculate that agriculture is directly responsible for 14% of global greenhouse gas (GHG) emissions in the form of CO₂ and equals and indirectly accounts for an additional 17% of emissions when land use and conversion for crops and pasture are included in the calculations, with a total of 31% (Raich and Tufekcioglu 2000). Nitrifying and denitrifying bacteria the primary contributors of N₂O emissions from soil, and their metabolic activity and thus N₂O production can be dependent on the physicochemical properties (Neill et al., 2005). Despite its widespread application, conventional tillage has been reported to play a negative role in the improvement of soil (Mathew et al., 2012; Fagundes et al., 2019; Lazzaretti et al., 2019) by altering its biological and physicochemical properties (Centurion et al., 2001; Viana et al., 2011). However, the contributions of agricultural management systems and the biochemical properties of soil to GHG emissions are yet to be comprehensively studied. The total cultivated area in the province is estimated to be 730,000 hectares, with approximately 33.4% of this being irrigated, while the remaining 65.6% consists of dry land. The primary crops produced in Golestan, making this region one of the most significant agricultural areas in the country. This study specifically examines the arable lands in the central parts of the province where agricultural activities are concentrated. As a result, this study hypothesized that tillage systems alter the biochemical properties of soil and thus GHG emission dynamics and sought to assess soil conservation and GHG emission management. Therefore, CH₄, N₂O, and CO₂ emissions and MC structure were analyzed under various agricultural and tillage systems for a case study of Golestan Province, Iran.

2. MATERIAL AND METHODS

2.1. Case-study region

Kordkuy County located at coordinates of 54.10°N and 36.47°E in the east of Golestan Province was selected as the case-study region. It has an average annual precipitation of 377 mm and an average annual temperature of 20°C. Humidity and the average annual evaporation of Kordkuy County are 66.41% and 4.0 mm, respectively. The region has a Udic moisture regime and a Mesic thermal regime with Typic Hapludalf soil (Khormali and Kehl, 2011). Table 2 describes the agricultural management system of the region. Conservation tillage has been implemented in the region since 2017, spanning a period of eight years. A randomized complete

block design (RCBD) was employed to perform experiments in two treatments, CA and conventional tillage (CT) with three replicates.

Table 1. Description of treatments

Treatment	Fertilizer	Weed Management	Straw and Stubble Management	Tillage
CT	50-100 kg of potassium sulfate 50-100 kg of triple superphosphate 200-300 kg of urea	Plow, Disk Herbicide	Selling straw (Burning the remains of the previous crop)	Moldboard plow Disk
CA	50-100 kg of potassium sulfate 50-100 kg of triple superphosphate 200-300 kg of urea	Herbicide	At least 30% of the residue remains on the surface. (residue retention on the field: 1-4 ton/he)	NT

2.2. Measurements of soil properties

Soil samples from a depth of 0-30 cm were collected from farmlands subjected to the CA and CT management practices in autumn 2024. They were then dried in ambient air and sieved through a 2-mm mesh. 1:1 soil suspension was used to measure pH and electrical conductivity (EC) (e.g. Rhoades, 1996; Smith and Doran, 1996). Cation substitution with sodium acetate was employed to measure the cation exchange capacity (CEC) at a pH of 7.0 (Chapman, 1965). Moreover, the hydrometer method was performed to evaluate soil texture (Bouyoucos, 1962), and the cylinder method was utilized to calculate the bulk density (Carter *et al.*, 1986). The samples were wetted immediately for a number of tests, e.g., microbial respiration. Microbial respiration was measured by placing the samples at 25°C within a closed container. The produced CO₂ would be absorbed by NaOH and measured through the titration technique (Chhonkar *et al.*, 2007; Isermeyer, 1952). Chloroform extraction was used to evaluate microbial biomass (Jenkinson and Ladd, 1981) and microbial biomass nitrogen (MBN) (e.g. Asgharzadeh, 2010). Furthermore, the colony count method was used within an agar culture medium to measure the microbial population (Asgharzadeh, 2011).

2.3. GHG sampling and analysis

The CO₂, CH₄, and N₂O emissions of the samples were obtained using the static chamber method (Bowden *et al.*, 2000). The gas samples were collected six times randomly in early November 2024, immediately following tillage through dark static stainless steel cylindrical chambers with a height of 30 cm, a total surface area of 0.05 m², and a volume of 0.01 m³. The GHGs were sampled during 08:00-18:00 using vacuum pumps inside black silicone balloons at three intervals (30 min, 12 h, and 24 h). The moisture content and temperatures of air and soil inside the chambers were measured. A glass wool cover was applied to the cylinders to neutralize the impact of temperature changes, as shown in Fig. 1. Gas chromatography (GC) was employed to analyze the gas samples. The GHG concentrations were obtained as:

$$F = \frac{M \times P \times V \times \sigma v}{R \times T \times A} \quad (2)$$

where F is the flux rate (g/m².h), M denotes the molecular mass (CH₄=16 g/mol, CO₂=44 g/mol, and N₂O=44 g/mol), P is air pressure (101300 Pa), V represents the volume of the chamber (0.01 m³), A is the chamber-covered soil surface area (0.05 m²), σv is the linear regression slope of gas concentration variation within the chamber headspace during sampling (ppm/h), and T

is the internal absolute temperature of the chamber (K), and R is the gas constant ($8.314 \text{ m}^3\text{P/Kmol}$).



Fig. 1. Glass wool-covered chamber with a thermometer and a hygrometer

2.4. Experimental setup

This study used an RCBD with three replicates to analyze the experimental data as a factorial. The tillage practice (two levels) and sampling time (three levels) were the experimental factors, and the CO_2 , CH_4 , and N_2O concentrations would be the traits to be measured. The data were analyzed in SAS V. 9.2. Duncan's multiple-range test (DMRT) was used to compare the means at $p < 0.05$. Furthermore, Microsoft Excel V. 2016 was employed to graph the data.

3. Results

3.1. Physiochemical and biological properties of soil

It was found that tillage practices significantly impacted the SP, CEC, and EC of the soil, as shown in Table 2. It was found that CA resulted in a 0.26% lower topsoil pH than CT, which was significant ($p < 0.05$). The potential soil acidity (H^+) was inversely correlated with pH. High H^+ content (acidity in soil) was argued to be stratified in the upper soil layer (Franzluebbers, 2002). This suggests that soil organic carbon (OC) and pH are inversely correlated as crop residues that accumulate in the upper layer release organic acids (Hickman, 2002).

CA resulted in a 7.1% higher SP value than CT. Tillage was reported to impact volumetric moisture content in the order of moldboard plow (14.3%) $\approx$ double disk (16.2%) $<$ NT (19.5%), highlighting that chisel plow was almost the same as double disk and moldboard plow systems (Rius et al., 2022). Furthermore, the tillage systems were observed to have mixed impacts on the moisture content. The average air-filled porosity experienced a reduction in the order of moldboard plow (48.4%) $\approx$ chisel plow (48.8%) $\approx$ double disk (46.8%) $>$ NT (41.1%). Tillage systems influenced the air-filled porosity on twelve dates. The CA and CT treatments produced a bulk density of 1.38 and 1.36 g/cm, respectively. The long-term implementation of the NT system raised the bulk density and produced more compact soil (Chen et al., 2012). This does not facilitate the flow of nutrients to the deeper layers and impacts crop root growth and the absorption of nutrients, leading to a crop yield decline (Ji et al., 2013). Klik and Rosner (2020) reported that a soil tillage reduction enhanced BD, regardless of the soil texture at depths of 0-20 cm under three long-term tillage management practices.

It was found that CA raised the CEC and OC of the soil. Conservation tillage was found to enhance CEC by 69.2% and OM by 40.8%. This is in agreement with earlier works (Sa et al., 2017; Steinbach and Alvarez, 2006; Powlson et al., 2014). Hence, CA may enhance fertility in soil by protecting its structure from tillage (Williams et al., 2016; Kiani et al., 2025). Maize cultivation under long-term CA enhances soil organic matter (SOM) in light of the high residue input. The management system, edaphoclimatic conditions, and crop type are determinants of the high residue input and the consequent elevation of CEC and OC (Lal, 2015). Maize cultivation is a strong determinant of OC enhancement as the residue of maize has a low decomposition rate. Maize plays a key role in preserving high SOM content since it has a greater C/N ratio than soybean, decelerating decomposition and promoting accumulation (Broder and Wagner, 1988; Wegner et al., 2015). This is essential as OC content is high in productive regions and plays a key role in ensuring soil quality and effective productivity. According to Table 2, the treatment systems produced significantly different biological parameters, i.e., MBC, MBN, soil respiration (SR), and MC ($p < 0.01$). The CA treatment maximized microbial activity, resulting in 59.2%, 67.2%, 52.1%, and 353.6% higher MC, MBN, SR, and MBC relative to the CT treatment, respectively (Table 2). This is consistent with Nair and Ngouajio (2012), demonstrating that the introduction of crop residue changed OM, and multiple soil biology parameters, e.g., MBN, MBC, SR, and MC, were involved in its composition. A part from residue quality and quantity, the physical properties of soil, e.g., aggregates and pore space, and environmental factors may also impact the interactions between microorganisms (Six et al., 2000). Straw can function as a source of nutrients and energy to promote bacterial growth by providing polyphenols, vitamins, proteins, and carbohydrates (Singh et al., 2008; Bai et al., 2018). The results show that the retention of straw positively contributes to the abundance of bacteria and the BC composition, as shown in Fig. 2. It was reported that BC abundance and bacterial diversity could be remarkably enhanced through long-term straw retention techniques (Yuan *et al.*, 2011). The results showed that BC composition and bacterial diversity enjoyed superb improvements upon straw retention. Zhao *et al.* (2018) demonstrated that BC abundance and soil properties had mixed correlations based on redundancy analysis. It was found that microorganism proliferation could be enhanced in arable land by raising the C/N ratio of soil (Liu et al., 2016). The present study identified mixed relationships between dominant BCs and the environmental parameters of soil, e.g., OC, C/N ratio, and TN. Tillage impacts microclimate and OM input availability and may alter microbial species in soil (Zuber *et al.*, 2016). NT treatment leads to greater moisture content and lower soil temperatures relative to plow tillage, representing a more effective approach for microbial growth and colonization (Wang *et al.*, 2016, 2017). Although a number of works reported that NT treatment raises microbial activity compared to conventional tillage approaches (Hungria *et al.*, 2009; Silva et al., 2013; Guo et al., 2016), there have been reports arguing that NT does not significantly contribute to microbial diversity in soil (Li *et al.*, 2018a).

Table 2. Physicochemical and biological properties of soils (Lowercase letters compare treatments by LSD's test at 5 % probability)

Treatment	pH	EC (dSm ⁻¹)	SP (%)	Sand (%)	Silt (%)	Clay (%)	Texture
CA	7/63 ^b	0/93 ^a	33 ^b	31 ^a	62/66 ^a	22 ^a	Silty loam
CT	7/65 ^a	0/74 ^b	35/5 ^a	29 ^b	47 ^a	24 ^a	Loam
Treatment	CEC (CmolKg ⁻¹)	MBC (mg kg ⁻¹ soil)	SR (mg Co ₂ gr ⁻¹ soil.day ⁻¹)	MBN (mg kg ⁻¹)	MC (mol gr ⁻¹)	Total C (%)	OC (%)
CA	19/3 ^a	871/66 ^a	21/65 ^a	0/92 ^a	22/33×10 ^{6a}	4/53 ^a	1/69 ^a
CT	11/4 ^b	192 ^b	14/2 ^b	0/55 ^b	14×10 ^{6b}	3/1 ^b	1/2 ^b

CA = Conservation Agriculture; CT = Conventional Tillage; EC=Electrical Conductivity; SP=Saturation Percentage; CEC=Cation Exchange Capacity; OC=Organic Carbon; Bd=Bulk density; MBC=Microbial Biomass Carbon; SR=Soil Respiration; MBN=Microbial Biomass Nitrogen; MC=Microbial Community; TOC=total organic carbon; Lowercase letters compare treatments by LSD's test at 5 % probability.

3.2. GHG emission rates

The CH₄, N₂O, and CO₂ emissions from the surfaces of soils were comparatively analyzed under various management systems. The GHG emissions were monitored at three intervals (30 min, 12 h, and 24 h) following the installation of the chambers. The sampling time and CO₂ emissions were not significantly different in the treatment systems. The CO₂ flux remained steadily small at 28.4 g/m²h under the CT treatment, as shown in Fig. 2. Tillage regulates the soil structure and changes C storage and emission (Abdalla et al., 2013). Semi-humid and temperature soils lose carbon mostly in the form of CO₂, which is primarily induced by the mineralization of OC. Tillage increases the CO₂ emission by raising soil disturbance (Lu and Liao, 2017). The surface layer of soil loosens under classic hoe-type plow, leading to dominant disruptions. As the moisture content, bulk density, and temperature of soil change, the oxygen content of loosened soil rises and mineralizes OC, inducing carbon losses. The production of CO₂ in soil arises from the respiration of roots and OM microbial decomposition, and the moisture content and temperature of soil are key determinants (Davidson and Janssens, 2006). Tillage practices may alter the physicochemical properties of soil and CO₂ production and dynamics. Earlier works reported that conservation tillage decreases CO₂ emissions compared to conventional tillage, particularly with NT being the primary treatment system (Abdalla et al., 2019; Reicosky, 1997; Ussiri and Lal, 2009). Researchers have proposed a variety of explanations for the adverse impacts of conservation tillage on CO₂ emissions. He et al. (2011) held that conservation tillage may support C stocks by restricting temperature elevations and minimizing aggregate disruptions. It diminishes soil disturbance and the access of microorganisms to OC pools. As a result, the decomposition of OC and C loss through leaching or CO₂ fluxes are reduced (Six et al., 2002). Nevertheless, it is also possible that conservation tillage accelerates CO₂ emissions by increasing water availability and thus promoting microbial activity (Plaza-Boadilla et al., 2014b).

Furthermore, the sampling time and GHG fluxes did not significantly differ between the treatments. As shown in Fig. 2, CH₄ showed a steadily small flux of approximately 0.038 g/m²h under conventional management. The management systems may impact GHG emissions by, for example, altering OC, soil fertility, and moisture retention (Tate et al., 2015). This CH₄ flux

pulse following a shift in the management system is yet to be explained. First, methanotrophs (a sensitive group of microorganisms) regulate CH_4 fluxes, and a disturbance arising from a management shift may induce a lack of methanotroph-methanogen balance and increase CH_2 emissions in comparison to CT treatments, while the MC of soil underwent a shift to a new steady state (Scheffer *et al.*, 2001). NT is a globally popular treatment adopted to mitigate soil erosion and promote soil health in the long term; however, it has highly inconsistent effects on the CH_4 emission from soil. Furthermore, given climate variability, it is crucial to assess multiple aspects of agricultural systems under the framework of a “climate-smart” agricultural strategy (Paustian *et al.*, 2016). The N_2O flux was steadily small at $0.017 \text{ gr/m}^2\text{h}$ under conventional agriculture. It was reported that conservation tillage resulted in a potential reduction of 15-40% in N_2O emissions relative to conventional tillage (Drury *et al.*, 2006; 2011). Organic matter was further demonstrated to strongly contribute to the regulation of GHG emissions (Zuber and Villamil, 2016). The return of plant residues resulted in a 32.1% reduction in NO emissions and a 12.9% elevation in N_2O emissions. This aligns with the hypothesis that fresh crop residues provide labile OC substrates and anaerobic conditions due to decomposition, enhancing denitrification (Chen *et al.*, 2013). On the other hand, aged organic matter led to a 2.6-15.9% decline in NO and N_2O emissions, as shown in Fig. 2. This can be mainly explained by increased N substrate immobilization. The inconsistent impacts on GHG emissions require effective crop residue management to minimize N_2O emissions. Microbial nitrification and denitrification are responsible for producing N_2O in soil (Liu *et al.*, 2014; Nivelle *et al.*, 2016). Denitrification can be dependent on a number of factors at various scales. The use of nitrogenous fertilizers is a key determinant in soil management. Hence, nitrogenous fertilization impacts the dynamics of N_2O emissions to a great extent (Zuber and Villamil, 2016), accounting for a fertilizer loss of nearly 1.25%. Tillage systems and plant residues are also key GHG emissions determinants in agricultural management. The tillage treatment alters a set of belowground environmental parameters and residue distributions in soil (Kabiri *et al.*, 2016). N_2O emission elevations can be explained by enhanced denitrification and/or microbial nitrification (Lafuente *et al.*, 2020).

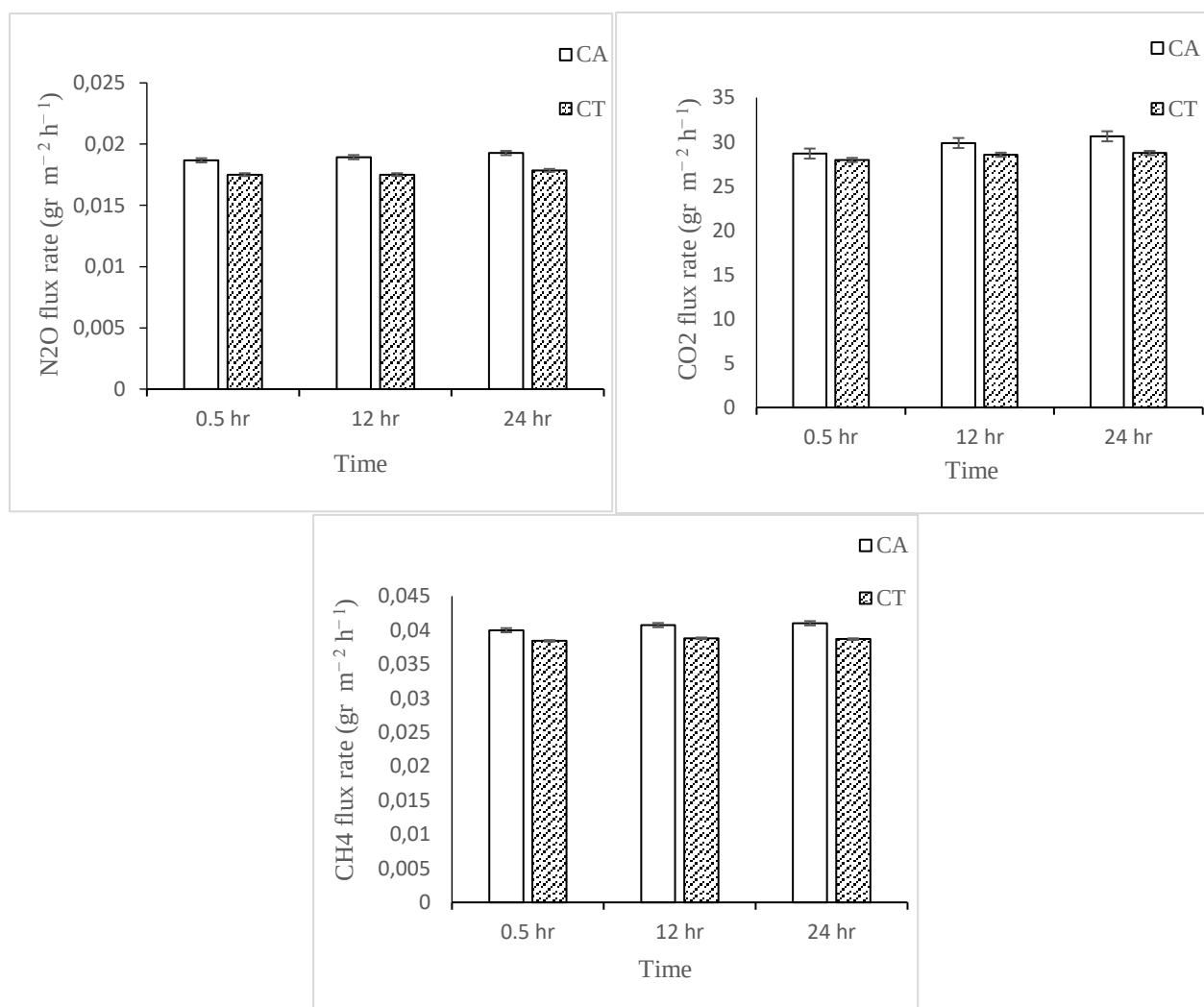


Fig. 2. GHG emissions under different tillage treatments at three sampling times. CA=Conservation Agriculture; CT=Conventional Tillage. Lowercase letters compare treatments by LSD's test at 5 % probability.

CONCLUSIONS

This study found significant changes in the biological and physicochemical properties of soil upon shifts between tillage systems and residue management. A methodology was proposed to quantify GHG emissions from silty clay loam farmlands treated by various management systems. It was concluded that OC, CEC, and bulk density increased under CA, whereas pH and SP declined. Moreover, CA had substantially inconsistent impacts on soil nutrients.

CA maximized total C. A shift from CT to CA increased the biological factors by 52.11-353.6%. Furthermore, CO₂, CH₄, and N₂O emissions increased insignificantly under CA. GHG emissions and soil properties were also observed to be correlated insignificantly. There is extensive literature on the impacts of conservation tillage on GHG emissions, indicating that, in particular, NT would remarkably hinder CH₄, N₂O, and CO₂ emissions; however, reports have been inconsistent in some cases. A set of factors appear to play a key role in this respect, e.g., soil structure, cropland type, local weather, plant residue retention, crop type, and intricate biogeochemical mechanisms. It is vital to implement a comprehensive strategy that synergistically incorporates key factors in order to ensure effective conservation agriculture, which was not the case in the case-study region. This study adopted a short- and medium-term

approach (two to five years). Therefore, it would be impossible to track the variations that develop over time. This is particularly crucial since the contributions of local environmental parameters to CA effectiveness continue to be growingly appreciated. The analysis of short-term soil health can provide insights into challenges that may not become apparent in the short term. Long-term investigations into GHG emissions and responses to climate variability remain yet to be conducted. Future research can fill this gap to help farmers make informed decisions and acquire practical adaptability.

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EFFECT OF SEWAGE SLUDGE BIOCHAR AMENDMENT ON SANDY SOIL QUALITY IN OUARGLA REGION (ALGERIA)

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ABSTRACT

This work aims to study the effect of biochar produced from sewage sludge by pyrolysis at a temperature of 300°C on the quality of sandy soil in Ouargla region. Three application rates of biochar (1%, 2%, and 5%) were studied. A pot experiment was conducted over a 90 days period under semi-controlled conditions. The results revealed an increase in EC, C/N ratio, water retention capacity (WRC), and organic carbon of the sandy soil with the increase in biochar application rate. A decrease in initial bulk density (BD), final bulk density, total nitrogen, and soil pH was also observed with the increase in biochar dose. The results indicated that the application of biochar significantly improved the physical properties of the tested sandy soil (bulk density and water retention capacity), particularly at the 5% dose. The application of sewage sludge biochar to sandy soil significantly modified the soil's organic carbon, organic matter, and pH: increasing organic carbon and organic matter, while decreasing pH with increasing biochar application rate. Future field studies are indispensable to determine if the same effects of biochar observed in pots can be reproduced.

Keywords: Soil quality, Sand, Biochar, Sewage sludge, Ouargla.

1. INTRODUCTION

In the northern Algerian Sahara, soils are primarily of aeolian origin and mainly consist of sands; this is true for the soils in Ouargla region. These soils, given their characteristics, have poor physical and chemical qualities. A possible solution to improve their properties is to add organic matter to these soils to enhance both their physical and chemical properties (Koull and Halilat, 2016). Ouargla region is characterized by light soils with a predominance of sand and a particulate structure. They are characterized by a low organic matter content, low biological activity, alkaline pH, high salinity, and good aeration (Rouvillois-Brigol, 1975). Water stress and salinity are important factors that greatly threaten crop growth and productivity. Improving soils affected by salinity is a major challenge that must be addressed before crop production in these areas becomes feasible (Abd El-Mageed et al., 2021).

Organic amendments are heterogeneous materials that provide a source of soil organic matter and maintain or restore chemical, physical, biological, and ecological functions. Specifically, increasing soil organic matter can influence the soil microclimate, microbial community structure, biomass turnover, and nutrient mineralization. The search for locally sourced alternatives is ongoing, as many forms of organic amendments can be costly or geographically limited (Amoah-Antwi et al., 2020).

Biochar is considered a stable carbon source capable of enhancing soil quality (Ojeda et al., 2015). It has proven to be an alternative for waste disposal and a nutrient source, acting as a soil amendment (Alves et al., 2021). Biochar is a black, carbon-rich, stable, and solid product derived from the pyrolysis of biomass materials. Although it has long been known as a soil carbon sequestrant, biochar is also recently advocated for agricultural applications to improve

soil quality; thus, its agronomic value is being evaluated (Igalavithana et al., 2016). Biochar can enhance the physical and chemical properties of soil and increase agricultural productivity systems through direct and indirect effects on crop growth and soil quality. Additionally, biochar can help mitigate climate change directly by sequestering stable carbon compounds in the soil and potentially indirectly by increasing carbon uptake by trees (Diatta et al., 2020). The application of biochar has been proposed as a viable measure to rehabilitate soil fertility (Elkhilifi et al. 2021).

The stable carbon pool, including aliphatic carbon, aromatic carbon, and functional groups, is the essential component of biochar and determines their functionality as a soil amendment. The content and properties of stable carbon are strongly influenced by the feedstock and pyrolysis temperatures (Xing et al., 2021).

The production of sludge represents the final and unavoidable step in wastewater treatment. Most of the impurities present in the incoming wastewater, or the products resulting from microbial transformation, are concentrated in the sludge. As wastewater treatment becomes more widespread, sludge production is bound to increase (Robert et al., 1994). Using sludge from wastewater treatment plants proves to be an alternative for improving the biophysico-chemical properties of soils (Idder et al., 2012). The main limit in using sludge in agriculture is related to its content of persistent organic micropollutants and, more importantly, trace mineral elements that can be toxic (Robert et al., 1994).

Sewage sludge is considered a good feedstock material for biochar production (Huang et al., 2017). The application of sludge-derived biochar is a promising approach to improve soil fertility by increasing phosphorus content, immobilizing heavy metals, and providing carbon sources available for soil microbes, thereby stimulating microbial biomass (Xing et al., 2021).

The application of biochar has affected several soil properties related to three important soil functions: nutrient release, water storage, and carbon storage. These functions are regulated by soil disaggregation processes (loosening, swelling, micro-cracking) that primarily occur during the initial phase of soil-water contact (Ojeda et al., 2015). However, the value of biochar for production systems in arid regions has been less studied, and the characteristics of some biochars may pose challenges for their use in these soils (Diatta et al., 2020).

The term soil quality refers to the soil's ability to fulfill its three main functions: economic productivity, environmental regulation, and the provision of aesthetic and cultural values (Agegnehu et al., 2017).

Given the importance of organic amendments in Saharan agriculture and their scarcity, our research aims to valorize residual sludge from wastewater treatment plants in the arid regions of the Algerian Sahara by analyzing the effect of its biochar on the physical, physico-chemical, and biochemical properties of local agricultural soils, thereby contributing to improving soil fertility and promoting sustainable agriculture in these regions. The objective of our study is to evaluate the initial impact of adding residual sludge biochar at three application rates on the physical, physico-chemical, and bio-chemical properties of sandy soil in Ouargla region. We hypothesized that the addition of biochar will increase water retention capacity (WRC), organic carbon, and total nitrogen, decrease electrical conductivity (EC) and bulk density (BD), and maintain pH and C/N ratio at acceptable levels in the sandy soil of the Ouargla region.

2. MATERIALS AND METHODS

2.1. Experimental Site

The experimental work was carried out in the semi-controlled greenhouse of the agricultural exploitation of the Ouargla University, located southwest of the city of

Ouargla, approximately six kilometers from the city center (31°57' North, 5°20' East, 134 m altitude).

2.2. Soil and Sewage Sludge used

The soil used in this experiment is a sandy soil with a predominance of coarse sand (52.75%) (Table 1), collected from the dunes in Ain El Beida area (31°96' North, 5°39' East). According to Hamdi-Aissa (2001), the dunes are aeolian accumulations of fine-grained sands probably derived from Mio-Pliocene red sands. After collection, the soil was air-dried for three days and sieved to 2 mm. Table 1 presents the main basic physical and chemical properties of the soil.

Table 1. Basic physical and chemical properties of the soil used in the experiment

	Granulometry (%)			OC (%)	OM (%)	N tot (%)	C/N	pH	EC (dS/m)	WRC (%)
	Coarse sand	Fine sand	Silt + Clay							
Soil	52,75	43,70	2,21	0,62	1,07	0,03	20,8 7	6,76	2,46	9,34

The soil is very saline (Aubert, 1978), very low in organic matter (Soltner, 2014), very low in total nitrogen, and has a neutral pH (Baize, 2000).

The sewage sludge used as raw material was obtained from the wastewater treatment plant (WWTP) of Touggourt, which only treats domestic wastewater in drying beds. The implemented treatment system is the low-load activated sludge biological process.

The sludge collected from the WWTP was air-dried at room temperature for five days, then dried at 105°C for 24 hours in an oven. Subsequently, it was ground in a knife mill and sieved with a 1 mm sieve.

2.3. Biochar Production

The biochar used for the experiment was produced by pyrolysis of the sewage sludge. The ground and sieved sludge was placed in porcelain crucibles with appropriate covers and pyrolyzed under limited oxygen conditions in a muffle furnace. The pyrolysis was carried out at a temperature of 300 °C and maintained at this temperature for one hour, converting 86.56% of the sludge into biochar. The residence time and pyrolysis temperature were set according to the study by Wang et al. (2018).

The most commonly used method for producing biochar is slow pyrolysis due to its relatively higher yield and better quality biochar (Kumar et al., 2020). Producing biochar at lower temperatures can help mitigate the potential negative effects of raw materials (Diatta et al. 2020). Slow pyrolysis uses a longer residence time (about 1 hour) and a slower heating rate (less than 10°C/min), which are preferred for a high biochar yield and its surface properties due to sufficient time for the repolymerization of condensable compounds (Antonangelo et al., 2021). At temperatures below 500°C, the produced biochar tends to have more oxygenated functional groups, which are preferable for the immobilization of inorganic pollutants (Enaime et al., 2020).

Once the pyrolysis process was complete, the biochar was removed from the furnace and cooled to room temperature. The biochar samples were then weighed and stored in sealed plastic boxes until analysis and soil incubation. An analysis of the biochar was conducted, and the results are presented in Table 2.

Table 2. Basic properties of biochar used

Biochar	Result	Method used
pH	5.87	Electrometric method with a biochar/water ratio of 1/10 (Singh et al., 2017).
EC (dS/m)	6.77	Singh et al. (2017) according to a ratio (biochar/water = 1/10).
Organic matter	48.89	Incineration at 750°C for 6 hours (Enders and Lehmann, 2017).
Total nitrogen (%)	4.29	The Kjeldahl method.
Na ⁺ (ppm)	1668.59	Flame spectrophotometer dosage.
K ⁺ (ppm)	609.06	Flame spectrophotometer dosage.
Ca ⁺⁺ (ppm)	1640.77	Flame spectrophotometer dosage.
WRC (%)	28.00	Richards' membrane press (1965).

2.4. Design and conduct of the experiment

The biochar incubation experiment was conducted in pots, which are PVC cylinders (6 cm in diameter and 25 cm in height) with a capacity of 1 kg. The bottoms of the pots were closed with a fine mesh screen to support the soil and allow aeration. The experiment consisted of incubating biochar in the soil for three months, from January 13, 2021, to April 13, 2021. The incubation duration was set according to studies by Basso et al. (2013) and Glab et al. (2016; 2018).

Soil-biochar mixtures were prepared with the following biochar application rates (equalizing the biochar mass as a percentage of the total sample mass): 1% (SB1%), 2% (SB2%), and 5% (SB5%). A control treatment (CT) without biochar addition was also tested.

The experimental design adopted was a completely randomized design with a single classification criterion (biochar application rate) and four replicates for each treatment. The experimental design included four blocks, in which the four treatments were repeated, resulting in a total of 16 samples. The soil was mixed by hand with biochar to obtain the treatments.

The mixtures and the control were packed into cylindrical pots. Using demineralized water, moisture was maintained at 60% of the soil's water content (Elkhlifi et al., 2021) on a dry weight basis by weighing and adding water to the pots three times a week. The pots were covered with perforated aluminum foil to maintain moisture and minimize water evaporation while allowing air circulation.

2.5. Soil Analysis

At the end of the trial, soil height measurements were taken to calculate the final bulk density. Then, physico-chemical analyses were carried out after drying the samples from all pots. The samples were analyzed for organic carbon, total nitrogen, pH, electrical conductivity (EC), and water retention capacity (WRC).

Carbon content was determined using the Walkley-Black method, which is based on the cold oxidation of organic carbon by potassium dichromate in an acidic medium. The percentage of organic matter was approximately estimated by multiplying the carbon content by 1.724.

$$\text{OM (\%)} = \text{C (\%)} \times 1.724$$

Total nitrogen (%) was determined by the Kjeldahl method, which involves hot digestion of organic matter with concentrated sulfuric acid (H_2SO_4) in the presence of a catalyst. The extraction solution is distilled with excess soda and titrated with H_2SO_4 (0.05 N).

The C/N ratio, indicating the rate of organic matter decomposition, was derived.

The pH measurement was performed by the electrometric method on a fine soil suspension with a soil-water ratio of 1:2.5 (Mathieu and Pieltain 2003). Electrical conductivity, expressed in dS/m, was measured using a soil-water ratio of 1:5 (Aubert 1978). Soil pH and EC were measured using a multi-parameter meter.

The distance between the top of the soil surface and the top of the column was measured to estimate the free space volume of each column. The soil volume was then determined by the difference from the total column volume. Bulk density was calculated by dividing the initially oven-dry soil mass by the soil volume. This approach assumes no change in soil mass during incubation, and the obtained value corresponds to the average bulk density of the column (Basso et al., 2013).

Water retention capacity was determined using the Richards membrane press (1965).

2.6. Statistical Analysis

All analytical data were expressed as mean $\pm$ standard deviation. Data were subjected to one-way analysis of variance (ANOVA) with a single classification criterion (biochar application rate). Analyses were performed using XLSTAT 2014 software. The significance of differences between treatment means was examined by one-way ANOVA. Mean separation was achieved using the Tukey HSD test. A non-parametric Kruskal-Wallis test was applied, often used as an alternative to ANOVA when the normality assumption is not met. The Bonferroni-corrected significance level was calculated with a defined probability of 0.05.

3. RESULTS AND DISCUSSION

3.1. Effects of sewage sludge biochar on the physical properties of sandy soil

3.1.1. Bulk density (BD)

Bulk density depends on soil structure and is an indicator of soil compaction, aeration, and ease of root development (Celik et al., 2004).

The application of sewage sludge biochar showed a decrease in the bulk density of sandy soil with increasing in the biochar rate (Fig. 1). This indicates that biochar treatments create porous spaces (Novak et al., 2016). The incorporation of biochar into the soil provides a loosening effect by introducing additional micropores and creating hosting macropores, thereby reducing the soil's bulk density (Guo, 2016).

The addition of biochar to sandy soil causes a decrease in the initial BD from 1.55 g/cm³ (control) to 1.50 g/cm³ (at 5% biochar). Meanwhile, a decrease in the final BD from 1.58 g/cm³ (control) to 1.51 g/cm³ (at 5% biochar) was observed after 3 months of incubation.

Abel et al. (2013) reported that the addition of biochar leads to a decrease in the BD of sandy soils. Several authors have revealed that soil BD decreases with the increase in biochar rate (Hardie et al., 2014; Peake, 2015; Glab et al., 2016). BD decreases with the increase in total carbon content (Glab et al., 2018).

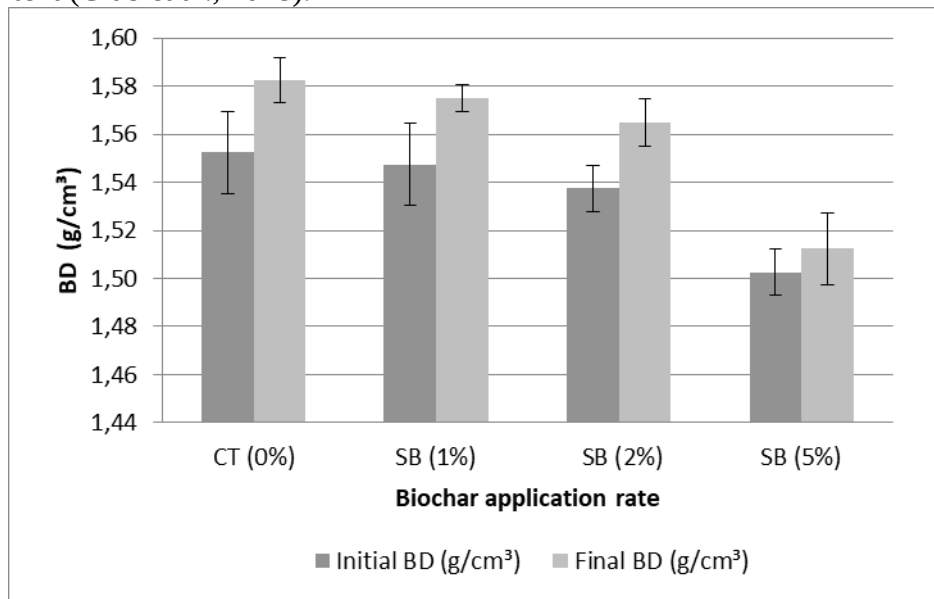


Fig. 1. Initial and Final Bulk Density (BD) of the Soil

The results obtained show that the initial bulk density is lower than the final bulk density in all treatments (Fig. 1). The increase in bulk density was attributed to recompaction by particles deposited in the leachate waters (Novak et al., 2012). However, the rate of compaction decreased with the increasing application of biochar (Mukherjee and Lal, 2013). The bulk density of the soil increased over time for all columns and was significantly lower for biochar-amended soils compared to non-amended soils (Laird et al., 2010).

ANOVA showed a significant main effect of the biochar dose on the initial BD ($P < 0.0001$, $F = 10.6087$). There was no change in the initial BD between the 1% biochar treatment and the control. The 2% biochar treatment reduced the initial BD compared to the control and the 1% biochar treatment, but did not produce a significant result according to Tukey's test. The same test revealed that the initial BD significantly decreased with the 5% biochar dose compared to the control and the 1% and 2% doses ($P < 0.05$).

After 3 months of incubation, there was no significant interaction effect on the final BD between the 1% and 2% doses and the control. The final BD of the soil at the 5% biochar dose was significantly lower than the final BD of the control. Bulk density was only affected by the sewage sludge biochar amendment at the 5% dose. According to Basso et al. (2013), the addition of biochar to sandy soil significantly reduced the soil bulk density after 91 days of incubation.

Organic matter amendments generally increased the soil organic matter concentration, leading to a decrease in bulk density (Celik et al., 2004). The decrease in soil bulk density increases soil porosity and aeration and can have a positive effect on root and microbial respiration (Basso et al., 2013).

3.1.2. Water Retention Capacity (WRC)

Increasing the water retention capacity of sandy soils will help improve water use efficiency in agricultural production and could be

essential for providing sufficient energy and food to a growing global population (Basso et al., 2013).

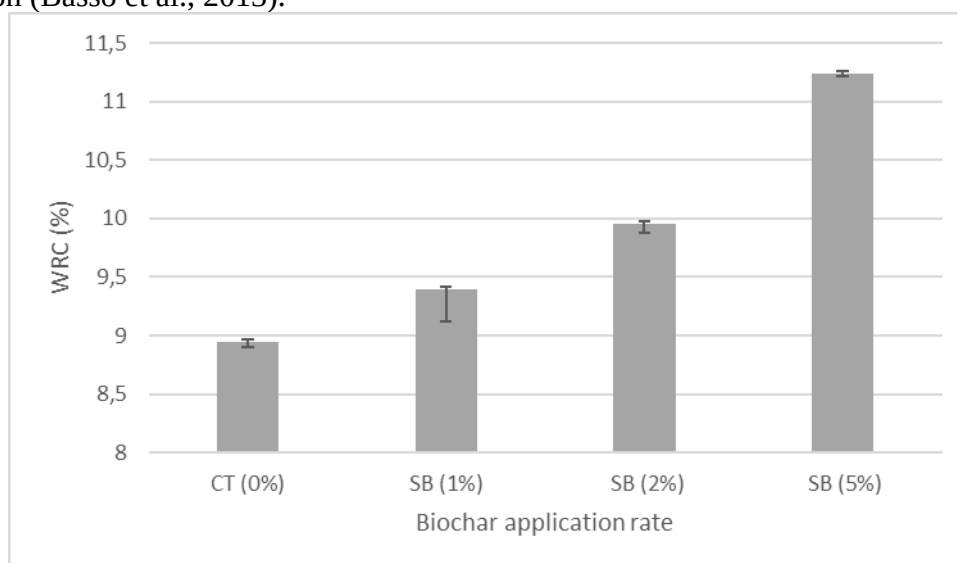


Fig. 2. Water Retention Capacity (WRC) of the Soil

According to Figure 2, the soil amended with biochar shows a significant effect on increasing the WRC. As the biochar application rate increases, the WRC of sandy soil also increases. The 5% treatment achieved the highest WRC value, while the lowest WRC was observed in the control. The WRC in the 1% treatment increased by 5.14%, the 2% treatment by 11.29%, and the 5% treatment by 25.72% compared to the control WRC.

Some studies have reported the positive effect of biochar amendment on WRC (Herath et al., 2013; Baronti et al., 2014; Peake, 2015; Glab et al., 2016).

Biochar application can influence soil porosity and, consequently, water retention through three mechanisms: (1) direct contribution of pores within the biochar, (2) creation of retention pores between the biochar and surrounding soil aggregates, and (3) enhancing the persistence of soil pores due to increased aggregate stability (Hardie et al., 2014). For coarse-textured soils, biochar application improves soil water retention capacity (Guo, 2016).

Kruskal-Wallis showed a significant main effect of biochar amendment on soil WRC ($P < 0.05$). The Bonferroni test revealed that, compared to the control, WRC was significantly increased only with the highest biochar dose (5%). Biochar treatments did not show significant differences in WRC among themselves. The application of sewage sludge biochar improved the WRC of sandy soil compared to the control; however, the extent of this effect depended on the biochar application rate.

The observed differences in water content between the biochar treatments and controls were primarily due to the high porosity of biochar, which could have physically retained more water (Basso et al., 2013).

The ability of biochar to increase soil moisture retention capacity may enhance crop yields under water stress during critical periods of the growing season (Laird et al., 2010). The additional WRC generated by biochar application could help reduce the frequency of irrigation during dry periods and allow plants to survive longer (Herath et al., 2013).

Adding organic matter from various sources to the soil has been one of the most common rehabilitation practices to improve soil physical properties (Celik et al., 2004). Biochar application rates of 1% to 2% (by weight) can significantly improve soil physical quality in

terms of bulk density (BD) and water retention capacity (WRC). The effects of biochar on soil physical properties depend on several factors, such as the biomass or feedstock type, pyrolytic conditions, application rate, and environmental conditions (Mukherjee and Lal, 2013). The effect of biochar amendment on improving soil physical properties depends on the soil type, biochar quality, and amendment rate and may change over time (Guo, 2016).

3.2. Effects of sewage sludge biochar on the physico-chemical properties of sandy soil

3.2.1. pH

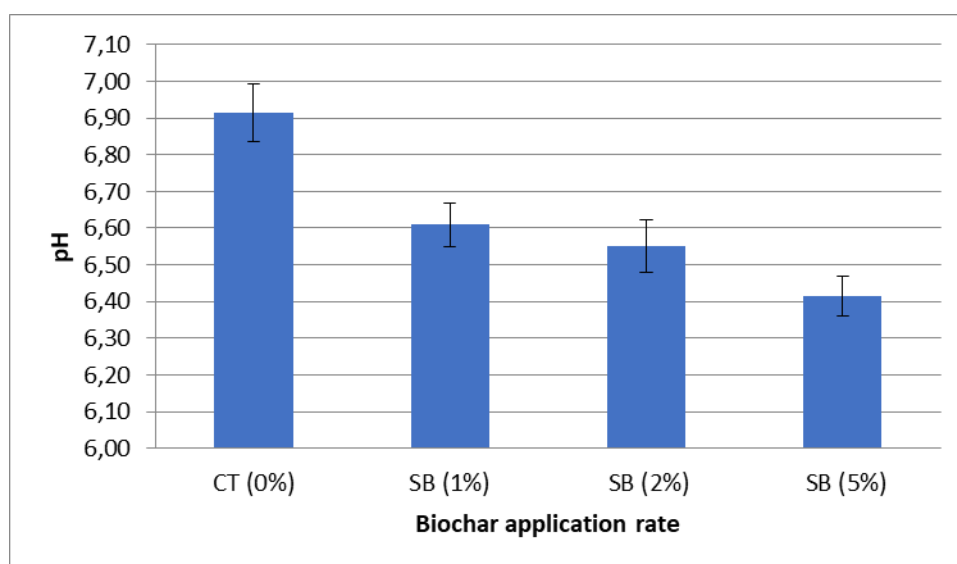


Fig. 3. Soil pH

The results showed that the soil pH was significantly affected ($P < 0.0001$, $F = 40.1573$), decreasing from 6.92 to 6.42 with the increase in biochar rate from 0 to 5%, with the maximum reduction observed at the 5% biochar dose (Fig. 3). The acidic substances produced by the oxidation of biochar and organic matter may have caused the pH decrease (Liu and Zhang, 2012). Ojeda et al. (2015) found that soil pH decreased with the application of slow-pyrolysis sludge biochar.

The soil pH decreased by 0.31, 0.37, and 0.5 units with 1%, 2%, and 5% biochar amendments, respectively, compared to the control. The pH of the sewage sludge biochar used in this study is lower than that of sandy soil and can therefore serve to reduce pH and increase soil acidity.

According to Baize (2000), the observed soil pH values were considered neutral (with 1% and 2% biochar) and slightly acidic (with 5% biochar).

The Tukey test revealed a significant difference between the 1% and 5% biochar additions but no significant difference between the 1%, 2%, and 2%, 5% treatments.

The tested soil pH decreased with the increasing rate of co-composted biochar, which could be attributed to the higher cation exchange capacity of the co-composted biochar (Abdel-Mageed et al., 2021).

However, Laird et al. (2010), Zhang et al. (2012), and Alves et al. (2021) observed an increase in soil pH after biochar application. The liming effect of biochar is related to ash fractions, surface functional groups, biochar application rates, and soil type (Xing et al., 2021). The pH enhancement (increase) effect brought by biochar can be attributed to soil acidity (Sekar, 2012).

3.2.2. Electrical Conductivity (EC)

Electrical conductivity is the total amount of ions in solution (Soltner, 2014). An excessive amount of salt in the soil can have a detrimental effect on plants and the soil itself (Mathieu and Pieltain, 2003).

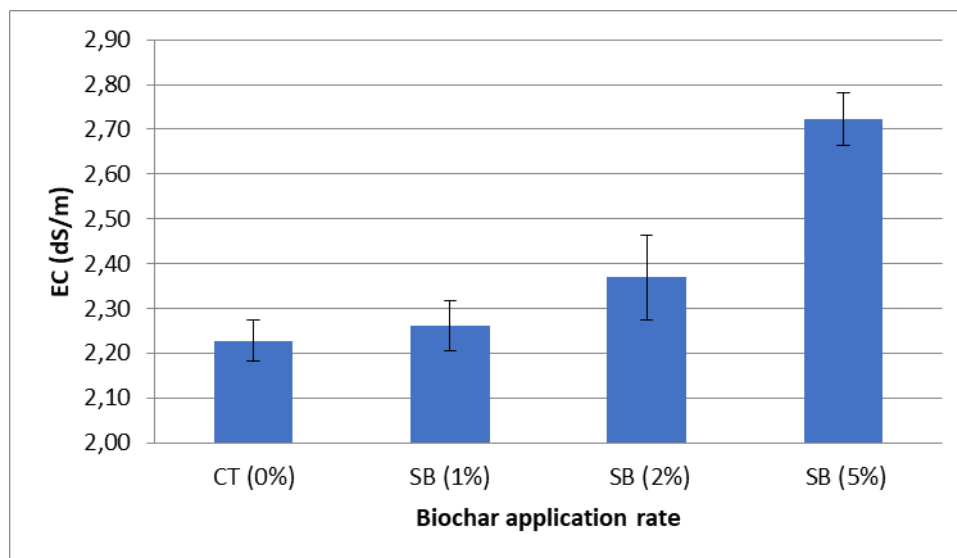


Fig. 4. Soil Electrical Conductivity

The application of sewage sludge biochar significantly increased the soil EC from 2.23 to 2.72 dS/m, with an increase of 121.97% (Fig. 4). The high EC of the biochar (6.77 dS/m) likely influenced the soil EC. A decrease in EC under the control treatment might indicate that the demineralized water used for irrigation leached salts out of the pots.

Soil EC was highest in the 5% biochar treatment and lowest in the control (Fig. 4). The salinity in all treatments falls within the very saline category (Mathieu and Pieltain, 2003).

Kruskal-Wallis showed a significant main effect of biochar amendment on soil EC ($P < 0.05$). The Bonferroni test revealed that, compared to the control, EC was significantly increased only with the highest biochar dose (5%). There were no significant differences in EC among the biochar treatments themselves.

Treatments incorporating biochar significantly increased salinity compared to the unamended control (Gwenzi et al., 2016). The application of poultry litter biochar significantly increased EC (Chan et al., 2008). However, Jones et al. (2012) reported that electrical conductivity was not significantly influenced by biochar additions.

3.3. Effects of sewage sludge biochar on the bio-chemical properties of sandy soil

3.3.1. Effects of biochar on organic Carbon, organic matter, and total Nitrogen

Organic matter is an important source of nutrients for plants, and knowing its total content in the soil provides information on its fertilizing potential (Mathieu and Pieltain, 2003). It increases the water retention capacity of sandy soils (Soltner, 2014).

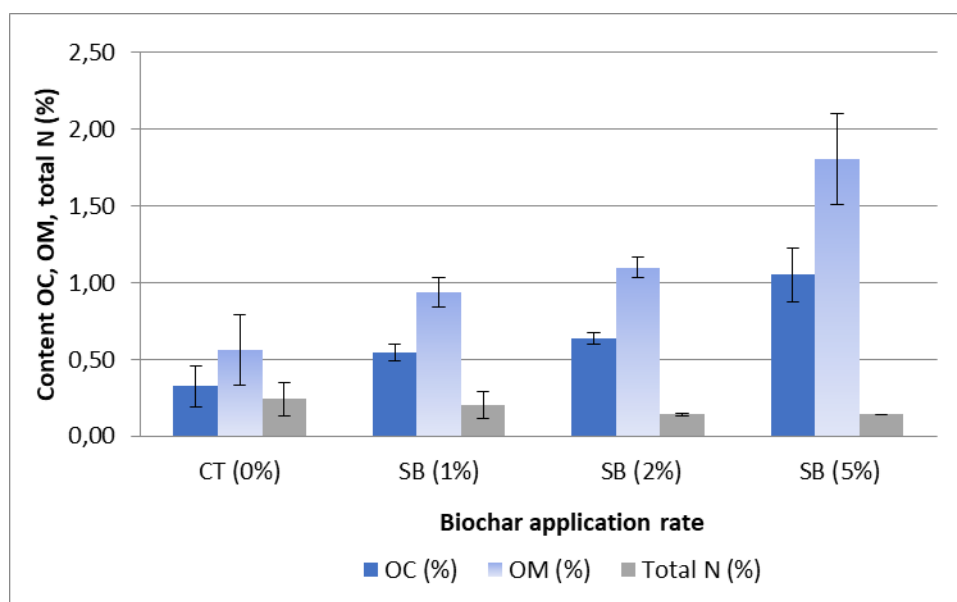


Fig. 5. Soil organic Carbon, organic matter, and total Nitrogen

The content of organic carbon and organic matter improved under all biochar treatments, with the maximum increase observed at the 5% dose. The application of biochar increased organic carbon and organic matter by 1 to 5%, with an increase corresponding to the higher biochar dose (Fig. 5). Similar results were reported by Chan et al., 2007; Chan et al., 2008; and Zhang et al., 2012.

Biochar treatments at 2% and 5% doses significantly increased these parameters compared to the control. The increase was 221.1% and 95.1% for the 5% and 2% application doses, respectively ($P < 0.05$). However, the 1% biochar treatment did not significantly increase organic carbon and organic matter compared to the control ($P > 0.05$).

The increase in organic carbon content due to biochar application in the soil is consistent with the findings of Van Zwieten et al., 2009; Jones et al., 2012; Agegnehu et al., 2016; Sadaf et al., 2017; and Alves et al., 2021.

The enhancement of soil organic carbon stock and stability through biochar application provides both agronomic and ecological benefits, offering a win-win solution to the growing concerns of food security and climate change (Amoah-Antwi et al., 2020).

The application of various decorated biochars has shown significant improvement in soil organic matter due to the dissolution of organic carbon in biochar-amended soils (Elkhlif et al., 2021).

Total nitrogen content is a good fertility index, provided it is interpreted according to the C/N ratio (Mathieu and Pieltain, 2003). There is a decrease in total nitrogen with increasing biochar application doses (Fig. 5), though this decrease is not significantly different from the total nitrogen in the control ($P > 0.05$).

In general, the appropriate application of biochar sludge-based with high-ash content on sandy or acidic soils is highly recommended to improve soil nitrogen fertility (Xing et al., 2021).

Studies have shown the positive effect of biochar amendment on soil total nitrogen content. The application of poultry litter biochar significantly modified total nitrogen (Chan et al., 2008). Higher amounts of N were found in the 5% biochar treatments compared to conventional fertilization (Alves et al., 2021). The addition of biochar significantly increased total N, relative to the respective initial soil nutrient content (Agegnehu et al., 2016). Total N content increased

under biochar amendments, more or less proportionately to the amendment rates (Zhang et al., 2012). Soil total N content significantly increased by 0.6%, 4.7%, and 6.9% for biochar treatments of 5, 10, and 20 g kg⁻¹, respectively (Laird et al., 2010).

However, Jones et al. (2012) suggest that biochar had no significant effect on soil nitrogen cycling in either the short or long term. Sekar (2012) found that biochar does not reduce nutrient leaching.

3.3.2. C/N ratio

The C/N ratio is a widely used indicator to characterize the overall organic matter present in the soil (Baize, 2000). The C/N ratio reflects the intensity of biological activity (Soltner, 2014).

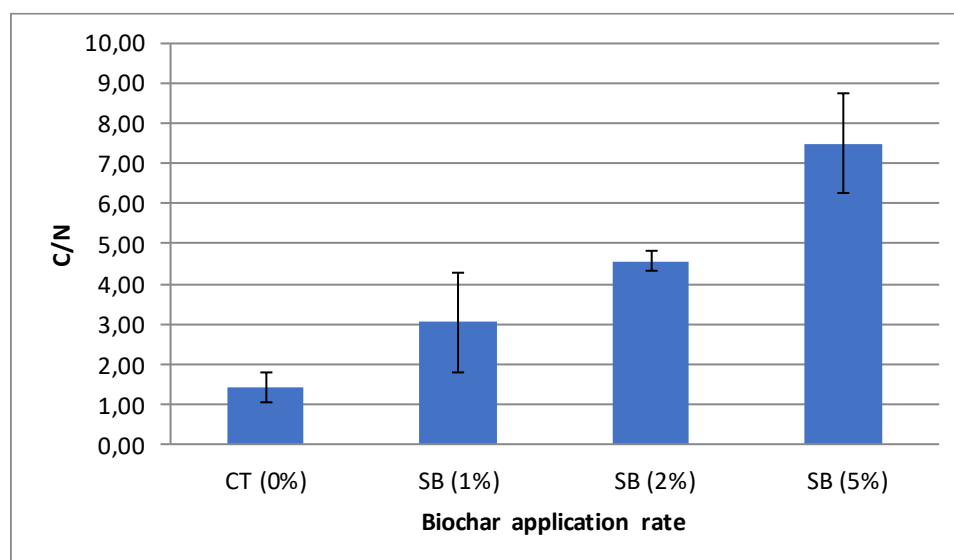


Fig. 6. Soil C/N ratio

The C/N ratio of the soil increased with the application of biochar from 0 to 5% (Fig. 6). ANOVA indicated a significant main effect of biochar dose on the C/N ratio ($F = 33.0060$, $P < 0.0001$). The Tukey test revealed that the C/N ratio significantly increased with 2% and 5% doses compared to the control. There was no significant interaction effect between the 1% dose and the control. The 5% biochar treatment showed a significantly higher C/N ratio compared to the 1% and 2% doses.

A high biochar amendment rate of 40 t ha⁻¹ resulted in an elevated C/N ratio (14–16 under 40 t ha⁻¹ compared to 13 under 20 t ha⁻¹), which could lead to nitrogen limitation for methanogenic bacteria (Zhang et al., 2012).

4. CONCLUSIONS

The results of this study showed that the addition of sewage sludge biochar affected soil characteristics, leading to changes in the physical, physico-chemical, and bio-chemical properties of sandy soil. Changes in soil characteristics amended with biochar, such as decreased pH and increased organic carbon, EC, and WRC, were consistent with the characteristics of the biochar used in this study. There was higher WRC, EC, organic carbon, and C/N ratio and lower pH and total nitrogen in the biochar-amended treatments than in the control. The results indicated that

biochar application significantly improved the physical properties of the tested sandy soil (bulk density and water retention capacity), especially at the 5% dose. This material can be recommended as a soil amendment that improves the quality of sandy soils.

Valorizing the accumulated sewage sludge in treatment plants by converting it into biochar and assessing its impacts on agro-pedological potentials will enable us to promote the use of non-conventional organic resources for the development of Saharan soils, increase the profitability of treatment plants, and thus achieve sustainable agricultural development that favors the conservation of the Saharan environment through the recycling of waste water treatment products.

Further research is recommended to improve the conditions for sewage sludge biochar production, fully quantify the long-term benefits (field trials) of using this biochar, and test other agricultural waste for biochar production.

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COMPARATIVE TESTING OF SUNFLOWER HYBRIDS IN THE SOIL AND CLIMATIC CONDITIONS OF THE TUTRAKAN REGION

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ABSTRACT

The field experiment was carried out in the area of the North-East Bulgaria during the period 2022–2024. The test was performed by means of a block method with four replications; experimental field area – 25 m² after wheat as winter predecessor. The following sunflower hybrids were tested; LG5665, LG50510, LG5478 and LG5662. The aim of the investigation was to determine the production potential of the tested sunflower hybrids in the region of North-East Bulgaria. The analysis of the results showed that the highest values of the productivity elements were reported for the LG56.65 hybrid and the lowest – for the LG56.62 hybrid. The LG56.65 hybrid was most suitable for growing under the conditions of North-East Bulgaria; it gave maximum average seed yield (275 kg/da) during the three years of testing.

Keywords: sunflower, hybrids, seed yield, crude fat seed yield.

INTRODUCTION

To ensure a sustainable high level of productivity in sunflower cultivation, the most reliable and effective approach is to apply a strategy based on combining hybrids with different growing season lengths and diverse economic qualities, since only through such a combination of genetic diversity can yield stability be achieved under variable climatic and soil conditions (Georgiev et al., 2009; Iliev, 2004; Safahani et al., 2014; Krizmanic et al., 2003; Ribeiro and Raiher, 2013, Delibaltova, 2015).

In our country, new hybrids with high productive capabilities and valuable economic qualities are constantly being tested, which is why their study in different regions of the country is essential for obtaining maximum yields and good quality plant production (Adel M. Ali, 2002).

Despite the fact that sunflower is a crop with good ecological plasticity, the choice of a hybrid should be made specifically for agro-ecological regions in order to realize its maximum productive potential in the relevant soil and climatic conditions.

Therefore, in order to fully use the productive potential of the hybrid as a factor for obtaining high yields, the correct choice of the most suitable for each individual agro-ecological region is of great importance (Nenova et al., 2005; Penchev et al., 2006; Saldjiev, 2004). This creates the need for systematic studies of sunflower hybrids in different regions of the country (Nenova et al., 2007; Tahsin and Yankov, 2006; Tahsin, 2012; Yankov et al., 2009).

The aim of the study is to establish the productive potential of several sunflower hybrids in the region of Northeastern Bulgaria.

MATERIAL AND METHOD

In the period 2022–2024, a field experiment was conducted in the land of the village of Tsar Samuil, Tutrakan Municipality - Northeastern Bulgaria. The oilseed sunflower hybrids LG5665, LG50510, LG5478 and LG5662 were included for comparative testing.

The experiment was set up using a block method in four replications with a harvest plot size of 25 m² after a predecessor wheat. The sunflower was grown under non-irrigated conditions using the technology adopted for the region on a leached chernozem soil type. To achieve the goal of the study, the following indicators were taken into account: plant height, flower head diameter, number of seeds in one flower head, mass of 1000 seeds, seed yield. The experimental data were processed using the analysis of variance (Anova) method, and the differences between the variants were analyzed using the Duncan (1995) multi-rank test.

The main climatic factors determining the growth, development and productivity of corn are temperatures and precipitation, their combination and distribution during the growing season.

The studied period (2022–2024) covers years whose average daily temperature values during the sunflower growing season are very close to each other and slightly higher than those recorded during the multi-year period, but different in terms of precipitation, which affected the growth and development of plants.

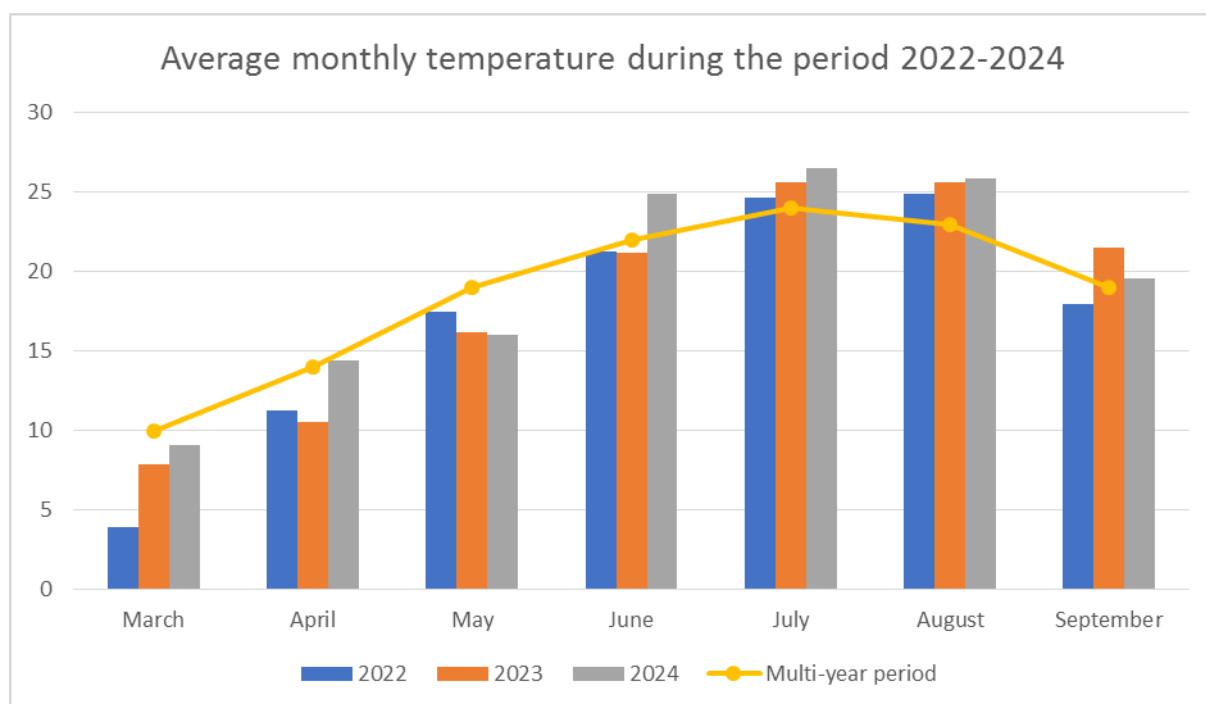


Figure 1. Temperature distribution during the period 2022–2024

The amount of precipitation during the growing season of sunflower is as follows: 2022 - 389.8 mm, 2023 - 289.6 mm, 2024 - 367.21 mm, with a multi-year norm of 404 mm. The amount of precipitation during the critical phases for plants (budding, flowering and the beginning of grain filling) has a significant impact on the productivity of the tested hybrids. In this regard, the most favorable is 2022, the amount of precipitation is 389.8 mm and the yields

obtained from all hybrids are the highest compared to other years. In the economic year 2024, the amount of precipitation during these phases is insufficient to realize the productive potential of the tested hybrids and obtain lower yields.

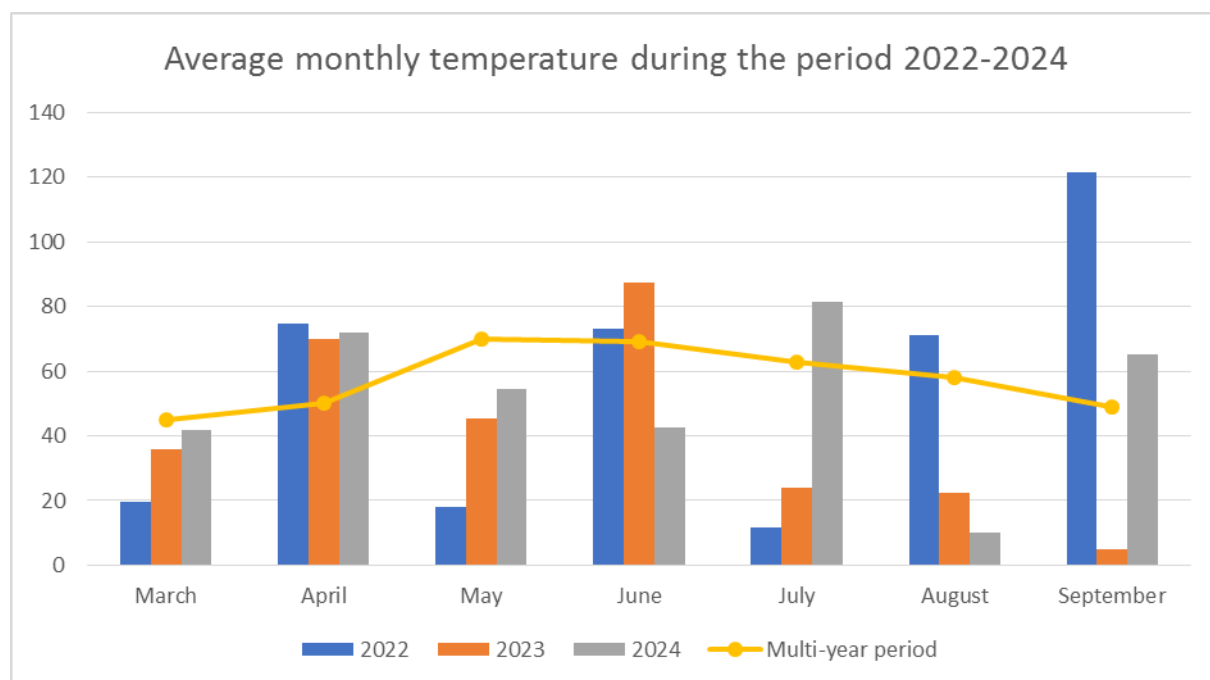


Figure 2. Distribution of precipitation during the period 2022–2024

RESULTS AND DISCUSSION

The obtained average values for plant height and structural elements of yield are presented in Table 1. The data show that the tested sunflower hybrids differ in terms of plant height compared to the standard (LG5665). Statistically proven, the plants of the LG5478 hybrid have the highest height (176.75 cm). In the other two hybrids (LG50510 and LG5662), the differences are not proven and are within the statistical error.

The analysis of the cotyledon diameter shows that the LG50510 hybrid has mathematically proven lower values than the standard (at LSD 0.1%), and in the LG5478 hybrid, the cotyledon dimensions are 20.06 cm and exceed the standard by 8.26%. Hybrid LG5662 has values that are very close to the standard (LG5665) and the differences between them are unproven.

Table 1. Height of plants and structural elements of the yield, average during the period 2022–2024

Hybrid	Height of the plant (cm)	Diameter of the disk (cm)	Number of seeds per disk	Weight of 1000 seeds (g)
LG5665	161,66	18,53	834,83	51,89
LG50510	160,54	17,4***	812,33	50,69
LG5478	176,75***	20,06***	877,92**	55,54**
LG5662	168,4	18,72	872,75*	53,52
LSD 5%	7,23	0,64	28,79	2,18
LSD 1%	9,66	0,86	38,46	2,91
LSD 0.1%	12,64	1,12	50,37	3,81

The indicator of the number of seeds in one cotyledon for the tested hybrids varies from 812.33 pieces for Hybrid – LG50510 to 877.92 pieces for hybrid – LG5478. The LG5478 hybrid outperforms the control by 43 pieces. Statistically proven, the LG5478 hybrid outperforms all other hybrids.

The mass of 1000 seeds for the tested sunflower hybrids varies from 50.69 g for LG50510 to 55.54 g for LG5478. Proven differences in this indicator compared to the standard are only for the LG5478 hybrid (at LSD 1%).

The favorable combination of weather conditions in 2022 is a prerequisite for obtaining higher seed yields compared to 2023 and 2024 (Table 2). The obtained values range from 306 kg/ha for the LG56.65 hybrid to 328 kg/ha for LG54.78. This hybrid has been proven to exceed the LG56.62 and LG50.510 hybrids in terms of seed yield by 11 and 14 kg/ha, respectively.

In the second experimental year (2023), the yields obtained from the tested hybrids were higher compared to 2024. Mathematical processing of the data shows that the differences between all the studied hybrids are significant. The highest yields were recorded for the LG56.65 standard.

The lowest sunflower yields were recorded in the third experimental year (2024) and ranged from 210 kg/da for the hybrid LG54.78 to 235 kg/da for LG56.62.

Table 2. Seed yield – kg/da

hybrid year	LG 56.65 M*	LG 56.62	LG 50.510	LG 54.78
2022	306 ^a	317 ^a	314 ^a	328 ^a
2023	290 ^b	219 ^c	265 ^b	276 ^b
2024	230 ^c	235 ^b	228 ^c	210 ^c
Average of the period	275	257	269	271

On average for the study period (2022–2024), the LG56.65 hybrid with a yield of 275 kg/da outperformed all studied hybrids by 1.5 to 6.5%. The lowest yield was recorded for the LG56.62 hybrid – 257 kg/da, which is 12 to 18 kg/da behind the others.

Table 3. Analysis of variance or seed yield

Tests of Between-Subjects Effects								
Dependent Variable: Y								
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power ^b
Corrected Model	80210,488 ^a	11	7291,863	199,957	,000	,984	2199,530	1,000
Intercept	3458607,809	1	3458607,809	94841,842	,000	1,000	94841,842	1,000
H	2187,973	3	729,324	20,000	,000	,625	59,999	1,000
X	66513,175	2	33256,587	911,961	,000	,981	1823,922	1,000
H * X	11509,340	6	1918,223	52,601	,000	,898	315,609	1,000
Error	1312,816	36	36,467					
Total	3540131,113	48						
Corrected Total	81523,304	47						

a. R Squared = ,984 (Adjusted R Squared = ,979)

b. Computed using alpha = ,05

The results of the analysis of variance (Table 3) show that the variation in sunflower seed yields is determined by both weather conditions over the years (98.1%) and hybrids (62.5%). There is a well-proven interaction between the tested factors (hybrid x year) – 89.8%.

CONCLUSIONS

The most suitable for the conditions of Northeastern Bulgaria is the hybrid LG 56.65 M, which gives the maximum average seed yield (275 kg/da) in all three years of the study.

The structural elements of the yield in the tested sunflower hybrids have the highest values in the hybrid LG 54.78.

The lowest seed yield was obtained from the hybrid LG56.62 (257 kg/da).

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INFLUENCE OF CHEMICAL MUTAGENS ON THE TULIP PLANT

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ABSTRACT

Tulip, being an important ornamental plant, generally requires lengthy and laborious procedures to develop new varieties using traditional breeding methods requires. The high popularity of tulips has led to the need to cultivate new varieties with new shapes and colors, which is also suitable for production. The use of induced mutagenesis techniques is one of the most important methods for the creation of new varieties. The impact and influence of chemical mutagens on ornamental plants is quite important, specifically in our study on the tulip plant (*Tulipa L.*). In our treatments we used chemical mutagens EMS (Ethyl methanesulfonate) and MMS (Methylmethane sulphonate) with three doses each of them. The treatments of the tulip bulbs were done according to the protocols described by the IAEA. Bulbs after treatment with chemical mutagens were planted in the greenhouse in the M1 generation. Treatment with the second dose of chemical mutagen EMS resulted being the most positive, influencing the leaf length of tulip plant compared to the control. The second dose of the chemical mutagen MMS has the greatest influence on the width of the tulip petals. In the materials obtained from treatment with chemical mutagens MMS and EMS, in general the average value of plant height was positively affected and was higher than the untreated control. Treatments with chemical mutagens MMS second dose, MMS third dose, EMS second dose and EMS third dose influenced the height growth of tulip plant.

Keywords: Chemical mutagens, Mutations, Tulip

INTRODUCTION

Tulip (*Tulipa L.*), being an herbaceous ornamental plant (i.e., family *Liliaceae*), is cultivated flower plants worldwide, especially in Europe (Pourkhaloee et al., 2018). Considering a high commercial value due to its various colour, long flowering period, and pleasing fragrance, it has been regarded as the national flower by many countries, such as Turkey, Holland, and Iran (Mu et al., 2020). Tulip (*Tulipa L.*), are also widespread in Albania. The tulip is a perennial plant, characterized by underground reserve organs (bulbs). Tulip plant is planted in the months of October-November (Hernández-Muñoz et al., 2019). It blooms in the spring season in April and May, but depending on the temperature conditions, in warm temperature they bloom even earlier (Okazaki, 2005; Van Rossum et al., 1998).

The use of mutagenesis technique has marked a new revolution in the creation and improvement of new plants with desired traits and characteristics (Juodkaite et al., 2008). This technique has a wide use in the creation of agricultural crops, in fruit cultivation and horticulture. The use of both mutagenesis techniques is of interest, but the technique of induction using chemical agents has a higher frequency of induction of desired mutations

compared to the technique of induction using physical agents (Ibrahim et al., 2018). In our study we used chemical induced mutagenesis to improve leaf length, width of the tulip petals as well as the average value of plant height.

MATERIAL AND METHOD

In our treatments, we used chemical mutagens EMS (Ethyl methanesulfonate) and MMS (Methylmethane sulphonate) each applied in three different doses. The treatments of the tulip bulbs were done according to the protocols described by the IAEA (FAO/IAEA, 2018). The selected plant material in this study were tulip bulbs (Noman et al., 2017). Dormant tulip bulbs have been treated with chemical mutagens according to the recommendations of specialized manuals on how to use doses in different ornamental plants, taking into account in the calculation of doses that the dose of the chemical mutagen is a product of the concentration of the mutagen, temperature and exposure time of the material in it (FAO/IAEA, 2018; Ylli et al., 2018). Knowing the structure of the bulbs material is very important during the treatment process (Melsen et al., 2021).

The treatment was performed with these mutagens with three doses each: EMS (0.1%, 0.2%, 0.3%) and MMS (0.050 M, 0.075 M, 0.100 M). The tulip bulbs were treated with these solutions for one hour (Popescu, 2012). The bulbs, after the respective treatments for each mutagen, are rinsed for two hours in a row with running water to remove the residues of the chemical mutagens. In addition to them, the control is kept in running water for two hours. The process of rinsing after treatment is very important, as it has to do with stopping the further entry of mutagenic substances into the tulip bulbs (Podwyszynska et al., 2003).

Plant materials, bulbs, after rinsing, are placed on filter paper to remove part of the water. Bulbs after treatment with chemical mutagens were planted into flowerpots ($L \times W \times H = 49 \times 20 \times 18$ cm) in the greenhouse in the M1 generation (Kodym et al., 2003). In the first mutant generation we analyzed the influence of chemical mutagens on morphological traits such as height of tulip plants, length and width of tulip leaves as well as on the width and length of tulip petals (Mba, 2013).



Figure 1. Treatment of tulip bulbs with chemical mutagens

RESULTS AND DISCUSSION

The experiment was conducted with three replicates for each mutagen treatment dose, as well as for the control. Bulb materials treated with three different doses of the chemical mutagens EMS and MMS were observed throughout all vegetative growth stages (Kodym et al., 2003).

Over a period of four weeks, successive measurements of tulip plant height were

recorded at different time intervals. The collected data were then analyzed to assess the effects of the chemical mutagens on plant height (FAO/IAEA, 2018; Ylli et al., 2018).

Table 1. The height measurements of treated plants compared to the control

No. of measurements	CO	EMS ₁	EMS ₂	EMS ₃
1	8.8	5.8	8.3	7.2
2	12.8	9.9	17.6	15
3	22	11	27	22.5
4	26	14	26.7	26
SD	7.9	3.4	8.9	8.4

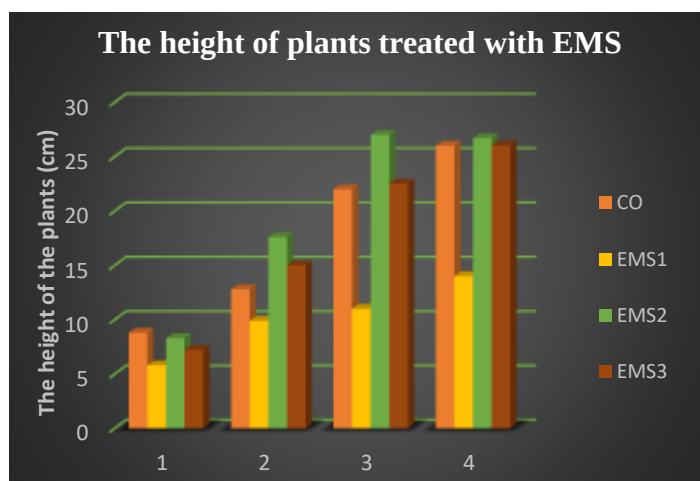


Figure 2. The height of plants treated with the chemical mutagen EMS

The greatest influence on the height of tulip plants treated with the chemical mutagen EMS was exerted by the second and third doses of EMS (0.2% and 0.3%, respectively) at different stages of vegetative development (Figure 2). However, there are large differences between these two doses of mutagens (Ylli et al., 2011). The second dose of the chemical mutagen EMS has a greater influence than the third dose of the chemical mutagen EMS on the height of tulip plants.

Another trait analyzed was the length of tulip leaves. Measurements were taken on different days over a three-week period for all leaves of plants treated with chemical mutagens, and the results were compared with those of untreated control leaves (Podwyszynska et al., 2010).

Table 2. Measurements of leaf length in tulip plants treated with EMS compared to the control

Average	CO	EMS ₁	EMS ₂	EMS ₃
1	13.4	7.4	12.7	11.69
2	14.5	9.7	13.6	12.7
3	15.6	10.4	14.4	12.72
SD	1.1	1.6	0.9	0.6

As can be seen from the Table 2, the second dose of the chemical mutagen EMS has resulted in the chemical mutagen with the most positive influence on the length of the leaves of tulip plants. Followed by the third dose of the chemical mutagen EMS, which has a significant positive influence compared to the control, but has a lower influence on the length of the tulip leaves compared to the second dose of the chemical mutagen EMS. Whereas the influence of the first dose of the chemical mutagen EMS is almost negligible compared to the untreated control.

Table 3. Measurements of leaf width of tulip plants treated with the chemical mutagens EMS and MMS compared to the control

No. of measurements	CO	MMS ₁	MMS ₂	MMS ₃	EMS ₁	EMS ₂	EMS ₃
1	6	5	4.2	5.7	4	6.2	6
2	7.5	5.7	7.1	6.6	5	7	6.2
3	12.6	7.4	7.5	7.2	6.3	14.5	6.5
SD	3.5	1.2	2	1	1.2	4.6	0.3

As can be seen from the summary table (Table 3), the first dose of the chemical mutagen MMS has a greater influence than the first dose of the chemical mutagen EMS on the increase in leaf area. Also, the second dose of the chemical mutagen EMS has a positive impact compared to the control on the increase in leaf area. From the data in the table, it is also noted that the third dose of the chemical mutagen MMS has a greater influence than the third dose of EMS.

At the end of the tulip flowering period, the plants were cut and the petals were taken to measure their width. Measurements were taken for all petals on each flower and the data were analyzed to see how the chemical mutagens influenced the width of the tulip petals. As can be seen from the Figure 4, compared to the control, the first dose of the chemical mutagen MMS has the greatest influence on the width of the tulip petals for the first dose.

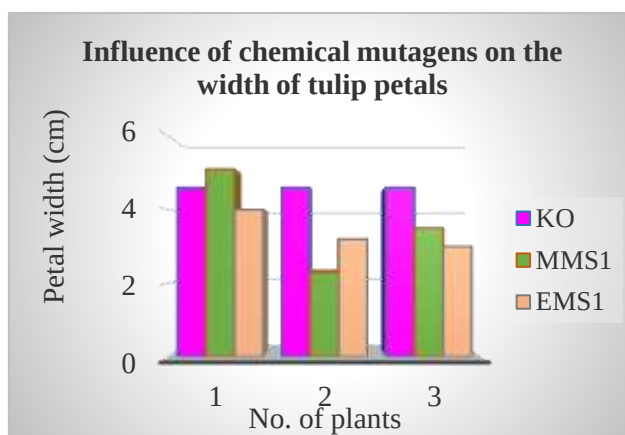


Figure 4. Influence of the first dose of chemical mutagens on the width of tulip petals

As can be seen from the Figure 5, compared to the control, the chemical mutagen MMS has a greater influence on the width of the tulip petals for the second dose of the first two tulip plants. In the flowerpot treated with the second dose of the chemical mutagen MMS, there are only two tulip plants, while in the flowerpot treated with the second dose of the chemical mutagen EMS, there are four tulip plants.

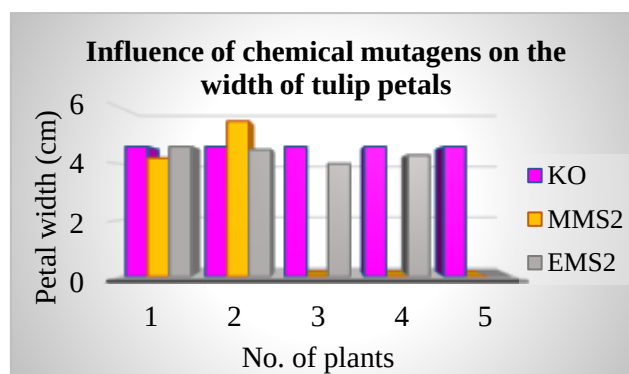


Figure 5. Influence of the second dose of chemical mutagens on the width of tulip petals

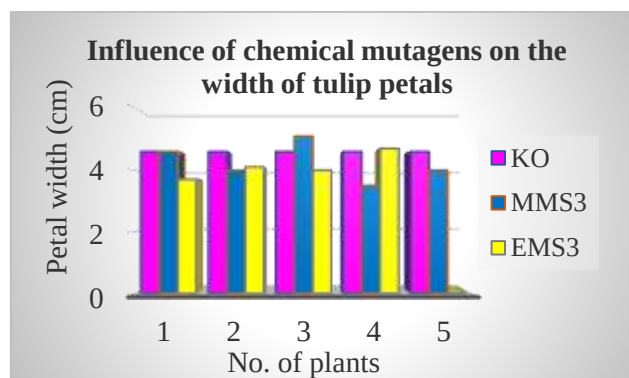


Figure 6. Influence of the third dose of chemical mutagens on the width of tulip petals

In the case of the third dose of chemical mutagens compared to untreated control plants, it results that the influence on the width of tulip petals for treatments with the third dose of chemical mutagens MMS and EMS does not have a very large difference between the two

mutagens. As can be seen in the Figure 6, the third dose of chemical mutagens MMS and EMS have almost the same positive impact on almost all petals of tulip plants.

CONCLUSIONS

Based on the study results, in the materials treated with the chemical mutagens MMS and EMS, the average plant height was generally positively affected and was higher than that of the untreated control. Treatment with the second and third doses of the chemical mutagens MMS and EMS influenced the height growth of tulip plants. Among these, the second dose of EMS had the most positive effect on leaf length compared to the control. Meanwhile, the second dose of MMS had the greatest influence on the width of tulip petals.

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INFLUENCE OF MUTAGENS ON PHYSIOLOGICAL PARAMETERS OF BEAN PLANT

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ABSTRACT

Beans (*Phaseolus vulgaris*) is a major legume plant with high nutritional and economic importance. The aim of the study is the influence of treatments with physical and chemical mutagens on the plant of *Phaseolus vulgaris*, to improve its physiological features influencing the length and width of the bean leaves, as well as the size of the stem of the bean plants. Selected seeds of *Phaseolus vulgaris* variety Shijak were prepared for treatment with mutagens after their selection. For the treatment of bean seeds, both physical and chemical mutagenesis are used. The protocols recommended by the IAEA were used for the treatment of the Shijak bean seeds. Irradiation of biological seed materials was carried out at the Institute of Applied Nuclear Physics. Bean seeds are irradiated with Cs-137 gamma radiation, in three doses: 50 Gy, 100 Gy, 150 Gy and in addition to them, the control. Bean seeds were also treated with chemical mutagens and we used four mutagens: EMS (Ethyl methanesulfonate), dES (Diethyl sulfate), dMS (Dimethyl sulfate), MMS (Methylmethane sulphonate). For each of them, we did the treatment with three doses: Diethyl sulfate - dES 0.0025M, 0.005M, 0.010M; Ethyl methanesulfonate - EMS 0.1%, 0.2%, 0.3%; Dimethyl sulfate - dMS 0.001M, 0.0015M, 0.0025M; Methylmethane sulphonate - MMS 0.05M, 0.075M, 0.1M. Bean seeds treated with these mutagens based on IAEA protocols have been placed in various experiments in the laboratory, greenhouse and field plot. The treatment with physical mutagens presents a higher average value for the length of the stem and the root. The use of mutagenesis on *Phaseolus vulgaris* seeds has had an influence on the change of leaf morphology depending on the type of mutagen and the doses used. For the plant materials treated with chemical mutagens, it was observed that the three compound leaves have different impacts, but there was a positive correlation and the leaves were longer in length compared to the control. Leaves appear elongated compared to leaves of plants treated with physical mutagens at all three doses. For the physical mutagen we had plants that the mutagen affected both leaf length and width values leading them to a circular leaf shape.

Keywords: *Phaseolus vulgaris*, Physical mutagen, Chemical mutagen

INTRODUCTION

Mutation induction techniques are diverse, but in general they are divided into two major groups based on the type of mutagenic agents used for changes at the genetic level in chemical and physical induction techniques (Sofkova et al., 2021). Physical and chemical mutagens have been used successfully in plant breeding programs to artificially generate genetic variations for the development of new varieties with improved traits (Velu et al., 2012). Mutant crop varieties

are more adaptable to the environment, require less agricultural contributions, and are therefore more economical to grow and contribute to more environmentally friendly agriculture.

Phaseolus vulgaris exhibits high genetic variability and is an important crop for agricultural production (Horn et al., 2017). However, due to the numerous climatic changes that have occurred in recent years, bean production has experienced a significant decline (EFSA Journal, 2021). Appreciating the bean as a very convenient food, has been shown a special care in the selection and preservation of cultivars that have adapted to the ecological conditions of the areas where they are located (Mba et al., 2013). Bean production decrease has led to many studies to power the elimination of performance losses in field (Singh et al., 2005).

MATERIAL AND METHOD

The selected plant material was Shijak variety bean (*Phaseolus vulgaris*) seeds obtained from the National Seed and Seedling Entity which is the Genetic Bank of Albania. The experimental work was based to special protocols of the IAEA. The experimental work is organizing respectively in the Mutagenesis Laboratory, in an experimental field in the Fieri district (Albania) and in the greenhouse, part of the Department of Biotechnology, Faculty of Natural Sciences, University of Tirana.

In the greenhouse experiment the seeds were planted in pots with soil with control of temperature and humidity (FAO/IAEA, 2018; Ylli et al., 2018). For the treatment of the bean seeds was used the gamma radiation with Cs-137, which were applied in three different doses, 50 Gy, 100 Gy and 150 Gy (FAO/IAEA, 2018). The irradiated seeds and the control material were planted in the experimental field in randomized complete block (3m x 0,7m) and four replications, while in the greenhouse the seeds were planted in two replications.

Bean seeds were also treated with chemical mutagens and we used four mutagens: EMS (Ethyl methanesulfonate), dES (Diethyl sulfate), dMS (Dimethyl sulfate), MMS (Methylmethane sulphonate). For each of them, we did the treatment with three doses: Diethyl sulfate - dES 0.0025M, 0.005M, 0.010M; Ethyl methanesulfonate - EMS 0.1%, 0.2%, 0.3%; Dimethyl sulfate - dMS 0.001M, 0.0015M, 0.0025M; Methylmethane sulphonate - MMS 0.05M, 0.075M, 0.1M. The seeds were treated with this mutagenic solution for one hour, based in FAO/IAEA protocols (FAO/IAEA, 2018; Stamo et al., 2012; Ylli et al., 2013). Bean seeds treated with these mutagens based on IAEA protocols have been placed in various experiments in the laboratory, greenhouse and field plot.

RESULTS AND DISCUSSION

Based on the study results, measurements were done according to the described protocol for all material taken from the experimental field in Fieri district and the greenhouse in Tirana. The analyzes of the bean plants materials *Phaseolus vulgaris* irradiated with three doses of gamma radiation 50 Gy, 100 Gy, 150 Gy as well as the control were done in the Induced Mutations Laboratory of the Biotechnology Department. Comparing them with each of the mutagens used, it shows that the physical mutagen had a higher development in the bean plant (Kodhelaj et al., 2021; Ylli et al., 2019).

Measurements were made on all three bean leaves at the same level and on each plant. From the collected values, statistical analyzes were performed, looking at the correlation between doses and physiological indicators. The values of length and width came almost decreasing at the same percentage rate as for length and width. The use of mutagenesis on *Phaseolus vulgaris* seeds has had an influence on the change of leaf morphology depending on the type of mutagen and the doses used (Kodhelaj et al., 2017; Ylli et al., 2013). Leaves appear elongated compared to leaves of plants pretreated with physical mutagens at all three doses. For

the physical mutagen we had plants that the mutagen affected both leaf length and width values leading them to a circular leaf shape.

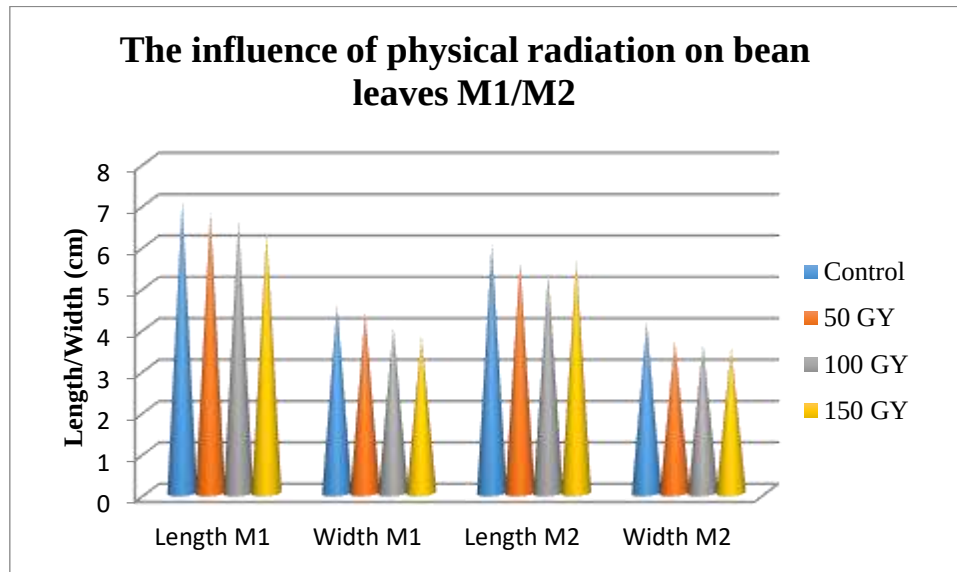


Figure 1. The graph of the impact of physical mutagens on leaf length/width in M1

The use of mutagenesis on *Phaseolus vulgaris* seeds has had an influence on the change of leaf morphology depending on the type of mutagen and the doses used. For the mutagen dMS, the presence of plants with two leaves and an increase in leaf length were observed at the third dose of this mutagen. While in width the leaves have almost not changed. For the mutagen EMS a positive correlation was again observed in the length of the leaves which are increasing up to the third dose of this mutagen, while in the width of the leaf we have no influence of this mutagen.

For the treatments with the mutagen MMS again, the width of the leaf was not affected, but the length was again affected by the highest dose followed by the lowest dose used. While the mutagen dES had a very low impact on the leaf width, while the lower dose of the mutagen had a greater impact on the leaf length. For the plant materials treated with chemical mutagens, it was observed that the three compound leaves have different impacts, but there was a positive correlation and the leaves were longer in length compared to the control as it appears in the photos. Leaves appear elongated compared to leaves of plants pretreated with physical mutagens at all three doses.



Figure 3: The influence of physical mutagens on leaf size

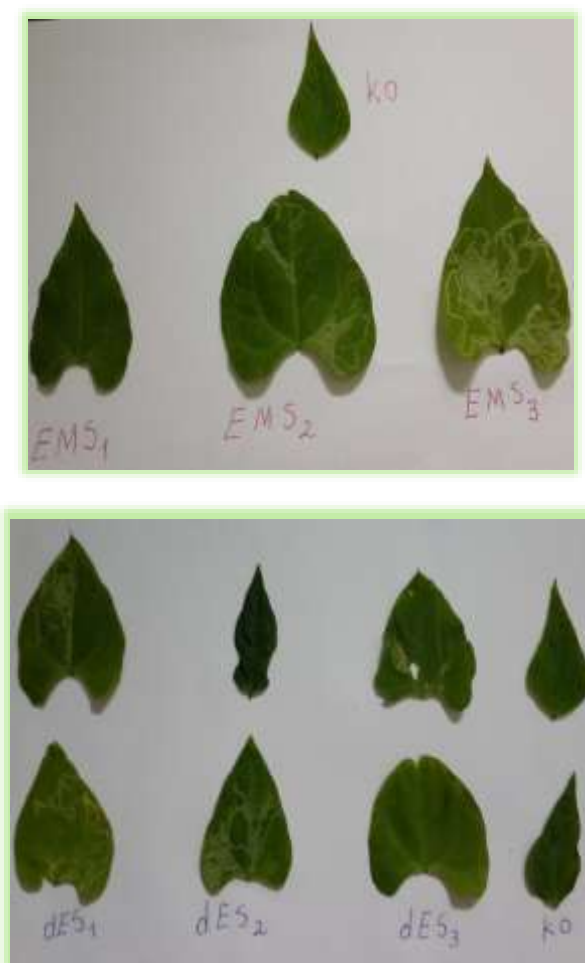


Figure 4: The influence of chemical mutagens on leaf size

The leaves are shown to maintain the same parameters and appear almost as rounded leaves unlike the leaves of plants pre-treated with chemical mutagens which will be treated following the results. But this decrease that we see from the graph is relatively low. To analyze the relationship between the doses used and the measurements made in leaf width and length, we use linear regression analysis where we focus on two very important elements such as the R^2 and correlation coefficients shown in the table below. The aim in the regression analysis is to describe the length or width of the leaves depending on the doses of physical mutagen used. All the data shown by the statistical analysis will show how the physical mutagen has affected the physiological indicators.

Table 1. Linear regression for leaf length/physical radiation in M1

Model	R	R^2	Adjusted R^2	Std. Error of the Estimate
1	.160 ^a	.026	.025	1.66093

a) Predictors: (Constant), Doses of mutagen used

In this table are presented the values of coefficients R and R^2 which show a low degree of correlation and only 2.6% of the length can be explained by the doses of the physical mutagen used.

Table 2. Analysis of variance for leaf length after physical irradiation treatment in M1

Model	Sum of Squares	Df	Mean Square	F	Sig.
1 Regression	87.025	1	87.025	31.546	.000 ^b
Residual	3304.915	1198	2.759		
Total	3391.940	1199			

a. Dependent Variable: Leaf Length

b. Predictors: (Constant), Doses of mutagen used

Table 3. Correlation coefficients for the relation between physical radiation and leaf length in M1

Model	Unstandardized Coefficients		Standardized Coefficients	T	Sig.
	B	Std. Error	Beta		
1 (Constant)	7.031	.080		87.639	.000
Doses of mutagen used	-.241	.043	-.160	-5.617	.000

Dependent Variable: Leaf length

From the coefficients shown in table B, we can create a linear equation where we would have $y=7.031-0.241x$; where y =leaf length and x =physical radiation dose. Noting also the p value which is less than 0.05 where we see in the ANOVA table means that there is a relationship between length and doses used. Based on the derived equation we can argue that with an increase of one unit of physical mutagen doses we would have a decrease in leaf length by 24.1%. The same situation occurs with leaf width.

Table 4. Linear regression for leaf width after treatment with physical irradiation in M1

Model	Change Statistics					Durbin-Watson
	R Square Change	F Change	df1	df2	Sig. F Change	
1	.040 ^a	50.299	1	1198	.000	.378

a. Predictors: (Constant), Doses of mutagen used

b. Dependent Variable: Leaf length

Model	Sum of Squares	Df	Mean Square	F	Sig.
1 Regression	103.045	1	103.045	50.299	.000 ^b
Residual	2454.280	1198	2.049		
Total	2557.325	1199			

a. Dependent Variable: Leaf length

b. Predictors: (Constant), Doses of mutagen used

Table 6. Correlation coefficients for the relation between physical radiation and leaf width in M1

Table 5. Analysis of variance for leaf width after treatment with physical radiation in M1

In this case, only 4% of the width can be explained by the doses of physical radiation used and by the correlation coefficients where we build the equation $y=4.572-0.262x$; where y =leaf width and x =physical radiation dose. Here too, according to the significant value, we see that there is a relationship between the two variables since the value is smaller than the confidence value, $p<0.05$. While according to the equation, we say that with an increase in doses by one unit, the values of leaf width will decrease by 26.2%. In both cases we would have a decrease as we saw from the graph and the percentage of decrease would be almost the same for both indicators.

Model	Unstandardized Coefficients		Standardized Coefficients	T	Sig.
	B	Std. Error	Beta		
1 (Constant)	<u>4.572</u>	.069		66.131	.000
Doses of mutagen used	<u>-.262</u>	.037	-.201	-7.092	.000

a. Dependent Variable: Leaf length

CONCLUSIONS

For the mutagen dMS, the occurrence of plants with two leaves and an increase in leaf length were recorded at the third dose of treatment. In contrast, leaf width remained almost unchanged. In the case of the mutagen EMS, a positive correlation was again observed for leaf length, which continued to increase up to the third dose of this mutagen, while leaf width was not significantly affected. For treatments with the mutagen MMS, leaf width showed no significant variation, whereas leaf length was positively influenced, with the highest dose producing the greatest effect, followed by the lowest dose. The mutagen dES exhibited a minor effect on leaf width, whereas its lower dose exerted a more pronounced influence on leaf length.

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EXAMINING THE IMPACT OF RURAL TOURISM ON RURAL DEVELOPMENT: THE EXAMPLE OF KUYUCAK VILLAGE

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ABSTRACT

Rural development is the improvement of socially, economically, and culturally disadvantaged conditions of those living in rural areas compared to urban areas. Rural tourism is one of the types of activities that support rural development. This study examines the effect of lavender production on rural tourism in Kuyucak Village of Isparta province. The main material of the study consists of data from 74 residents of Kuyucak village who are involved in lavender production and rural tourism, collected through purposive sampling method and surveys. Rural tourism takes place during the lavender production season in Kuyucak village. Within the scope of the study, the majority of the local population (89%) engages in lavender production, as well as participating in rural tourism activities such as guiding nature walks, photo shoots in lavender fields, production of local foods and lavender-based products, and operating guesthouses. It has been determined that with the start of lavender production in the village, the promotion of the village and economic development have been supported. However, due to the limited lavender production period and the lack of other supporting activities in the village afterwards, it is stated that visitors generally stay for 3-5 hours (58.1%). It is recommended to extend visit durations with different tourist activities and to spread this model to other rural areas.

Keywords: Rural tourism, Rural development, Lavender, Kuyucak village.

INTRODUCTION

Rural tourism has emerged as a significant idea, gaining global recognition and undergoing thorough examination due to its beneficial impacts on rural development processes. This form of tourism, while not novel, experienced significant growth post-World War II, becoming globally prominent in the late 1970s and early 1980s (Aydın, 2012; Lane et al., 2022). Rural tourism originated from the back-to-nature movement in England and then disseminated to industrialized European nations (Çeken et al., 2012). Historically, it has served as a developmental instrument for addressing challenges faced in rural regions of Europe (Emekli et al., 2007).

Rural regions have seen transformation in recent years as a result of global crises, particularly in emerging nations. Urbanization and modernization have resulted in the eradication or integration of traditional agricultural and rural culture, while conventional industries such as fishery, forestry, and agriculture have approached depletion (Gao and Wu, 2017). Currently, tourism is distinguished as a sector capable of enhancing living conditions and generating new employment prospects (MacDonald and Jolliffe, 2003).

The notions of rural tourism and rural development are significantly interconnected and mutually reinforcing (Singhania et al., 2022). Rural tourism is acknowledged as a fundamental instrument in rural development. Rural tourist development offers an auxiliary income stream for individuals engaged in traditional industries (Çeken et al., 2012). This can curtail the

migration of local populations to metropolitan centers and ensure the natural environment remains monitored (Demircioğlu, 1993).

In recent years, rural tourism has become increasingly significant as a strategic instrument for promoting the socio-economic development of rural regions in both developed and developing nations. This form of tourism is acknowledged as a principal catalyst for economic diversification in rural areas, generating employment by leveraging local resources and cultural assets, while rejuvenating regional economies through diverse income streams (He et al., 2021). Rural tourism engages with various sectors, including food, textiles, and transportation, by generating direct revenue and employment for local residents; it also fosters local craftsmanship and enhances the economy through a multiplier effect. The active involvement of local populations in tourist endeavors fosters beneficial outcomes for income equality and economic robustness (Fong and Lo, 2015). Endorsed by local, national, and international initiatives, rural tourism possesses the capacity to draw individuals to rural regions globally (Soykan, 1999). This somewhat inhibits or diminishes the migration of youth to major urban centers.

The effects of rural tourism are not limited to economic dimensions; they also encompass socio-cultural and environmental development. In this context, favorable consequences include enhancements in infrastructure, health and education services, the creation of new work chances, elevated income levels, and increased participation of women in economic activities (Ahipaşaoğlu and Çeltek, 2006). Although rural tourist activities primarily consist of short-term experiences, these interactions can still exert enduring impacts on regional development.

The impact of tourism on rural development must be sustainable. To guarantee sustainability, it is imperative to enact economically, socially, and culturally suitable good policies (López-Sanz et al., 2021). These policies must pertain to the efficient utilization of historical, ecological, cultural, and agricultural resources within the region (Çeken et al., 2012). Rural tourism is regarded as a mechanism that can aid in the sustainable development of economically undiversified rural regions.

Recent years have witnessed a notable growth in studies pertaining to rural tourism and rural development, with this subject regularly explored by researchers. This demonstrates the significance and prominence of the topic among academic literature. In Türkiye, the significance of rural tourism is being highlighted in projects aimed at fostering rural development. In this setting, lavender farming exemplifies a significant junction between rural tourism and development. Lavender has been extensively employed in human civilization historically, serving the cosmetics, pharmaceutical, and aromatherapy industries due to its antibacterial and therapeutic attributes. Indigenous to the Mediterranean basin, lavender has evolved into both an agricultural and tourism hub in areas like Kuyucak Village in Isparta, owing to its capacity to thrive in calcium-rich and arid soils. This research has designated Kuyucak Village in Isparta province as a case study to investigate the impact of rural tourism on rural development. Kuyucak Village attracts attention due to its tourism potential derived from lavender cultivation, effectively illustrating the connection between rural development and tourism.

MATERIAL AND METHOD

A survey was done in Kuyucak Village, Isparta province, to assess the influence of rural development on rural tourism. As to TURKSTAT (2023) data, the total population of Kuyucak village in Isparta province, designated as the focus for the survey study, is 274 individuals. A survey was done with 74 individuals from March to May 2024 with a purposive sample method.

The study analyzed the assessments of local residents who were directly or indirectly impacted by rural tourism initiatives, including those stemming from lavender cultivation. The evaluation encompassed participation in alternative activities stemming from lavender production in the village, the attainment of economic sufficiency, the implementation of

promotional strategies, the impact of alternative lavender products, and the prospects for rural tourism. The study data were provided as proportions and percentages. An interview was conducted with the officials of the Keçiborlu District Directorate of Agriculture and Forestry. Evaluations were conducted on rural tourism pertaining to Kuyucak Village, based on face-to-face interviews with the institution and local residents.

RESULTS

The demographic data of the local residents of Kuyucak Village who participated in the study is displayed in Table 1. 55.6% of the participants are male, while 44.4% are female. The participation of the young population in the study is notably low at 3.8%. During the lavender producing season, young individuals engage in temporary jobs in the village and thereafter migrate to urban areas during the off-season. The primary cause of this is the scarcity of economic possibilities for youth in the village during the off-season. The 26-35 age demographic comprises 16.9% of the participants and typically resides in the same household as elder family members, indicating their involvement in agriculture alongside their families. Upon assessing the educational status in the village, it was determined that the prevalence of high school and lower education levels is significant, at 80.7%. The primary cause of this phenomenon is the geographical isolation of rural areas from urban centers, coupled with the age of the village inhabitants and the exceedingly low enrollment rates in educational institutions. Participants possessing associate degrees and higher educational qualifications generally engage in employment within the public and commercial sectors. 78.1% of the participants had lived in the village for over 21 years. The majority are directly engaged in lavender-related activities. Of the participants, 48.1% only cultivate lavender, while 21.9% sell harvested lavender plants and related items. Several vocations are indirectly influenced by the rural tourism activities associated with lavender in the region. The participants include café and tent owners (7.5%), tourist guides, photographers (5.0%), and automobile rental providers (1.9%).

Table 1. Demographic characteristics (%)

General		General	
Gender		Monthly average income	
Female	44.4	Less than 20,000 ₺	13.1
Male	55.6	Between 20,100 - 30,000 ₺	26.9
Age		Between 30.100 - 40.000 ₺	33.8
Between 18-25	3.8	Between 40.100 - 50.000 ₺	13.7
Between 26-35	16.9	Between 50.100 - 60.000 ₺	8.8
Between 36-45	14.4	60,100 ₺ and above	3.7
Between 46-55	22.5	Occupation	
Between 56-65	33.8	Lavender producer	48.1
66 and above	8.8	Lavender product seller	21.9
Education		Public/private wage earner	11.3
Literate and primary education	35.7	Cafe, tent, etc. operator	7.5
High school	45.0	Guide, photographer, etc.	5.0
Associate degree, bachelor's and above	19.3	Vehicle rental (tour)	1.9
		Other	4.4

The initiation of rural tourism in Kuyucak village has led the local populace to identify economic benefits (36.3%) and enhancements in the village-region's visibility (30.6%) as the

principal advantages. This indicates that the primary aims of rural tourism are well comprehended by the local population. The 20% social welfare indicates that rural tourist operations positively impact the social life of local residents (Table 2).

Numerous supports exist for the advancement of rural tourism within rural development, chiefly IPART. 87.5% of participants reported benefiting from rural development support, which they indicated contributes significantly to the economy (96.3%).

Table 2. Rural tourism contribution and satisfaction perception (%)

Rural tourism contribution	Economic development	36.3
	Promotion of the village/region	30.6
	Social welfare	20.0
	Job opportunity	11.9
	Social status	1.3
Benefiting from rural development supports	Yes	87.5
	No	12.5
Satisfaction status from rural tourism activity	Satisfied	94.4
	Not satisfied	5.6
Belief that rural tourism activities provide economic contribution	Provided contribution	96.3
	Did not provide contribution	3.7

In addition to generating direct economic revenue from lavender activities, alternative livelihood sources have been established in the region utilizing lavender. In addition to lavender cultivation, local residents provide traditional dishes (35.6%), photography services in lavender fields (26.3%), and guiding services (15%). Lavender, local culture, and natural beauty are integrated within commercial value (Table 3).

Table 3. Rural tourism income adequacy status (%)

Adequacy of livelihood sources	Adequate	79.4
	Not adequate	20.6
Income sources other than lavender production	Local foods	35.6
	Photo shooting in lavender fields	26.3
	Guiding nature walks	15.0
	Advertising activities for tourists	11.3
	Guesthouse/hotel management	8.8
	Transportation	3.1

Table 4 presents the lodging status and promotional framework of tourists in the region. The duration of stay influences the region's economic revenue. Visitors to Kuyucak village typically engage in day excursions lasting 3 to 5 hours or a full day without overnight lodging. The limited percentage of overnight stays (8.8%) may result from insufficient housing alternatives or a scarcity of alternative activities during extended visits. 86.9% of local vendors of lavender by-products advertise their offerings via social media or regional stands. Lavender crowns/flowers (40.6%) and lavender plants (23.1%) rank as the most favored products among visitors. The proportion of value-added products (oil, soap, cream, jam, and tea) is 17.5%. This circumstance signifies significant advancements in the marketing of both processed and unprocessed lavender products.

Table 4. Visitor behaviors and product marketing structure (%)

Tourist accommodation duration	3-5 hours	58.1
	1 day without accommodation	33.1
	1 night with accommodation	7.5
	2-3 day tour	1.3
Promotion of lavender by-products	Done	86.9
	Not done	13.1
Products purchased by tourists	Lavender crown/flower	40.6
	Lavender plant	23.1
	Lavender oil	18.1
	Lavender soap	7.5
	Lavender cream	5.6
	Lavender jam	2.5
	Lavender tea	1.9

Table 5. Rural tourism perception (%)

	Strongly Disagree	Disagree	Undecided	Agree	Strongly Agree	AVERAGE
Kuyucak village is worth seeing because of the beautiful view of the lavender fields.	0.6	1.9	3.7	46.3	47.5	4.4
The village people should be included in rural tourism activities because rural tourism reflects traditional village culture.	1.2	1.9	8.1	38.8	50.0	4.3
Kuyucak has the conditions to meet the expectations of incoming tourists.	1.2	-	13.1	37.5	48.2	4.3
Kuyucak has sufficient potential in terms of alternative tourism types.	1.2	2.5	14.4	40.0	41.9	4.2
Kuyucak has adequate tourist activities. However, it needs to be developed.	0.6	2.5	16.2	37.5	43.2	4.2
Kuyucak has improved its infrastructure facilities thanks to rural tourism.	0.6	1.2	15	40.0	43.2	4.2
Transportation to Kuyucak village and its surroundings is convenient and adequate.	1.2	2.5	16.2	35.6	44.4	4.2
Sufficient promotions and information are provided before coming to Kuyucak village.	1.2	1.9	13.7	40.6	42.5	4.2

Table 5 presents the evaluations concerning the local population's perspective of rural tourism. The primary impetus for rural tourism in Kuyucak village is the aesthetically pleasing lavender fields. Consequently, the local populace concurs that the village ranks among the appealing destination locales (avg. 4.4). The local populace concurs on the imperative of engaging the community in this process, as rural tourism embodies the culture (88.8%). Consensus exists that the village can fulfill guest expectations (85.7%) and that alternative tourism activities are available (81.9%). This suggests that the local populace anticipates the

future with optimism and that rural tourism endeavors will persist. A consequence of rural development initiatives is the enhancement of regional infrastructure. A significant portion of the populace believes that infrastructure has improved due to tourism (83.2%) and that regional promotional efforts persist (83.1%).

CONCLUSIONS

Kuyucak Village, recognized as the "lavender capital," excels in lavender cultivation, a practice that has been conducted in Türkiye for than a century. The festivals held at the onset of the lavender season annually enhance the region's tourism potential. In contrast to the village population of roughly 274, according to the 2022 TURKSTAT data, Kuyucak attracts thousands of visitors annually to its lavender fields. The tourist count increased from 600,000 in 2022 to 700,000 in 2023. Research on the local population indicates pleasure with the growing influx of guests and demonstrates that participants hold favorable views on rural tourism. In this context, it is determined that tourism activities in the village have attained a scale that can directly influence the income level of the local inhabitants, alongside agricultural output.

While the Provence region of France is the most recognized place for lavender fields worldwide, Kuyucak village in Isparta, Türkiye, plays a significant role in lavender production. Isparta, historically referred to as the "land of roses," exemplifies rural growth in Kuyucak, attributed to the prevalence of lavender fields and the rural tourism stimulated by this cultivation. The lavender plant has been identified as the catalyst for rural tourism growth in Kuyucak, with public investments being utilized efficiently. Furthermore, officials indicated in interviews that lavender farmers traveled internationally to France in 2017 and 2019 to study analogous production practices.

Ample work possibilities must be established in the village to deter the exodus of the youth. To prolong visitors' stays, incentive mechanisms must be implemented for projects like home boarding houses, ensuring that the public derives tangible advantages from these services.

Diverse tourism activities, including a range of evening events, should be cultivated to enhance visitor diversity and attendance, while the promotion of the village to both domestic and international tourists should be intensified through collaboration with travel companies.

In conclusion, attaining rural development via rural tourism is quite significant. The advancement of rural development will substantially aid in diminishing regional development gaps in Türkiye and enhancing social welfare. In this framework, rural tourism is regarded not merely as a form of tourism but also as a crucial source of employment for rural regions and an efficient instrument for sustainable development.

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SHEDDING LIGHT OF SPIRULINA PLATENSIS ON MICROBIOTA AND LIFE QUALITY

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ABSTRACT

A healthy and balanced diet is known to strengthen the immune system. Also, it is especially important to include antioxidant-rich foods in the daily diet, such as phytochemical compounds, flavonoids, essential oils, phenolic compounds, and some natural plants. Due to their rich content, natural antioxidants have become even more important in maintaining health, improving life quality, and combating various diseases, especially pandemics today.

Recent studies have revealed that humans have their own microbiota ecosystem, and this system plays a crucial role in maintaining a balanced physiological process. The microbiota, which begins at birth and develops with age, is particularly affected by nutrition, environmental factors, and stress. Considering that the gut microbiota provides a multifaceted communication within the human body through neural, endocrine, immunological, and metabolic pathways, it's clear that microbiota production and balance for a healthy life become important.

Since the last century, in addition to various diseases, environmental factors, such as insufficient water, climate change, or new viruses, have contributed to infectious diseases and epidemics. In this context, the World Health Organization has reported that 80% of the world's population uses herbal products for a protect their life. Nowadays, among natural products, there is an increasing tendency towards *Spirulina platensis* and its extracts especially against diseases and for improving life quality. This study aimed to review the effects of *S. platensis* supplementation on gut microbiota function, metabolism, and life quality.

Keywords: Microbiota, life quality, *Spirulina platensis*, stress

INTRODUCTION

The gut microbiota influences the host's energy balance through several mechanisms, including the provision of energy from nondigestible food components and effects on intestinal transit, energy intake, and energy expenditure. In particular, the intake of nondigestible carbohydrates alters the species severity of the gut microbiota in both the short and long term. At the same time, individual differences in colonic microbiota composition also influence responses to dietary changes (Flint et al., 2012).

Spirulina platensis supplementation is accepted as an alternative food by World health Organization and Food Drug Administration. This important food has been taken as a daily intake as well as treatment in recent years. It was reported that *S. platensis* and its extracts can change the capacity of gut microbiota with both microbiota counts and severity. Particular C-phycocyanin which a natural protein in *S. platensis* can improve the gut health (Zhang et al., 2022b). Some researchers indicated that the phycocyanin can increase the *Faecalibaculum*, *Lactobacillus*, *Romboustia*, and *Clostridium*, and can decrease *Enterococcus*, *Bacteroides*, and

Parabacteroides after chemotherapy. Also, some researchers reported that *S. platensis* can treat the intestinal tissue via epigenetic mechanisms (Luna et al., 2018).

Recently, specific components have been interested for improving effects on gut microbiota. The beneficial relationship between the host and the microbiota emerges in the digestion of dietary fiber. These fibers are only broken down by the microbiota's fibrolytic fermentation, converting them to short-chain fatty acids in the colon and exhibiting anti-inflammatory properties (Yao and Rock, 2015). Considering the lack of literature on the effects of *S. platensis* on the gut microbiota, this review summarizes and discusses information about the systemic consequences of *S. plantensis* in relation to the regulation of the gut microbiota and health.

Gut Microbiota

Gut microbiota is an ecosystem consisting of microorganisms such as bacteria, archaea, fungi and viruses that live in the digestive system of animals and humans. It has many effects, including colonization, resistance to pathogens, protection of the intestinal tissue, metabolize dietary and pharmaceutical compounds, control of immune function, and even effects on behavior via the gut-brain axis.

The count of a human gut microbiota is approximately between ten thousand and one hundred billion. This flora is established at birth and changed through the whole life. There is a balance between host and microbial community via their diversity, specifies composition, metabolic outputs, genetical differences and/or host's pathogenicity (Flint et al., 2012). Understanding the role of microbiota in the maintenance of health observes the nutrition importance during life. It's known that nutrition impact on gut environment as well as microbiota population.

Many species in the gut microbiota have been observed which belonged to the genera Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, Escherichia and Lactobacillus (Searls, 2005; Beaugerie and Petit, 2004; Khanna and Tosh, 2014, Guarner and Malagelada, 2003). All these species are common patterns of microbiome community in which composition evaluating whole life. As the microbiome composition changes, the composition of bacterial proteins produced in the gut also changes. Enzymes involved in fermentation, methanogenesis, and some metabolisms (arginine, glutamate, aspartate, and lysine) have been reported in high concentrations in the gut microbiota of adult humans. In contrast, the dominant enzymes in the microbiomes of newborns have been reported to be involved in cysteine metabolism and fermentation pathways (Yasunenko et al., 2012).

Emphasizing maintaining a well-functioning digestive system contributes to a well-rounded understanding of gut health. However, the framework required to assess and achieve optimal gastrointestinal health is complex. Daily influences, the individual's clinical condition, and well-being all play a role in this assessment.

Microiota and nutrition

The gut microbiota contributes to digestion and immune modulation by playing a role in the gut-brain axis, where microbial metabolites (such as short-chain fatty acids and neurotransmitters) influence brain function and behavior. The gut-brain axis is known as the biochemical signaling that occurs between the gastrointestinal tract and the central nervous system. However, this definition is expanded to include the role of the intestinal flora in interactions, and is also used to describe paradigms that explicitly include the intestinal flora (Wang and Kasper 2014).

The gastrointestinal tract is densely innervated by neurons located both within and outside the intestine (enteric). Therefore, the microbiota regulates many functions in the body and works in conjunction with the central nervous system. Microbiota produce various metabolites which influence the neurons in the central nervous system via enteric neurons, thus regulating the host's physiology and metabolism (Herrema et al., 2016; Yang and Chiu, 2017). At the same time, symbiotic relationship between host and microbiota has various beneficial effects such as digestion of complex carbohydrates and maturation of the immune response. Most microbial activities are functions of the entire gut microbiota community, rather than being attributed to a single species. The balance and outcome of the community determine the net contribution to health or disease.

Lifespan-increasing approaches, such as dietary energy restriction (reducing food intake without causing malnutrition) and reducing insulin-like stimuli, affect metabolism, and are affected by it. Also, metabolism and aging are closely related. The immune system's homeostatic balance, including pro-inflammatory and anti-inflammatory immune system functions, shifts, and low-grade systemic inflammation occurs with aging. This process affects various biological functions, including the gastrointestinal ecosystem (Martin et al., 2017).

Excessive dietary fat and sugar are known to increase the growth of pathogenic microorganisms. In other words, exogenous fatty acids contribute to the proliferation of certain lactobacilli (Streptococci, Lactococci, Clostridia). Given that excessive fat and sugar consumption is associated with chronic inflammatory diseases, it has been reported that a high-fat and sugar diet can lead to a decrease in mucus membranes and the proliferation of *Helicobacter Pylori* and *Entamoeba Histolytica* in the intestine (Wasielewski et al., 2016).

Secretory IgA in the intestine provides protection against potentially pathogenic enterobacteria and directly determines the intestinal microbial population. However, a high-fat diet has been observed to increase intestinal permeability and oxidative stress, leading to the chronic, low-grade inflammation seen in obesity and increasing the risk of glucose intolerance and type 2 diabetes (Liu et al., 2017). Diet can reduce metabolic endotoxemia and inflammatory toxins. Therefore, diet plays a role in preventing overweight and obesity and reducing chronic inflammation.

The alteration of the number and composition of bacteria in the microbiota, disrupting their balance is known as dysbiosis, and it negatively impacts health and hinders the immune system's proper functioning. Dysbiosis alters intestinal permeability, leading to disease (Falony et al., 2016; Berbers et al., 2017). The association between disease and dysbiosis and the resulting changes in bacterial composition make the microbiota an important target for improving health. Dysbiosis and impaired intestinal permeability lead to many diseases, including obesity, type 2 diabetes, cardiovascular diseases, chronic inflammation, anxiety, depression, autism, inflammatory bowel disease, HIV, Hepatitis infections.

Spirulina platensis and microbiota

Spirulina platensis is a microalga belonging to the Cynobacteria family. It has been shown that *S. platensis* improves the intestinal absorption due to its cell wall's lack of cellulose, which in turn improves intestinal function and digestion. Furthermore, studies have shown that Spirulina can impact on the proliferation of harmful microorganisms while promoting the growth of beneficial ones like as *Lactobacillus* (Richmond, 1992; Pulz and Gross, 2004; Seyidoglu et al., 2017). Its high protein content, polysaccharide and pigment structure make these plants special. Its richness in vitamin B12 and beta-carotene, as well as its vitamin E, thiamine, cobalamin, and inositol content, is particularly important. Furthermore, studies have reported that its beta-carotene, lutein, and zeaxanthin contents also have supportive effects in

cancer treatments. In addition, researchers have revealed that the polysaccharides it contains also increase immunity (Seyidoglu et al., 2017; Alves et al., 2025).

Considering the important compounds of *S. platensis*, the effectiveness of *S. platensis* on intestinal microbiota and health is of considerable interest (Alves et al., 2025). A study with mice feeding *S. platensis*, gut microbiota composition improved (Rasmussen et al., 2009). They found that while *Clostridium* population increased *Bacterioides* populations decreased. Another study Zhang et al. (2022a) showed the increase of *Lactobacillaceae* and *Helicobacteraceae* populations, production of SCFAs and thereby reducing effect of the gut permeability with *S. platensis*. In addition, the anti-inflammatory effects of *S. platensis* in gut microbiota was interested in studies (Yu et al., 2020). The rats feeding by *S. platensis* has a decrease in IL-6, TNFalpha and lipopolysaccharide levels. Also, a decrease in intestinal microbiota was shown by *Bacteroidetes* and *Enterobacteriaceae* family (Yu et al., 2020).

S. platensis and probiotic combining effects on gut health have been interested for gut microbiota. Researchers reported that *S. platensis* combined with *Lactobacillus* improved the gut microbiota composition by increasing important genus as *Erysipelatoclostridium*, *Roseburia*, *Enterococcus*, *Bifidobacterium*, *Coriobacteriaceae* (Barros de Medeiros et al., 2022; Liu et al., 2022). The results showed that the combining effects can boost their effects on host and develop new healthy products. The importance of these combining effects were from rich dietary fiber and phenolic compounds which enhanced the effects on gut microbiota and thereby host health.

DISCUSSION AND CONCLUSION

The gut microbiota interacts particularly with the central nervous system and the immune system. This bidirectional interaction can lead to various stimuli in human physiology, resulting in changes in the microbiota. In recent years, the microbiota is called the second brain. It develops in utero and affects the central nervous system, and signals from the central nervous system also affect the intestinal microbiota.

Nutrition plays a prominent role in the formation of the intestinal microbiota, and it is argued that altering the permeability of the microbiota can alter the composition of the bacteria present, leading to many significant diseases. Therefore, restoring the intestinal microbiota through various interventions is considered an important intervention. Given that the relationship between health and the intestinal microbiota remains a mystery, microbiota transfers will inevitably become a hot topic.

Studies reported that *S. platensis* and its extracts have several important effects on health through various physiological mechanisms. Considering the fact that *S. platensis* contributes to metabolic functions and important responses such as oxidative and inflammatory, it is accepted that these effects of *S. platensis* will be effective in improving the gut microbiota and will be beneficial for a healthier life.

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FIVE-YEAR ASSESSMENT AND STATISTICAL EVALUATION OF OCCUPATIONAL RADIATION DOSES FOR CUSTOMS OFFICERS IN ALBANIA

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ABSTRACT

This study evaluates the occupational exposure to ionizing radiation among customs officers in Albania who work in proximity to X-ray scanning systems used for inspecting individuals and cargo at border checkpoints. The monitoring program involved approximately 120 customs officers stationed across six major customs in the country, where full-body and vehicle scanners are regularly operated.

Thermoluminescent dosimeters (TLD-100) were used to monitor personal doses, with readings performed using Harshaw TLD reader models 6600 and 4500 located in Institute of Applied Nuclear Physics. The selection of TLDs and equipment ensured reliable dose measurement under routine working conditions in customs environments.

The data collected was subjected to statistical analysis to determine the distribution of exposure across different customs points and among the personnel. The results showed that the average annual effective dose was approximately 1.47 mSv, which is significantly below the internationally recommended occupational exposure limit of 20 mSv per year (ICRP guidelines).

This study represents one of the first systematic evaluations of occupational radiation exposure among Albanian customs officers. The findings confirm that current operational practices and protective measures are adequate for maintaining radiation safety. However, continued surveillance, periodic training, and equipment calibration are recommended to ensure long-term protection.

Keywords: Occupational exposure, Thermoluminescent dosimetry, Custom checkpoints, Statistical analysis.

INTRODUCTION

The evaluation of occupational exposure for customs officers working with X-ray scanning systems at major border checkpoints in Albania was carried out through a systematic program of individual monitoring, laboratory measurements, and statistical assessment. Approximately 120 customs officers from the main customs sites, including Morine, Qafë-Thanë, Durrës, Kakavijë, and Kapshticë, were equipped with thermoluminescent dosimeters (TLD-100) that were worn during routine working hours in scanning areas. The dosimeters were collected every two months and analyzed at the Dosimetry Laboratory of the Institute of Applied Nuclear Physics, where personal dose equivalents $H_p(10)$ and $H_p(0.07)$ were determined. Measurements were performed using Harshaw TLD reader models 4500 and 6600, with the latter providing automated sequential reading of large numbers of dosimeters.

The five-year monitoring program showed that the average annual effective dose received by customs officers was about 1.47 mSv, with values across sites ranging from 0.7 to 2.2 mSv/year. The cumulative five-year averages were between 5.3 and 7.3 mSv, remaining well below the ICRP occupational limit of 20 mSv/year. Statistical analysis confirmed a lognormal distribution of doses, consistent with international findings, and demonstrated that the current radiation protection measures in Albanian customs checkpoints are effective in keeping occupational exposure at safe levels.

MATERIAL AND METHOD

At some of the customs in Albania, x-ray scans are performed on trucks and goods entering or leaving Albania. These customs are Morinë, Qafë Thana, Kakavijë, Kapshticë, Durrës and Mother Teresa Airport as in figure 1. Durrës is a maritime customs, Mother Teresa is an airport custom, while the others are land customs where employees are professionally exposed.

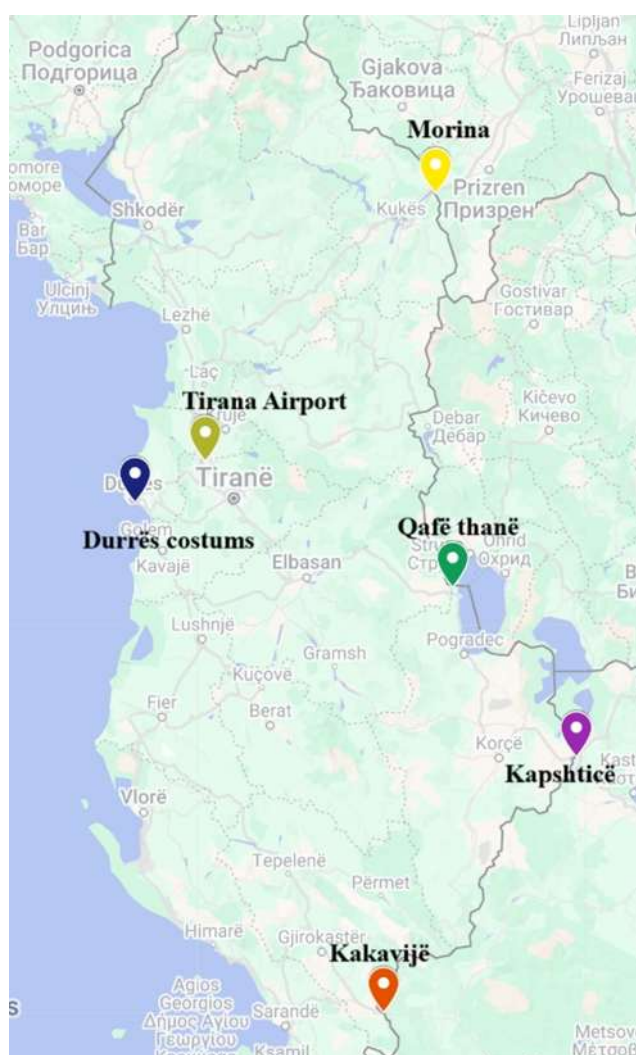


Figure 8. Albania checkpoints border where is implemented x-ray scanner

The main purpose of scanning is to detect and stop illegal goods entering and leaving Albania. Trucks are lined up to enter the scanning room, where a scanner with a movable arm scans the truck. Employees process the image and provide confirmation for customs clearance (figure 2 and 3).



Figure 9. X-ray scanner



Figure 10. X-ray checkpoint

The collected data were statistically processed to estimate annual average effective doses and to examine the distribution of exposures across sites and personnel during the five-year period from 2019 to 2023. Further statistical analyses were conducted using MATLAB software. To ensure the reliability of the results, officers with incomplete five-year monitoring records were excluded from the dataset. All procedures were implemented in accordance with national radiation protection regulations and international recommendations of the ICRP, which define an occupational dose limit of 20 mSv per year.

The dosimetry laboratory is based on thermoluminescence dosimetry, which consists of the effects of temperature on the separation of electrons from trap energy levels (figure 4) created because of the presence of impurities in the crystal lattice. These energy levels are occupied by electrons excited due to the interaction of atoms with radiation and are released or de-excited as a result of the increase in the temperature of the crystal, consequently the oscillation of the semi-stable or trap energy levels. This causes the release of photons with lower energy due to energy losses between the valence, conduction and intermediate energy levels (IEC61066). Our system consists of two readers suitable for thermoluminescent dosimetry, of

the Harshaw model manufactured by thermofisher, Harshaw 4500 (manual) and Harshaw 6600 (Riyadh Ch. Abul-Hail, Murad K . Abdallah, 2018).



Figure 11. Harshaw 6600 plus reader

Both readers are supplied with gaseous nitrogen by means of a nitrogen generator which is used for protection from oxidation, heating of crystals and maintaining the purity of photonic signals.

Measurements are carried out once every 2 months, the dosimeters are collected from the service office and brought to the laboratory, where they are measured and delivered within the day with the relevant flat measurement. The dose is given together with bg because its value has a large fluctuation depending on the construction materials of the objects where the dosimeters are kept.

Calibration of the Harshaw 6600 and Harshaw 4500 readers was performed by golden cards at SSDL in Croatia and Greece accredited and recognized by the IAEA in the SSDL networks. These dosimeters, lately, were sent for irradiation in Jozef Stefan Institute (SSDL) (2025) and then measured using the Harshaw 6600PLUS reader. The recommended IEC 61066 protocols for preheating, reading, and annealing of TLD-100 detectors were applied, and background corrections were systematically included in the dose evaluation process.

To calibrate the reader, the best performing dosimeters, known as calibration or "Golden" doseimeters (Dmitriy Spartakovich Kim et al., 2019), were selected (1-2% of all doseimeters). The RCF and ECC was generated for some time-temperature profiles (TTP) corresponding to some types of dosimeters in use (Ervis Gega, Elida Bylyku, 2025). For example, for the TLD-100 dosimeters manufactured by Radcard, the recommended time-temperature profile is: preheat for 5 seconds at 170 °C, acquire with a temp rate of 25 °C/s, for 13 seconds up to 300 °C, and anneal for 5 seconds at 300 °C (Omer Kouakou et al., 2019).

RESULTS AND DISCUSSION

International literature informs us that the annual effective dose for staff monitored with TLD (LiF:Mg,Ti) is usually < 1–3 mSv/year, well below the regulatory limit of 20 mSv/year (averaged over 5 years) (M.H. Nassef, A.A. Kinsara, 2017; Gholamhossein Pourtaghi, 2014). The variations mainly come from staff roles, workload and radiation protection practices. In 2023, about 265196 scans were performed, a huge increase compared to only 14655 in 2015. Global reports such as UNSCEAR confirm a long-term downward trend in occupational

exposures in medical fields, with typical average values around 0.3–0.5 mSv/year, while specific studies often report average annual doses between 0.2–1.5 mSv/year (B Basić et al., 2010).

The results obtained from the five-year monitoring program provide a comprehensive overview of occupational exposure among customs officers working with X-ray scanning systems in Albania. The systematic use of TLD-100 detectors and rigorous calibration procedures ensured reliable measurements of personal dose equivalents $H_p(10)$ and $H_p(0.07)$. The statistical processing of the dataset allowed for the identification of annual dose trends, inter-site variations, and overall compliance with international safety standards. Before examining the site-specific results, it is important to highlight that the recorded doses represent realistic operational conditions, as dosimeters were worn continuously during work shifts in proximity to scanning units. This approach eliminates the possibility of underestimation and supports the robustness of the findings. Furthermore, the lognormal distribution observed in the data aligns with patterns commonly reported in occupational radiation studies, reflecting variability in workload, scanning frequency, and individual work practices. The discussion that follows integrates these findings with both national regulatory limits and international reference values, thereby providing a critical perspective on radiation safety management in customs environments.

Table 1. Average annual equivalent dose in mSv for 5 years

Customs checkpoint	Hp(10mm) (mSv)					
	2019	2020	2021	2022	2023	5 years
Morinë	1.29	1.23	0.72	1.51	1.30	5.33
Qafë thanë	1.21	1.36	1.05	2.02	1.68	6.76
Durrës	1.03	1.25	1.18	2.15	1.79	7.33
Kakavijë	1.29	1.20	1.00	2.10	2.03	6.53
Kapshticë	1.37	1.19	1.05	2.19	1.98	7.15

The analysis of the five-year monitoring data (Table 1, figure 6) provides clear evidence of occupational radiation exposure levels among customs officers stationed at major border checkpoints in Albania. Table 1 summarizes the annual average effective doses $H_p(10)$ recorded between 2019 and 2023, while Figure 5 illustrates the site-specific variations. Across all sites, the annual average dose per officer ranged between 0.7 and 2.2 mSv with average dose 1.47mSv/year for 5 years with standard deviation 0.45mSv, with cumulative five-year averages between 5.3 and 7.3 mSv. These results confirm that the recorded exposures are well below the ICRP occupational dose limit of 20 mSv/year, thus ensuring compliance with both national and international standards.

When compared with international data, the Albanian results fall within the lower to medium range of occupational exposures. These findings demonstrate that while customs officers in Albania are exposed to measurable occupational doses due to routine use of full-body and vehicle scanners, the levels remain within internationally acceptable ranges. The observed variations across sites are likely attributable to differences in scanner usage frequency, equipment type, and staffing patterns. Importantly, the systematic monitoring program and robust statistical evaluation confirm the adequacy of current radiation protection measures,

while also underlining the need for continuous surveillance and periodic staff training to ensure sustained safety standards.

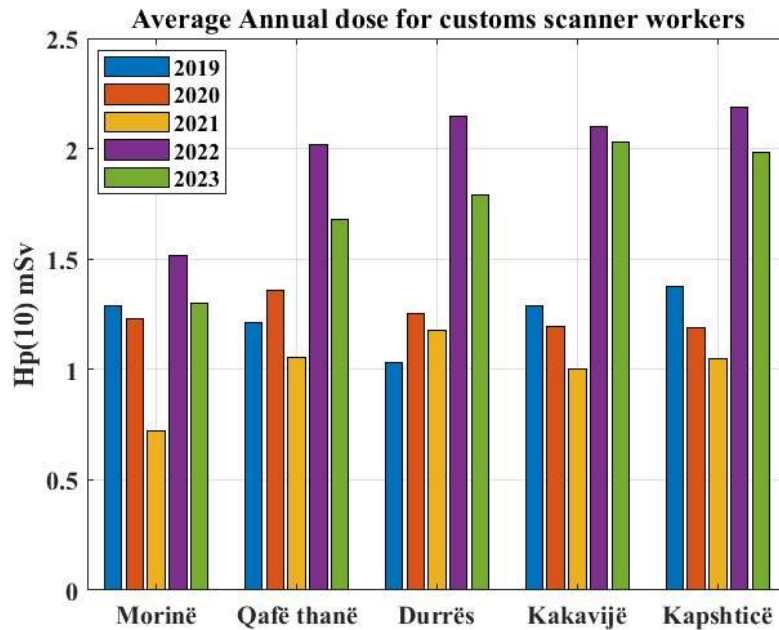


Figure 12. Average annual equivalent dose for five years

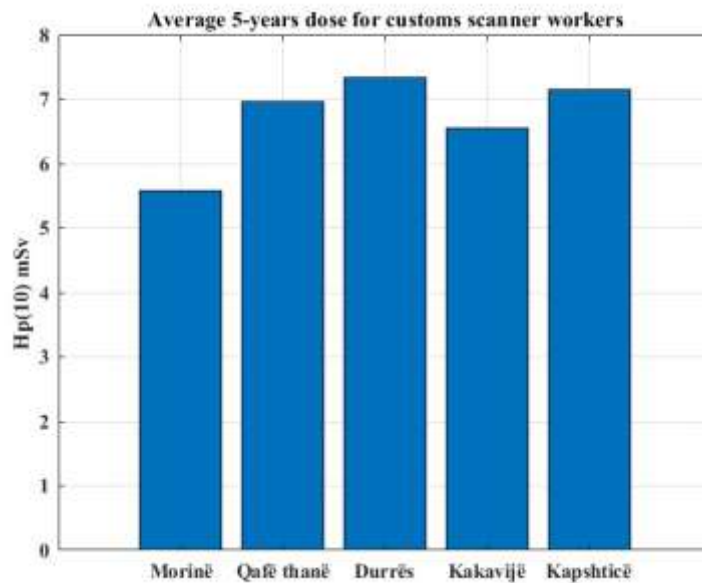


Figure 13. Average five years dose for five different border x-ray checkpoints

We have also calculated the collective effective dose given according to the formula (ICRP, 2007):

$$S(E_1, E_2, \Delta T) = \int_{E_1}^{E_2} E \left[\frac{dN}{dE} \right]_{\Delta T} dE \quad (1)$$

Where S is the collective effective dose for the energy interval $[E_1, E_2]$ and for the period ΔT and E is the individual effective dose. dN/dE is the distribution of the number of individuals according to the effective dose (i.e. how many people receive a dose between E and $E+dE$). The integral shows that the collective dose is the sum (integral) of all individual doses weighted by the number of individuals. Written in simplified form:

$$S = \sum_i \bar{H}_i N_i \quad (2)$$

Where $\bar{H}_i$ is the average value of subgroup i and N_i is the number of individuals in that subgroup (UNSCEAR, 2000). In table 2 the effective collective dose is with unit man Sievert multiplied by 10^3 or we can write man mSv.

Table 3. Collective effective dose

	2019			2020			2021			2022			2023		
	$\bar{H}_i(\text{mSv})$	N_i	S	$\bar{H}_i(\text{mSv})$	N_i	S	$\bar{H}_i(\text{mSv})$	N_i	S	$\bar{H}_i(\text{mSv})$	N_i	S	$\bar{H}_i(\text{mSv})$	N_i	S
Morinë	1.3	14	18.0	1.2	14	17.2	0.7	14	10.1	1.5	17	25.7	1.3	15	19.5
Qafë thanë	1.2	14	17.0	1.4	14	19.0	1.1	14	14.7	2.0	16	32.3	1.7	15	25.2
Durrës	1.0	15	15.5	1.3	16	20.0	1.2	16	18.8	2.1	16	34.3	1.8	16	28.7
Kakavijë	1.3	14	18.1	1.2	18	21.5	1.0	18	18.1	2.1	16	33.6	2.0	13	26.4
Kapshticë	1.4	14	19.2	1.2	15	17.8	1.0	17	17.8	2.2	16	35.0	2.0	16	31.8
Total		71	87.8		77	95.6		79	79.5		81	160.9		75	131.5

Statistical testing confirmed that the dose data followed a lognormal distribution, as verified through chi-square goodness-of-fit tests at a 95% confidence level. Additional optimization of histogram bin sizes was performed according to the Freedman–Diaconis rule.

The hypothesis H_0 on the dose distribution was tested with a successful case for the Lognormal distribution. We performed bin optimization using Freedman and Diaconis's rule (David Freedman, Persi Diaconis, 1981). We found the Interquartile Range using Matlab $\text{IQR}=\text{iqr}(\text{AA2023})$;

$$H = \frac{2\text{IQR}}{n^{\frac{1}{3}}} \quad (3)$$

Where H is size of bin, from where we can write the number of bin as:

$$N_{bin} = \frac{ecc(ii)_{max} - ecc(ii)_{min}}{H} \quad (4)$$

We defined and tested some of the distributions with MATLAB which we initially approved with easyfit:

`pd = fitdist(AA2023,["Lognormal"]);`

From the following formula we obtain the evaluation of the hypothesis raised which is based on the chi square test

$$\chi^2 = \sum \frac{(O - E)^2}{E} \quad (5)$$

Which is done using kode: `[h,p,stats] = chi2gof(AA2023,'cdf',pd,'Alpha',0.05,'NBins',NBIN)` when we took `chi2stat: 2.3459` and `df: 3`.

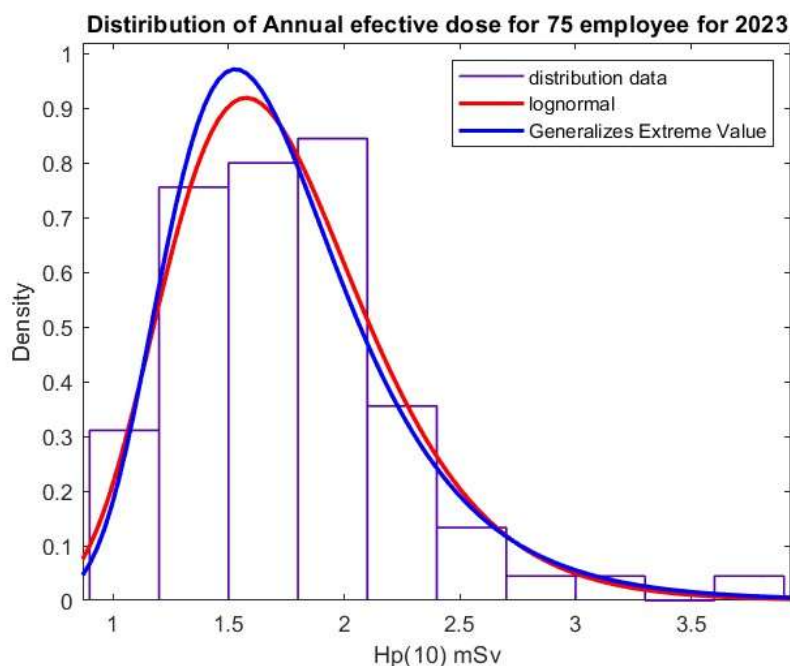


Figure 14. Fiting distribution of annual effective dose for last year of study

Which resulted that for the lognormal distribution our values are more suitable for $\alpha=0.05$ for the year 2023 where 80 customs employees from Durrës, Kakavijë, Kapshticë, Qafë Thanë Morinë were studied. Rinas airport was not taken into consideration because the method of operation and the scanners used are different. Also, employees who have not worked the full 5 years under review have been filtered, which has led to a reduction in the total number.

Cargo workers at Mother Teresa Airport in Tirana during the study period 2019 to 2022 have not had regular measurements, from one measurement per year, not according to the rule of bimonthly measurements, referring to the fading effect we cannot take these values into account. The year 2023 has had accurate measurements, a total of 6 pieces for 15 employees where the average annual dose results to be 1.6mSv.

CONCLUSIONS

This five-year assessment of occupational radiation exposure among customs officers in Albania provides the first systematic evaluation of dose levels associated with the operation of X-ray scanning systems at major border checkpoints. The findings demonstrate that the mean annual effective dose of approximately 1.47mSv for year with standard deviation 0.45 mSv is significantly below the ICRP occupational limit of 20 mSv/year. Even in years of increased operational workload, the measured doses remained within safe regulatory boundaries, confirming the adequacy of existing radiation protection practices.

The observed lognormal distribution of the dataset is consistent with international reports on occupational exposures, reflecting natural variability in workload, scanner operation, and individual work habits. When benchmarked against global literature, the exposure levels of Albanian customs officers are comparable to those recorded in diagnostic radiology personnel and moderately higher than those of radiotherapy staff, but still well within the range of internationally reported values.

The results underscore the effectiveness of the national monitoring program, which combines reliable dosimetric methods, strict calibration protocols, and robust statistical

analysis. At the same time, the study highlights the importance of sustaining continuous surveillance, regular equipment maintenance, and periodic staff training to ensure long-term safety.

In conclusion, the occupational exposure of customs officers in Albania remains low and compliant with international standards. Future work should focus on strengthening the national database of radiation doses, broadening coverage to include additional customs sites such as airports, and enhancing radiation safety culture through ongoing professional development. Such measures will not only reinforce the current protection framework but also provide a resilient foundation for managing occupational radiation risks in line with international best practices.

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EVALUATION OF THE CYTOTOXIC EFFECTS OF BEE VENOM (*APIS MELLIFERA*) ON HEP3B HEPATOCELLULAR CARCINOMA CELLS

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ABSTRACT

Bee venom is a product that attracts attention due to its many biological effects, primarily antioxidant and anticancer effects, thanks to the bioactive components it contains with unique pharmacological properties. The biologically active components in its content, such as melittin, apamin, and phospholipase A2, are the main substances responsible for these effects. The HEP3B cell line is a widely used model in in vitro studies of hepatocellular carcinoma. This study was conducted to investigate its cytotoxic effects on HEP3B hepatocellular carcinoma cells. Bee venom was applied to HEP3B cells at different concentrations, and cell viability was measured using the MTT assay after 24-hour incubation. In cytotoxicity analyses, bee venom was found to reduce cell viability in a dose-dependent manner, and IC₅₀ values were found to change over time. In conclusion, bee venom was shown to be both a natural pharmacologic and potential antitumor agent, and it was concluded that further studies are needed to understand the molecular mechanisms of this property.

Keywords: Bee venom, Melittin, Cytotoxicity, HEP3B cells, Hepatocellular carcinoma

INTRODUCTION

Bee venom is a biological toxin secreted by the honeybee (*Apis mellifera*), containing numerous pharmacologically active peptides, enzymes, and various components (Gajski et al., 2024). Melittin, one of these components, constitutes a large part of bee venom (Chen et al., 2016). Melittin, an amphipathic peptide that can cause cell lysis by increasing membrane permeability, has attracted considerable attention in recent years for its anti-inflammatory, antimicrobial, and anticancer effects and is increasingly being studied in various disease models (Cui et al., 2024; Stela et al., 2024; Zhang et al., 2024). In addition, apamin, a small neurotoxic peptide, inhibits Ca²⁺-dependent K⁺ channels in nerve cells; phospholipase A2 initiates the release of biologically active lipid mediators by hydrolyzing membrane phospholipids (Burke et al., 2009; Messier et al., 1991). The synergistic interactions of these bioactive components demonstrate that bee venom is a potent biological agent capable of regulating numerous biological processes at the cellular level. In addition to its well-known use in apitherapy for conditions such as arthritis and chronic pain, recent research has increasingly focused on the potential applications of bee venom in the field of oncology (Supriya et al., 2025). Melittin has been reported to induce apoptosis by modulating key signaling pathways, including NF-κB, MAPK, and PI3K/Akt, and to inhibit tumor growth in various cancer cell models. Additionally, bee venom has been shown to exhibit anti-angiogenic effects, suppress genes associated with

metastasis, and enhance the efficacy of traditional chemotherapeutic agents when used in combination therapies (Huh et al., 2010). These multifaceted effects make bee venom and its components attractive candidates for the development of new anticancer agents.

Hepatocellular carcinoma (HCC) is the most common primary liver cancer worldwide and poses a significant health burden due to its high mortality rate. Currently, HCC treatment involves surgical resection, liver transplantation, and systemic therapies; however, the prognosis is generally poor, necessitating new treatment strategies (Mauro, et al., 2025; Yang et al., 2019). Despite recent advances in molecularly targeted therapies and immunotherapies, recurrence rates remain high and survival outcomes are not at the desired level; this situation highlights the need for alternative or complementary treatment approaches. Natural products and bioactive compounds are gaining increasing attention as potential sources of new anticancer agents due to their ability to target multiple molecular pathways and their lower incidence of side effects compared to traditional chemotherapies. The HEP3B cell line is one of the in vitro models used to study the molecular mechanisms of HCC and to evaluate the cytotoxic or antiproliferative effects of new therapeutic agents (Qiu et al., 2015). In particular, elucidating the relationship between the antioxidant properties and cytotoxic effects of bee venom may contribute to the development of new combination strategies aimed at controlling oxidative stress in liver cancer. The investigation of the effect of bee venom on these cells is important for a better understanding of its anticancer potential. In this context, determining the antioxidant properties of bee venom and evaluating its cytotoxic effects on HEP3B cells sheds light on the pharmacological profile of bee venom and establishes the scientific basis for revealing its role as an adjunctive agent in liver cancer treatment.

MATERIAL AND METHOD

This in vitro experimental study was conducted between July and August 2025 at the Cell Culture Laboratory of the Department of Medical Biology and Genetics, Faculty of Medicine, Ankara Medipol University. The bee venom used in the study was collected using the electroshock method from bees raised in the Kızılcahamam region of Ankara in June 2025. The device used was designed so that when it came into contact with the bees, it would not harm them and would allow them to release their venom onto the glass plate without losing their stingers.

Bee venom solutions were prepared at concentrations ranging from 0.01 to 3.78 mg to investigate their cytotoxic effects on HEP3B hepatocellular carcinoma cells. Stock solutions were prepared in phosphate-buffered saline (PBS, pH 7.4) and sterilized by passing through a 0.22 μm membrane filter. Solutions were freshly prepared each day of the experiment and diluted to appropriate doses.

Bee venom solutions were prepared in PBS (pH 7.4), sterilized by passing through a 0.22 μm membrane filter, and diluted with EMEM prior to treatment to achieve final concentrations of 0.1–100 $\mu\text{g/mL}$. The HEP3B cell line (ATCC HB-8064) was cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded at 1×10^4 cells/well into 96-well plates and allowed to adhere overnight. Cells were then treated with bee venom at the specified concentrations for 24 hours, and untreated cells were used as the control group.

Cell viability at the end of incubation was determined by adding 10 μL of MTT solution (5 mg/mL) to each well and dissolving the formazan crystals formed after 4 hours of incubation with dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm, and cell viability (%) was calculated relative to the control group. IC_{50} values were determined using non-linear regression analysis.

The data obtained are expressed as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad® Prism 8.0 (GraphPad Software, San Diego, CA, USA) software, and differences between groups were evaluated using one-way ANOVA and Tukey post-hoc test. A $p < 0.05$ value was considered statistically significant.



Figure 1. Collecting bee venom with an electroshock device



Figure 2. Scraping bee venom from a glass plate surface

RESULTS AND DISCUSSION

In this study, bee venom exhibited a significant cytotoxic effect on HEP3B cells. The half-maximal inhibitory concentration (IC_{50}) of bee venom was calculated as 3.37 mg/mL, indicating a potent cytotoxic response. Cell viability decreased significantly at low and medium concentrations, but viability was partially higher at the highest dose (3.78 mg/mL). This unexpected finding can be interpreted as melittin causing rapid cell lysis at high doses, thereby preventing the MTT substrate from accurately reflecting metabolic activity. Similar non-monotonic dose-response curves have been reported previously in peptide toxin studies, and

this situation is thought to stem from the effect of necrotic cells on MTT stainability (Hoogstraten et al., 2022); Lagarde et al., 2015).

In the literature, it has been shown that bee venom disrupts cell membrane permeability by forming pores, induces lysis and apoptosis, creates oxidative stress by increasing ROS production, and initiates caspase-3 activation by disrupting mitochondrial membrane potential. (Kong et al., 2016; Li et al., 2023; Wang et al., 2024; Zhang, et al., 2024). Our study did not directly examine these mechanisms; however, it is thought that the observed cytotoxic effect may be related to one or more of these pathways, particularly the mitochondrial pathway, which may play an important role. Future RT-qPCR, Western blot, and immunohistochemistry analyses evaluating markers of apoptotic and necrotic pathways (caspase-3, BAX/Bcl-2 ratio, PARP degradation) will contribute to elucidating this mechanism.

The potential of melittin in cancer treatment has been extensively discussed in the literature. Alonezi et al. (2016) reported that melittin treatment significantly reduced viability in cisplatin-resistant ovarian cancer cells and that combination therapy produced a synergistic cytotoxic effect. (Alonezi et al., 2016). This finding suggests that melittin is an important therapeutic candidate not only due to its antiproliferative effect alone, but also due to its ability to enhance the response to chemotherapy. This approach may contribute to reducing drug-related side effects by achieving equivalent efficacy with lower doses of chemotherapy. Although combination therapy with chemotherapy drugs was not administered in our study, it was demonstrated that rye poison produced a significant cytotoxic effect in HEP3B cells, and it was revealed that this could provide preliminary information for future combination therapy studies.

The therapeutic use of bee venom is limited due to toxicity and the risk of allergic reactions. Therefore, the development of targeted and controlled release systems is of critical importance. The melittin nano-liposome formulation developed by Mao et al. (2017) suppressed tumor growth in HCC cells and in vivo mouse models and exhibited lower hemolytic toxicity compared to free melittin (Mao et al., 2017.) Such nanoformulations can improve the pharmacokinetic profile of melittin, providing high concentrations in the target tissue while reducing systemic side effects. Additionally, carrier systems based on liposomes, dendrimers, or polymeric nanoparticles can reduce the immune response and enable more controlled release. The cytotoxicity findings obtained in our study provide preliminary information for the development of such safe and targeted melittin formulations.

Our findings support the potential of melittin as an anticancer agent in HCC cells and provide important preliminary data for further research. However, limitations of our study include the use of only in vitro MTT assays and the absence of mechanistic analyses. Future studies should elucidate melittin's effects on apoptotic and necrotic pathways, its combination synergy with chemotherapy drugs, and its safety profile in in vivo tumor models. This would enable melittin to be introduced into clinical practice, both as a standalone treatment and as an adjuvant therapy option, particularly for multidrug-resistant tumors

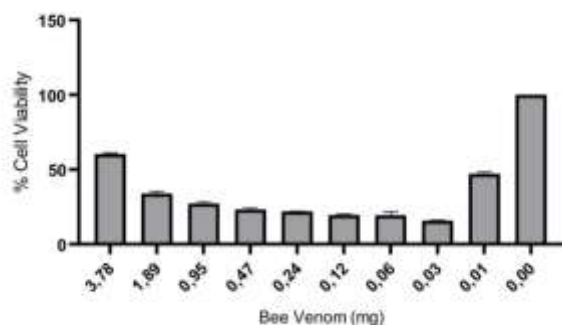


Figure 3. Hep3B cell viability percentages for different concentrations of bee venom

CONCLUSIONS

This study determined that bee venom exhibits dose-dependent cytotoxic effects on HEP3B hepatocellular carcinoma cells, with an IC_{50} value of 3.37 mg/mL. Our findings suggest that bee venom and its major peptide, melittin, may have promising anticancer potential; however, these results are limited to in vitro MTT assays and do not provide definitive evidence regarding the underlying molecular mechanisms. The variability in cell viability at high concentrations also indicates the need for more controlled studies. Future preclinical studies using in vivo tumor models will enable the development of bee venom for clinical research, the enhancement of its safety with targeted delivery systems, and the demonstration of its synergistic efficacy in combination studies with chemotherapeutic agents.

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HISTOLOGICAL EFFECTS OF PRUNUS AMYGDALUS VAR AMARA ON PANCREATIC TISSUE IN DIABETIC RATS

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ABSTRACT

This study evaluates the histopathological impact of *Prunus amygdalus var amara* (bitter almond) on pancreatic tissue in diabetic rats, contextualizing its ethnobotanical use for diabetes management against potential toxicity. Despite traditional applications, concerns persist regarding cyanogenic compounds in the plant, necessitating a histological assessment of pancreatic damage.

Pancreatic samples from alloxan-induced diabetic rats (four groups: healthy control, diabetic untreated, glibenclamide-treated, and plant-treated) underwent paraffin-embedding histology. Tissues were fixed in 10% formalin, dehydrated via ethanol gradients, cleared in toluene, embedded in paraffin, and sectioned (5 µm). Hematoxylin-eosin (H&E) staining distinguished cellular structures. Microscopic analysis (Leica) assessed histoarchitectural changes, focusing on islets of Langerhans, acini, and ducts.

- Control group (A): Normal pancreatic structure with intact acini, islets, and ducts.
- Diabetic groups (B, C): Partial beta-cell destruction in islets, sparing acini.
- Plant-treated group (D): Severe necrosis of endocrine/exocrine pancreas, lymphocytic infiltration, vascular inflammation, and ductal destruction.

The partial beta-cell loss in diabetic groups aligns with alloxan's pancreatotoxicity. However, group D's extensive damage suggests synergistic toxicity from cyanogenic compounds in *P. amygdalus*, exacerbating alloxan effects. Notably, hypoglycemia observed in plant-treated rats likely stems from extra-pancreatic mechanisms (e.g., intestinal malabsorption), not insulin secretion. These findings caution against using cyanogenic plants in diabetes despite transient glycemic benefits. Future studies should quantify cyanogens, assess multi-organ toxicity, and compare almond varieties' safety profiles.

P. amygdalus var *amara* exhibits antidiabetic potential but induces severe pancreatic toxicity at 28 mg/kg, underscoring risks of traditional use. Comprehensive toxicological profiling and exploration of non-pancreatic mechanisms are imperative for safe therapeutic development.

Keywords: diabetes- pancreatic tissu -bitter almond

INTRODUCTION

Toxic plants contain various chemical substances, such as alkaloids, cyanogenic glycosides, or protein toxins, which can cause histological damage in the target organs of the human or animal body. These effects vary depending on the plant, the dose ingested, and the duration of exposure, but they often involve mechanisms such as enzyme inhibition, inflammation, cellular necrosis, or apoptosis. For example, cyanogenic glycosides release cyanide, which blocks the mitochondrial respiratory chain, leading to tissue hypoxia and histological damage in organs such as the liver, kidneys, or pancreas. Alkaloids, on the other hand, can disrupt cell membranes or neurotransmitters, causing neuronal degeneration or inflammations. These alterations are often studied in plant toxicology to understand the risks of accidental or intentional poisoning (World Health Organization, 1993; Barceloux, 2008).

The bitter almond tree (*Prunus amygdalus* var. *amara*) is a variety of the almond tree whose seeds contain amygdalin, a cyanogenic glycoside that, under the action of digestive or bacterial enzymes, breaks down into hydrocyanic acid (cyanide). The latter is highly toxic and can affect the pancreas by inhibiting cytochrome c oxidase, a mitochondrial enzyme essential for cellular respiration. Histologically, this manifests as necrosis of the pancreatic acinar cells, inflammatory infiltration (hemorrhagic pancreatitis), and degeneration of the islets of Langerhans, which can lead to acute pancreatic failure. Cases of severe poisoning have been documented, with estimated lethal doses of 50-300 mg of amygdalin per kg of body weight (Bolarinwa et al. 2014; EFSA, 2016).

The objective of this study is to determine the effects of a dose of *Prunus amygdalus* var. *amara* seed on the pancreas of rats rendered diabetic by alloxan.

Explain the mechanisms of the hypoglycemia observed in the diabetic rats after administration of a dose of 28 mg/kg of body weight.

MATERIALS AND METHODS:

Histological study:

Materials:

Equipment:

Incubator set at 56°C (nüve incubator EN 055)

Paraffin embedding station (Leica)

Cooling plate (Leica EG1150C)

Microtome (LEICA RM 2125RT)

Water bath (Leica)

Hotplate set at 37°C (Jouan)

Microscope (Leica)

Reagents:

Formalin 10%

Ethanol 70%, 90%, 100%

Toluene

Paraffin

Harris's Hematoxylin

Eosin 1.5%

Mounting medium (HistoRAL)

Small Materials / Sundries:

Cassettes

Forceps

Slides and coverslips

Diamond-tipped pen

Microtome blades

Flat-bottom metal molds

Slide trays / Slide racks

Methods:

Histological techniques involve a series of sequential operations. The method we employed, using paraffin embedding, consists of the following steps:

1- Pancreas Collection:

At the end of the experiment, the rats were sacrificed under anesthesia and secured to a cork board on their dorsal side by fixing all four limbs with pins (Figure). A small incision was made in the perineal area to allow the insertion of a dissecting probe. Following this, the abdominal cavity was dissected.

The pancreases are isolated to observe any histopathological changes or tissue abnormalities (lesions, colour, consistency, regeneration, etc.).

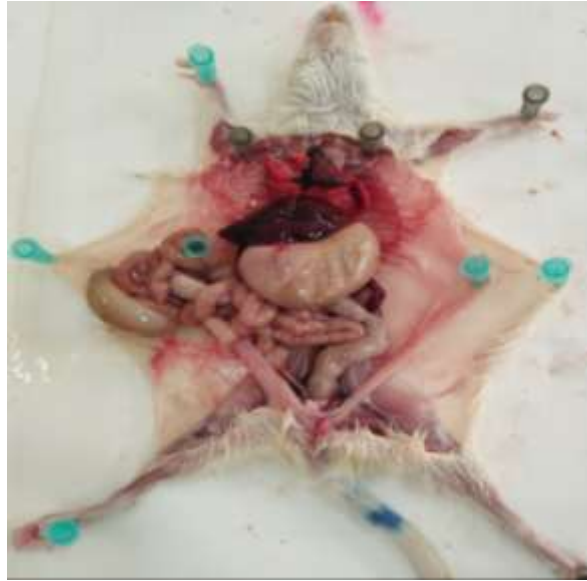


Photo 01: Rat dissection (BOUKHEMACHA.F, 2019)

2- Fixation:

The collected pancreases are washed with physiological saline and immediately immersed in a sufficient volume of fixative liquid, which is formalin (10%).

The organs are cut, and the resulting tissue pieces are placed in numbered, well-identified plastic cassettes for each group.



Photo 02: Workstation where tissue sampling and fixation are performed (BOUKHEMACHA.F, 2019)

3- Washing, Dehydration, and Clearing:

Before proceeding with dehydration, the fixed tissue pieces are washed under running water to remove excess fixative.

The pieces are then dehydrated by gradually passing them from the washing solution to absolute alcohol through successive baths of 70°, 90°, and 100% alcohol, each lasting 2 hours. This is followed by clearing, where the pieces are placed in a paraffin solvent—toluene in our case—for 2 hours.



Photo 03: Cylinders where dehydration, clearing, and impregnation are carried out (BOUKHEMACHA.F, 2019)

4- Paraffin Embedding and Block Preparation (Embedding):

The cassettes are immersed in two successive baths of liquid paraffin inside an incubator set at 56°C for 15 minutes and 12 hours, respectively; this allows the paraffin to infiltrate the samples. After embedding, the cassettes are cooled, resulting in a solid block for each sample. The paraffin blocks are sectioned using a microtome to obtain very thin 5 μ m thick slices from each sample, preventing cell overlapping. The section is placed and adhered onto a glass slide, then dried on a hotplate set at 37°C for 15 minutes, followed by incubation in an oven set at 56°C for at least 2 hours.



Photo 04: Incubator set at 56°C on the left - Cooling plate on the right (BOUKHEMACHA.F, 2019)

5- Deparaffinization and Staining:

Sections are stained using the topographical staining method HEMATOXYLIN EOSIN, the principle of which is the staining of nuclei by an aluminum lake (hemalum) and of background structures by a single acidic dye (eosin).

This empirical staining method allows for the distinction between alpha and beta cells of the endocrine pancreas. Alpha cells (secreting glucagon) are stained pink and are far less numerous than the blue-stained beta cells (secreting insulin). Typically, alpha cells are distributed at the periphery of the islets. Delta cells (secreting somatostatin) are not visible with this stain.

The slides are immersed in 2 successive baths of toluene for 7 and 5 minutes respectively, followed by 3 successive baths of decreasing ethanol concentrations (100%, 90%, 70%) for 1 minute each, and then rehydrated in water for 3 minutes.

The slides are then placed in a bath of Harris's hematoxylin for 45 seconds, followed by a water rinse for 3 minutes.

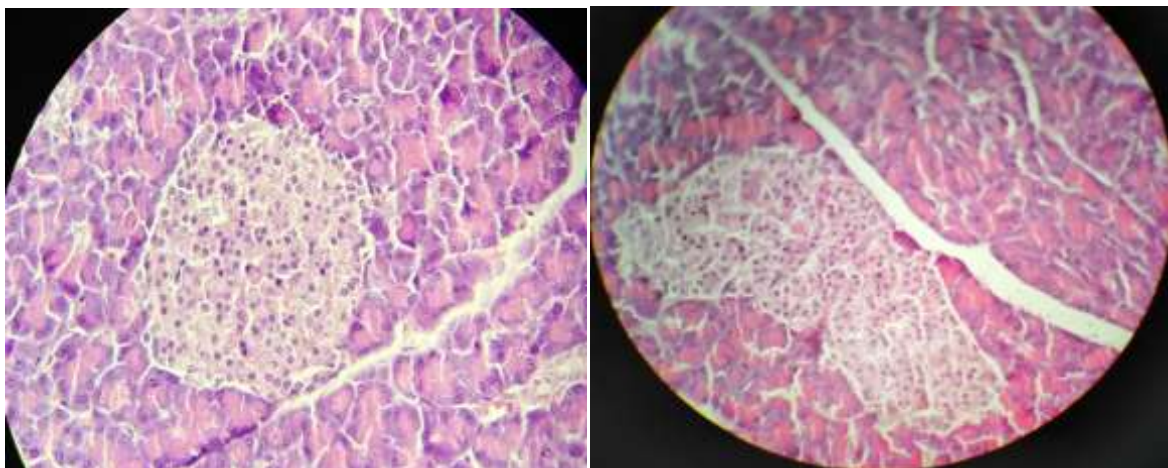
Next, the slides are placed in an eosin bath for 5 minutes, followed by 3 successive baths of increasing ethanol concentrations (70%, 90%, 100%) for 30s, 30s, and 1 minute respectively to dehydrate them. Finally, they are placed in 2 successive toluene baths for 5 minutes each, then mounted using resin and covered with a coverslip.

6- Microscopic Visualization:

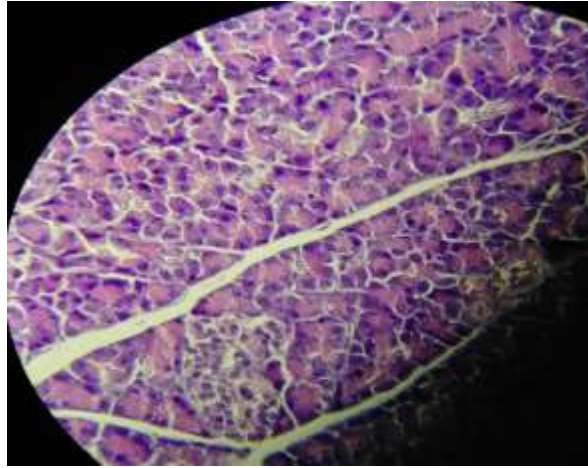
Microscopic visualization is performed using a "LEICA" optical microscope, and photographs of the various samples were taken.

Results and discussion:

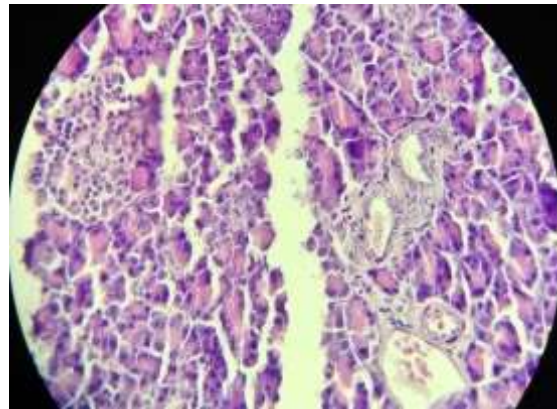
Histological study:



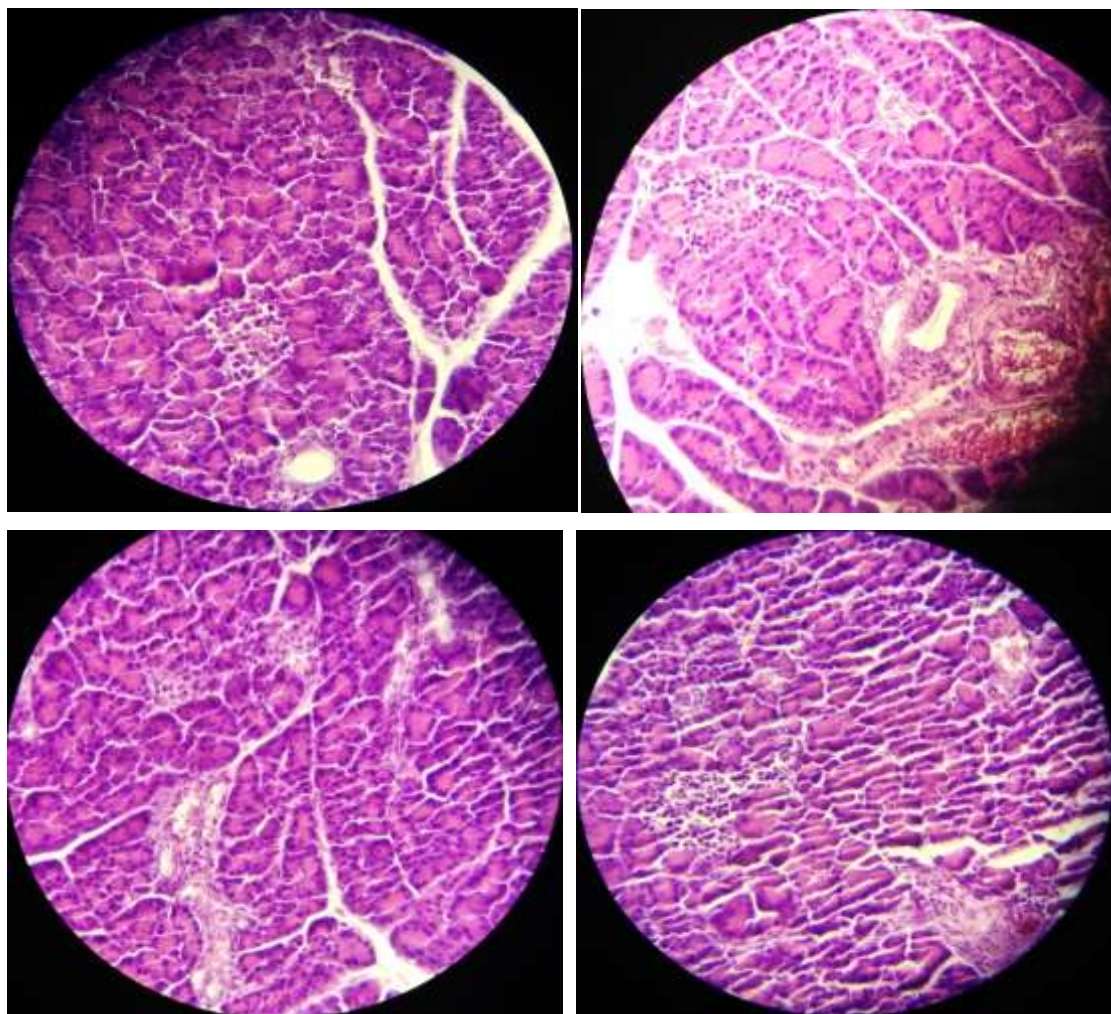
(A)



(B)



(C)



(D)

Photo 05: Pancreatic histology (stained with Hematoxylin-Eosin) of healthy and diabetic rats: A (Healthy group: negative control), B (Untreated diabetic group: positive control), C (Diabetic group treated with Glibenclamide: reference group), D (Diabetic group treated with the studied plant). (BOUKHEMACHA.F, 2019)

A: Negative Control: Normal (healthy) rats:

Microscopic observation shows: acini, Langerhans islets, and excretory ducts with a normal appearance, and an absence of signs of infiltration.

B: Positive Control: Untreated diabetic rats:

Microscopic observation shows: partial destruction of the beta cells in the Langerhans islets, with intact acini.

C: Reference Group: Diabetic rats treated with the reference molecule: an oral antidiabetic drug, glibenclamide:

Microscopic observation shows: partial destruction of the beta cells in the Langerhans islets, with intact acini.

D: Test Group (*Prunus amygdalus var amara*): Diabetic rats treated with the seed of the *Prunus amygdalus var amara* species at a dose of 28 mg/kg:

Microscopic observation shows:

- Severe necrosis of the Langerhans islets
- Inflammation of the blood vessels (vasculitis)
- Significant lymphoid cell infiltration
- Destruction of the excretory ducts
- An effect even on the exocrine pancreas (destruction of acini)

Discussion:

For the negative control group: No lesions were present; the endocrine and exocrine pancreas had a normal appearance, consistent with healthy rats.

For the two groups: positive control and reference group: The partial destruction of the beta cells in the Langerhans islets (insulin-producing cells) is explained by the pancreatotoxicity of alloxan.

For the test group: Diabetic rats treated with the species *Prunus amygdalus var amara*: The total destruction of the endocrine and exocrine pancreas can be explained by the toxic effect of alloxan on one hand, and the toxic effect of the plant, which is rich in cyanogenic compounds, on the other hand, even at a low dose and over a short treatment duration.

Note: The decrease in blood glucose is due to an extra-pancreatic mechanism (possibly intestinal malabsorption) and not an intra-pancreatic mechanism.

CONCLUSIONS:

At the end of our study, it appears that:

- The histological examination of the pancreas confirmed the toxicity of the plant, manifested by the destruction of acini, vasculitis, lymphoid infiltration, severe necrosis of the Langerhans islets, and even destruction of the excretory ducts, following 7 days of daily administration of a 28 mg/kg dose of the *Prunus amygdalus var amara* seed.

As future perspectives, we plan to:

Conduct a histological examination of other organs, namely the stomach, liver, and kidney, to better understand the mechanisms of the antidiabetic effect.

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ETHNOBOTANICAL SURVEY OF ANTIDIABETIC PLANTS IN THE THENIET EL HAD REGION, TISSEMSILT PROVINCE

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ABSTRACT

An ethnobotanical survey was conducted on **260** individuals (men and women, both diabetic and non-diabetic, aged over 18) in the Theniet El Had region (Tissemsilt province) over two months (January and February 2019) to document medicinal plants used in traditional phytotherapy as antidiabetics. Data was collected using questionnaires, and plant identification was based on the Algerian floras of **Quezel** and **Santa** (1962-1963) and **Ozenda** (2004). The data was processed using **Excel**.

The survey identified **52 medicinal plants**, both native and imported, belonging to **24 families**, with a total of **261** citations. Among them, 21 plants were mentioned only once, while 6 plants received more than 10 citations. These popular plants are: *Artemisia herba-alba* (45 citations), *Cinnamomum verum* (33), *Olea europaea* (27), *Trigonella foenum* (20), *Tecrium polium* (17), and *Marrubium vulgare* (12). Their popularity appears to be linked to their effectiveness, traditional use, and low cost.

The most commonly used plant parts in the study, in descending order, are: **leaves** (25 times), **seeds** (8times), **stems** (2times), and **fruits, fruit juice, flowers, floral buds, bulbs, sap, rhizomes, bark, and scales** (each mentioned once). The main preparation methods are **decoction** or **infusion** of the leaves, seeds, and aerial parts.

This research highlights the diverse range of plants used in traditional medicine for diabetes treatment in the region and underscores the importance of these plants in local healthcare practices, often attributed to their accessibility and traditional knowledge.

Keywords: Survey-antidiabetic-plants

INTRODUCTION

Diabetes is a major global public health problem, including in Algeria (WHO, 2016). To manage this condition, a significant portion of the population uses traditional herbal medicine (Boudjelal et al., 2013).

This practice is particularly common in regions where traditional medicine remains a primary level of healthcare.

In Algeria, previous ethnobotanical studies conducted in regions such as Sidi Bel Abbès have documented this usage (Miara et al., 2018).

These studies identified frequently cited plants, such as *Olea europaea* L. (olive tree) and *Artemisia herba-alba* (Miara et al., 2018).

Scientific research has confirmed that many medicinal plants contain promising active compounds, such as flavonoids with antioxidant properties (Bentabet et al., 2020). However, knowledge of these traditional remedies is often local and fragmented. It risks being lost with passing generations and urgently needs to be documented.

The Theniet El Had region in the wilaya of Tissemsilt is an ideal location for a new investigation. This area is home to the Theniet El Had National Park, renowned for its exceptional biodiversity and its forest of Atlas cedars (Belouahem et al., 2011).

This unique ecosystem is likely to host a diverse medicinal flora, valued by local know-how that remains largely unstudied. No systematic study has been published specifically on the antidiabetic plants from this precise locality.

The present study therefore aims to fill this gap.

Its main objective is to document and identify the plant species used against diabetes by the local population. It will document the methods of preparation (decoction, infusion...), the parts of the plant used (leaves, roots...), and the traditional dosages applied.

This collection effort is essential for preserving a threatened ethnobotanical heritage.

The results will constitute a valuable database for the region.

They could guide future phytochemical and pharmacological research in the laboratory (Bentabet et al., 2020).

The ultimate goal is to scientifically validate the efficacy of these traditional remedies.

This study thus contributes to the valorization of local natural resources.

In the long term, it could participate in the discovery of new active compounds for improved antidiabetic drugs.

MATERIALS AND METHODS:

An ethnobotanical survey was conducted, using a questionnaire, over a two-month period: January and February 2019. The survey was conducted among 260 diabetic and non-diabetic individuals. This involved random sampling of both diabetic and non-diabetic persons.

Description of the Study Area:

This study was carried out in the Theniet El Had region, in the wilaya (province) of Tissemsilt. It is located southwest of the capital. The wilaya is situated in the high plains region and is bounded as follows:

To the north, by the wilaya of Ain Defla.

To the west, by the wilayas of Relizane and Chlef.

To the east, by the wilaya of Médéa.

To the south, by the wilayas of Tiaret and Djelfa.

Regarding the questionnaire; it was developed by Dr. Elassenouni, Dr. Benmokhtar, and Prof. Smati, and subsequently validated by two professors: in epidemiology (Prof. Ait Oubelli Kamel) and in diabetology (Prof. Aissa Boudiba).

The survey questionnaire form comprises 4 sections for collecting:

I. Information about the interviewee: (age, sex, weight, academic level, profession, place of residence, and languages spoken)

II. Information about the illness: (Diabetic or not, duration of diabetes, type of diabetes, treatment - follow-up and complications, controlled biological parameters, associated pathologies, diet, physical activity, family history of diabetes)

III. Information on traditional medicine and diabetes treatment: (Knowledge or lack thereof of plants known to be anti-diabetic, through which means? Reason for use)

IV. Information on medicinal plants used as anti-diabetics by the local population:

(Vernacular name of the plants, plant type, parts used, preparation method and duration of use, frequency of plant use, effect on glycemic control, impact on medical treatment, observed side effects, and usage precautions)

-The questionnaire was administered in the waiting rooms of various departments at: hospitals of Theniet El Had (wilaya of Tissemsilt).

For the Listing of Plants and References:

Initially, a list of vernacular names of medicinal plants used by this population was created. The taxonomic identification of the plants and the definitive determination of their botanical names, their French names, and their families were carried out by referring to documents: the Flora of Algeria [Quezel and Santa, 1962-1963], Flora and Vegetation of the Sahara [Ozenda 2004].

The plant families were classified according to the number of citations in descending order.

For processing the survey data, we used software, namely Excel; an important tool for processing survey data. After entering the questions and answers collected from the interviewees, we proceeded to analyze the gathered data.

Fiche N° / / - / /	ENQUETE ETHNOBOTANIQUE SUR LES PLANTES MEDICINALES UTILISEES COMME ANTIDIABETIQUES DANS LA REGION DE	Date de l'enquête / / - / / - / /	Page 1/2
I- PROFIL DE L'INFORMATEUR			
Date de naissance :	/ / - / / - / /	Q5- Prenez-vous votre traitement correctement ? • 1- Oui ; • 2- Non	/ /
Age (ans) :	/ /	Si non, pourquoi ?	
Sexe : • 1- Masculin ; • 2- Féminin	/ /	Q6- Faites-vous régulièrement un contrôle médicale et/ ou biologique ?	
Poids (Kg) :	/ / , / /	• 1- Oui ; • 2- Non	/ /
Niveau académique : • 0- Analphabète • 1- Primaire • 2- Secondaire • 3- Universitaire gradué • 4- Universitaire post-gradué	/ /	Si oui, quels sont les paramètres dosés ? • Glycémie : 1- Oui ; 2- Non • Hémoglobine glyquée : 1- Oui ; 2- Non • Cholestérol : 1- Oui ; 2- Non • Triglycérides : 1- Oui ; 2- Non • Urée : 1- Oui ; 2- Non • Créatinine: 1- Oui ; 2- Non • Autres, précisez :	/ / / / / / / / / / / / / /
• Profession :		Q7- Avez-vous souffert de : • Troubles de la vue : 1- Oui ; 2- Non • Troubles rénaux : 1- Oui ; 2- Non • Troubles card. et de circulation: 1- Oui ; 2- Non • Hypertension artérielle : 1- Oui ; 2- Non • Pied diabétique : 1- Oui ; 2- Non • Autres, précisez :	/ / / / / / / / / /
Région de résidence : • Wilaya : • Commune :	/ / / /	Q8- En plus du diabète, êtes-vous atteints d'une autre maladie ? • 1- Oui ; • 2- Non Si oui, la(ou les)quelle(s) ?....	/ / / /
Langues parlées : • 1- Arabe : 1- Oui ; 2- Non • 2- Amazighe : 1- Oui ; 2- Non • 3- Français : 1- Oui ; 2- Non • 4- Autre(s), précisez :	/ / / / / / / /	Q9- Suivez-vous actuellement un régime alimentaire à cause du diabète ? • 1- Oui ; • 2- Non * Si oui, Ce régime a été imposé par le médecin ? • 1- Oui ; • 2- Non * Quels sont les aliments que vous ne mangez pas ?	/ / / / / /
II- INFORMATION SUR LE DIABETE			
Q1- Êtes-vous diabétique ? • 1- Oui ; • 2- Non → Si oui,	/ /	Q10- Êtes-vous fumeur ? • 1- Oui ; • 2- Non Si oui, combien de cigarettes/ jour ?	/ /
Q2- Quel type de diabète ? • 1- Diabète type 1 • 2- Diabète type 2 • 3- Diabète gestationnel • 4- Autre, précisez :	/ /	Q11- Un membre de votre famille a-t-il le diabète ? • 1- Oui ; • 2- Non Si oui, qui ?	/ /
Q3- Quelle est la date du diagnostic de diabète ? / / - / / - / /		Q12- Pratiquez-vous régulièrement du sport ou de la marche ? • 1- Oui ; • 2- Non Si oui, dans quelle fréquence ?	/ /
Q4- Quel traitement suivez-vous ? • 1- Diététique : 1- Oui ; 2- Non • 2- Insulinothérapie : 1- Oui ; 2- Non • 3- Antidiabétiques oraux : 1- Oui ; 2- Non précisez les antidiabétiques oraux	/ / / / / /		

Fiche N° / / - / - /	ENQUETE ETHNOBOTANIQUE SUR LES PLANTES MEDICINALES UTILISEES COMME ANTIDIABETIQUES DANS LA REGION DE	Date de l'enquête / / - / - /	Page 2/2
III- MEDECINE TRADITIONNELLE ET TRAITEMENT DU DIABETE			
Q1- Connaissez-vous des plantes médicinales réputées antidiabétiques ?			
• 1- Oui ; • 2- Non	/ /		
Si oui, par quel biais ?			
• Héritage familial / Expérience des autres dans votre entourage : 1- Oui ; 2- Non	/ /		
• Herboriste / Tradipraticien : 1- Oui ; 2- Non	/ /		
• Pharmacien / Médecin : 1- Oui ; 2- Non	/ /		
• Culture générale : 1- Oui ; 2- Non	/ /		
• Formation professionnelle : 1- Oui ; 2- Non	/ /		
Q2- Si vous êtes diabétique, avez-vous déjà utilisé des plantes médicinales antidiabétiques ?			
• 1- Oui ; • 2- Non	/ /		
Si oui, quand est ce que vous les avez utilisées ?			
• 1- Avant d'avoir recours à la médecine conventionnelle	/ /		
• 2- Après échec de la médecine conventionnelle	/ /		
• 3- En complément de celle-ci	/ /		
* Pourquoi vous soignez-vous avec les plantes médicinales ?			
• 1- Elles sont sans danger	/ /		
• 2- Elles sont plus efficaces que les traitements médicamenteux commercialisés	/ /		
• 3- Par habitude	/ /		
IV- PLANTES MEDICINALES UTILISEES COMME ANTIDIABETIQUES PAR LA POPULATION LOCALE			
Q1- Quel est le nom vernaculaire ?			
.....			
Q2- Quel est le nom scientifique ?			
.....			
3- Quel est le type de plante ?			
• 1- Spontanée	/ /		
• 2- Cultivée	/ /		
• 3- Provenant d'une autre région du pays (préciser)	/ /		
.....	/ /		
• 4- Importée	/ /		
• 5- Inconnu	/ /		
Q4- Quelle(s) parties(s) utilisez-vous ?			
• Racine : 1- Oui ; 2- Non	/ /		
• Tige : 1- Oui ; 2- Non	/ /		
• Feuille : 1- Oui ; 2- Non	/ /		
• Fleur / inflorescence : 1- Oui ; 2- Non	/ /		
• Fruit : 1- Oui ; 2- Non	/ /		
• Graine : 1- Oui ; 2- Non		/ /	
• Plante entière : 1- Oui ; 2- Non		/ /	
• Autres (préciser).....		/ /	
Q5- Quelle est la période de récolte :			
• 1- Printemps : 1- Oui ; 2- Non	/ /		
• 2- Été : 1- Oui ; 2- Non	/ /		
• 3- Automne : 1- Oui ; 2- Non	/ /		
• 4- Hiver : 1- Oui ; 2- Non	/ /		
Q6- Quels sont la forme d'emploi et le mode de préparation ? (donnez la recette de la préparation)			
.....			
.....			
.....			
.....			
Q7- Quel est le mode d'administration :			
• 1- Oral	/ /		
• 2- Autre, précisez :	/ /		
Q8- Quelle est la dose utilisée ?			
Posologie (quantité / jour) :			
Q9- Quelle est la durée d'utilisation :			
Q10- Quelles sont les associations possibles ?			
.....			
.....			
Q11- Quel est le résultat de l'emploi des plantes médicinales ?			
• 1- Guérison ; • 2- Amélioration	/ /		
• 3- Stabilisation ; • 4- Inefficacité	/ /		
Q12- Quelles sont les variations du bilan glucidique ?			
• 1- Diminution des glycémies	/ /		
• 2- Diminution de l'HbA1c	/ /		
• 3- Augmentation de l'HbA1c	/ /		
• 4- Effet stationnaire	/ /		
Q13- Quel est l'impact sur le traitement médical ?			
• 1- Réduction des doses	/ /		
• 2- Arrêt du traitement	/ /		
• 3- Pas de changement	/ /		
Q14- Quels sont les effets secondaires observés ?			
.....			
.....			
Q15- Quelles sont les précautions d'emploi de la plante?			
.....			
.....			

Photo 01: Questionnaire form (BOUKHEMACHA 2019)

RESULTS AND DISCUSSION:

The results, presented in graph form, are as follows:

I. **Anthropological** (age, sex, and weight), Sociological, and Educational Data (academic level, profession):

II. Data Related to the Disease:

Type of diabetes, duration, conventional treatments, and complications.

III. Data Related to Plants Used in the Treatment of Diabetes:

Scientific name, vernacular name, family, frequency of citation, part used, and mode of preparation.

52 listed plants belonging to 24 different families, some of which are spontaneous, others are imported.

The list of plants is given in the following table:

Out of 261 citations, 52 medicinal plants belonging to 24 families were listed and ranked by number of citations. 21 of these plants were cited only once, and 06 plants from this list had more than 10 citations. Their popularity could be attributed to tradition, their effectiveness, and their low cost.

These plants are: *Artemisia herba-alba* (45 citations), *Cinnamomum verum* (33 citations), *Olea europaea* (27 citations), *Trigonella foenum-graecum* (20 citations), *Teucrium polium* (17 citations), *Marrubium vulgare* (12 citations), *Allium sativum* (8 citations), *Thymus vulgaris* (8 citations).

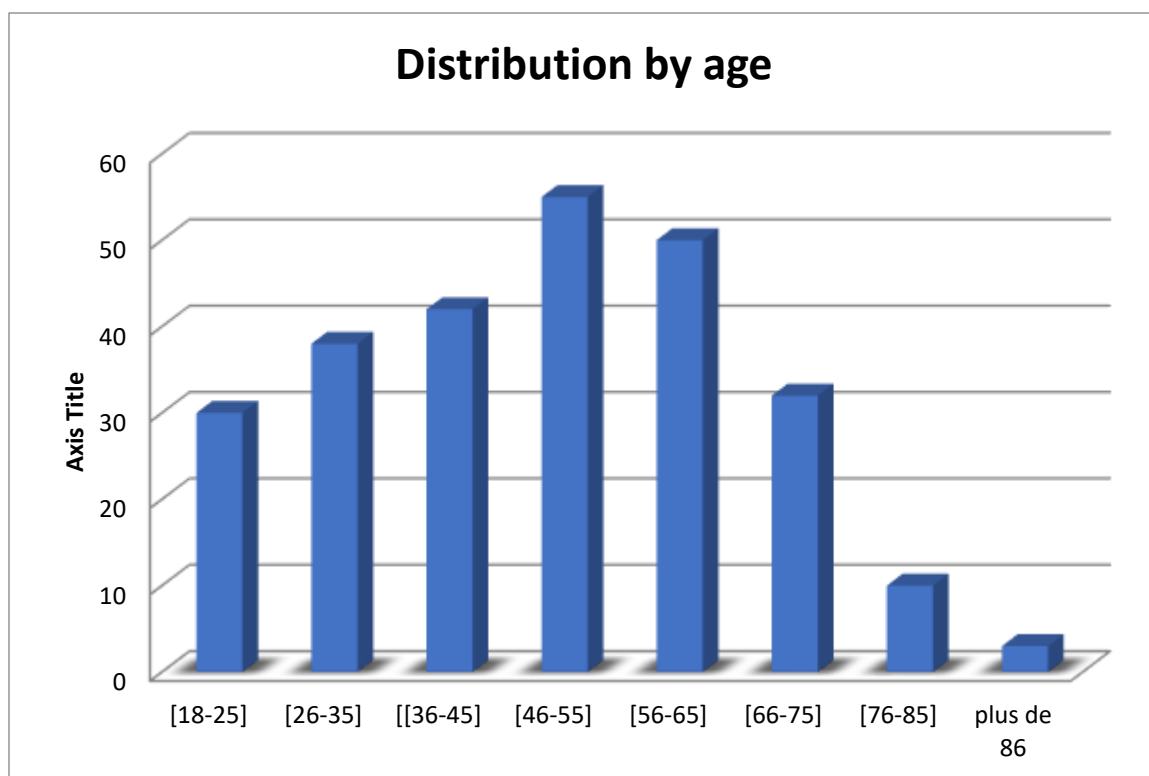
Although in many citations more than one part of the plant is used, the most frequently used plant parts in our study, in descending order, are: leaves (25 times), seeds (8 times), stems (2 times), fruits (1 time), fruit juice (1 time), flowers (1 time), floral bud (1 time), bulb (1 time), sap (1 time), rhizome (1 time), bark (1 time), scales (1 time).

In this study, decoction or infusion of leaves, seeds, and aerial parts are the main modes of preparation.

Based on the collected information, the use of medicinal plants remains either a standalone treatment or a complementary one to conventional medications.

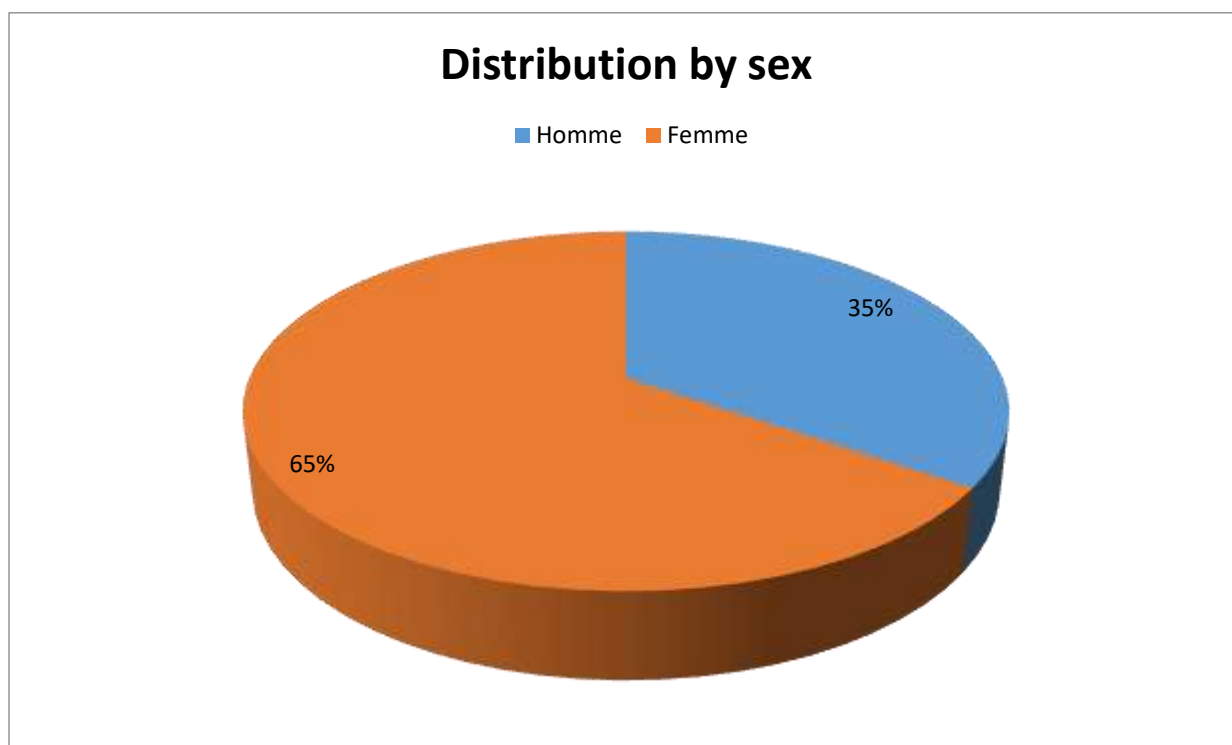
Based on the results of the ethnobotanical survey and bibliographic data, we have selected the plant for our experimental study: the Bitter Almond tree (*Prunus dulcis* var. amara).

Bitter Almond was selected for research into antidiabetic activity because, on one hand, the plant is used by the target population despite its toxicity, and on the other **hand, we noted** from our bibliographic research that there are few studies on its antihyperglycemic effect.

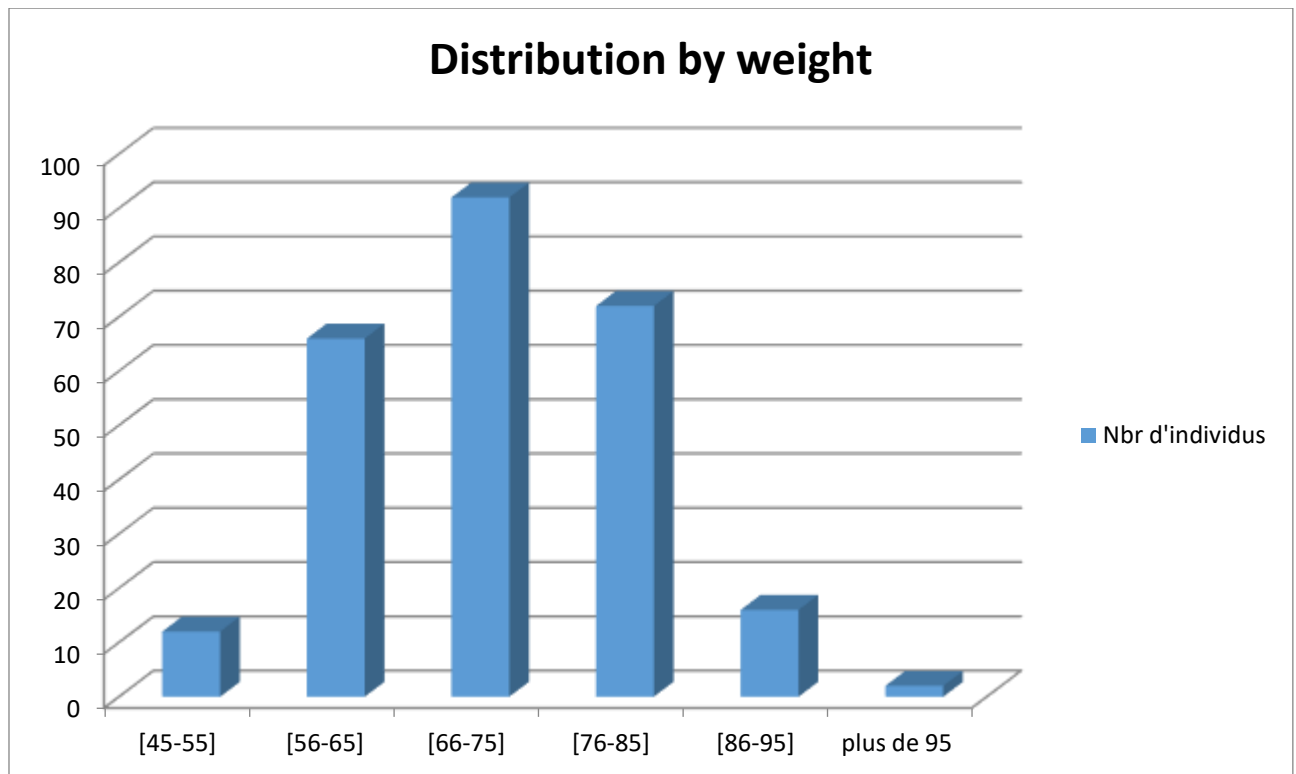


Distribution by Age: The age of the studied population ranged from 18 to 93 years, with an average age of 55 years.

The frequency of diabetics in the studied population increases with age, with the age group most affected by diabetes being between 46 and 60 years.

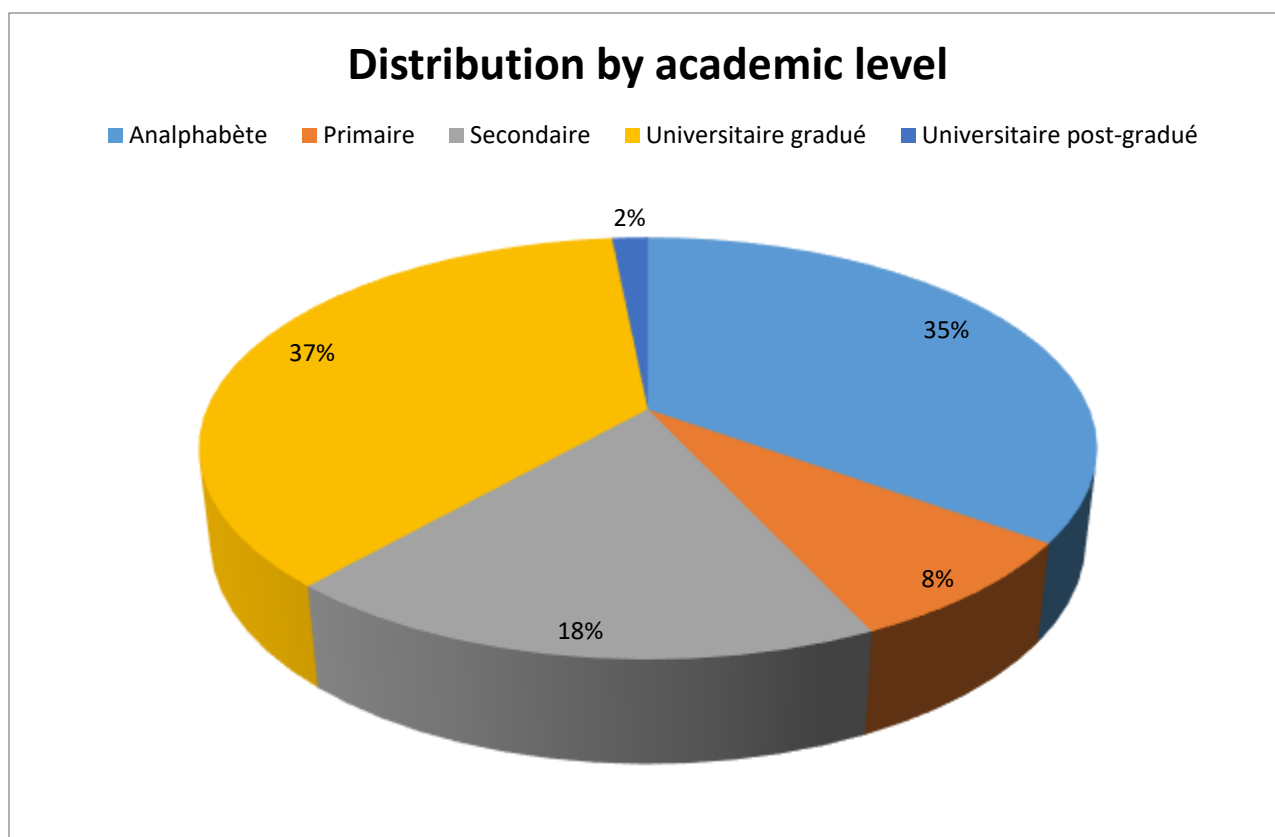


Distribution by Sex: The distribution of respondents by sex showed an overall predominance of women (169 women, representing 65% of the total studied population) compared to men (91 men, representing 35%), i.e., a female/male ratio of 1.85. (Women were more cooperative and sociable, while men were more rushed and less cooperative).

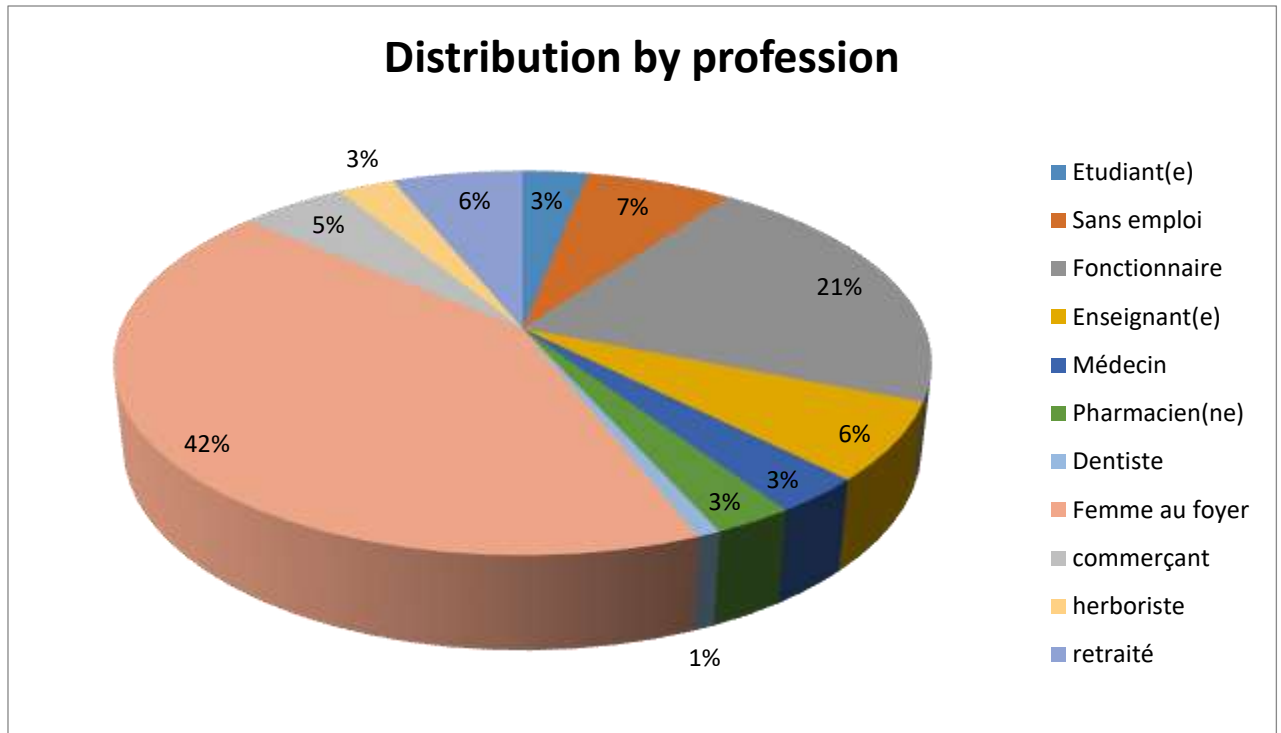


Distribution by Weight: It is observed that the most significant weight range is between 66 and 76 kg, followed by 76 to 85 kg, then 56 to 65 kg, while individuals with a weight greater than 86 kg represent only a small percentage.

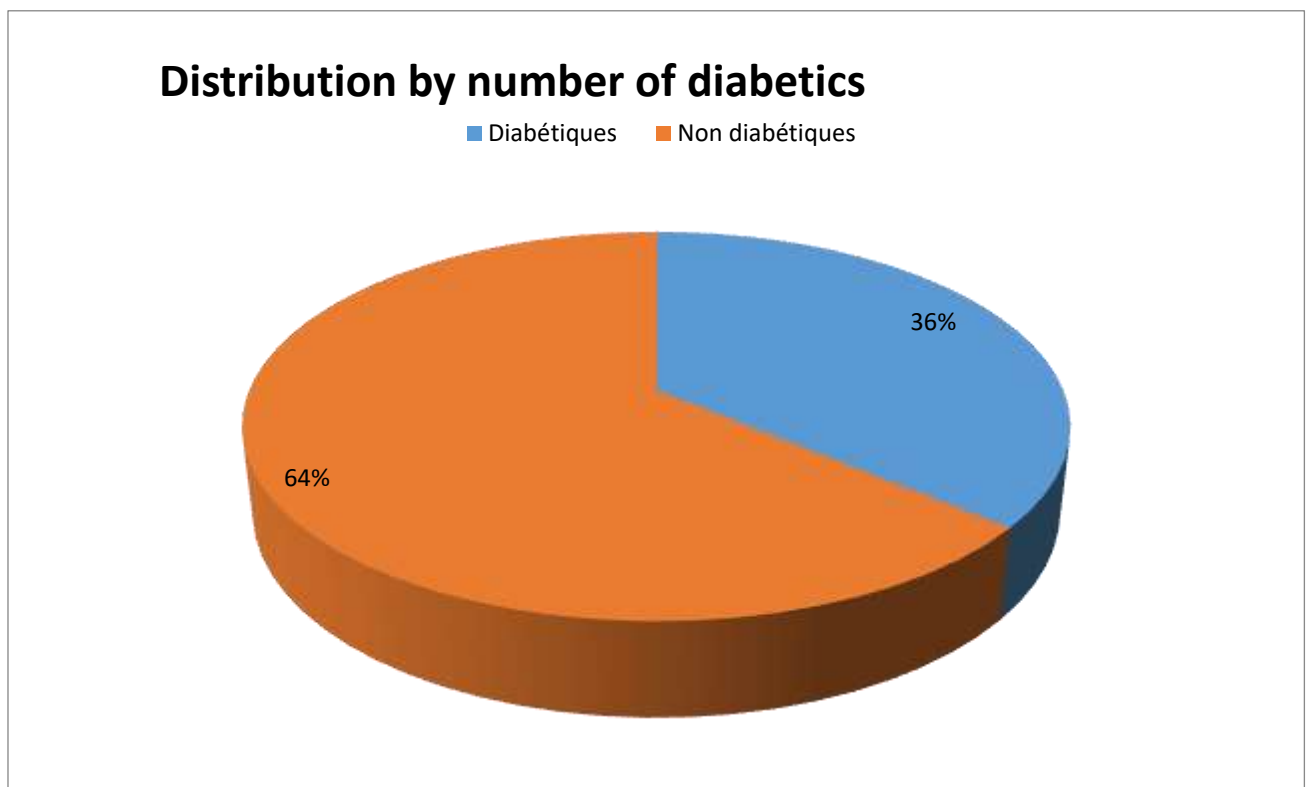
This is positive for the studied population. This can be explained by the fact that the randomly selected sample are not all diabetics, and also because the majority of diabetics follow a diet and engage in physical activity.



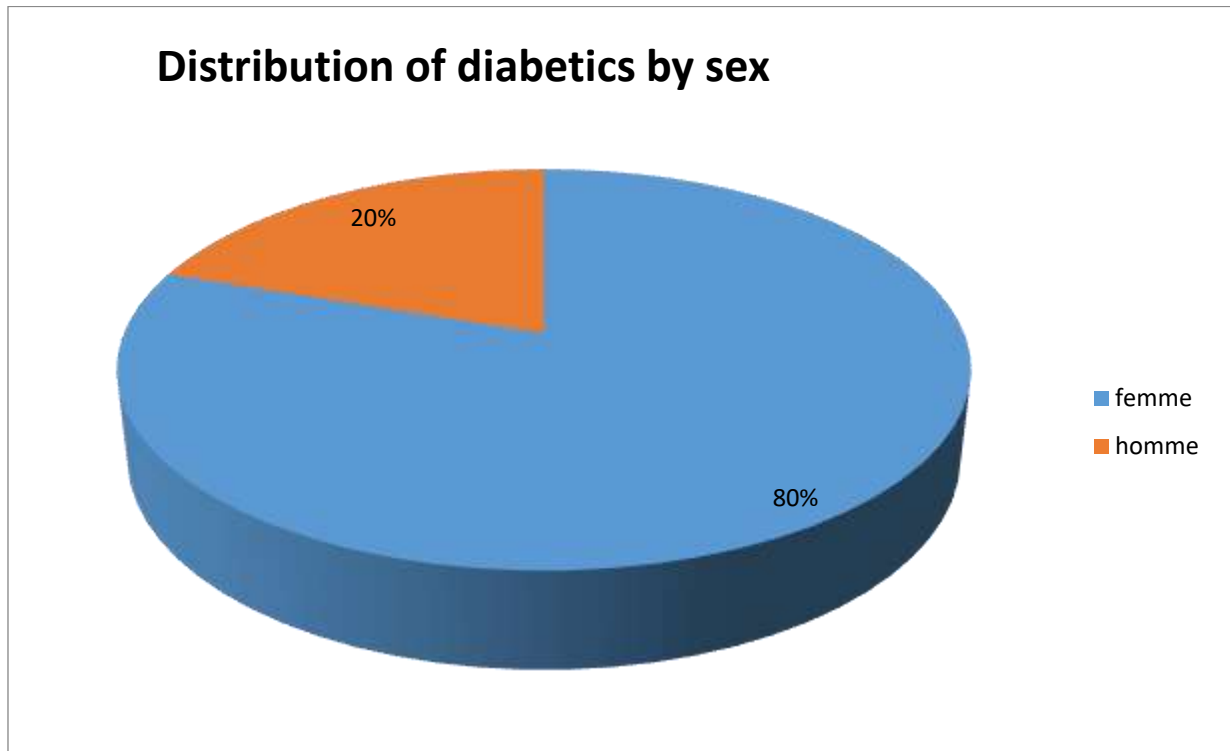
Distribution by Academic Level: Here we observe a percentage of 37% illiterates, followed by 35% with a university level, 18% with a secondary level, and finally university graduates and post-graduates with 8% and 2% respectively.



Distribution by Profession: We observe 42% housewives (whether illiterate or not), followed by 21% civil servants (regardless of their academic level), 7% unemployed, 6% teachers. Students, doctors, pharmacists, and dentists represent only a small percentage.

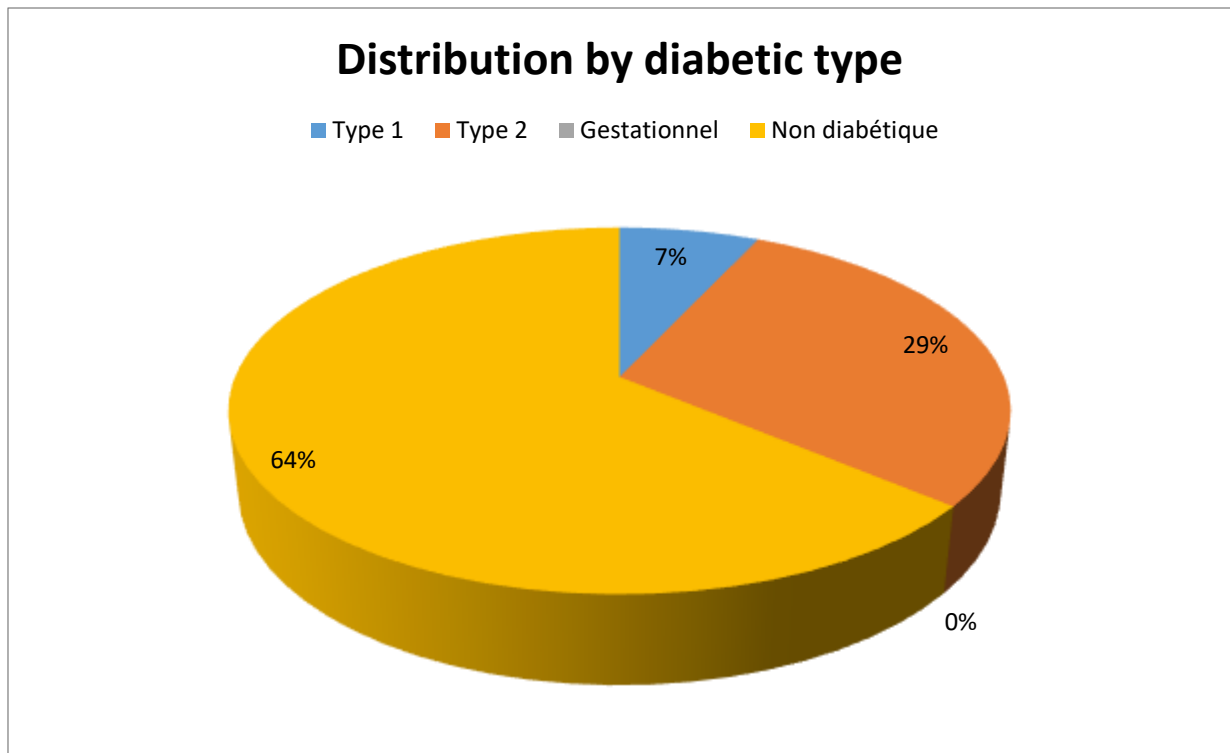


Distribution by Number of Diabetics: 64% of the surveyed population are non-diabetics and 36% are diabetics.



Distribution of Diabetics by Sex: Among the 36% of diabetics surveyed (96 men and women): 20% are men (19, of which 4 Type I and 15 Type II) and 80% are women (77, of which 14 Type I and 63 Type II).

Risk factors such as obesity and sedentary lifestyle, generally more prevalent in women, may explain this difference between the two sexes. However, sex as such is not considered a risk factor for both types of diabetes.



Distribution by Type of Diabetes: We note a percentage of 29% for Type 2 diabetes and 7% for Type 1 diabetes. Similarly, we did not record any cases of gestational diabetes or other types of diabetes.

Plant	Scientific name	Family	Number of citat°	Part used	Mode of préparation
L'armoise	<i>Artemisia herba-alba</i>	Lamiaceae	45	leaves	Infusion
Cinnamon	<i>Cinnamomum verum</i>	Lamiaceae	33	Stem	Decoction
Olive tree	<i>Olea europaea</i>	Oleaceae	27	leaves	Infusion
Fenugreek	<i>Trigonella foenum</i>	Fabaceae	20	Seeds	Decoction
Germander	<i>Teucrium polium</i>	lamiaceae	17	Leaves	Infusion
Le Marrube	<i>Marrubium vulgare</i>	Lamiaceae	12	Leaves	Infusion
Garlic	<i>Allium sativum</i>	Amaryllidaceae	08	bulb	Eat raw
thyme	<i>Thymus vulgaris</i>	Lamiaceae	08	Leaves	Infusion
La sauge	<i>Salvia officinalis</i>	Lamiaceae	05	Leaves	Infusion
Lemon	<i>Citrus limon</i>	Rutaceae	05	fruit	Decoction
Lavender	<i>Lavandula stoechas</i>	Lamiaceae	05	Leaves	Infusion
Mint	<i>Mentha spicata</i>	Lamiaceae	05	Leaves	Infusion
Mulberry	<i>Morus sp</i>	Moraceae	05	Leaves	Infusion
Bitter almond	<i>Prunus amygdalus</i> var <i>amara</i>	Rosaceae	04	Seeds	Powder to mix with honey
ivette musquée	<i>Ajuga iva</i>	Lamiaceae	04	Leaves	Infusion
Colocynth	<i>Citrullus colocynthis</i>	Cucurbitaceae	03	Seeds	To suck
Bitter lupine	<i>Lupinus albus</i>	Fabaceae	03	Seeds	Decoction
Ginger	<i>Zingiber officinale</i>	Zingiberaceae	03	rhizome	Decoction
Aquilaria	<i>Aquilaria sp</i>	Thymelaeaceae	03	Leaves	Infusion
L'écorce du grenadier	<i>Punica granatum</i>	Lythraceae	03	Bark	Decoction
Curcuma	<i>Curcuma longa</i>	Zingiberaceae	02	Stem	Decoction
Zygophyllum	<i>Zygophyllum sp</i>	Zygophyllaceae	02	Aerial part	Decoction
Aristolochie	<i>Aristolochia sp</i>	Aristolochiaceae	02	Leaves	Decoction
Coriander	<i>Coriandrum sativum</i>	Apiaceae	02	Leaves	Infusion
Globularia	<i>Globularia sp</i>	Plantaginaceae	02	Leaves	Infusion
Diss gras	<i>Ampelodesma mauritanicus</i>	Poaceae	02	Leaves	Decoction

Celery	<i>Apium graveolens</i>	Apiaceae	02	Leaves	Infusion
Myrtle	<i>Myrthus communis</i>	Myrtaceae	02	Leaves	Infusion
Henna	<i>Lawsonia inermis</i>	Lythraceae	02	Leaves	Decoction
chardon-marie	<i>Silybum marianum</i>	Asteraceae	01	Flower bud + Leaves	Decoction
Black cumin or Black seeds	<i>Nigella sativa</i>	Renonculaceae	01	Seeds	Decoction
indian Costus	<i>Saussurea Costus</i>	Asteraceae	01	Bark	Decoction
Oleander	<i>Nerium oleander</i>	Lauraceae	01	Leaves	Foot bath
Rue or herb of grace	<i>Ruta graveolens</i>	Lamiaceae	01	Leaves	Infusion
Dorema	<i>Dorema ammoniacum</i>	Apiaceae	01	Gum	To suck
Onion	<i>Allium sepa</i>	Amaryllidaceae	01	Scales	Decoction
Watercress seeds	<i>Nasturtium officinale</i>	Brassicaceae	01	Seeds	Decoction
Fennel seeds	<i>Foeniculum vulgare</i>	Apiaceae	01	Seeds	Decoction
Clove	<i>Syzygium aromaticum</i>	Myrtaceae	01	Flower bud	Decoction
Nono,pomme- chien	<i>Morinda citrifolia</i>	Rubiaceae	01	fruit juice	For drinking
Karouvaipilai	<i>Murraya koenigii</i>	Rutaceae	01	Leaves	Infusion
Aloe vera	<i>Aloe vera</i>	Aloeveraceae	01	Sap	For drinking
Vervain	<i>Verbena officinalis</i>	Lamiaceae	01	Leaves	Infusion
Absinthe	<i>Artemisia absinthium</i>	Asteraceae	01	Leaves	Infusion
Bay laurel	<i>Laurus nobilis</i>	Lauraceae	01	Leaves	Infusion
L'alfa	<i>Stipa tenacissima</i>	Poaceae	01	Leaves	Decoction
Bitter melon	<i>Momordica charantia</i>	Cucurbiataceae	01	Leaves	To suck
Rosmary	<i>Rosmarinus officinalis</i>	Lamiaceae	01	Leaves	Infusion
Arroche des jardins	<i>Atriplex hortensis</i>	Amaranthaceae	01	Leaves	Infusion
Chamomille	<i>Chamaemelum nobile</i>	Asteraceae	01	Flower	Infusion

Table 01: Listing of plants used as antidiabetic by the population

CONCLUSIONS

At the conclusion of our study, the following key findings emerge:

-The ethnobotanical survey conducted in the region of Theniet El Had, in the wilaya of Tissemsilt, among 260 people, allowed us to identify 52 plants belonging to 24 different families.

-Some plants are used in the treatment of diabetes despite their toxicity, as is the case with *Prunus amygdalus var. amara* (bitter almond), the plant on which our study focused.

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LITERATURE REVIEW OF GROWING CONDITIONS OF NATURAL LINDEN STANDS (*Tilia cordata* Mill., *Tilia platyphyllos* Scop. and *Tilia tomentosa* Moench.) IN BOSNIA AND HERZEGOVINA

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ABSTRACT

The *Tilia* complex in Bosnia and Herzegovina does not constitute a large percentage of forest trees and their stands in the overall forest ecosystem fund, but it has significant vitality and potential for future commercial production of wood needed for industry and various social needs. In addition, linden trees provide significant shade and nutrients for beneficial insects, which is why the importance of these trees in terms of protecting the local climate of forests and cities and the survival of insect populations is extremely high. This article reviews the literature dealing with the natural conditions of growth and development of linden plant communities in Bosnia and Herzegovina. The basis for selecting the vegetation or phytocoenological areas to be studied was information from the "MOE-Scholarship" - DBU report on the conducted scientific research on linden in Bosnia and Herzegovina, i.e. care was taken to describe the natural habitat conditions of linden forest stands in those localities where the trees described in that report were found and measured. The results of the research indicate that the conditions of natural habitats where *Tilia* trees grow are very heterogeneous, and that in Bosnia and Herzegovina there are various types of soil, such as mosaic, cambisol and gley soils. In addition, soil moisture conditions and the amount of precipitation are also very different from one part of the country to another, which is why planting forest trees in some parts of Bosnia and Herzegovina is difficult or inadvisable, while in some areas it is better to plant linden trees (forests at all). In terms of rockiness, the natural habitats are also very different, so it is possible to find linden forests on extremely shallow lands on limestone rocks, but also in river canyons, for example on sandstone or as they emerge from the rocks. This research should help in understanding the habitat conditions in which noble tree species grow, but at the same time it can also search for potential solutions for future nursery production of these species, which is why this research can be considered significant from the aspect of forestry production.

Keywords: *Growing conditions, Natural stands, Linden, Tilia cordata, Tilia platyphyllos, Tilia tomentosa, Bosnia and Herzegovina*

1.INTRODUCTION

The literature on linden in Bosnia and Herzegovina mainly originates from the time of the Socialist Federal Republic of Yugoslavia, of which the most prominent is the book by Herman (1971) from the University of Zagreb (Croatia), in which the main linden species (*Tilia cordata* Mill., *Tilia platyphyllos* Scop. and *Tilia tomentosa* Moench.) in the former country of Yugoslavia are described in great detail. Similar to most European countries, writing on linden has stagnated for several decades, while in Bosnia and Herzegovina there have been almost no scientific articles on linden in the past 30 years or so. The rare research papers on silver-leaved linden in the Western Balkan countries have been mainly published in the neighboring Republic of Serbia (due to the existence of a large linden complex on Fruska Gora Mountain), while in Bosnia and Herzegovina, an article by Vejzagić, Ballian and Memisević-Hodžić (2021) was

published in 2021, which deals with the morphological diversity of *T. tomentosa* trees in natural forest populations in the country, with control populations in the Republic of Serbia. Interestingly, the article states that *T. tomentosa* also grows in river canyons, which indicates a stable distribution of this species in thermophilic habitats of the Western Balkans.

As part of the scholarship program for young scientists - researchers, called "MOE scholarships", which enabled research at institutes in Germany, the first post-war research on small-leaved (*T. cordata* Mill.), large-leaved (*T. platyphyllos* Scop.) and silver-leaved (*T. tomentosa* Moench.) lindens was carried out (DBE.de, 2021), which indicates the existence of significant differences in the morphological and genetic characteristics of the three main linden species in Bosnia and Herzegovina, with numerous hybrids and morphological varieties. The existence of differences in morphological characteristics between silver-leaved lindens in Bosnia and Herzegovina and in Serbia is also indicated by the research of the authors Vejzagić, Ballian and Memisević-Hodžić (2021), who described the typical appearance of *T. tomentosa* leaves in Bosnian and Herzegovinian populations in a published article. The type of fruit with multiple bracts, which was found in *T. tomentosa* trees in the thermophilic forests in Herzegovina area, was first described by Vejzagić (2022), in a Short communication within Forestry journal of Forestry faculty from Banja Luka. Although the linden was considered to be relatively well researched and described in detail in Europe, since clones of its hybrids were massively used for planting, as described in an article by Pigott (1992), it is suspected that there are still many unexplored aspects of the genus *Tilia*. A significant contribution to the understanding of the behavior of the genus *Tilia* from the onset of the Ice Age to the present day has obviously been made by authors such as Fineschi et al. (2003), who have shown the essence of why *Tilia* clones are still so successfully used in planting today, since these are trees whose pollen, according to pollen maps, once covered the entire European continent. There are very few written documents on usage of linden clones in Bosnia and Herzegovina, and it is known that Bosnia and Herzegovina has a problem with the lack of statistical data, which are very important for planning future climate change mitigation strategy (Mueller and Hofmann, 2022). According to the Oral statement of the "Visoko" Forestry unit (2020), a large number of linden clones were planted by the former Yugoslav People's Army, during voluntary afforestation campaigns in the previous century, thanks to which a large number of trees still adorn the hills around Visoko, as well as numerous other municipalities and cities in Bosnia and Herzegovina. In this paper, it should be shown what the natural conditions of growth and development of *Tilia* trees are in natural habitats where large groups or forest stands of linden grow (currently mature linden trees and stands), that is, what the existing quality literature in Bosnia and Herzegovina says about soil conditions, rockiness, precipitation and annual temperatures. It is believed that this knowledge could help in understanding the conditions of the habitats in the context of climate change, that is, in understanding the potential for the linden to regenerate from nature on these habitats or possibly take over the primacy over time.

2.MATERIAL AND METHOD

Research obtained main information about the seven different ecological-phytocenological areas in Bosnia and Herzegovina, that are interesting in Forestry because of the existence of forest stand structures or interesting individual trees from the aspect of morphology, leaf hairiness or "gene pool". Since the simple review method of the given information was used, the main or key terms in the research are related to: 1) growing conditions, 2) natural stands, 3) linden species 4) bosnian-herzegovinian forest territory. The report (DBU.de, 2021) of the first post-war article on three linden species in Bosnia and Herzegovina (*Tilia cordata* Mill., *Tilia platyphyllos* Scop. and *Tilia tomentosa* Moench.) was used as a background for this research, where the description of the main habitat conditions in

the natural forests / locations of the researched trees was gathered from the relevant scientific literature in Bosnia and Herzegovina as follows: 1) doctoral thesis Kvesic (2018), and 2) Stefanovic (1983).

Author Kvesic (2018), in which PhD dissertation on *Acer campestre*, the basic characteristics of the ecological-phytocenological areas of bosnian-herzegovinian forests are very thoroughly described, gathered previously relevant literature and brought all the most important information on climate, altitudes, plant communities and humidity conditions to one place. It is important to note that the ecological-vegetation communities of the forest populations, where the three main linden species in Bosnia and Herzegovina also grew as individual or group trees, were described decades earlier by Stefanovic *et al.* (1983), whose research work serves as fundament for many forestry studies.

Main informations on potential for planting *Tilia* were founded in the “Republika Srpska Afforestation Master Plan” (Govedar and Mataruga, 2021) while the main information for the *Tilia* frost sensitivity was extracted from the “Afforestation Manual” (Visnjic, 2024) (supported by the Association of Forestry Engineers and Technicians of Bosnia and Herzegovina), were different tree species used for afforestation in Bosnia and Herzegovina are presented.

3.RESULTS AND DISCUSSION

This chapter is divided into two parts, the first of which deals with a review of information on the natural conditions of forest ecosystems for several different ecological-phytocoenological areas. The second part of the chapter points out the importance of preserving the existing “gene pool” of *Tilia* trees in natural ecosystems.

3.1.Brief review of most important data on seven ecological-phytocenological area in Bosnia and Herzegovina

This sub-chapter provides brief reviews on main information about the seven different ecological-phytocenological areas in Bosnia and Herzegovina: 1) Northern Bosnia area, 2) Lower Drina area, 3) West Bosnian-Karst-Dolomite area, 4) Central Bosnian area, 5) Zavidovici-Teslic area, 6) Sub-Mediterranean-mountainous area, and 7) Sub-Mediterranean-montane area.

3.1.1.Basic information about the Northern Bosnia ecological-phytocenological area

Kvesic (2018) described in detail the Northern Bosnian area/region as a special ecological vegetation region in Bosnia and Herzegovina, which is characterized by an altitude amplitude of 80 to 980 meters above sea level. The climate is moderately continental, with about 55% of the total precipitation during the growing season. Potential evapotranspiration is greater than precipitation during the growing season (0.91), while other parameters indicate continental and xerothermal climate. The vegetation period lasts 180-200 days. From the aspect of geomorphology, the northern Bosnian region is characterized by the presence of alluvial plains of the Sava River and the lower reaches of the Vrbas River, the Ukrina and the Bosna River with its tributaries Usora and Spreca, as well as deluvial terraces. There is a raised hilly relief, mainly of Tertiary sediments, which are mostly covered by carbonate-free loess. Pseudogley dominates as a soil type in this area, while district cambisol can also be found on tertiary sediments. There are also combinations of eu-gley and semi-gley, rendzine on marly soils, chanco-cambisol on limestone and independent soil areas of fluvisol and pelosol. These soils are not favorable from the aspect of water-physical properties, due to their heavy mechanical composition. Dystric cambisols occur on sloping terrains, which are more favorable from the aspect of water-physical properties. The North Bosnian area is characterized by

widespread forests of sessile oak (*Q. petraea*) and hornbeam (*C. betulus*), while in favorable locations there are also present forests of European beech or *Fagetum-montanum*, as well as sessile and Austrian oak (*Q. cerris*) forests. In the lowland areas, there are more present pedunculate oak (*Q. robur*) forests, and hornbeam forests without previously described mixtures.

3.1.2. Basic information about the Lower Drina ecological-phytocenological area

Kvesic (2018) provides an overview of the basic characteristics of the Lower Drina region, which is characterized by an altitude range of 80 to 1020 meters above sea level. The area is characterized by a predominantly continental climate. Alluvial plains and deluvial terraces occur in low areas. About 55% of annual precipitation falls in the growing season, while the ratio of precipitation and potential evapotranspiration is **very unfavorable** (0.82). The vegetation period lasts 190-210 days. Alluvial plains characterize the part from which the Majevisa mountain rises, while south of Zvornik city this area turns into a mountain massif above the Drina river canyon. The building consists of alluvial sediments, Eocene flysch, andesitic-dacite eruptives, and Paleozoic and Triassic sediments. Automorphic and hydromorphic soils are the most common. Forests of pedunculate oak and hornbeam are very common, followed by forests of Hungarian (*Q. frainetto*) and Austrian oak (*Q. cerris*), as well as sessile oak forests with hornbeam, and European beech forests (secondary phytocenoses are acidophilic mountain beech forests).

3.1.3. Basic information about the West Bosnian-Karst-Dolomite ecological-phytocenological area

West Bosnian-Karst-Dolomite area as an area that mostly belongs to the mountain range from 300 to 800 meters above sea level, which is characterized by insufficiently clear penetrations of the Mediterranean and continental climate (Kvesic, 2018). The subalpine zone has an amplitude of 800-1900 meters above sea level. Kvesic was exploring information about the plant communities written by Drinic, according to which the influence of the continental climate prevails in this area in the winter, but the Mediterranean climate has a stronger influence in the summer. In the growing season, a smaller part of the annual precipitation falls, however, the proportions of precipitation and evapotranspiration are still favorable. The vegetation period lasts 120-200 days, depending on the altitude. The area is built from different limestone-dolomite massifs and plateaus, while in places there are traces of glacial activity. The presence of dolomite is more frequent compared to silicate rocks, and district cambisols are mostly found on the silicate substrate. There are also very shallow soils, with a pronounced skeletal structure and a heavy mechanical composition, but there are also deeper soils, which are deluvially colluvial, as well as carbonate-free soils on limestone, which, depending on the position and size, **can meet the conditions necessary for raising forest tree nurseries**. The author cites the following phytocenological communities as the most common: 1) *Piceo-Abieti-Fagetum* (European beech and Silver fir forests with Norway spruce, 2) *Abieti-Fagetum* (European beech and Silver fir forests), 3) *Piceo-Pinetum illyricum* (Scots pine and Norway spruce forests, with Silver fir, 4) *Piceetum montanum inversum* (frosty Norway spruce forests of intrazonal character), 5) *Pinetum nigrae dinaricum* (Austrian pine forests, which grow in warmer habitats), 6) *Pinetum silvestris dinaricum* (Scots and Austrian pine forests, which grow in slightly cooler habitats), 7) *Fagetum subalpinum* (subalpine European beech forests), and 8) *Pinetum mugi* (scrub mountain pine forests). In the lower parts of this ecological-vegetation area grow sessile oak and hornbeam forests (*Quercus-Carpinetum*), European beech forests (*Fagetum montanum illyricum*), forests of European beech and maple (*Aceri obtusati-Fagetum*), forests of oak and European hop-hornbeam (*Quercus-Ostryetum* / *Orno-Ostryetum*), and forests of oriental hornbeam (*Carpinetum orientalis*).

3.1.4. Basic information about the Central Bosnian ecological-phytocenological area

Unlike the previous ecological-vegetation area, the Central Bosnian ecological-vegetation area is characterized by an altitude amplitude of 300 to over 2200 meters above sea level. The mountain climate dominates, and in the period from July to September, and from December to July, the presence of a continental climate in places near the city of Zenica can be felt. In the other months, the effects of the Mediterranean climate are pronounced, and in the growing season, about 48% of the annual precipitation falls. The ratio of precipitation and potential evapotranspiration is **not very unfavorable**, and it reaches around 0.92. The vegetation period lasts about 180-200 days. From the aspect of geomorphology, three units are distinguished within this area, namely: - the massif of the Central Bosnian Slate Mountains (mostly built of crystalline slates and rhyolites), - the undulating terrain of the Jurassic fleece, and - the Sarajevo-Zenica basin. Because of this, the land cover of the entire area is very heterogeneous and complex, i.e. isolated homogeneous soils can also be found, on which **it is possible to organize nursery production**. In this area, the forests of sessile oak and hornbeam (*Quercus-Carpinetum*) are mostly found, dominantly on hilly terrains in the valley of the river Bosna, and between the towns of Sarajevo and Zenica, while around the town of Travnik these forests are only sporadically represented. Residues of the pedunculate oak and hornbeam (*Carpinus betuli-Quercetum roboris*) forests can be found, as well as forests with a predominantly acidophilic character, known as *Luzulo-Fagetum*. *Blechno-Fagetum* forests are also found in fragments, while European beech and Norway spruce forests (*Piceo-Abieti-Fagetum*) dominate large areas. The subalpine zone is associated with forests of subalpine beech (*Fagetum subalpinum*) and subalpine spruce in cold locations (*Piceetum subalpinum*). Above them is a sum of scrub mountain pine trees (*Pinetum mugi*). A special feature of this area is the existence of a phytocenosis of green alder (*Athyrio-Alnetum viridis*), which is a glacial relic. Researchers can also find forests of silver fir and Norway spruce (*Abieti-Piceetum silicolum*) in this area, but also forests of Scots pine and Norway spruce (with silver fir) (*Piceo-Pinetum silicolum*), which represent secondary phytocenoses. Among the thermophilic phytocenoses, the author emphasizes European beech and maple forests (*Aceri obtusati-Fagetum*) and oak and European hop-hornbeam forests (*Quercus-Ostryetum carpinifoliae*), which are represented on limestone-dolomite outcrops. It is also possible to find thermophilic species of oriental hornbeam (*C. orientalis*). (Kvesic, 2018)

3.1.5. Basic information about the Zavidovici-Teslic ecological-phytocenological area

Describing the Zavidovici-Teslic area, Kvesic (2018) states the presence of heterogeneous orography and the height amplitude from 250 to 1328 meters above sea level. The region is dominated by a moderate-continental climate, and in the period July-August the strongest influence of the Mediterranean climate is felt. In the growing season, about 56% of the annual precipitation falls, and the scale of precipitation and potential evapotranspiration is favorable from a climatic point of view. The vegetation period lasts about 180-190 days. The relief is largely made of serpentinized peridotite, then of eruptives and cherts, while limestones are less common. The presence of distric cambisol is characteristic of acid silicate rocks, while the presence of eutric cambisol is characteristic of silicate rocks. Pseudogley soils can also be found, as well as mosaics of chalcocomelsols and chalcocambisols, which are very xerothermic and **not suitable for nursery production** of forest tree species. Among the forest phytocenoses, it is possible to find basophilic pine forests (*Erica Pinetum nigrae serpentinicum*, E. P., and *Erica-Quercetum petraeae*), then acidophilic sessile oak forests (*Quercetum petraeae montanum*), European beech and silver fir forests with Norway spruce (*Abieti-Fagetum*

siliciolum), Scots pine and Norway spruce forests (with silver fir) (*Piceo-Pinetum*), silver fir and Norway spruce forests (*Abieti-Piceetum*), and the remains of pedunculate oak and hornbeam forests (*Quercu-Carpinetum*) (on deluvial terraces). Only the peripheral parts of this ecological-vegetation area have the presence of beech habitats alternating with oak forests.

3.1.6. Basic information about the Sub-Mediterranean-mountainous ecological-phytocenological area

Describing the Sub-Mediterranean-mountainous area, the author (Kvesic, 2018) states that it is located in the higher parts of the Mediterranean-Dinaric region, where the height amplitude is 1800-2000 meters above sea level. Orographically, this is a distinctly mountainous area, which has a wide subalpine zone. Some parts of the area exceed an altitude of 2000 meters above sea level. The entire area is influenced by the Mediterranean climate, and about 36% of the total average annual precipitation falls in the growing season. The ratio of precipitation and evapotranspiration **is favorable** and reaches 1.06. The vegetation period lasts 120-190 days. Karst erosion and glaciations occur in the outer part of the Dinarides. The area is built of limestone and dolomite, while the karst fields contain tertiary sediments. The source parts of the Neretva were built from Jurassic fleece. There are various soil combinations, in the mosaic type of calcomelanosol-chalcocambisol-luvisol on a dolomite substrate, then rendzine complexes, as well as calcomelanosols-chalcocambisols on a dolomite and limestone substrate, as well as dystic and eutric cambisols and semigleys. Since the soils are generally quite shallow and the rockiness area is huge, changes in soil moisture are frequent which makes the area therefore **not ideal for nursery production** of forest tree species. Among the forest phytocenoses, European beech and silver fir forests (*Abieti-Fagetum*), secondary beech forests (*Fagetum montanum illyricum*) and oak stand structures are encountered on the surfaces exposed to the West and South.

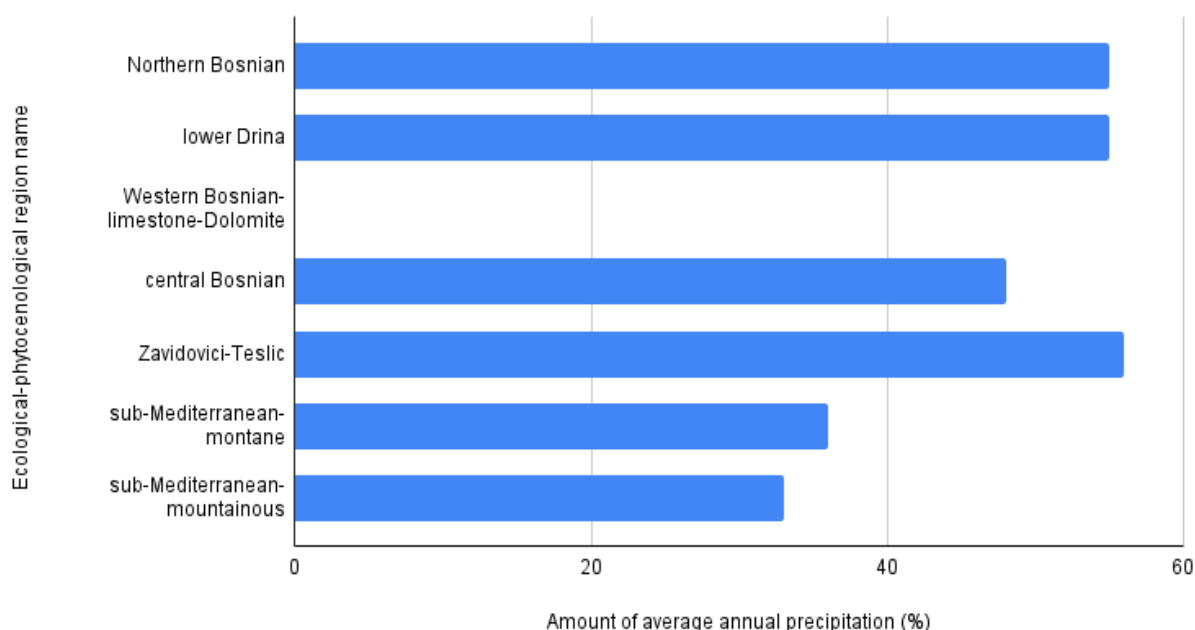
3.1.7. Basic information about the Sub-Mediterranean-montane ecological-phytocenological area

Describing the Sub-Mediterranean-montane area, Kvesic (2018) stated that the altitude ranges from 750 to 1200 meters above sea level. Orographically, this ecological-vegetation area is located in a hilly-mountainous zone, which is characterized by unfavorable meteorological conditions. The impact of the changed Mediterranean climate is felt, and in the growing season, about 33% of the average annual precipitation falls. The ratio of water income and expenditure is **unfavorable**, and reaches 0.86. Geomorphologically, this area is characterized by the southern slopes of the Dinaric mountains as well as smaller mountain units connected by plateaus and several smaller karst fields, which are made of limestone and dolomite. The soil types are mosaic like: 1) calcomelanosol-chalcocambisol-luvisol, 2) calcomelanosol-luvisol on limestone, 3) red like soil ("*Terra-rossa*"), while regosol and rendzina complexes can be found on clay sediments, - chalcocambisol and "*Terra rossa*" mosaics on limestone and dolomites, - lithosol and calcomelanosol mosaics on limestone (less common), while independent calcomelanosol can be found near fluvisol areas.

3.2. The importance of preserving the existing "gene pool" of *Tilia* trees in natural ecosystems.

Bearing in mind all the data provided in the sub-chapter 3.1., Graph 1 was created, which indicates the differences between the average annual amount of precipitation and potential evapotranspiration, according to Kvesic (2018) (in accordance with Stefanovic, 1983).

Amount of average annual precipitation (%) in the ecological-phytocenological regions in Bosnia and Heregovina



Graph 1. The amount of average annual precipitation in relation to the potential degree of evapotranspiration in different ecological-phytocenological areas in Bosnia and Herzegovina.

Comment: The reason for the absence of data for the Western Bosnia-limestone-Dolomite area is that it represents an extremely complex area, both from the aspect of the amount of annual precipitation, and from the aspect of the use of soil moisture in the most diverse parts of the terrain, where a large number of phytocenological communities are spread. Since this information is important from the aspect of the potential establishment of nurseries or *Tilia* plantations in the future, there is enough time to perform these analyzes in the future.

The graph implies a high degree of average annual precipitation in the Zavidovici-Teslic ecological-phytocenological area, while the lowest amount of this degree is in the Sub-Mediterranean-mountainous ecological-phytocenological area.

In this part of the article it is important to emphasize why the statements about the favorability of the natural habitat soils / conditions for installing plant nurseries were bolded. Although most of forest experts indicate that linden production is successful in any nursery, since the plant nurseries are well-stocked with the water, and that the main problem for *Tilia* growing is actually the dormancy of the seed, the climate change driven drought and need in the future to rationalize water spending is indicating that funding plant nurseries of certain plant species in their natural habitats / preferable conditions by these plants, what may help foresters to spend less water if having other habitat conditions optimised for those species (air temperature, soil type, adequate rockiness, enough humidity and soil depth, enough shadow from other surrounding plants such surrounding forests that regulates local forest climate and so on). If analysing the Master Plan for Afforestation for Republika Srpska (Bosnia and Herzegovina), there is huge potential for *Tilia* to be planted in different areas of the country. The foresters should take care of *Tilia* sensitivity to frost (Visnjic, 2024), since almost all the

local tree species in Bosnia and Herzegovina are sensitive to frost in early age.

The survival of linden as a species (*Tilia cordata* Mill., *Tilia platyphyllos* Scop. and *Tilia tomentosa* Moench.) is officially not endangered in Bosnia and Herzegovina, which does not exclude the need of monitoring unnecessary cuts. The fragmentation of the habitat of linden trees has greatly increased due to the anthropogenic factor in the past, because linden wood is very attractive and useful in agriculture, viticulture, carving and making various objects. Large linden habitats in the countries of the Bosnia and Herzegovina (DBU.de, 2021) are linden forests in Cazinska Krajina, the Una River Valley, in the Neretva River Canyon, and in the neighborhood (linden forests on Bilogora, Croatia and Fruška Gora, Serbia Republic), where about 22(23) species (Herman, 1971), subspecies, hybrids, pheno-forms and varieties of linden were identified and described. The Ice Age contributed to the fragmentation of the once widespread linden habitat throughout Europe (Fineschi et al., 2013, Klug, 2000), and the drop in average annual temperature caused numerous problems for the germination and fruiting of the large-leaved linden (DBU.de, 2021). The small-leaved linden tolerated the drop in temperature somewhat more easily and in some regions of the north it still grows in ancient habitats. The southernmost parts of Europe and warmer refuges from the north became ideal for the continuation of the gene flow of *Tilia* in Europe, following a line across the Balkan Peninsula, Asia Minor, and up to Iran. Linden not only continued to reproduce more successfully in the southern parts of Europe, but also adapted its reproduction in the remaining fragmented habitats of northern Europe to the new climatic conditions. Until the end of the coldest period of the Ice Age, its habitats were slowly taken over by the much more resistant European beech, and *Tilia* thus remained an indicator of ancient forests and ecosystems as man once knew. If analysing the results of the report on linden research in Bosnia and Herzegovina (DBU.de, 2021), it can be seen that only a few trees were sampled in the wider area of Čapljina, whose linden are characterized by special morphological features in terms of hairiness and resistance to heat and a high drought index. A legitimate question arises whether in the past there was illegal cutting of trees on the wider territory of Bosnia and Herzegovina, which could eventually lead to their eradication. From the aspect of preserving the *gene pool*, it is important that the remaining linden trees are used for propagation, and that heat-resistant linden varieties, which are characterized by the presence of hairs on the front side of the leaves, eventually start growing in nurseries. Such a move would help the preservation and restoration of the Čapljina linden, that is, the restoration of perhaps an entire provenance.

The key to distinguishing the behavior of species within the genus *Tilia* in the complex of linden families in Bosnia and Herzegovina is in monitoring transitions based on hybrid clusters. The importance of clonal groups and their research is reflected in the complexity of the long-term goals of afforestation of bare forests in Bosnia and Herzegovina, i.e. the permanent struggle with the harmful effects of climate change (planting linden, especially large- and silver-leaved, in drought-affected areas), as well as in the complexity of afforestation of dry areas in Europe. The chance of Bosnian forestry from a commercial point of view could lie in the production of highly resistant linden seedlings for the purpose of afforestation in countries such as Germany. Vejzagic (2022) points to the potential of producing highly resistant seedlings of silver-leaved linden, and recommends that the effect of *T. tomentosa* on fauna (rodents and other animals in the forest) be further investigated. Climate change is more than ever the focus of molecular research of different tree species, such as the evolvability-heritability research project, because the current and burning issue in the *Forest Genetics* field is discovering species resistant to high temperatures-driven pathogen and insect diseases in forest trees and drying affected forest stands. If it is taken into consideration that lindens can produce a stable amount of seeds in very dry years, in which, in addition to high temperatures, very low air humidity is expressed, this makes species from the genus *Tilia* ideal for

afforestation in the future. National forestry industries in countries abundant with *Tilia* may benefit greatly from additional research on linden and potential mass production of furniture wood, because of *Tilia* drought resilient potential, which is similar to ash (*Fraxinus spp.*). Some German Genetics related research already indicated that ash has the potential of a "tree of the future" (BLW, 2024). When searching for more resistant individuals in *Tilia* bastards, such *Tilia x europaea*, which is widely used in Europe in parks, alleys and gardens planting, special attention should be paid to deeper analysis, showing whether bastards with *T. cordata* as a first parent or bastards with *T. platyphyllos* as a first parent are more or less resistant to certain diseases and disorders caused by the complex health conditions of forest ecosystems. Although numerous scientific literature in Northern European countries state that *T. x europaea* as a bastard of these two species does not come from nature in forests, according to Herman (1971), this bastard occurs naturally in the countries of the former Socialist Federative Republic of Yugoslavia. Therefore, there is an interest in investigating whether in Bosnia and Herzegovina there are special suitable conditions for this resistant bastard to occur naturally.

According to Austin (2007), ecological conditions, together with the anthropogenic factor, influence the shaping of the natural distribution of species. According to Brown (1984), the density of plant populations is higher in the central part of their distribution, while towards the edges of their ecological amplitude, populations become fragmented. Habitat fragmentation of plant populations often leads to a reduced effective size of plant populations (Young et al. 1996), and fragmented populations are characterized by reduced genetic diversity (Leimu et al. 2006). According to Eckert *et al.* (2008), this was observed when comparing populations from the edges and populations from the central parts of their distribution. According to Frankham (2003), lower genetic diversity of populations is characterized by reduced ability of populations to survive and/or adapt (Young *et al.* 1996). The risk of negative consequences of habitat fragmentation is correlated with the ability of populations to spread (Thomas, 2000). Kvesić (2018) states that, according to Travis and Dytham (1999), the ability of populations to spread depends on the ability to colonize new habitats and exchange genes between populations. Therefore, the ability of populations to spread affects the degree of their extinction and genetic drift within existing populations. Rajora *et al.* (2002) and Johnson *et al.* (2009) claim that the ability to spread populations often causes "inbreeding" through self-fertilization and inbreeding (Kvesić, 2018). Reduced heterozygosity, as a consequence of inbreeding (Kvesić, 2018), facilitates the expression of harmful genes and increases the risk of extinction (Newman and Pilson 1997, Reed and Frankham 2003). Kvesić (2018) states that forest tree species are characterized by intense gene flow but also an intense anthropogenic factor, which is reflected in the management of forest resources. Because of this, research into the impact of habitat fragmentation on the genetic diversity of populations is difficult, and therefore it is necessary to conduct numerous studies in order to find specific knowledge about the sensitivity of forest populations to the fragmentation of their habitat (Chybicki *et al.* 2014, Kvesić 2018).

By reviewing the data previously presented in this article, and compare it with the DBU.de report (2021), it can be seen that the majority of forestry companies interesting for potential nursery production are located or can be located in the North Bosnia region, the Lower Drina region, the West Bosnia-Limestone-Dolomite region, the Central Bosnia region, the Zavidovici-Teslic region, Sub-Mediterranean-montane area and Sub-Mediterranean-mountainous area. Based on the data provided by Kvesic (2018) and Stefanovic (1983), there is a preliminary estimation, that linden nursery conditions (for potential future mass afforestation of drought habitats) should be investigated in the Western-Bosnian-Limestone-Dolomite region (localities with deep soils) and Central Bosnia (isolated localities with deep soils). For the sub-Mediterranean-montane area, it has been estimated that the establishment of nurseries for the mass production of linden seedlings is a non-supporting idea, due to the

unfavorable slope of the terrain, which is reflected in the presence of dolomite and limestone rocks, on which there are fragmented soils in the form of a mosaic. Poor soil conditions are predisposed by natural matrix (rock), and poor humidity also represents a barrier to plant nursery production of most of the forest tree species. Other interesting ecological-phytocenological areas that were taken into consideration (table 1) are not clear whether the conditions are favorable for raising larger nurseries for plantation mass production of linden seedlings. A combination of pseudogley soils and conditions that correspond to the growth of the oak and hornbeam ecosystem may come into consideration (Northern Bosnian area), since large linden forests are already growing in that area. According to DBU.de (2021), *T. tomentosa* populations have already been sampled for research analysis in Bosanski Petrovac and Sanski Most, which support the idea of investigating plant nursery organisation (specifically for *Tilia*) on the terrain conditions characterized in the study. A detailed analysis would be needed in order to see how the influence of increased moisture in the soil would affect the transplanting of seedlings grown on such soil to other habitats, which are not characterized by the presence of pseudogley soils. If the proposal to build nurseries for the mass production of linden could not be made on the basis of an analysis of the ecological-phytocenological conditions of the areas managed by certain forestry companies in Bosnia and Herzegovina, quality studies should be conducted on how much the vegetation conditions and climatic characteristics of these areas have changed in the last few decades, since the base for rezoning were published by the forestry expert Stefanovic (1983) at a time when climate change did not have drastic effects on the extension of the vegetation period and disturbances in the humidity in the air and land.

Table 1. Ecological growth conditions of forest ecosystems in different ecological-phytocenological regions in Bosnia and Herzegovina (created on the basis of Stefanovic, 1983 and Kvesic, 2018 information).

Region name	Climate type	Height amplitude (m)	T.A.P. (%)	W.C.R	N.V.D	Dominant soil type	Dominant phytocenological community
Northern Bosnian	continental	80-980	55	0,91	180-200	Pseudogley	<i>Quercus-Carpinetum</i>
lower Drina	continental	80-1020	55	0,82	190-210	auto- and hydromorphic soils	<i>Quercus-Carpinetum</i>
Western Bosnian-limestone-Dolomite	mediterranean+continental	300-1900			120-200	limestone soil types	<i>Piceo-Abieti fagetum</i>
central Bosnian	continental+mediterranean	300-2200	48	0,92	180-200	heterogeneous complex soils	<i>Quercus-Carpinetum</i>
Zavidovici-Teslic	temperate continental+mediterranean (VII-VIII)	250-1328	56		180-200	serpentinized peridotite soils	<i>Erico-Pinetum</i>
sub-Mediterranean-montane	mediterranean+mountainous	1800-2000	36	1,06	120-190	limestone, dolomite and soil mosaics	<i>Abieti-Fagetum</i>
sub-Mediterranean-mountainous	alternating mediterranean	750-1200	33	0,86		soil mosaics	Various vegetation

Comment: *T.A.P. - Total Annual Precipitation), **W.C.R. - Water Change Ratio -

Ratio that shows proportion of Total Annual Precipitation in comparison to potential evapotranspiration in the certain ecological-phytocenological region, for period of one year),
*** N.V.D. - Number of vegetational days in a year. Light green color represents solid conditions and light orange color represents inadequate conditions for funding plant nurseries.

The graph 1 implies a high degree of average annual precipitation in the Zavidovici-Teslic ecological-phytocenological area, while the lowest amount of this degree is in the Sub-Mediterranean-mountainous ecological-phytocenological area. So, also from the aspect of water income and expenditure ratio it is observed that continental parts of Bosnia and Herzegovina are more suitable for preliminary investigation of linden nursery funding preconditions. Taking into account the report DBU.de (2021) it is visible that Bosnia and Herzegovina has potential preconditions for investigating the possibilities of linden nursery funding in different areas, which may be abundant with soil humidity, since certain populations of *Tilia* already exist in such areas. It is also recommended to investigate the preconditions for organizing linden nursery production in the southern region of the country, since DBU.de (2021) shown that *Tilia* can survive drought habitat conditions thanks to existence of metamorphosed parts of its leaves, which appears microscopically in form of hairs.

All aforementioned indicates that *Tilia* is very interesting from the aspect of climate change and that changes in soil evaporation and air temperatures oscillations including heat waves should be explored in addition, to study whether climate conditions in the natural habitats of the tree species deteriorate or stagnate. By finalizing the review of the available literature in this article, we approach the summary of conclusions.

4.CONCLUSION

Conducted research shows that many linden stands grow naturally in seven different ecological-phytocenological zones in Bosnia and Herzegovina: 1) Northern Bosnia area, 2) Lower Drina area, 3) West Bosnian-Karst-Dolomite area, 4) Central Bosnian area, 5) Zavidovici-Teslic area, 6) Sub-Mediterranean-mountainous area, and 7) Sub-Mediterranean-montane area. The analyses of the brief descriptions of natural conditions that dominate these areas shows that linden are growing in diverse regions, characterized by various soil, climate and vegetation indicators. Although the linden in Bosnia and Herzegovina can be found in mountainous, montane, (sub)mediterranean, valley and hilly areas, the production of mass plant material is not possible everywhere. Because of the intensification of the climate change processes, it is important to seek innovative solutions to spend less water in funding plantations and producing plantlings itself. Saving water in the future seems to be the upcoming challenge which could be well managed by choosing future nurseries locations for certain species according to the best growing conditions for those species in the autochtone habitats. It has been observed that *Tilia* has interesting potential for future explorations in Bosnia and Herzegovina, and this article supports further explorations on habitat and climate impact on natural stands.

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EFFICACY OF THE USE OF TOPICAL ATROPINE AS AN ALTERNATIVE THERAPY IN PREVENTING THE PROGRESSION OF MYOPIA IN CHILDREN 0–14 YEARS OLD – A LITERATURE REVIEW

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ABSTRACT

Introduction: The global prevalence of childhood myopia has increased dramatically over recent decades, becoming a major public health concern. Early-onset myopia often leads to higher degrees of refractive error and a higher risk of ocular complications in adulthood. Pharmacological interventions, particularly low-dose topical atropine, have recently gained attention as effective methods to slow myopia progression. **Aim:** This literature review aims to evaluate the efficacy, safety, and clinical applicability of topical atropine as an alternative therapy for preventing myopia progression in children aged 0–14 years. **Methodology:** A comprehensive literature search was conducted using PubMed, Scopus, and ScienceDirect databases for studies published between 2010 and 2024. Randomized controlled trials, cohort studies, and meta-analyses assessing atropine concentrations between 0.01% and 1% were included. The primary outcomes analyzed were spherical equivalent refraction (SER) change, axial length elongation, and reported side effects. **Results and Discussion:** The majority of studies demonstrate that low-dose atropine (0.01–0.05%) significantly reduces myopia progression with minimal side effects such as transient photophobia or blurred near vision. Comparative analyses suggest that lower concentrations maintain therapeutic benefits while minimizing rebound effects upon discontinuation. Variability in outcomes between ethnic groups and treatment durations was also noted. **Conclusion:** Current evidence supports the use of low-dose topical atropine as an effective and safe intervention for controlling myopia progression in children. However, standardized treatment protocols and long-term follow-up studies remain essential to optimize dosage and safety profiles.

Keywords: *myopia control, topical atropine, pediatric ophthalmology, literature review, refractive error*

1. INTRODUCTION

Myopia (nearsightedness) is one of the most common ocular disorders worldwide, with an alarming rise in prevalence over the past few decades. It is estimated that by 2050, approximately half of the world's population will be myopic (Holden et al., 2016). The onset of myopia during childhood is particularly concerning, as early manifestation often leads to rapid progression, higher degrees of refractive error, and a greater lifetime risk of developing sight-threatening complications such as myopic maculopathy, retinal detachment, and glaucoma.

Traditional management of myopia has primarily focused on optical correction through spectacles or contact lenses; however, these methods do not address the underlying pathophysiology or the progression of the condition (Holden et al., 2016; Jonas et al., 2019; Lanca & Saw, 2022). Recent studies indicate that while specialized optical devices—such as multifocal contact lenses or defocus-incorporated multiple segment (DIMS) spectacles—can

reduce myopia progression by approximately 30–50%, they cannot halt axial elongation or the biological mechanisms driving myopia (Huang et al., 2015; Wu et al., 2019).

In contrast, pharmacological approaches—particularly low-dose atropine therapy—have emerged as promising interventions for slowing myopia progression in children (Chia et al., 2016; Hansen et al., 2023; Yam et al., 2020). Atropine, a non-selective muscarinic receptor antagonist, has been used in ophthalmology for over a century, primarily for pupil dilation and cycloplegia (Fu et al., 2022). Its exact mechanism in myopia control is not fully understood; however, evidence suggests that atropine acts by blocking muscarinic receptors in the retina and sclera, thereby modulating biochemical pathways that regulate ocular growth (Fu et al., 2022; Song et al., 2019). Animal and human studies have demonstrated that atropine reduces axial elongation and scleral remodeling independent of its cycloplegic effects (Wu et al., 2021; Wang et al., 2023).

Nevertheless, the optimal concentration that balances efficacy, safety, and tolerability remains under active investigation (Wang et al., 2023; Gong et al., 2022; Yam et al., 2020). Lower concentrations (0.01–0.05%) have shown comparable efficacy to higher doses but with fewer side effects, less photophobia, and minimal rebound upon discontinuation (Chia et al., 2016; Hansen et al., 2023; Wu et al., 2021).

This literature review aims to summarize current evidence regarding the efficacy, safety, and clinical applicability of topical atropine in controlling myopia progression among children aged 0–14 years, based on published randomized controlled trials, longitudinal studies, and meta-analyses (Chia et al., 2016; Hansen et al., 2023; Wang et al., 2023; Yam et al., 2020).

2. METHODOLOGY

A systematic literature review was conducted across three databases: PubMed, Scopus, and ScienceDirect. The search covered publications from January 2010 to June 2024 using the keywords: “myopia control,” “low-dose atropine,” “pediatric myopia,” “atropine 0.01%,” and “axial elongation.”

2.1 Inclusion Criteria

Studies involving participants aged 0–14 years with diagnosed myopia.

Randomized controlled trials (RCTs), cohort studies, and meta-analyses evaluating atropine concentrations ranging from 0.01% to 1%.

Studies reporting at least one of the following outcomes: change in spherical equivalent refraction (SER), axial length (AL), or incidence of adverse effects.

2.2 Exclusion Criteria

Studies involving non-human subjects or adult populations.

Non-English publications or conference abstracts without peer review.

Studies combining atropine with other pharmacological or optical interventions without separate outcome reporting.

2.3 Data Extraction

For each included study, data were extracted on study design, sample size, atropine concentration, duration of follow-up, and main outcomes related to myopia progression and safety.

3. RESULTS

3.1 Overview of Selected Studies

From an initial pool of 146 articles, 27 met the inclusion criteria. Among these, 12 were randomized controlled trials, 6 were prospective cohort studies, and 9 were meta-analyses. The sample sizes ranged from 60 to 1,200 participants, and follow-up durations ranged from 6 months to 5 years.

Table 1. Summary of Included Studies in the Literature Review

Study Type	Number of Studies	Sample Size Range (Participants)	Follow-up Duration	Key Focus / Outcome Measures
Randomized Controlled Trials (RCTs)	12	80 – 1,200	1 – 5 years	Efficacy and safety of atropine at varying concentrations (0.01–1%)
Prospective Cohort Studies	6	60 – 800	6 months – 3 years	Long-term effects and compliance in clinical settings
Meta-Analyses / Systematic Reviews	9	Aggregated (multi-study)	Based on pooled data	Quantitative synthesis of treatment efficacy and side-effect profiles
Total	27	60 – 1,200	6 months – 5 years	

3.2 Efficacy in Myopia Control

3.3

Across most studies, low-dose atropine (0.01–0.05%) demonstrated a statistically significant reduction in myopia progression compared to placebo or no treatment. The study by Chia et al. (2016) reported that atropine 0.01% reduced myopia progression by approximately 50–60% compared to controls, with minimal side effects. Similarly, the study (Yam et al., 2020) confirmed a dose-dependent response, where 0.05% atropine showed slightly better control than 0.01%, though with marginally higher photophobia rates.

Axial elongation, a key structural indicator of myopia progression, was also significantly reduced in atropine-treated groups. Meta-analyses by Wu et al. (2019) and Gong et al. (2022) concluded that atropine concentrations $\leq 0.05\%$ effectively inhibited axial elongation with fewer adverse events than higher doses.

Table 2. Summary of Key Findings on Low-Dose Atropine Efficacy in Myopia Control

Atropine Concentration(s)	Main Outcomes on Myopia Progression	Effects on Axial Elongation	Reported Side Effects	Study / Source
0.01%	Reduced myopia progression by 50–60% compared to controls.	Significant reduction compared to placebo.	Minimal side effects; mild photophobia in some cases.	<i>ATOM2 (Chia et al., 2016)</i>
0.01%, 0.025%, 0.05%	Dose-dependent effect: 0.05% more effective than 0.01% and 0.025%.	0.05% produced the greatest reduction in axial elongation.	Slightly higher photophobia at 0.05%, but overall well tolerated.	<i>LAMP Study (Yam et al., 2020)</i>
$\leq 0.05\%$	Demonstrated statistically significant slowing of refractive progression across multiple trials.	Consistent reduction in axial elongation across atropine concentrations $\leq 0.05\%$.	Lower incidence of adverse events than higher-dose atropine.	<i>Wu et al. (2019) – Meta-analysis</i>
0.01–0.05%	Confirmed effectiveness of low-dose atropine in reducing myopia progression compared with placebo/no treatment.	Significant inhibitory effect on axial elongation in pooled data.	Very low rates of photophobia and near-vision blur.	<i>Gong et al. (2022) – Meta-analysis</i>

3.3 Safety and Tolerability

The most commonly reported adverse effects were mild and transient, including photophobia, blurred near vision, and allergic conjunctivitis. These were dose-dependent and less frequent at concentrations $\leq 0.05\%$. Higher doses ($\geq 0.1\%$) were associated with greater discomfort and a higher risk of rebound myopia upon discontinuation.

Notably, several trials found no significant differences in visual acuity or pupil size between low-dose atropine and placebo groups after long-term use, suggesting a favorable safety profile suitable for pediatric populations.

3.4 Variability by Ethnicity and Duration

Differences in treatment efficacy were observed among ethnic groups. East Asian populations, particularly in Singapore, Taiwan, and Hong Kong, demonstrated higher baseline progression rates but also greater therapeutic response to atropine. Studies in European cohorts, though fewer, showed comparable trends but with slightly reduced efficacy (Lanca & Saw, 2020).

Duration of treatment also influenced outcomes: multi-year therapy provided better cumulative control, whereas abrupt discontinuation often led to temporary rebound effects. Gradual tapering schedules have been proposed to mitigate this phenomenon.

4. DISCUSSION

The recent and most of literature supports low-dose atropine as a first-line pharmacologic option for managing childhood myopia progression. Its mechanism of action, though not fully elucidated, likely involves inhibition of muscarinic receptors influencing scleral remodeling and ocular elongation.

4.1 Optimal Dosage and Regimen

Determining the optimal atropine concentration for controlling myopia progression in children remains a subject of ongoing research. Evidence from major clinical trials shows that **0.05% atropine is slightly more effective** than lower concentrations in slowing both refractive error progression and axial elongation. However, this stronger effect comes with **a higher likelihood of side effects**, such as mild photophobia and near-vision difficulties.

In contrast, **0.01% atropine has been consistently shown to offer the best balance between efficacy and tolerability**. Although it does not reduce myopia progression quite as strongly as 0.05%, it still provides significant clinical benefit while minimizing adverse effects. This makes it easier for children to continue using the medication over long periods, improving treatment adherence (Chia, A., et.al., 2016).

Both the **ATOM2 (Atropine for the Treatment of Myopia 2)** and **LAMP (Low-Concentration Atropine for Myopia Progression)** studies demonstrated that **0.01% atropine is effective, safe, and highly suitable for long-term use**, which is essential because myopia progression typically continues for several years during childhood. For these reasons, many clinicians consider 0.01% an optimal starting concentration and a practical choice for routine myopia management (Yam, J., et al. 2020).

4.2 Rebound and Long-Term Effects

Rebound myopia—progression after discontinuing atropine—is a notable concern, particularly with higher concentrations. Longitudinal studies indicate that the rebound effect is markedly less pronounced with 0.01% atropine (Chia et al., 2016; Yam et al., 2020). Continuous monitoring and stepwise withdrawal are therefore recommended for sustained benefit.

4.3 Clinical Implementation

From a public health perspective, low-dose atropine offers a cost-effective and scalable intervention, particularly in high-prevalence regions. However, its implementation requires careful patient selection, regular monitoring, and counseling to ensure adherence and manage side effects.

4.4 Research Gaps

Despite strong evidence, several gaps remain:
Standardization of protocols: Variability in dosing frequency, duration, and discontinuation strategies across studies complicates comparison.

Ethnic diversity: Most trials involve East Asian cohorts; more data from European and African populations are needed.

Combination therapies: Future research may explore synergistic effects of atropine with optical interventions (e.g., orthokeratology or multifocal lenses).

5. CONCLUSION

The current body of evidence robustly supports low-dose atropine (0.01–0.05%) as a safe, effective, and practical pharmacological intervention for controlling myopia progression in children. The treatment significantly slows both refractive and axial elongation changes with minimal side effects and a manageable rebound profile.

Future studies should aim to standardize dosing protocols, extend follow-up durations beyond five years, and include diverse populations to strengthen global applicability. As childhood myopia continues to rise worldwide, integrating atropine therapy into comprehensive myopia management programs could play a pivotal role in reducing the long-term burden of visual impairment.

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OPTIMIZATION OF CELLULASE PRODUCTION IN A PACKED BED BIOREACTOR

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ABSTRACT

Cellulose enzymes are central to lignocellulosic waste bioconversion to fermentable sugars, hence a basic element of sustainable biotechnological processes. While submerged fermentation, a widely used method of microbial enzyme production, holds substantive disadvantages, notably huge energy requirements and wastewater production, solid-state fermentation (SSF) offers several advantages concerning cost efficiency, enzyme productivity, and environmental friendliness, as a result. This study aimed at cellulase production carried out in a packed bed bioreactor under SSF mode by utilizing *Bacillus amyloliquefaciens* NRRL B-645. Three major operating parameters—air flow rate, temperature, and substrate amount—were recognized and optimized by using a Central Composite Design (CCD) method of analysis. Results indicated that CCD was a useful statistical tool for evaluating and improving cellulase production under bioreactor conditions.

Keywords: Cellulase, Solid-state fermentation, Packed-bed bioreactor, Process optimization, Agro-industrial wastes

INTRODUCTION

The use of fossil fuels, recurrent energy shortages, as well as challenges related to waste management, have fuelled the need for development of sustainable biotechnological solutions (Pham et al., 2022). Agricultural and food industry by-products of lignocellulosic origin are considered a considerable potential source for biofuel and other high-quality product production (Dobrzyński et al., 2022; Wang et al., 2024). However, their treatment often requires physical or chemical pre-treatment, which may bring about environmental challenges (Hassan et al., 2018; Dar et al., 2022).

Cellulases are enzymes that break down cellulose into glucose units and consist of three main components: endoglucanase, exoglucanase, and β -glucosidase. This class of enzymes holds a central position for affordable and efficient lignocellulosic waste conversion, representing some 20% of the world market of enzymes (Bhagat and Kokitkar, 2021; Singh et al., 2021). Bacteria species of the genus *Bacillus* are of particular interest for their high cellulolytic potential (Vu et al., 2022).

Although submerged fermentation is widely used for microbial enzyme production, it has certain disadvantages related to energy consumption and cost (Farinas, 2015; Poltronieri and D'Urso, 2016). Solid-state fermentation (SSF) has been developed as an alternative, offering a more sustainable approach due to its lower water requirements and greater economic feasibility (Gómez-Ramos et al., 2025; Perwez and Al Asheh, 2025).

This study investigated cellulase production via solid-state fermentation in a packed-bed bioreactor system using the *Bacillus amyloliquefaciens* NRRL B-645 strain. The bioreactor

operating conditions (air flow rate, temperature, and substrate amount) were optimized using Central Composite Design (CCD).

MATERIAL AND METHODS

Solid Substrates

In this study, hazelnut oil cake obtained from Altaş Yağ Sanayi A.Ş. (Ordu, Turkey), and maize bran obtained from Sunar Mısır Entegre Tesisleri Sanayi ve Ticaret A.Ş. (Adana, Turkey) were used. Both industrial by-products were ground and fractionated using ASTM standard sieves. The particle fractions in the -30+50 mesh size range were stored in airtight containers under appropriate conditions and used in fermentation experiments.

Microorganism

The bacterium *Bacillus amyloliquefaciens* NRRL B-645 was obtained from the NRRL (National Center for Agricultural Utilization Research, USA) culture collection.

Microorganism Growth and Preservation Medium

Luria–Bertani (LB) liquid and agar media were used for the cultivation and preservation of the microorganism. The media were prepared according to the standard formulation and sterilized in an autoclave (121 °C, 1.2 bar, 15 minutes) (Miller, 1972). Cells obtained from cultures grown in LB liquid medium were transferred to agar plates using spread and streak plating methods. The resulting stock cultures were stored at +4 °C and renewed every two weeks. For use in SSF, a colony of *Bacillus amyloliquefaciens* NRRL B-645 was taken from the stock culture and inoculated aseptically into 50 ml of LB liquid medium. The culture was incubated at 37 °C for 16 hours at 150 rpm.

Fermentation Medium

In a previous study, it was established that the most effective medium composition for cellulase production is as follows: 5.2 grams of maize bran, 4.8 grams of hazelnut oil cake, 4.8 mL of inoculum, 1.5 mL of a nitrogen source and 8.7 mL of tap water, for a duration of fermentation of 90 hours. The medium composition optimized was finally fed to a packed-bed bioreactor system at a ratio of solid:liquid of 1:1.5 (w/v).

Experimental Design

To optimize cellulase production in SSF, optimization of bioreactor operating parameters, air flow rate (L/min), temperature (°C) and substrate amount (g), were performed. The experimental levels of chosen parameters are listed in Table 1.

Table 1. Selected factors and their levels for bioreactor operating conditions

Independent variable	Factor	-1.68	-1	0	+1	+1.68
Air flow rate (L/min)	A	0.100	0.130	0.175	0.220	0.250
Temperature (°C)	B	31.0	32.6	35.0	37.4	39.0
Substrate amount (g)	C	10	12	15	18	20

The experimental design was developed using the Central Composite Design (CCD) as part of the Response Surface Methodology (RSM), and was carried out using Design-Expert 10 software. Cellulase activity (U/g) was chosen as the response variable. Analysis of variance (ANOVA) and response surface plots were also generated using the same software.

According to the Central Composite Design (CCD), a total of 20 experimental runs were cumulatively carried out including 8 factorial points, 6 axial points, and 6 center points. Total experimental runs (N) were estimated by a general formula based on CCD (Equation 1):

$$N = 2^n + 2n + n_c = 2^3 + 2(3) + 6 = 8 + 6 + 6 = 20 \quad (1)$$

In this instance, n represents the number of independent variables; 2^n is the number of factorial points; $2n$ is the number of axial points; and n_c is the number of times of replications of center point.

In analysing the experimental results, a second-order polynomial regression model was utilized for the investigation of cellulase production induced by independent variables. It is a typical model widely utilized for Response Surface Methodology (RSM) and represented by Equation (2) (Liu et al., 2020):

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j \quad (2)$$

In this equation, Y represents the response variable modelled by the regression equation. X_i and X_j are the independent variables used to evaluate their effects on the response. β_0 denotes the intercept term, while β_i , β_{ii} and β_{ij} represent the coefficients of the linear, quadratic, and interaction terms between the variables, respectively.

Enzyme Extraction

After undergoing solid-state fermentation, the fermentation medium was harvested from packed-bed bioreactors and then transferred to Erlenmeyer flasks. Extraction of the medium was carried out by sodium phosphate buffer (7.2 pH) and centrifugation. Supernatant recovered by this process acted as a crude solution of the enzyme for measuring cellulase activity.

In each extraction, buffer volume was adjusted to 10 mL per g of solid substrate.

Enzyme Activity Assay

Activity of cellulase was assayed by 3,5-dinitrosalicylic acid (DNS) method, measuring amounts of reducing sugars produced (Bernfeld, 1955). The analytical procedure for DNS method is summarized in Table 2.

Readings of each of the samples and controls were conducted in triplicate, and mean absorbance values were subsequently calculated. The net absorbance was also calculated by subtracting the mean absorbance of a control from that of the mean absorbance of a sample. Glucose equivalents of these absorbances were then ascertained by utilizing a standard curve.

One unit (U) of cellulase activity corresponds to an amount of enzyme that releases under routine assay conditions, 1 μ mol of glucose per minute.

Table 2. DNS-based analytical protocol for cellulase activity assay (Elsababty et al., 2022).

Reagent (mL)	Control (mL)	Sample (mL)
Carboxymethyl cellulose (CMC) solution (in sodium phosphate buffer, pH 7.2)	0.5	0.5
<i>Vortexed and incubated in a water bath at 50 °C.</i>		
Cellulase solution	-	0.5
<i>Incubated in a water bath at 50 °C for 30 minutes.</i>		
Color reagent (DNS)	1	1
Cellulase solution	0.5	-
<i>Incubated in a water bath at 50 °C for 10 minutes, then cooled to room temperature.</i>		
<i>Vortexed and absorbance was measured at 540 nm using a spectrophotometer.</i>		

RESULTS AND DISCUSSION

Ascertaining the statistical relevance of the model constructed for improving bioreactor operating conditions involved carrying out analysis of variance (ANOVA) applicability. Table 3 shows comparative statistical results for differing model types.

Based on the results of the ANOVA, the p-value of the F-test for the quadratic model proved to be less than 0.0001. Because this value is less than the threshold for significance of 0.05, the model proved to be statistically significant. The statistical strength of the model is also indicated by an R^2 of 0.9689, adjusted R^2 of 0.9409, and predicted R^2 of 0.8096. A value of R^2 nearing 1 shows a good fit of the model for the data and a high ability of the model to predict (Haaland, 1989). It has been, however, noted that for large numbers of model terms, or for a sample of too-small a sample, the adjusted R^2 value may be significantly less than R^2 (Hashemi et al., 2013). The acceptable degree of difference between adjusted R^2 and R^2 values obtained for cellulase validates the completeness of the model. The predicted R^2 value indicates how closely model predictions agree with results seen at a trial (Singh et al., 2017). Because of large degrees of error and variation normally encountered in biotechnology, a predicted R^2 value of 0.8096 is judged reasonable (Gois et al., 2020). The lack-of-fit p-value for the quadratic model proved to be 0.1160, a value larger than a normally accepted threshold for significance of 0.1.

Table 4 shows the p-values of the linear, quadratic, and interaction terms, as well as other statistical outcomes.

Table 3. Model evaluation results for the optimization of bioreactor operating conditions

Source	Sequential <i>p</i> -value	Lack of Fit <i>p</i> -value	Adjusted R^2	Predicted R^2	
Linear	0.5960	0.0002	-0.0590	-0.2397	
2FI	0.8994	0.0001	-0.2478	-0.9407	
Quadratic	<0.0001	0.1208	0.9409	0.8096	<u>Suggested</u>
Cubic	0.2045	0.1160	0.9585	-0.2207	

Table 4. ANOVA results for the optimization of bioreactor operating conditions

Source	Sum of Squares	dF	Mean Square	F-value	p-value Prob > F
Model	1.739E+006	9	1.933E+005	34.62	<0.0001
A - Air flow rate (L/min)	95609.81	1	95609.81	17.13	0.0020
B - Temperature (°C)	88566.70	1	88566.70	15.87	0.0026
C - Substrate amount (g)	10106.78	1	10106.78	1.81	0.2081
AB	52358.48	1	52358.48	9.38	0.0120
AC	13448.00	1	13448.00	2.41	0.1517
BC	2380.50	1	2380.50	0.43	0.5284
A²	6.388E+005	1	6.388E+005	114.45	<0.0001
B²	8.408E+005	1	8.408E+005	150.64	<0.0001
C²	2.616E+005	1	2.616E+005	46.87	<0.0001
Residual	55816.90	10	5581.69		
Lack of Fit	42158.52	5	8431.70	3.09	0.1208
Pure Error	13658.38	5	2731.68		
Cor Total	1.795E+006	19			
Mean	993.19				
R ²	0.9689				
Adjusted R ²	0.9409				
Predicted R ²	0.8096				
Standard Deviation	74.71				
C.V. %	7.52				
PRESS	3.418E+005				
Adequate Precision	15.5				

Parameters of model having their p-values less than 0.05 (A, B, AB, A², B², and C²) were recognized as significant model coefficients having impacts on cellulase activity. Their p-values indicate contributions of individual model coefficients to the response variable thereby revealing associations between independent variables.

The mean was calculated as 993.19, with a standard deviation of 74.71. The coefficient of variation (C.V.) was 7.52%, which generally indicates acceptable model reproducibility (Bandyopadhyay and Choudhury, 2018).

Precision value obtained was 15.5. As it greatly surpasses even the minimum acceptability measure of 4, it is believed that the model is trustworthy for obtaining precise outcomes for predefined parameters of designs and is statistically significant (Sharma and Kumar, 2017).

Fig. 1 illustrates the result of interaction between air flow rate (A) and temperature (B) on cellulase activity. The model identified it to be statistically significant for a p of 0.0120. The graph clearly illustrates both quadratic effects of both factors, which mean that cellulase activity reaches its optimum at an air flow of around 0.175 L/min and a temperature of 35 °C.

Present research reveals that air velocity exerts a direct influence on substrate temperature and substrate moisture. For packed-bed bioreactors, air velocity and these factors are closely intertwined for optimization of enzymatic production (Derakhti et al., 2012).

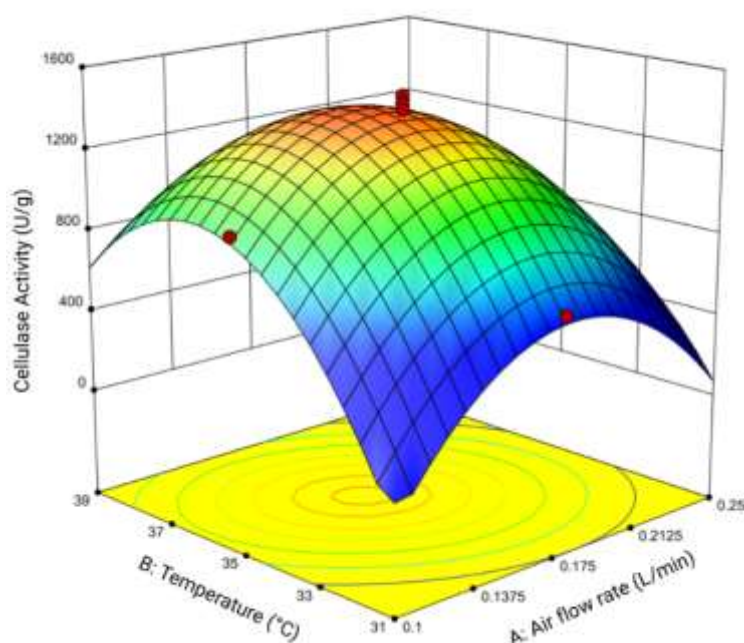


Figure 1. Response surface plot showing the interaction between air flow rate (A) and temperature (B) on cellulase activity.

CONCLUSIONS

As a result of the process optimization experiments conducted for cellulase production, the optimal bioreactor operating conditions were determined to be an air flow rate of 0.164 L/min, a temperature of 35.5 °C, and a substrate amount of 14.5 g. Under these conditions, the maximum predicted enzyme activity was 1415.31 U/g. Validation experiments performed under the same conditions measured cellulase activity at 1354.8 U/g, corresponding to 95.63% of the predicted value.

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SERRATIA MARCESCENS–ASSOCIATED CONJUNCTIVITIS IN THREE HORSES IN ROMANIA: A CASE SERIES WITH CLINICAL, MICROBIOLOGICAL AND MANAGEMENT IMPLICATIONS

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ABSTRACT

Bacterial conjunctivitis represents a relevant ocular disorder in equine medicine, producing discomfort, performance reduction, and in some cases economic impact. *Serratia marcescens*, an opportunistic Gram-negative bacillus, is rarely reported as a cause of primary equine conjunctivitis but is known for biofilm formation, environmental persistence and antimicrobial resistance (Hejazi & Falkiner, 1997; Mahlen, 2011). We report three cases of acute conjunctivitis due to *S. marcescens* in horses from the same farm in Romania. Diagnostic work-up included ophthalmic examination, cytology, culture and antibiogram. All isolates demonstrated sensitivity to fluoroquinolones and variable resistance to β -lactams, consistent with literature (Sader et al., 2020). Topical fluoroquinolone therapy produced clinical resolution within 10–14 days. These cases highlight the importance of targeted microbiological diagnosis, appropriate antimicrobial stewardship and farm-level biosecurity to prevent recurrence and spread.

Key words: *serratia marcescens*, equine ophthalmology, bacterial conjunctivitis, biosecurity, antimicrobial resistance

INTRODUCTION

Bacterial conjunctivitis in horses is common and typically associated with organisms such as *Staphylococcus* spp., *Streptococcus* spp., and *Pseudomonas aeruginosa* (Andrew et al., 2003; Brooks et al., 2000). Although *S. marcescens* is well documented as a cause of keratitis and endophthalmitis in human and veterinary medicine (Gonçalves et al., 2021; Cutler et al., 2020), conjunctival infections in horses remain infrequently described.

S. marcescens exhibits multiple virulence mechanisms including pigment production (prodigiosin), protease activity, biofilm formation and intrinsic antimicrobial resistance mechanisms (Venil et al., 2020; Shanks et al., 2013). Environmental survival allows contamination of water systems, medical instruments and equine facilities (Holcombe et al., 2020). Timely diagnosis is essential given the organism's ability to progress to ulcerative keratitis if untreated (Gilger, 2017; Whitley & Gilger, 2018).

Case Presentation

Three sport horses (6–11 years old) housed on the same farm presented with bilateral ocular discomfort following a period of high humidity and increased insect activity. All horses shared equipment, grooming tools and automatic waterers. No recent introduction of new horses occurred, but construction near the water system was noted, raising suspicion for environmental contamination.

Clinical signs included:

- Epiphora
- Mucopurulent discharge
- Conjunctival hyperemia & chemosis
- Blepharospasm

Img.1 Mucopurulent discharge



Img.2 Conjunctival hyperemia, chemosis



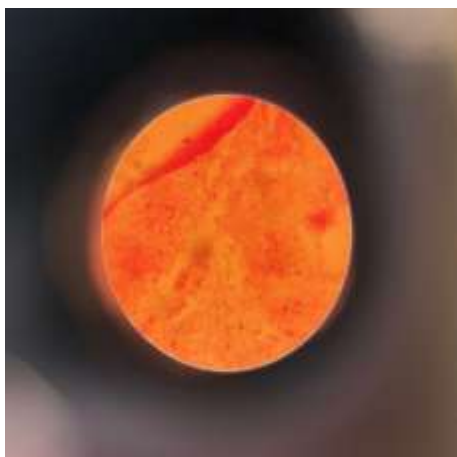
Fluorescein staining was negative in all cases. Schirmer tear test values were within normal limits.

Diagnostics

Cytology demonstrated neutrophilic inflammation with Gram-negative bacilli. Conjunctival swabs cultured on broth agar and MacConkey agar produced a distinctive feature is the intense red to brick-red pigmentation throughout the growth area. The pigment appeared mainly intracellular, with minimal diffusion into the surrounding agar. The colony surface was largely flat to slightly convex, and the texture appeared buttery to slightly mucoid. *Serratia marcescens* produces a characteristic red pigment, prodigiosin (Forbes et al., 2017)

The streak pattern indicated inoculation by surface streak on a slanted agar tube. No hemolysis or gas formation was observed in this medium.

Img. 1 Microscopic aspect of conjunctival sample



Img. 2 Bacterial growth on slant agar (*S. marcescens*)



Antibiotic susceptibility revealed sensitivity to fluoroquinolones (ciprofloxacin, ofloxacin) and variable β -lactam resistance, consistent with multidrug-resistant patterns reported in equine ocular isolates (Giuliano et al., 2022; Sader et al., 2020).

Treatment & Outcome

Topical fluoroquinolone ophthalmic solution was prescribed BID for 10–14 days. Significant improvement occurred by day 3, and full resolution was observed by day 14 in all horses. No recurrence occurred at 30-day reevaluation. Equipment disinfection and separate grooming tools were instituted.

DISCUSSION

The cluster suggests a shared environmental source, likely the communal water system or grooming equipment. *S. marcescens* is known for environmental biofilm persistence and resistance to disinfectants (Hejazi & Falkiner, 1997; Holcombe et al., 2020). Empirical β -lactam therapy may be ineffective. Similar to previous reports, fluoroquinolones achieved resolution (Cutler et al., 2020; Giuliano et al., 2022). Strict hygiene and targeted treatment prevented progression to keratitis, a known risk when *Serratia* affects the ocular surface (Kern, 2021).

These cases underline the need for culture-based treatment and surveillance in performance horses, particularly in farms hosting competition animals and frequent movement, increasing risk of international spread (Khor & Casanova, 2022).

CONCLUSION

S. marcescens should be considered an emerging equine conjunctival pathogen. Early cytology, culture and antibiogram are critical. Fluoroquinolones remain effective first-line therapy when culture confirms susceptibility. Biosecurity, water hygiene and equipment sanitation prevented recurrence. Continued antimicrobial resistance surveillance is essential.

Table 1. Clinical Features

Horse	Epiphora	Discharge	Chemosis
Horse 1	Yes	Yes	Mild
Horse 2	Yes	Yes	Moderate
Horse 3	Yes	Mild	Mild

Table 2. Microbiology & Antibiotic Profile

Test	Result	Interpretation
Culture	<i>S. marcescens</i>	Confirmed
Antibiogram	FQ sensitive; β -lactam variable	Targeted FQ therapy

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SURVEY ON THE RECOMMENDATIONS AND SAFE USE OF PLANT PROTECTION PRODUCT BY PHYTOPHARMACISTS

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ABSTRACT

Pesticides are widely used to protect agricultural production, but their application is associated with risks to human health and the environment. The awareness of stakeholders, particularly farmers and phytopharmacists, is considered essential for mitigating these risks. In this study, the knowledge and practices of phytopharmacists regarding pesticide use in Albania were evaluated. A structured survey with 23 questions was conducted between February and May 2025 across 10 districts. Of 100 phytopharmacists contacted, 95 completed the questionnaire. The findings revealed that a wide range of pesticides is stored in most outlets, with fungicides and insecticides representing the largest share of sales. Biological pesticides accounted for 36% of the sales volume but were less frequently traded due to higher costs and specific storage and application requirements. It was observed that 60% of respondents considered the pre-harvest interval in their recommendations, while 91% were familiar with its importance. Knowledge of hazard pictograms was generally adequate, except for those related to transport.

Keywords: Survey, pre-harvest, phytopharmacists, biological, chemical

INTRODUCTION

Pesticides play an important role in the development of agriculture, reducing crop losses and improving the yield and quality of agricultural products (Tudi et al. 2021). Population growth and climate change have contributed to the increase in pesticide use (Zhou et al. 2025, Yang et al. 2024), and higher global pesticide production is expected in the future (Global Agriculture. 2025,). Although pesticides are crucial for improving crop yields, their increasing use also causes numerous negative effects on the environment and human health (Zhou et al. 2025). Numerous problems have arisen from the inappropriate use of pesticides, including applications that deviate from label instructions (Beyuo et al. 2024; Labs 2022; Sachan et al. 2022) overuse or excessive dosing on different crops, failure to respect the pre-harvest interval (PHI) (EFSA 2025, Yami et al. 2025), and neglect of buffer zones or protected areas (EPA 2025; Human Rights Watch. 2018). Such practices significantly contribute to environmental degradation and pose health risks to both applicators and consumers. In recent decades, considerable efforts have been made to mitigate pesticide dependence (Hu 2020) through the design and implementation of policies aimed at reducing pesticide use. These measures include promoting non-chemical alternatives (Bakker et al. 2021), the widespread adoption of Integrated Pest Management (IPM) (European commission 2023; Pecenka 2021) and the implementation of biological and biotechnical control methods (Kevan & Shipp 2019). Retailers of pesticides also play a key role in disseminating these practices in Albania, providing recommendations on the safe use of plant protection products and guidance on the implementation of control methods during their routine work.

The objectives of the study were: (i) to identify the control measures recommended by phytopharmacists in the event of a phytosanitary problem; (ii) to determine the extent to which biological methods are recommended and the difficulties associated with their use; (iii) to assess knowledge about the pre-harvest interval (PHI) and risk pictograms; (iv) to evaluate the practical recommendation of biological control and biotechnical methods.

MATERIAL AND METHOD

The study was conducted through a survey consisting of 23 questions, during the period February–May 2025, in 10 districts of the Republic of Albania. The survey was carried out randomly by directly addressing retail sellers of plant protection products (PMBs) with a questionnaire. A total of 100 retailers out of 450 (according to the farmer portal agroalbania.al) across Albania participated, while 5 respondents refused to complete the survey. Questions 1 and 2 aim to identify the number of PMBs sold by retail sellers and the product groups most commonly traded. Questions 3 and 4 focus on how phytosanitary problems are managed, the measures taken, and the basis for PMB recommendations, including the applied methods. Questions 6 and 7 aim to assess the application of biological control and biotechnical methods for monitoring and reducing pest populations. Questions 8 to 12 explore how phytosanitary problems are identified in practice and the impact of state institutions and PMB distributors in supporting phytopharmacists. Questions 13–15 address the difficulties encountered in marketing biological plant protection products and the extent to which they are recommended. Questions 16 and 17 focus on the updates in phytopharmacists' knowledge. Question 18 asks phytopharmacists about their understanding of the concept of the pre-harvest interval (PHI). The final questions aim to assess phytopharmacists' knowledge regarding risk pictograms.

RESULTS AND DISCUSSION

Question 1 of the survey: *How many plant protection products do you have in your plant protection retail store?* (Alternatives: up to 10; 10–20; 20–30; over 30).

The results obtained from the first question of the survey are as follows: The majority of retailers (58 out of 95, or 61%) reported having over 30 different types of plant protection products available. About 25% of them (24 respondents) indicated that they have 20–30 products at their points of sale. Approximately 11% (10 respondents) reported having 10–20 products, while only 3% (3 respondents) stated that they have up to 10 plant protection products in their retail store (figure 1.1).

Question 2 of the survey: *Which groups of plant protection products do you sell the most?* (Alternatives: insecticides, fungicides, herbicides).

Among all the surveyed plant protection retail stores, the majority (about 51%, or 48 out of 95) reported selling fungicides and insecticides in equal amounts. About 23% indicated that they sell more insecticides and herbicides, while 13% (12 out of 95) reported selling mainly fungicides. Retailers that sell primarily insecticides account for about 9% of the total. One respondent did not answer this question (figure 1.2).

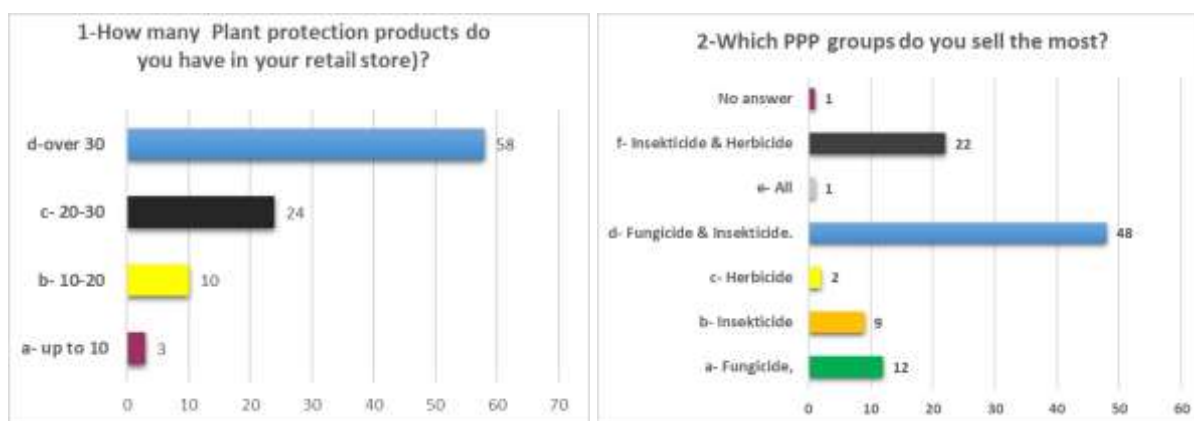


Figure 1. Graphs showing the amount of PPPs in the store and the pesticides that are sold the most.

Question 3 of the survey: *In case, a farmer comes to you with a disease or pest problem. What control measures do you recommend?* (Alternatives: I directly recommend a ppp (fungicide, insecticide); Another non-chemical measure).

Among all the surveyed approximately 55% (58 out of 95) reported directly recommending chemical fungicide or insecticide products, whereas the remaining respondents prefer to recommend non-chemical alternatives (about 45% or 43 out of 95) (figure 2.3).

Question 4 of the survey: *When you recommend a PPP to a farmer, what do you take into consideration?* (Alternatives: Profit rate; Pre Harvest Interval; Effectiveness; other (pollution)).

The largest number of respondents (34% or 32 out of 95) reported that their recommendations are based on both the pre-harvest interval and the effectiveness of the plant protection product (PPP). About 30% (or 28 out of 95) indicated that they rely mainly on product effectiveness, while 27% base their decisions solely on the pre-harvest interval. Considering the 34% who selected both pre-harvest interval and effectiveness, another 27% who chose only the pre-harvest interval, and 2% who indicated pre-harvest interval and profit, this brings the pre-harvest interval as a selection factor to a total of 63%. This very positive finding demonstrates awareness of food safety. Only 4% of pesticide retailers reported basing their decisions solely on the profit rate (figure 2.4).

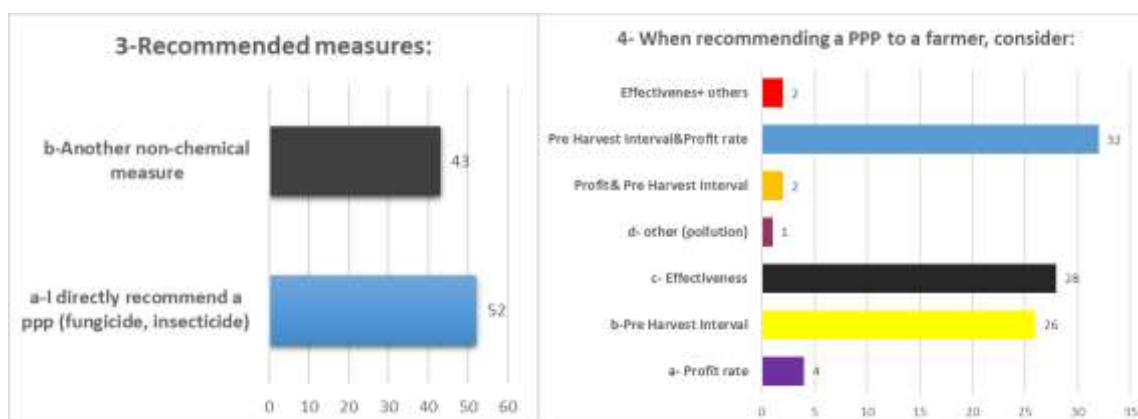


Figure 2. Graphs showing the recommended measures and the sources on which these recommendations are based.

Question 5 of the survey: *Among the disease and pest control measures, which do you consider most essential?.* (Alternatives: preventive; curative)

The largest number of respondents (89% or 84 out of 95) reported that preventive measures are the most essential. About 3% (or 3 out of 95) of respondents consider curative measures to be the most essential, while 7% (or 7 out of 95) believe that both preventive and curative measures are equally important. One respondent did not answer this question (figure 3.5).

Question 6 of the survey: *Do you sell biological plant protection products in the retail store?* (Alternatives: Yes; No)

The largest number of respondents (62%, or 59 out of 95) reported that they do not sell biological pesticides (plant protection products). About 36% (34 out of 95) of respondents stated that they sell biological plant protection products. Two respondents did not answer this question (figure 3.6).

Question 7 of the survey: *Do you sell traps for insect population reduction or monitoring?* (Alternatives: Yes; No)

The majority of respondents (51%, n = 48 out of 95) indicated that they sell traps used for insect population reduction or monitoring purposes. In contrast, 49% (n = 47 out of 95) reported that they do not sell traps (figure 3.7)

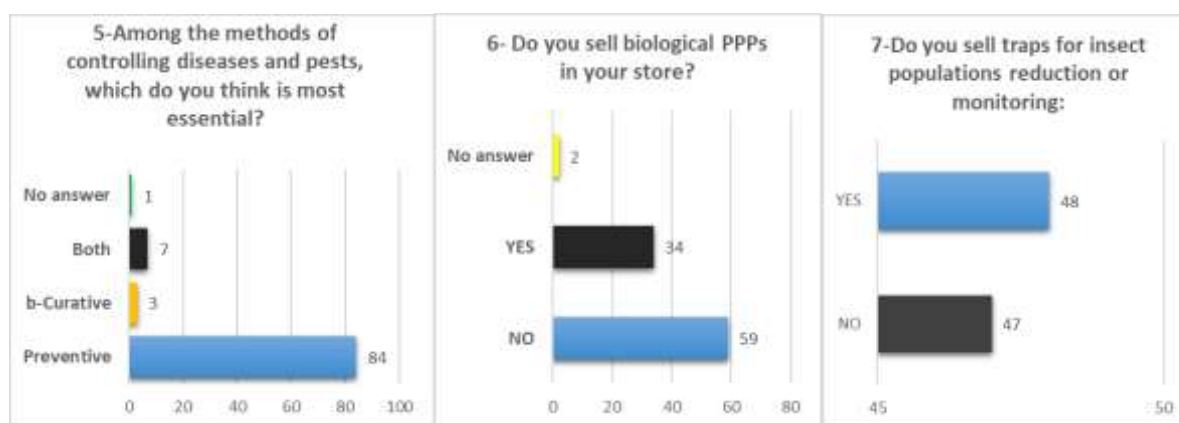


Figure 3, Graphs showing data on control measures, biological pesticide use, and traps use.

Question 8 of the survey: *How do you approach the identification of diseases or pests?* (Alternatives: Mostly, I go to the field or look at the photos or samples that the farmer brings; In most cases, I examine the photos or samples provided by the farmer; Mostly I go in the field; In most cases, the decision is made by the farmer; I rely exclusively on the farmer's descriptions).

According to the data obtained for question 8 of the survey, 42% (40 out of 95 respondents) of pesticide retailers reported that, in order to identify phytosanitary problems, they usually observe the situation in the field or examine photos or samples provided by the farmer. About 39% of ppp retailers do not visit the field; instead, they rely on the data, photos, or samples supplied by the farmer. The 16% of respondents stated that they primarily go to the field to assess the situation. In 1% of cases, the farmer decides, while 2% of respondents rely solely on the farmer's descriptions (figure 4.8).

Question 9 of the survey: *How do you approach new problems or difficulties?* (Alternatives: I act based on my knowledge and experience; I'm asking distribution companies for help; I am seeking assistance from regional agricultural specialists; I send the sample to the AUT plant protection laboratory)

The majority of respondents, approximately 74% (70 out of 95), act based on their own knowledge and experience. Among the respondents, 8 out of 95 seek assistance from

distribution companies, while 8 out of 95 send samples to the AUT Plant Protection (figure 4.9).

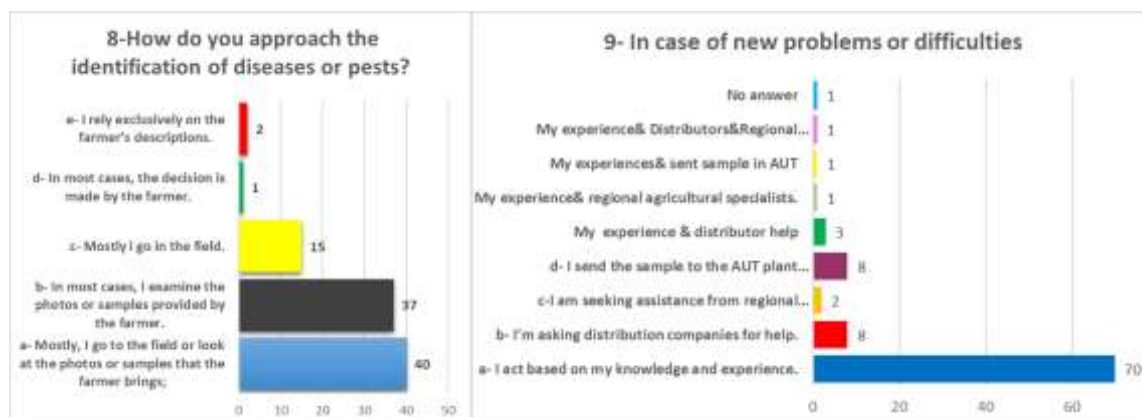


Figure 4. Graphs on identifying diseases, pests, and new phytosanitary problems.

Question 10 of the survey: *If you have sought assistance from PPP distribution companies. How satisfied are you with the outcome of the problem resolution?* (Alternatives: I didn't ask; Not at all; A little; Enough, A lot).

Approximately 43% of respondents (41 retailers) stated that they had not sought assistance from distribution companies. About 35% of respondents (33 individuals) reported being moderately satisfied, while nine respondents (9%) indicated that they were very satisfied with the support provided by the pesticide distributors (figure 5.10).

Question 11 of the survey: *If you have sought assistance from regional plant protection specialists. How satisfied are you with the outcome of the problem resolution?* (Alternatives: I didn't ask; Not at all; A little; Enough, A lot)

Approximately 71% of respondents (67 retailers) stated that they had not sought assistance from regional plant protection specialists. About 16% of respondents (15 individuals) reported being moderately satisfied, while seven respondents (7%) indicated that they were very satisfied with the support provided by regional plant protection specialists (figure 5.11).

Question 12 of the survey: *Do you have any information about the accredited Plant Protection Laboratory at Agricultural University of Tirana?* (Alternatives: Yes; No)

Approximately 58% of respondents (55 out of 95) are aware of the presence of this accredited Plant Protection Laboratory (figure 5.12).

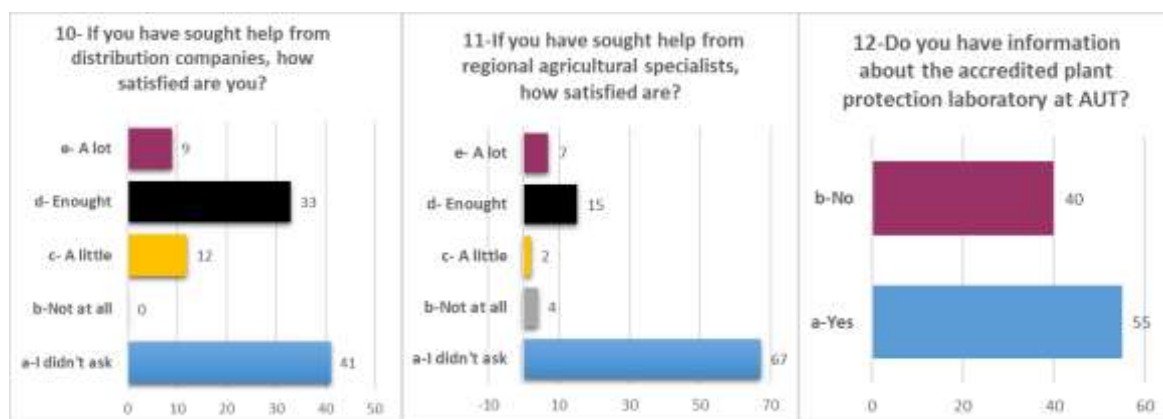


Figure 5. Charts presenting the evaluation of contributions from distributors and regional specialists, along with information on the plant protection laboratory.

Question 13 of the survey: *Regarding the use of biological versus chemical products: Which do you recommend more in your professional practice?*(Alternatives: Chemical; Biological; Chemical even though I have biological; Chemical because I don't have biological; equally).

Based on the data from question 13, the majority of respondents (approximately 72%, or 67 out of 95) recommend chemical control. Some respondents lack access to biological alternatives, while another 8% prefer chemical methods even though biological options are available. Approximately 25% of respondents recommend plant protection products of a biological nature (6.13).

Question 14 of the survey: Do you encounter any difficulties in selling biological fungicides or insecticides? (Alternatives: Yes; No).

Based on the data from question 14, the majority of respondents (65% or 62 out of 95) do not encounter difficulties in marketing biological PPPs, and 34% of them encounter difficulties. One respondent did not answer this question (6.14).

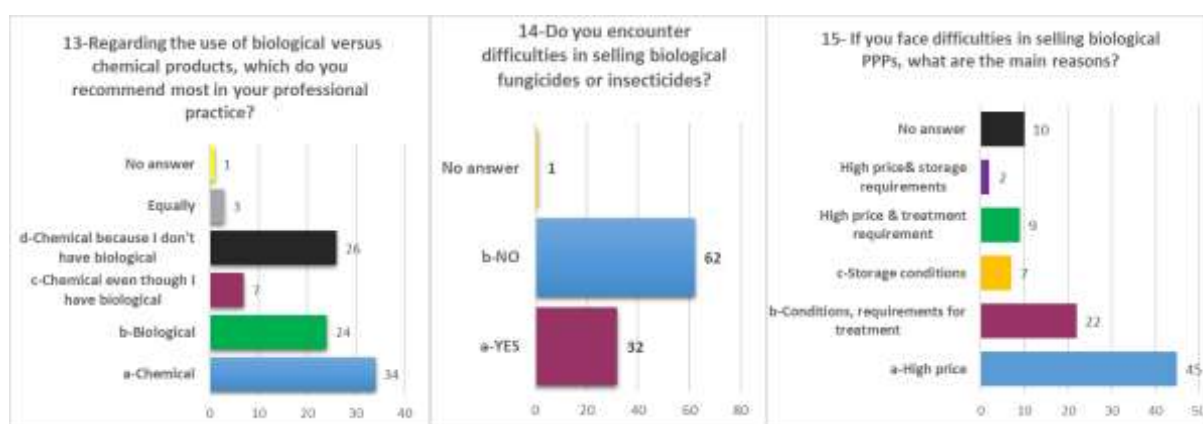


Figure 6. Graphs related to the use of biological products.

Question 15 of the survey: *If you encounter difficulties in selling biological plantprotection products (PPP), what are the reasons?* (Alternatives: High price; Conditions, requirements for treatment; Storage conditions).

Based on the data from question 15, it appears that 47% of respondents (45 out of 95) encounter difficulties due to the high price. 23% of respondents (22 out of 95) face difficulties related to specific application conditions, while 7% report challenges associated with special storage conditions. 11% of phytopharmacists did not answer this question. Furthermore, 10% of the reported difficulties stem from two combined factors: high price and treatment conditions, and 2% from the combination of high price and storage conditions (6.15).

Question 16 of the survey: *Have you participated in any training sessions recently?* (Alternatives: Yes; No).

From the survey data for question 17, it appears that 45% of respondents (43 out of 95) have participated in training, while 53% have not. Two respondents did not answer this question (fig. 7.16).

Question 17 of the survey: If you have participated in any training, who organized it? (Alternatives: From pesticide distribution companies; From various associations; From the Albanian authorities AM; From the Albanian authorities Agriculture Ministry AM).

The majority of respondents who had participated in training received this training from pesticide distribution companies (37% of respondents) (fig. 7.17).

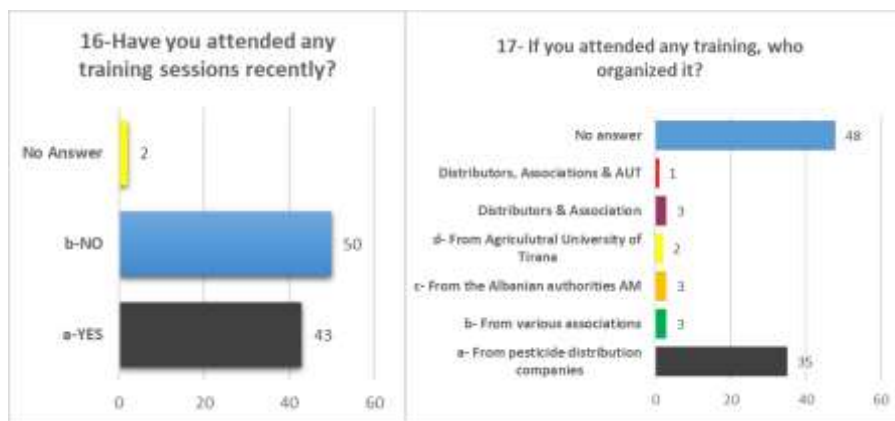


Figure 7. Graphs showing data on training participation and training organizers.

Question 18 of the survey: *How would you define the Pre-Harvest Interval (PHI)?* (Alternatives: The minimum interval between the final pesticide application and the harvest of the crop; The period you have to wait before applying the next treatment; Duration of effectiveness.).

A majority of respondents (91%, 86 out of 95) provided the correct answer, while 7% answered incorrectly and 2% did not respond to this question (fig.8.18).

Question 19 of the survey: *What do you understand by this pictogram (GHS 07)?*



(Alternatives: Indicates an environmental health hazard; Indicates a corrosive substance hazard; It indicates a risk to human health; It represents a serious risk to human health.).

Based on the survey data for question 19, 67% of respondents (64 out of 95) recognized this hazard pictogram, whereas 2% did not respond to this question (8.19).

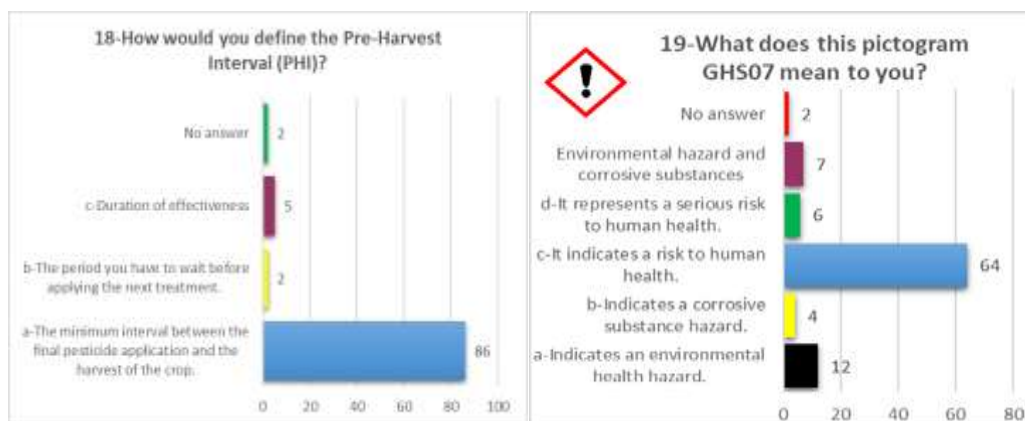


Figure 8. Graphs presenting answers about PHI and GHS 07 pictogram.

Question 20 of the survey: *What do you understand by this pictogram (GHS 09)?*



(Alternatives: Indicates an environmental health hazard; Indicates a corrosive substance hazard; It indicates a risk to human health; It represents a serious risk to human health.).

Based on the survey data for question 20, 92% of respondents (87 out of 95) recognized this hazard pictogram, whereas 3% did not respond to this question (fig.9.20).

Question 21 of the survey: *What do you understand by this pictogram (GHS 08)?*



(Alternatives: Indicates an environmental health hazard; Indicates a corrosive substance hazard; It indicates a risk to human health; It represents a serious risk to human health.).

Based on the survey data for question 21, 67% of respondents (63 out of 95) recognized this hazard pictogram, whereas 2% did not respond to this question (fig.9.21).

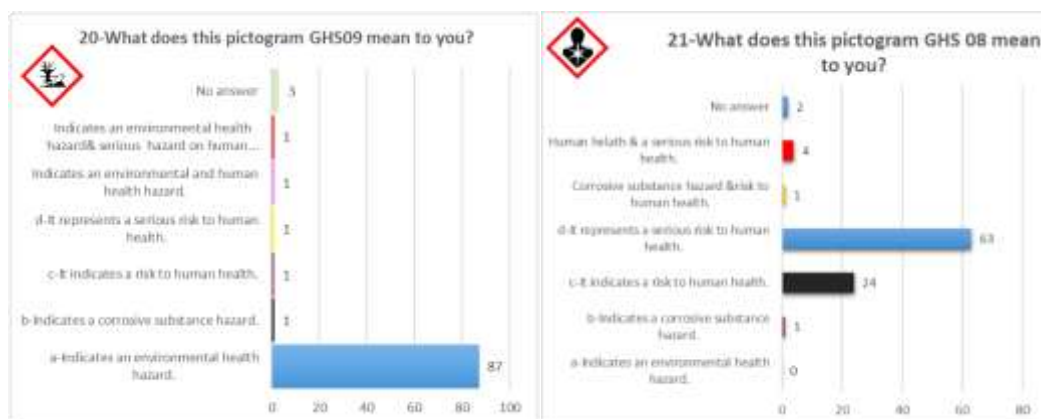


Figure 9. Graphs presenting answers about GHS 09 and GHS 08 pictograms.

Question 22 of the survey: *What do you understand by this pictogram (GHS 06)?*



(Alternatives: Acute toxicity (oral, inhalation, dermal); Chronic toxicity (oral, inhalation); Indicates a corrosive substance hazard; It represents a serious risk to human health.).

Based on the survey data for question 22, 61% of respondents (58 out of 95) recognized this hazard pictogram, whereas 3% did not respond to this question (fig.10.22).

Question 23 of the survey: *What do you understand by these pictograms?*



(Alternatives: We find them on the PPP label; It is not used for pesticides; To be used during pesticide transport).

Based on the survey data for question 23, 36% of respondents (34 out of 95) recognized this hazard pictogram, whereas 3% did not respond to this question (fig.10.23).

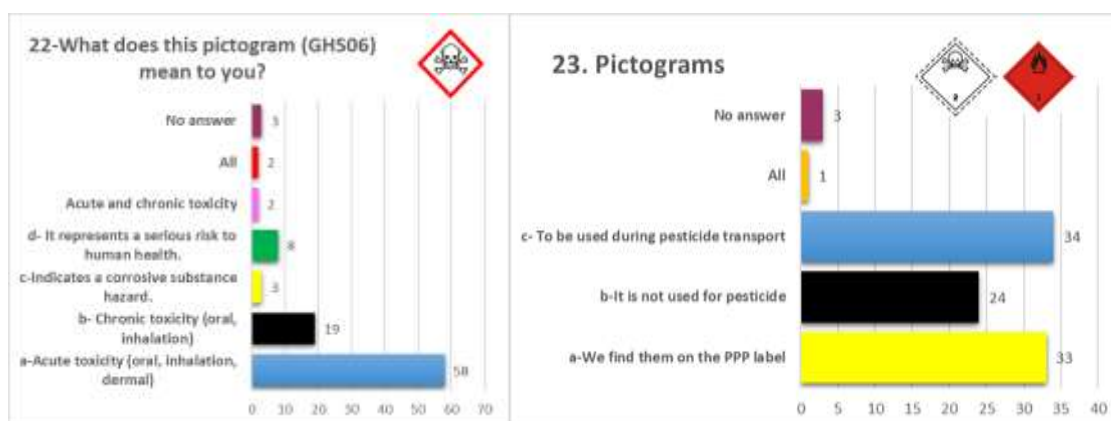


Figure 10 Graphs presenting answers about GHS 06 pictogram and transport pictograms.

CONCLUSIONS

The survey among phytopharmacist (pesticide retailers) highlights several important findings regarding the management and use of plant protection products (PPPs). Most pesticide retailer store maintain a diverse stock of over 30 PPPs, allowing effective product rotation to prevent resistance, with fungicides and insecticides representing the largest share of sales. Although chemical PPPs remain predominant (55%), many of pesticide retailers also recommend non-chemical measures. Notably, over 60% consider the pre-harvest interval, either alone or in combination with other factors, when recommending a PPP, and preventive measures are regarded as essential in control strategies. Biological PPPs and various traps hold a significant market share, but chemical PPPs are still preferred due to higher costs and specific application and storage requirements for biological products. In addressing problems or difficulties, Pesticide retailers (phytopharmacists) primarily rely on their experience and seek support mainly from PPP distributors. Finally, most respondents demonstrate good knowledge of the safe use and storage of PPPs, including hazard pictograms, though awareness of transport-related pictograms is limited. Overall, these findings emphasize the importance of continued training and support to promote safe and effective PPP use while encouraging the adoption of alternative and preventive measures.

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DIVERSITY AND IDENTIFICATION OF MITE POPULATIONS IN SELECTED WINE GRAPE CULTIVARS

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ABSTRACT

Vitis vinifera L. is one of the most important crops in the world economy, but is also affected by a wide range of mite pests, particularly those belonging to the families **Tetranychidae** and **Eriophyidae**. In vineyards, these pest populations are often kept below economic thresholds by predatory mites of the family **Phytoseiidae**. This study was carried out during 2025 in three wine grape vineyards in Tirana, Albania. Leaf samples were collected once per month, from May to September, from the cultivars **Sheshi i Bardhë**, **Sheshi i Zi**, and **Muscat Hamburg**. The aim was to identify and quantify mite populations associated with these grapevine cultivars. During the survey, mites from the families **Phytoseiidae** and **Tydeidae** were found, as well as typical symptoms of **Eriophyidae** infestations on grapevine leaves. Across all three wine grape cultivars, only one phytoseiid species, *Phytoseius finitimus* (Ribaga), was identified. Vineyard no. 2, corresponding to the cultivar Sheshi i Zi, showed the highest abundance of phytoseiid mites. Within phytoseiid populations, larval stages predominated, and the sex ratio was biased towards females.

Keywords: Grapevine, Phytoseiids, Tydeidae, Eriophyidae, Cultivar,

INTRODUCTION

Biological control, in addition to reducing dependence on chemical insecticides, promotes the conservation of biodiversity, a key element in building more balanced and sustainable agricultural systems (Curk et al. 2024, Freire et al. 2024, Pratisoli 2025). Phytoseiid mites are key natural enemies of pest species and often occur naturally in agroecosystems, where their populations can be supported through conservation practices rather than active intervention (Tixier et al., 2018; Aly, 2023). Their use represents an effective and sustainable alternative for integrated pest management (Duso et al. 2020, Vasquez et al 2023). The family *Phytoseiidae* includes many species of predators involved in the control of mite pests of crops all over the world (Tixer et al. 2013). In nature, a considerable number of natural enemies such as predatory mites and insects keep phytophagous mite populations under the economic damage levels (Duraj 2006). In European vineyards, these natural enemies play a key role in plant protection as their presence usually makes the use of acaricides unnecessary (Talebi et al. 2008; Tixer et al. 2013). The presence of phytoseiid mites on the grapevine shows better management from pests and diseases. Most species of this family are generalist predators; they can feed on their prey (especially the families *Tetranychidae* and *Eriophyidae*) but can also develop feeding on pollen, plant exudates, fungi and small insects (McMurtry & Croft 1997, Tixier et al. 2014). Each species has its specific biological features (Talebi et al. 2008). It is thus of interest to identify the species of useful mites of Phytoseiidae family on different grape cultivars.

Phytoseius finitimus is a generalist phytoseiid mite mainly recorded in the Mediterranean region on a variety of both cultivated and non-cultivated plants, such as grapevine, hazelnut, citrus, elm, etc. (Talebi et al. 2008) and is quite common in Mediterranean vineyards (Ahmad et al 2015; Pappas et al 2013; Tixier et al 2017) also present in Albania. The aim of the study was to identify and quantify mite populations associated with these grapevine cultivars. The specific objectives were: (1) to determine the abundance of *Ph. finitimus* in each cultivar; (2) to identify the sampling period with the highest number of phytoseiids; (3) to determine the ratio between developmental stages; (4) to determine the sex ratio; and (5) to assess the impact of phytophagous mites on phytoseiid populations.

MATERIAL AND METHOD

The study was carried out in three vineyards in 2025, involving three different wine grape cultivars. The vineyards are located on the outskirts of the city of Tirana, near Mount Dajti. Vineyard no. 1 is located at 41°19'01.4"N, 19°52'45.1"E and covers an area of 0.1 ha, (cultivated with Sheshi i Zi cultivar). Vineyard no. 2 is located at 41°18'56.2"N 19°52'37.0"E and covers an area of 0.25 ha, (cultivated with Sheshi i Zi and Sheshi i Bardhë cultivars). Vineyard no. 3 is located at 41°17'58.2"N 19°51'48.4"E and covers an area of 0.7 ha, (cultivated with Muscat Hamburg cultivar). The cultivation system used was double Guyot. For pest and disease protection, farmers applied plant protection products both during the vegetation period and as winter treatments. For this study, leaves were sampled once per month from May to September 2025, during the vegetative period. A total of 15 leaves (Girolami et al. 1989) per cultivar per sampling period were collected. Leaves were taken from within the rows, in the middle of the shoots (Duraj, 2000) avoiding the first row and the first three plants of the second row. Phytoseiid and Tydeid mites present on the leaves were counted under a stereomicroscope. Only mites of the *Phytoseiidae* family were mounted on microscope slides in Hoyer's medium for species identification. Species determination was performed using several identification keys for the *Phytoseiidae* family (Faraji et al 2011, Tixier et.al 2012, Tixer et al. 2013, Toyoshima et al 2016). Nomenclature of the setae followed the system of Lindquist and Evans, as adapted for the *Phytoseiidae* by Rowell, Chant, and Hansell (Rowell et al. 1978, Toyoshima et al. 2016).

RESULTS AND DISCUSSION

From the data collected in this study, it was found that only one mite species from the family *Phytoseiidae* was present: *Phytoseius finitimus* (Ribaga, 1904). In these vineyards, mites belonging to the families *Tydeidae* and *Eriophyidae* were also recorded.

Study results for vineyard no. 1, cultivar 'Sheshi i Zi'.

From the results obtained in **vineyard no. 1** (Figure 1), it was found that **only one mite species of the Phytoseiidae family, *Phytoseius finitimus***, was present. This species reached its **maximum abundance in July** with **1.87 ± 0.3 (SE) mites per leaf**, followed by **May** with **1.27 ± 0.3 (SE) mites per leaf**. In **September**, no phytoseiid mites were detected. Regarding other mite groups observed in this vineyard and cultivar, a **small number of Tydeidae mites** were recorded: **0.33 ± 0.06 (SE) per leaf** in May and **0.2 ± 0.06 (SE) per leaf** in July. Throughout all sampling periods, **phytoseiids clearly dominated** over Tydeidae mites, while **Tetranychidae mites were absent**. Typical **symptoms of gall formation (blister or erineum)** caused by **Eriophyid mites** were also present in this vineyard. The galls were **white inside**, indicating **active feeding sites** of the eriophyid mites (Al-Azzazy 2024). These symptoms were observed in **May, June, and July**. The presence of eriophyid mites has a significant impact on

phytoseiid populations ($y = 3.425x + 0.143$, $R^2 = 0.8524$), of which the main effect is attributed to the presence of eriophyids (statistically significant, $p = 0.025$), while the remaining variation is likely due to other factors, given that *Phytoseius finitimus* is a generalist predator (Papas et al. 2013) capable of feeding on different food sources (McMurtry et al. 2013).

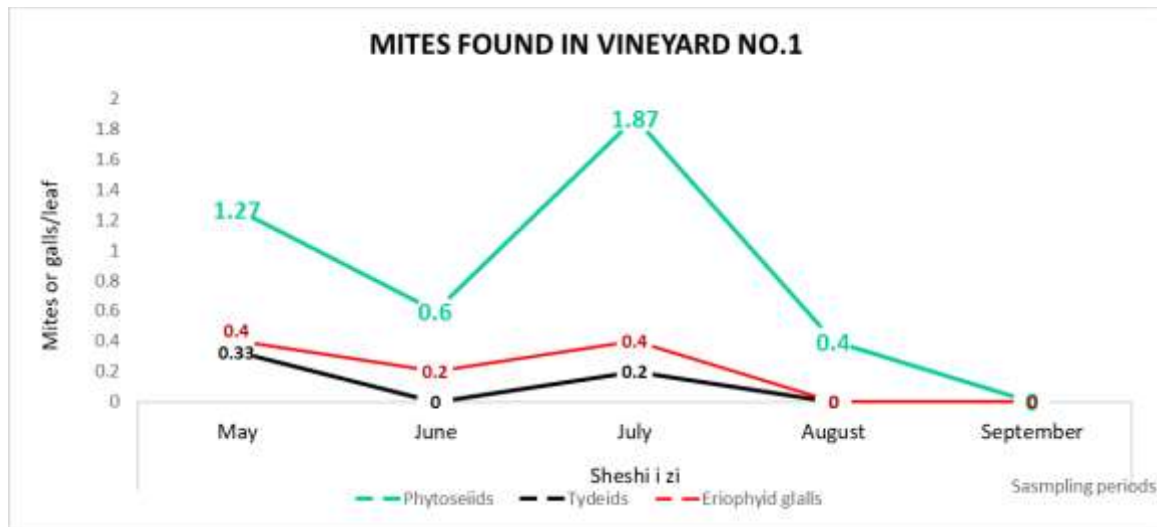


Figure 1. Mites present in the ‘Sheshi i Zi’ cultivar, vineyard no.1

In total, 8 tydeid mites and 68 phytoseiid mites were recorded in this vineyard throughout all sapling periods, consisting of 21 adults and 47 larvae. Within the populations of *Phytoseius finitimus* in the Sheshi i Zi cultivar of vineyard no. 1, the larval stages were dominant, followed by adult stages, while eggs were not observed in this cultivar. As shown in the following figures, July represented the month with the highest number of larvae (1.27 ± 0.22 SE per leaf) and adults (0.6 ± 0.09 SE per leaf). In September, no phytoseiid mites were found. Overall, considering the ratio among developmental stages, the larval stage dominated, comprising 69% of the total population, while adults represented 31%. Regarding the sex ratio, only females were recorded. In the end, an average of approximately 1 phytoseiid mite per sampling period per leaf was recorded, suggesting a potential role of this species in the biological control within the vineyard ecosystem.

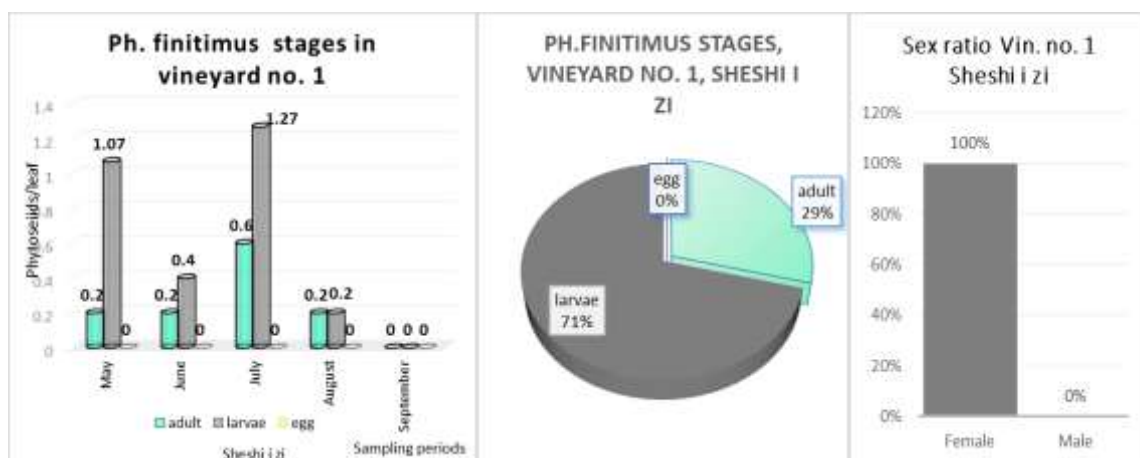


Figure 2. The ratio between egg, larval, and adult stages, and the sex ratio of *Phytoseius finitimus* in vineyard no. 1 (cultivar ‘Sheshi i Zi’).

Study results for vineyard no. 2, cultivars ‘Sheshi i Bardhë’ and ‘Sheshi i Zi’.

From the results obtained in vineyard no. 2, it was found that in both cultivars, only one species of the Phytoseiidae family, *Phytoseius finitimus*, was present. In both cultivars, the highest densities of phytoseiid mites were recorded during September. In the ‘Sheshi i Bardhë’ cultivar, the population reached an average of $2.8 \pm 0.4(\text{SE})$ *Ph. finitimus* per leaf, whereas in the ‘Sheshi i Zi’ cultivar, the maximum density reached $3.33 \pm 0.52 (\text{SE})$ individuals per leaf during the same period.

In contrast, the lowest population density in the ‘Sheshi i Bardhë’ cultivar was observed in May, with an average of $0.2 \pm 0.4 (\text{SE})$ individuals per leaf, while in the ‘Sheshi i Zi’ cultivar, the minimum occurred in August, with $0.47 \pm 0.52 (\text{SE})$ individuals per leaf.

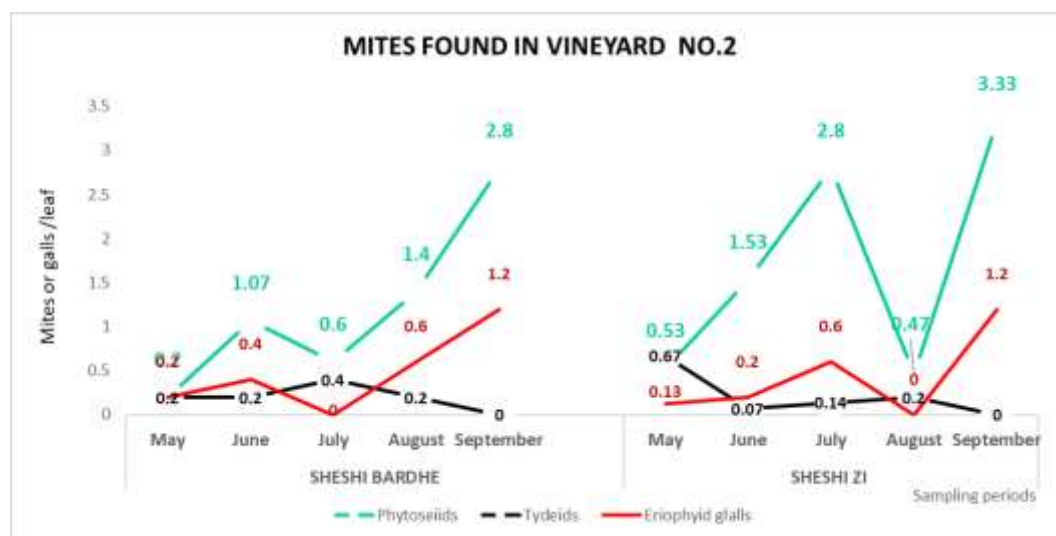


Figure 3. Mites present in the ‘Sheshi i Bardhë’ and ‘Sheshi i Zi’ cultivars, vineyard no.2

Tydeid mites were observed in both cultivars, and in all sampling periods, except for September. In cultivar ‘Sheshi i zi’, the highest density was recorded in May (0.67 ± 0.11 SE tydeids per leaf), while in the ‘Sheshi i Bardhë’ cultivar, the peak occurred in July (0.4 ± 0.06 SE tydeids per leaf). Tetranychidae mites were absent from both cultivars throughout the study. Additionally, typical symptoms of eriophyid mite infestation, in the form of galls, were observed in this vineyard. These symptoms appeared during all sampling periods except in July for cultivar ‘Sheshi i Bardhë’ and were absent in August for cultivar ‘Sheshi i zi’. In cultivars ‘Sheshi i Bardhë’ and ‘Sheshi i zi’ of vineyard no.2, the presence of eriophyid mites had a significant impact on phytoseiid populations (‘Sheshi i Bardhë’, $y = 2.0689x + 0.2209$, $R^2 = 0.9129$; ‘Sheshi i zi’, $y = 2.4873x + 0.6724$, $R^2 = 0.8674$). This effect is primarily attributed to the presence of eriophyids (statistically significant in ‘Sheshi i Bardhë’, $p = 0.01$; in ‘Sheshi i zi’, $p = 0.02$), while the remaining variation likely results from other ecological factors, including the generalist feeding behavior of *Phytoseius finitimus* (Pappas et al., 2013) and its ability to utilize multiple food sources (McMurtry et al., 2013).

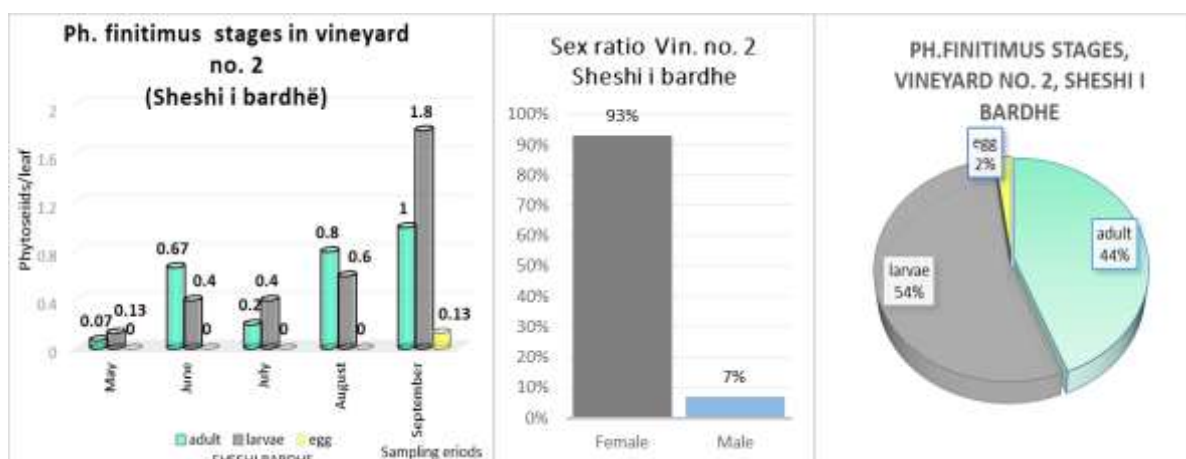


Figure 4. The ratio between egg, larval, and adult stages, and the sex ratio of *Phytoseius finitimus* in vineyard no. 2 (cultivar ‘Sheshi i Bardhë’).

In the populations of *Phytoseius finitimus* in the cultivar *Sheshi i bardhë* (vineyard no. 2), **larval stages were dominant**, followed by **adult stages**, while **egg stages were present in low numbers**. As shown in **Figure 4**, **September** was the month with the **highest number of larvae** (1.2 ± 0.26 (SE) per leaf) and **adults** (1 ± 0.16 (SE) per leaf). Eggs were detected **only in September** at 0.13 ± 0.02 (SE) per leaf, making this sampling period the **sole occurrence of the egg stage**. Overall, in terms of developmental stage ratios, **larvae comprised 54%** of the population, **adults 44%**, and **eggs 2%**. Regarding the **sex ratio**, females accounted for **93%** of the population. On average, **0.55 adults per leaf per sampling period** (total 41) and **0.67 larvae per leaf per sampling period** (total 51) were recorded in this cultivar. Consequently, as a **potential biological control agent**, an average of **1.2 phytoseiids per sampling period per leaf** was observed.

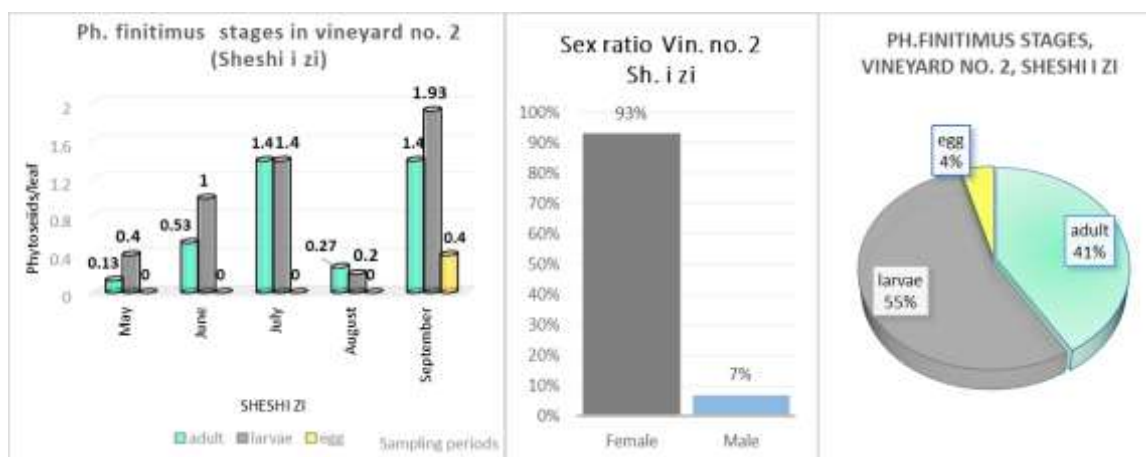


Figure 5. The ratio between egg, larval, and adult stages, and the sex ratio of *Phytoseius finitimus* in vineyard no. 2 (cultivar ‘Sheshi i Zi’).

In the *Sheshi i Zi* cultivar, populations of *Phytoseius finitimus* were dominated by larval stages, followed by adult mites, while the egg stage was present in low numbers, observed only in September. As shown in **Figure 5**, the highest larval densities were recorded in July (1.4 ± 0.28 (SE) larvae per leaf) and September (1.47 ± 0.28 (SE) larvae per leaf). Adult mites were less abundant than larvae, with the highest numbers observed in June and September (1.4 ± 0.25 (SE) adults per leaf). Eggs were recorded exclusively in September, with 0.4 ± 0.07 (SE) eggs per leaf.

Overall, considering the distribution among developmental stages, larvae comprised 55% of the population (74 individuals in total), adults 41% (56 individuals), and eggs 4% (6 individuals). Females dominated the population, accounting for 93% of adults, while males represented only 7%. On average, 0.75 adults and 0.99 larvae per leaf per sampling period were recorded in this cultivar. In total, the average number of phytoseiid mites per leaf per sampling period was 1.73, highlighting their potential role as biological control agents.

Study results for vineyard no. 3, cultivar ‘Muscat Hamburg’.

From the results obtained in vineyard no. 3 (Figure 6), it was found that only one mite species of the Phytoseiidae family, *Phytoseius finitimus*, was present. This species reached its maximum abundance in September with 1.53 ± 0.25 (SE) mites per leaf. In July, no phytoseiid mites were detected. Regarding other mites present in this vineyard, a considerable number of Tydeidae mites were recorded. Tydeidae were observed in all sampling periods except July, with the highest density recorded in September (0.67 ± 0.1 (SE) tydeids per leaf). Comparing the total numbers of mites, Phytoseiidae (33 individuals) were more abundant than Tydeidae (18 individuals).

Typical symptoms of eriophyid mite infestation, in the form of galls, were also observed in this vineyard during May, June, and September. The presence of eriophyid mites had a significant effect on phytoseiid populations ($y = 1.6875x + 0.0325$, $R^2 = 0.8361$), primarily attributable to eriophyid abundance (statistically significant, $p = 0.03$). The remaining variation likely results from other factors, particularly the generalist feeding behavior of *Phytoseius finitimus* (Pappas et al., 2013), which allows it to exploit multiple food sources (McMurtry et al., 2013).

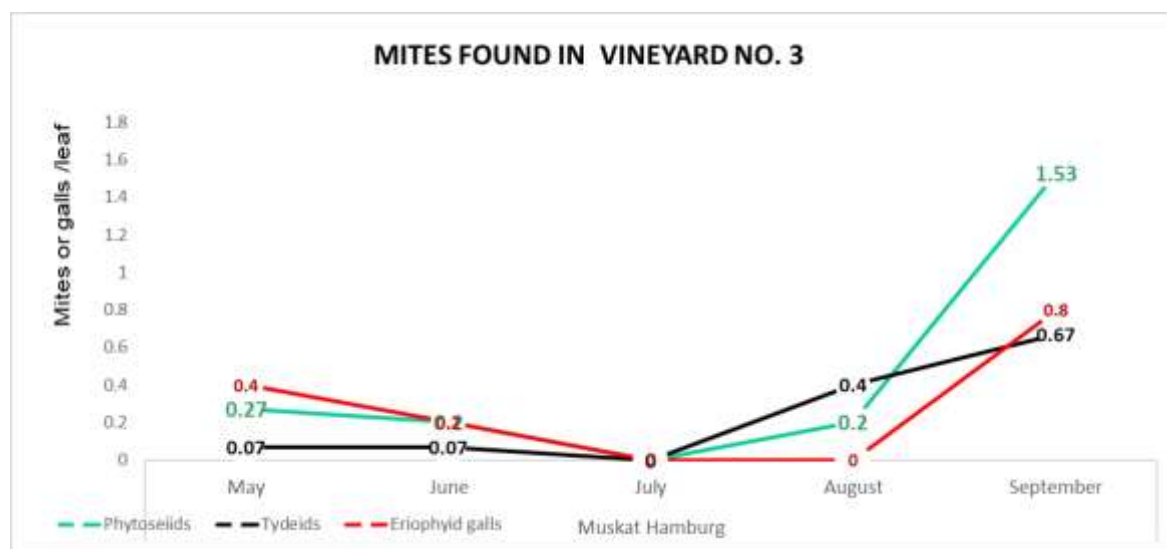


Figure 6. Mites present in the ‘ Muscat Hamburg ’ cultivar, vineyard no.3

In the populations of *Phytoseius finitimus* in the *Muscat Hamburg* cultivar (vineyard no. 3), the larval stages dominated, followed by the adult stage, while the egg stage was not detected in any of the samples collected during the study. As shown in Figure 7, September was the month with the highest number of larval stages (1 ± 0.16 SE per leaf) and adult mites (0.58 ± 0.09 SE per leaf). Overall, in terms of stage ratio, the larval stage accounted for 71% of the population, the adult stage for 29%, and the egg stage for 0%. On average, per sampling period in the *Muscat Hamburg* cultivar, 0.13 adults were recorded (10 in total), and 0.32 larvae (24 in total). Consequently, the average population density of phytoseiids was 0.44 individuals per

sampling period. Within the population of *Ph. finitimus*, females represented 90% of the individuals, whereas males comprised 10%.

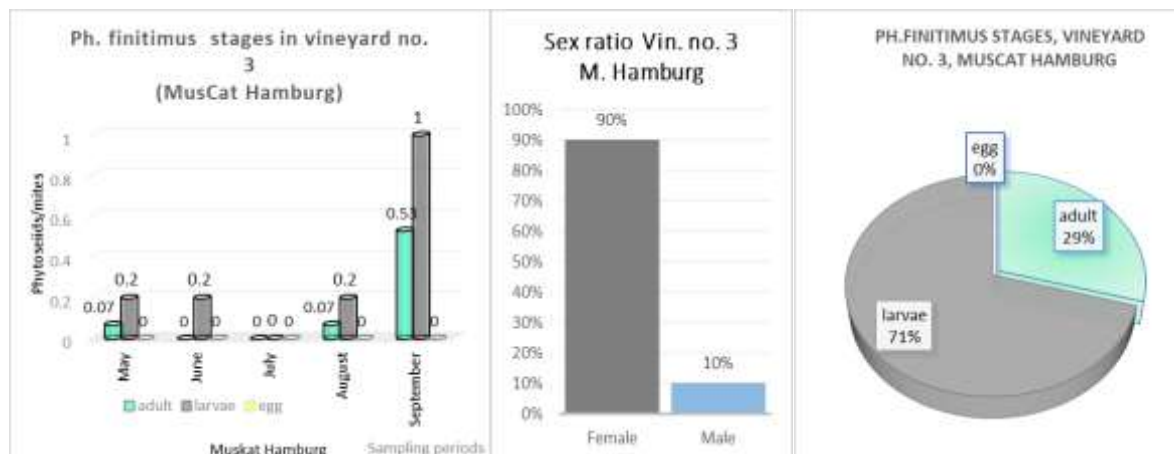


Figure 7. The ratio between egg, larval, and adult stages, and the sex ratio of *Phytoseius finitimus* in vineyard no. 2 (cultivar 'Muscat Hamburg').

CONCLUSIONS

From the study conducted in three wine grape vineyards managed by different farmers in Tirana, we identified the presence and activity of mites belonging to the families *Tydeidae*, *Eriophyidae*, and, most importantly, *Phytoseiidae*. During this survey, one phytoseiid species was identified: *Phytoseius finitimus*.

The Sheshi i zi cultivar had the highest number of phytoseiid mites per leaf, while the Muscat Hamburg was the least populated. Vineyard no. 2 showed the highest number of phytoseiids per leaf. The highest density of phytoseiids was recorded in September, reaching 3.33 individuals per leaf in the Sheshi i Zi cultivar (vineyard no. 2). May and September were the months with the greatest abundance of phytoseiids per leaf. Regarding developmental stages, larval stages dominated; egg stages were rarely recorded, and females prevailed over males in all samples. Eriophyid mites had a significant positive effect on *Ph. finitimus* populations.

The presence of *Phytoseiidae* mites across the vineyards and cultivars studied represents an important indicator for the natural biological control of phytophagous mites in grapevine ecosystems.

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CLINICAL AND HISTOPATHOLOGICAL STUDY BASED ON ENDOSCOPIC EVALUATION OF GASTRIC CANCER IN ALBANIA DURING 2024

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ABSTRACT

Gastric cancer remains one of the most lethal malignancies worldwide due to its clinically silent onset, significant variability in tissue pathology, and a tendency for detection at later stages of the disease. In Albania, the burden of this condition is further aggravated by delayed diagnosis and limited public awareness of early gastrointestinal symptoms. This retrospective study analyzed all biopsy-confirmed cases of gastric carcinoma diagnosed between January and December 2024 at a single Endoscopy Center in Tirana. Among 2,150 upper gastrointestinal endoscopies performed, 41 cases of gastric malignancy were identified, yielding a detection rate of 1.9%. Based on Lauren's classification, the predominant histologic subtypes were intestinal and diffuse adenocarcinomas. The majority of patients were male and over 45 years of age. Lesions were most commonly localized to the corpus and antrum–pylorus, with a smaller proportion involving the cardia and gastroesophageal junction. The leading presenting symptoms included epigastric pain, anorexia, weight loss, and iron-deficiency anemia, consistent with delayed clinical presentation and advanced local disease at the time of diagnosis. The results highlight the ongoing difficulty in achieving timely diagnosis and emphasize the urgent need for increased public awareness, timely referral for upper endoscopy, and standardized histopathologic assessment. Expanding access to diagnostic endoscopy and implementing early detection strategies may contribute to improved clinical outcomes and reduced mortality from gastric cancer in the Albanian population.

Keywords: Gastric cancer; Albania; Endoscopic diagnosis; Lauren classification; Histopathology; Early detection

INTRODUCTION

Gastric cancer remains a major global health concern, ranking among the most diagnosed malignancies and the fourth leading cause of cancer-related deaths worldwide, with over one million new cases and approximately 770,000 fatalities reported annually (Lin et al., 2024; International Agency for Research on Cancer, 2022). Although incidence rates have declined in some high-income countries, current predictive models suggest that gastric cancer will remain a major contributor to global disease burden through 2040, particularly in low- and middle-income countries undergoing demographic and epidemiologic transitions (Morgan et al., 2020; Lin et al., 2024). The disease exhibits marked biological heterogeneity, influenced by molecular alterations, environmental exposures, and host immune responses. The revised WHO classification of gastrointestinal tumors now emphasizes an integrative diagnostic approach, combining morphological and molecular features. Nevertheless, the Lauren classification

remains clinically relevant for distinguishing between intestinal and diffuse subtypes, each with distinct pathogenesis, epidemiology, and clinical course (Nagtegaal et al., 2020; Kushima, 2022; Lauren, 1965).

Persistent infection with *Helicobacter pylori* is the most well-established and modifiable risk factor, initiating chronic gastric inflammation and genetic alterations that contribute to the multistep progression from non-atrophic gastritis to atrophy, intestinal metaplasia, dysplasia, and ultimately carcinoma (Duan et al., 2025). Evidence from meta-analyses confirms that *H. pylori* eradication significantly reduces the long-term risk of developing gastric cancer, reinforcing its central role in primary prevention strategies (Ford et al., 2020). In addition, various dietary and lifestyle factors—such as high consumption of salted or smoked foods, low intake of fresh fruits and vegetables, tobacco use, and excessive alcohol intake—have been identified as contributors to increased gastric cancer risk (Maddineni et al., 2022; Bouras et al., 2022; Pu et al., 2024; Liu et al., 2022). A concerning trend is the rising incidence of gastric cancer in younger adults, often presenting with diffuse histology, minimal environmental associations, and a more aggressive clinical trajectory (Li, 2020). Advancements in diagnostic technologies—including high-definition endoscopy, virtual chromoendoscopy, risk stratification tools, and molecular biomarkers—are improving early detection rates. However, the global implementation of these tools remains uneven, particularly in resource-limited settings (Xia & Aadam, 2022; Matsuoka & Yashiro, 2023; Leja, 2024).

In Albania, gastric cancer remains a prominent public health concern. According to GLOBOCAN 2024 data, it is the third most frequently diagnosed malignancy in the country, accounting for 716 new cases (8.9%), and ranks as the second leading cause of cancer-related mortality with 560 deaths (11.3%). Data from the National Cancer Registry (2015–2019) indicate a rising incidence, a higher prevalence among males, and a predominance of cases diagnosed in individuals over 60 years of age. These trends reflect both delayed detection and the lack of structured early detection programs (ISHP/INSTAT, 2020). The high mortality-to-incidence ratio highlights the frequency of late-stage diagnosis, when treatment options are limited and prognosis is poor.

Endoscopic examination, accompanied by targeted biopsy, remains the gold standard for diagnosing gastric cancer. It enables direct visualization of suspicious lesions and provides tissue for histopathological evaluation, facilitating definitive diagnosis (Latorre et al., 2024; Lee et al., 2010). The correlation between lesion morphology, anatomical location, and histological subtype is clinically relevant, influencing prognosis and guiding therapeutic decisions, including endoscopic resection, surgical intervention, or systemic therapy (Kim et al., 2019; Kim & Seo, 2022; Li et al., 2022).

Within this framework, the present study—Clinical and Histopathological Study Based on Endoscopic Evaluation of Gastric Cancer in Albania During 2024—aims to provide an updated overview of the clinical features, endoscopic findings, and histopathological characteristics of gastric cancer in the Albanian population. By synthesizing data from both endoscopic and pathological evaluations, this research seeks to support earlier diagnosis, inform clinical decision-making, and contribute to the development of evidence-based national strategies for prevention and timely detection.

MATERIAL AND METHOD

This retrospective descriptive study was conducted at the La Vita Endoscopic Clinic in Tirana, Albania, over a 12-month period from January 1 to December 31, 2024. The primary objective was to characterize the clinical, endoscopic, and histopathological features of gastric cancer diagnosed during this interval.

All adult patients (≥ 18 years) who underwent diagnostic esophagogastroduodenoscopy (EGD) during the study period were considered for inclusion. A total of 2,150 upper endoscopic examinations were performed. Biopsies were obtained in 932 patients (43%) for various indications, including evaluation for *Helicobacter pylori* infection, systematic gastric mapping according to the Sydney Protocol, assessment of atrophic gastritis, intestinal metaplasia, and dysplasia for OLGA/OLGIM staging (MAPS III ESGE Guideline Update, 2025), investigation of eosinophilic esophagitis, and duodenal sampling for suspected celiac disease.

Among the biopsied cases, 41 patients were diagnosed with gastric carcinoma and were included in the analysis. Cases of duodenal carcinoma ($n = 1$) and esophageal carcinoma ($n = 5$) were excluded. For each confirmed gastric cancer case, the following demographic and clinical variables were recorded: age, sex, place of birth and residence, presenting symptoms, and reason for referral. Endoscopic lesion characteristics were also documented, including anatomical location within the stomach (cardia, fundus, corpus, antrum, or pylorus) and macroscopic appearance.

All endoscopic examinations were performed using the Olympus EVIS EXERA III – Series 190 system, which offers high-definition imaging and Narrow Band Imaging (NBI) technology to enhance mucosal and vascular visualization, thereby facilitating the detection of subtle neoplastic changes.

Histopathological diagnoses were established according to the 2019 WHO Classification of Tumors of the Digestive System, and tumors were further categorized using the Lauren classification (intestinal vs. diffuse type). Tumor differentiation was graded as well, moderately, or poorly differentiated.

Statistical analysis was performed using Python (version 3.x). After organizing the dataset, descriptive statistics were generated, and relationships between variables were examined using correlation and group comparison tests appropriate to data characteristics. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

During the study period (January 1 to December 31, 2024), a total of 2150 esophagogastroduodenoscopy examinations were performed at La Vita Endoscopic Clinic, in Tirana, Albania. Biopsy samples were collected from 932 patients (43%) based on clinical indications such as suspected mucosal lesions in the esophagus, stomach, or duodenum; investigation of *Helicobacter pylori* infection; systematic gastric sampling following the Sydney classification system; and assessment of mucosal changes including atrophy, intestinal metaplasia, and dysplasia, using the OLGA/OLGIM staging frameworks. Additional indications included biopsy of gastric polyps, evaluation of eosinophilic esophagitis, and duodenal sampling for potential celiac disease. Out of the total cohort, 41 patients were diagnosed with gastric carcinoma and subsequently included in this study.

5.1 Demographic Characteristics

Among the 41 patients identified with gastric carcinoma, 30 (73%) were male and 11 (27%) were female, illustrating a significant male predominance—a trend documented extensively in global cancer research (IARC, 2022). The average age at diagnosis was 62 years, with a range spanning from 25 to 81 years. Most cases (27 out of 41; 65.9%) were observed in individuals aged between 60 and 75, suggesting a higher prevalence in late adult life. Patients under the age of 40 were relatively rare (4/41; 9.8%), as were those older than 75 years (4/41; 9.8%). These findings are in line with established demographic patterns, where gastric cancer is more frequently seen in men and increases in prevalence with age, likely due to persistent *Helicobacter pylori* infection and other host-related risk factors (Smyth et al., 2020; IARC, 2022). Although rare, the presence of gastric cancer in patients as young as 25 underscores the importance of considering malignancy even in younger adults during clinical evaluation.

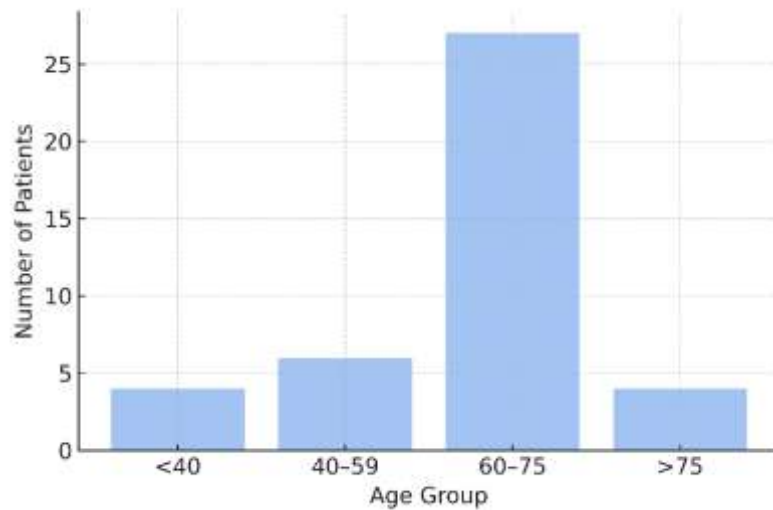


Figure 1. Age distribution of gastric cancer patients (n = 41)

5.2 Clinical Presentation

In our patient group, the most reported symptoms were anorexia (17/41; 41.5%), weight loss (15/41; 36.6%), epigastric discomfort or pain (15/41; 36.6%), and anemia (12/41; 29.3%). Signs of upper gastrointestinal bleeding, including melena, were present in 6 patients (14.6%), and 7 individuals (17.1%) experienced nausea and/or vomiting. Less frequently, symptoms such as dyspepsia, generalized fatigue, dysphagia, abdominal pain, and back pain were reported in smaller numbers. Two patients were evaluated postoperatively after previous surgical intervention for gastric perforation and were referred for diagnostic confirmation and staging.

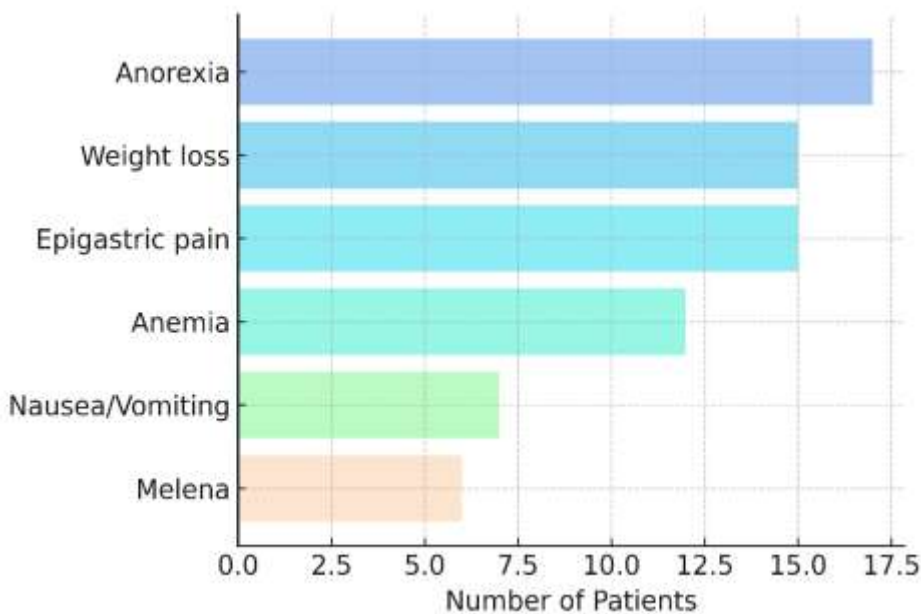


Figure 2. Distribution of clinical presenting symptoms

5.3 Tumor Localization

Endoscopic evaluation was performed to determine tumor location, with classification based on the primary anatomical region of the stomach involved. Lesions confined to a single area were categorized as cardia/fundus (upper), corpus (middle), or antrum-pylorus (lower). Tumors

involving two or more adjacent gastric regions (e.g., cardia and corpus, or corpus and antrum) were considered indicative of locally advanced disease. Tumors arising at a post-gastrectomy anastomosis site were classified separately due to their distinct clinical and pathological characteristics. Among the cohort, five patients presented with a linitis plastica phenotype, characterized by diffuse gastric wall thickening and reduced compliance. One case was limited to the corpus and was included in that group, whereas the remaining four exhibited broader regional involvement and were therefore assigned to the locally advanced category. Tumor localization data for the entire study population is summarized in Table 1.

Table 1. Anatomical localization of gastric cancer cases

Localization Category	Definition	n (%)
Corpus	Lesions confined to the gastric corpus	6 (14.6%)
Antrum–pylorus	Tumors located in the distal stomach (antrum and/or pylorus)	11 (26.8%)
Cardia / Gastroesophageal Junction (GEJ)	Tumors involving the cardia and/or GE junction	6 (14.6%)
Tumors involving multiple gastric regions	Tumors affecting two or more adjacent gastric areas	16 (39.0%)
Post-gastrectomy Anastomosis	Tumors arising at prior surgical anastomosis sites	2 (4.8%)

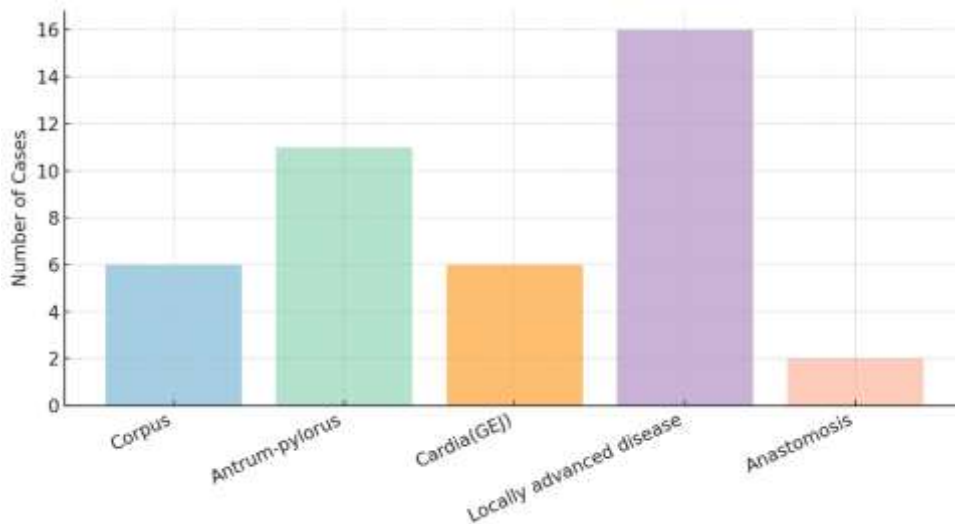


Figure 3. Distribution of gastric cancer localization across anatomical regions

The anatomical distribution observed in this cohort reflects the influence of two distinct but coexisting carcinogenic pathways, well-documented in global and regional gastric cancer epidemiology. Distal gastric cancers (antrum–pylorus) remain strongly associated with *Helicobacter pylori*–induced chronic atrophic gastritis and intestinal metaplasia, following the Correa cascade of gastric carcinogenesis (Correa, 1992; Correa & Houghton, 2007). This

pathway continues to dominate in high *H. pylori* prevalence areas, such as Albania and the Western Balkans (Abengoza et al., 2021).

Conversely, proximal and cardia/gastroesophageal junction (GEJ) tumors are increasingly linked to obesity, metabolic syndrome, and chronic gastroesophageal reflux disease (GERD), reflecting dietary Westernization and sedentary lifestyle patterns particularly over the past two decades (Smyth et al., 2020; ESMO Clinical Practice Guidelines, 2022). A rising incidence of proximal gastric and GEJ adenocarcinomas has been documented in Central and Eastern Europe, mirroring epidemiological trends previously observed in Western countries (Kim et al., 2018; Abengoza et al., 2021).

The high proportion of locally advanced tumors (39%) in this study suggests delayed diagnosis, likely due to the insidious onset of nonspecific symptoms and the absence of structured national screening programs for gastric cancer in Albania (ISHP & INSTAT, 2020). Similar patterns of late-stage presentation have been documented across Southeastern and Eastern Europe, reflecting broader system limitations in early detection (Bray et al., 2024; Abengoza et al., 2021; Leja, 2024).

Among the cases with a linitis plastica growth pattern, only one tumor was confined to a single anatomical region (corpus), while the remaining four involved multiple gastric regions. This distribution reflects the characteristic diffuse infiltrative behavior of poorly cohesive carcinoma, corresponding to the Laurén diffuse type, in which tumor cells permeate the gastric wall with extensive submucosal spread and progressive loss of distensibility (Lauren, 1965; WHO Classification of Tumours Editorial Board, 2022). Such presentations require heightened diagnostic consideration, particularly in younger patients or in individuals with a family history suggestive of hereditary predisposition, where germline *CDH1* mutation testing may be clinically indicated according to contemporary hereditary diffuse gastric cancer guidance (Blair et al., 2020; NCCN Clinical Practice Guidelines, 2025).

While this study categorized tumors using conventional anatomical regions (cardia/GEJ, corpus, antrum–pylorus), the observed distribution can also be interpreted within the framework of the nPTD classification, which subdivides the stomach into proximal (P), transitional (T), and distal (D) zones based on physiological lymphatic flow and functional anatomy (Kinami et al., 2016). Although not applied directly in this cohort, this system may provide valuable guidance for optimizing resection extent and lymphadenectomy, particularly in early-stage disease where function-preserving gastrectomy is considered.

5.4 Histopathological Characteristics

Based on Lauren classification, 26 out of 41 cases (63.4%) were classified as intestinal type, 10 cases (24.4%) as diffuse type, and 5 cases (12.2%) exhibited a mixed intestinal–diffuse phenotype.

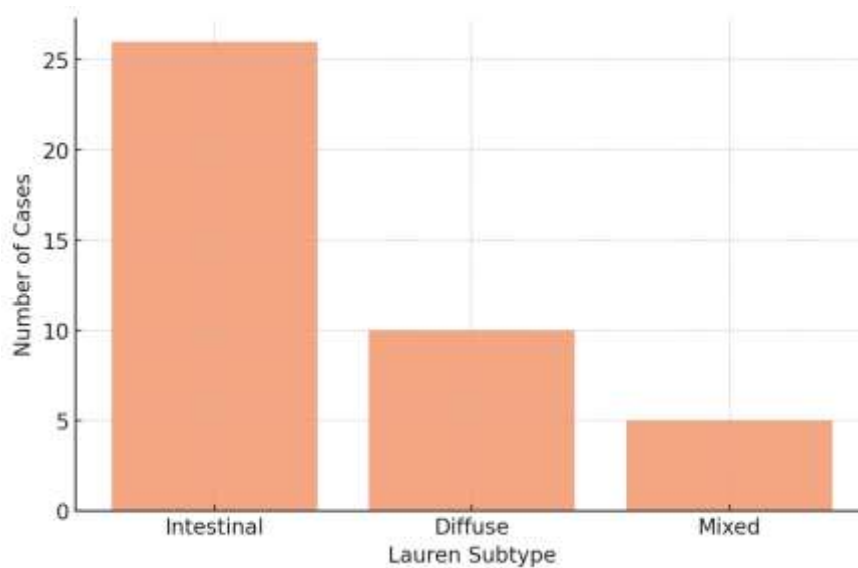


Figure 4. Lauren Histopathological Classification

Regarding histologic grade, poorly differentiated adenocarcinoma (G3) represented the largest proportion of tumors (23/41; 56.1%), followed by moderately differentiated (G2) (15/41; 36.6%) and well-differentiated tumors (G1) (3/41; 7.3%).

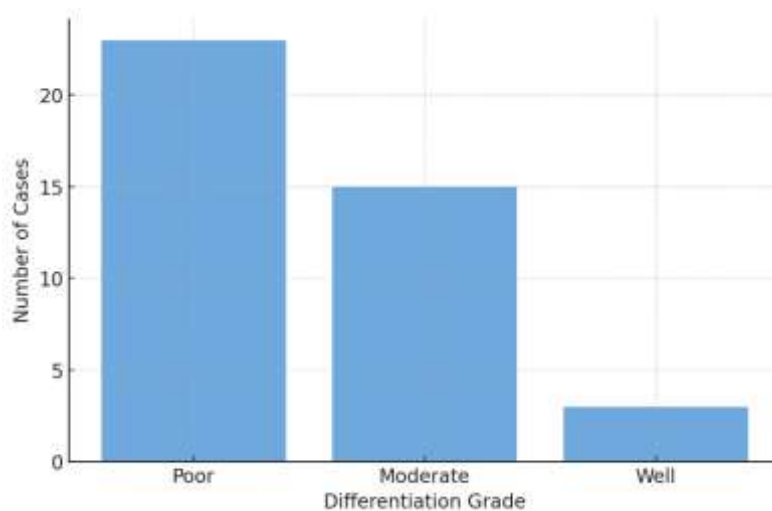


Figure 5. Tumor Differentiation Characteristics

Signet-ring cell morphology was identified in 10 cases, predominantly within the diffuse and mixed Lauren subtypes, which aligns with their characteristically infiltrative growth pattern (WHO Classification of Tumours Editorial Board, 2022). Additionally, mucinous adenocarcinoma, defined by $\geq 50\%$ extracellular mucin according to WHO criteria, was observed in 2 cases, both of which were classified within the diffuse subtype (WHO, 2019; Kushima, 2022).

Table 2. Histopathological Profile of Gastric Adenocarcinoma Cases

Histopathological Feature	N (%)	Differentiation Grade	N (%)
Lauren subtype		Well differentiated (G1)	3 (7.3%)
• Intestinal	26 (63.4%)	Moderately differentiated (G2)	15 (36.6%)
• Diffuse	10 (24.4%)	Poorly differentiated (G3)	23 (56.1%)
• Mixed	5 (12.2%)		
Signet-ring morphology	10 (24.4%)		
Mucinous phenotype	2 (4.9%)		

These findings reveal a predominance of histological features commonly associated with aggressive biological behavior, particularly within the diffuse and mixed subtypes of gastric adenocarcinoma. While the intestinal subtype remains the most frequent in this cohort, its distribution is consistent with the classical Correa cascade, wherein *Helicobacter pylori*–induced chronic atrophic gastritis and intestinal metaplasia progress toward the development of intestinal-type gastric cancer (Correa, 1992).

However, the notable proportion of diffuse and mixed histological types (36.6%), coupled with a high prevalence of poor differentiation (56.1%) and signet-ring cell morphology (24.4%), suggests a more clinically aggressive tumor phenotype. Diffuse-type gastric carcinoma is typically associated with loss of intercellular adhesion, frequently driven by CDH1 (E-cadherin) pathway dysfunction, and is characterized by infiltrative growth with a higher likelihood of peritoneal dissemination (Lauren, 1965; WHO, 2019; Blair et al., 2020).

The presence of signet-ring cell carcinoma in five patients aged ≤ 40 years is particularly noteworthy, given the established association of this morphology with the hereditary diffuse gastric cancer (HDGC) syndrome. In individuals younger than 50 years, especially those with a relevant family history or coexisting bilateral lobular breast cancer, referral for genetic counseling and consideration of germline CDH1 mutation testing is recommended according to current international HDGC guidelines (Blair et al., 2020; NCCN Clinical Practice Guidelines, 2025).

Additionally, the low proportion of well-differentiated tumors (7.3%) further supports the notion that gastric cancer in this clinical population is often diagnosed at a biologically advanced stage. This observation is consistent with earlier findings of late clinical presentation and reflects the absence of population-based early detection strategies in Albania and other countries in the Balkan region (MAPS III ESGE/EHMSG/ESP Guideline Update, 2025).

Table 3. Summary of Correlation Analyses

Correlation	Statistical Test	p-value	Interpretation
Age × Lauren subtype	Kruskal–Wallis	0.145	No significant age differences between Lauren subtypes.
Age × Differentiation grade	Kruskal–Wallis	0.864	No association between age and differentiation grade.
Age × Signet-ring presence	Mann–Whitney U	0.0085	Signet-ring tumors occur in significantly younger patients.
Localization × Lauren subtype	Chi-square	0.253	No significant association between anatomical site and Lauren subtype.
Localization × Signet-ring	Chi-square	0.003	Signet-ring morphology is more common in locally advanced disease
Localization × Differentiation grade	Chi-square	0.066	No significant association (trend toward poorer differentiation in advanced disease)

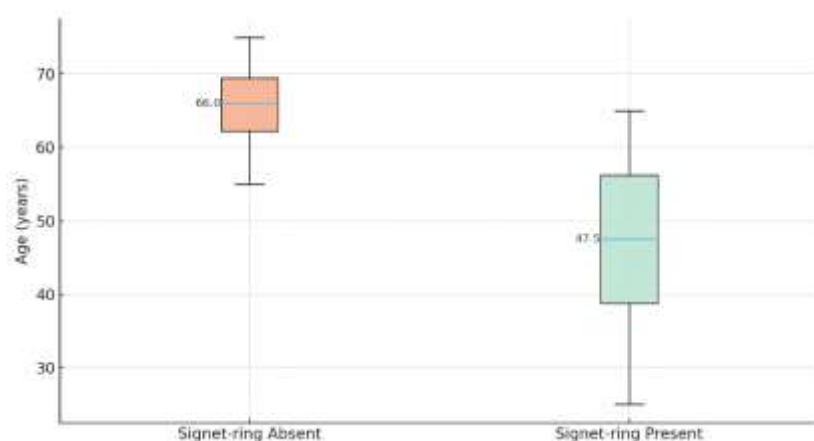


Figure 6. Age distribution of patients by presence of signet-ring cell morphology

A statistically significant association was observed between younger age and the presence of signet-ring cell carcinoma ($p = 0.0085$). As shown in Figure 6, patients with signet-ring morphology exhibited a lower median age compared to those with non-signet-ring tumors, which were predominantly diagnosed in older individuals. This distribution is consistent with

established evidence showing that diffuse-type gastric cancer, particularly signet-ring variants, occurs more frequently in younger patients and may follow distinct molecular and hereditary pathways, including CDH1 (E-cadherin) dysfunction, impaired intercellular adhesion, and familial clustering (Lauren, 1965; Li, 2020; WHO, 2019). Clinically, this age-related pattern is especially relevant in settings lacking structured screening programs, where younger patients may be under-recognized and consequently present with more advanced disease. These findings highlight the importance of heightened diagnostic vigilance in symptomatic younger individuals, even in the absence of a known family history.

In addition, a statistically significant association was identified between tumor localization and signet-ring morphology ($p = 0.003$). Tumors with locally advanced involvement more frequently demonstrated mixed or signet-ring histology, reflecting a more infiltrative biological phenotype. This pattern aligns with established characteristics of diffuse-type gastric carcinoma, including loss of E-cadherin-mediated intercellular adhesion and a propensity for submucosal and transmural spread, which allow extensive gastric wall involvement even when mucosal alterations remain subtle (Lauren, 1965; Kushima, 2022). While other correlations did not reach statistical significance, the observed trends suggest biological relevance that may be underpowered in the current sample. Larger, multicenter cohorts will be needed to validate these findings and refine clinicopathologic stratification, particularly among younger patients and those with high-risk histologic subtype.

Overall, these findings reinforce the importance of recognizing distinct clinicopathologic patterns, particularly in younger patients and in tumors with signet-ring morphology, to support more timely diagnostic and therapeutic decision-making. The observed association between younger age and signet-ring features further suggests that earlier endoscopic evaluation and selective genetic assessment (e.g., CDH1 testing) may be warranted in appropriate clinical contexts to facilitate timely diagnosis and personalized management.

CONCLUSIONS

This study confirms that gastric cancer in our cohort primarily affected older adults, with a male predominance, and often presented with non-specific symptoms, contributing to diagnostic delay. While tumors were distributed across all gastric regions, a significant number of cases involved contiguous gastric areas, reflecting advanced local spread at diagnosis. The intestinal type was the most common, whereas diffuse and signet-ring cell carcinomas were more frequently seen in younger patients and associated with poor differentiation. These findings emphasize the importance of earlier endoscopic evaluation of persistent upper gastrointestinal symptoms, as well as the need for standardized biopsy protocols, consistent histopathologic classification, and the implementation of structured gastric mapping strategies (e.g., MAPS III) to improve early detection, staging accuracy, and clinical outcomes within the Albanian healthcare context.

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COLORECTAL CANCER IN ALBANIA: EPIDEMIOLOGICAL TRENDS AND DIAGNOSTIC INSIGHTS IN 2024

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ABSTRACT

Colorectal cancer (CRC) is the third most diagnosed malignancy worldwide and the second leading cause of cancer-related death, according to WHO and GLOBOCAN 2022. While high-income countries have reduced mortality through structured screening programs, many low- and middle-income countries, including Albania, still face rising incidence due to the lack of screening infrastructure, low health literacy, and limited access to preventive services. Albania has neither a population-wide CRC screening program nor a national cancer registry, making clinical practice data essential for understanding disease patterns. We conducted a retrospective descriptive study to examine all colonoscopies performed during 2024 at a high-volume diagnostic center in Tirana (n = 1,789). Among these, 92 histologically confirmed CRC cases and 618 colorectal polyps were identified. Data were stratified by age, sex, tumor location, histological subtype, and symptoms. Polyp detection rate, CRC prevalence, and symptom distribution were evaluated to assess diagnostic burden and preventive potential. Findings demonstrate a substantial CRC burden in Albania, with diagnoses largely symptom-driven and frequent detection of polyps, including in younger patients. These results highlight missed opportunities for early detection and emphasize the urgent need for national CRC screening programs, public education initiatives, and structured risk-based prevention strategies.

Keywords: Colorectal cancer, Albania, Epidemiology, Colonoscopy, Screening, Public health

INTRODUCTION

Colorectal cancer (CRC) ranks among the most frequently diagnosed cancers globally and represents a major contributor to cancer-related deaths. According to GLOBOCAN 2022, over 1.9 million new cases and approximately 900,000 deaths were reported globally, ranking CRC third in incidence and second in mortality among all cancers (International Agency for Research on Cancer; 2022). The global distribution of CRC varies considerably, with the highest incidence observed in Australia, New Zealand, Europe, and North America, and the lowest in Africa and South Asia (Morgan et al., 2023). Such regional disparities are largely attributed to lifestyle and dietary habits, socioeconomic differences, and the existence or absence of structured screening programs (Doubeni et al., 2012). Although CRC incidence rises significantly after the age of 50, recent studies report a worrying increase in early-onset CRC (under 50 years of age), linked to genetic predisposition, obesity, metabolic syndrome, dietary factors, sedentary lifestyle, alcohol and tobacco use, and gut microbiota alterations (Sung et al., 2025; Murphy, Campbell, & Gunter, 2021). Countries with structured screening programs have achieved notable reductions in mortality, whereas nations with limited resources, including Albania, continue to experience late-stage diagnoses and higher mortality.

In Albania, colorectal cancer (CRC) has become an increasingly significant public health issue. Based on GLOBOCAN 2022 data (IARC, 2022), CRC ranks fourth among all cancers in both incidence and mortality, with 522 new cases (6.5% of all cancers) and 292 deaths (5.9% of cancer-related mortality). Data reported by the Institute of Public Health for the period 2015–2022 reveal a continuous rise in CRC incidence, accounting for approximately 6–7% of newly diagnosed cancers each year (Ylli et al., 2023). Over the past decade, the number of CRC cases has grown notably, shifting from previously low national rates compared with neighboring countries. This trend likely reflects both lifestyle-related risk factors and the introduction of fecal occult blood testing (FOBT) as part of early detection programs. Although the FOBT is recommended for individuals aged 35–70 years as part of a routine check-up, the extent of its practical implementation remains unclear and still insufficiently developed.

Epidemiological patterns show a sharp rise in CRC risk after age 50, peaking between 60 and 69 years, with an average diagnosis age of 63. Around 85% of patients are older than 50 years, while risk levels plateau beyond 70. For individuals under 50, incidence rates are similar between sexes, but after this age, rates among men become progressively higher—nearly twice those of women after 60. Mortality data indicate that CRC accounts for about 6.2% of all cancer deaths, more frequently in men (53%) than women. Despite the overall increase in mortality between 2015 and 2022, age-standardized mortality has remained relatively stable. Hospitalizations due to CRC have also risen, reflecting more diagnosed cases engaging with the healthcare system. Collectively, these findings underscore CRC's evolution from a relatively uncommon disease to a major public health challenge in Albania (Ylli et al., 2023).

The present study analyzes all colorectal cancer cases diagnosed in a major private diagnostic center in Tirana during 2024, providing updated epidemiological insights and diagnostic patterns. Findings demonstrate a substantial CRC burden in Albania, with diagnoses largely symptom-driven and frequent detection of polyps, including in younger patients. These results highlight missed opportunities for early detection and emphasize the urgent need for national CRC screening programs, public education initiatives, and structured, risk-based prevention strategies. Ultimately, this work aims to support evidence-based health policy, contribute to the development of a more comprehensive national CRC registry, and promote interventions that reduce mortality and improve patient outcomes.

MATERIAL AND METHOD

This is a retrospective descriptive study conducted at one of the most specialized private diagnostic centers in Albania for gastrointestinal diseases, located in Tirana, over the course of the year 2024. The study included all patients who underwent colonoscopy during the study period, totaling 1,789 individuals. For each patient, detailed information was collected regarding the clinical indications for the procedure. This included gastrointestinal symptoms prompting the examination, previous pathological findings from imaging studies such as abdominal ultrasound or computed tomography (CT), routine screening in individuals over 50 years of age, positive fecal occult blood tests, and a documented family history of colorectal cancer. This comprehensive approach allowed the identification of both symptom-driven and opportunistic screening cases. Demographic data, including age, sex, place of residence, and place of birth, were recorded. Endoscopic findings, including results from colonoscopy and sigmoidoscopy, were systematically documented, along with histopathological outcomes from biopsy samples. We assessed the prevalence of colorectal polyps among patients who underwent colonoscopy. All endoscopic procedures were performed using the Olympus EVIS EXERA III – Series 190 system. This advanced endoscopic platform provides high-definition imaging, a wide field of view, and Narrow Band Imaging (NBI) technology, enhancing mucosal contrast and allowing detailed visualization of subtle lesions. The system is widely recognized

for its reliability and diagnostic accuracy in clinical gastroenterology practice. Data collection and management were performed following standard ethical and clinical guidelines, ensuring patient confidentiality and data integrity. Anonymized data were subsequently analyzed to assess patterns of colorectal cancer according to age, sex, clinical indications, and other demographic factors. This methodological framework provides a comprehensive overview of the epidemiological and diagnostic profile of colorectal lesions in Albania and facilitates evidence-based recommendations for improving early detection and risk-stratified screening strategies.

RESULTS AND DISCUSSION

During the study period (January–December 2024), a total of 1,789 colonoscopies were performed at the Gastroenterology diagnostic center La Vita, in Tirana, Albania. Among these, 92 cases (5.1%) were diagnosed with colorectal cancer (CRC) confirmed by histopathological examination. Additionally, 618 colorectal polyps were detected, and 32 patients (34.8%) with CRC presented with synchronous polyps.

1. Demographic Characteristics

Of the 92 CRC patients, 50 were male (54.3%) and 42 were female (45.7%), showing a mild male predominance.

CRC incidence increased sharply with age: 81 patients (88%) were aged ≥ 50 years, and 11 patients (12%) were under 50 years. No case occurred below age 40, whereas 11 patients (12%) were between 40–49 years, 48 (52%) between 50–69 years, and 33 (36%) were ≥ 70 years old.

Table 1. Baseline Characteristics of the Study Population

Characteristic	n	%
Total patients	92	100
Male	50	54.3
Female	42	45.7
<40 years	0	0
40–49 years	11	12.0
50–69 years	48	52.0
≥ 70 years	33	36.0

These findings are consistent with data reported by the Albanian Institute of Public Health (Ylli et al., 2023). As shown in Figure 1, the incidence increases with age, peaking between 60 and 70 years. Moreover, the report indicates that, after the age of 50, colorectal cancer occurs more frequently in males than in females.

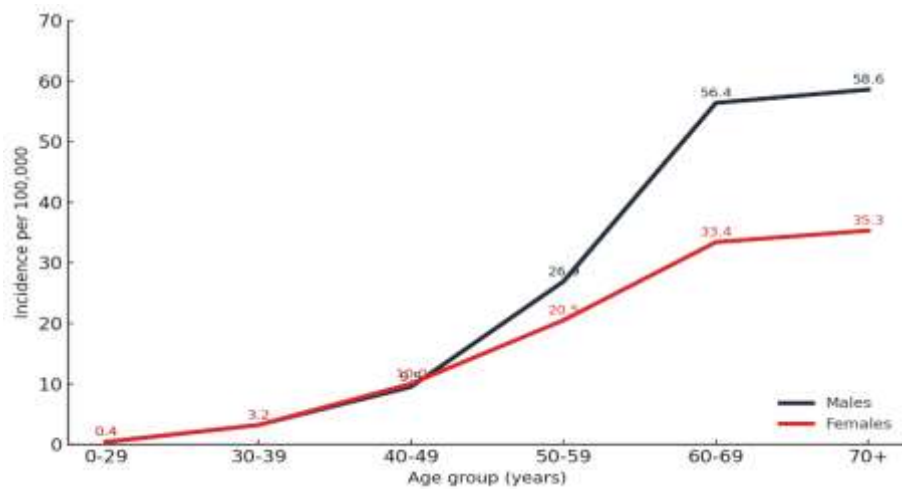


Figure 1. Annual incidence (/100,000) of colorectal cancer by age group for the period 2015–2022 (ISHP) Our data confirm that CRC remains primarily a disease of older adults but with a notable minority (12%) occurring before age 50, consistent with international trends of increasing early-onset CRC incidence.

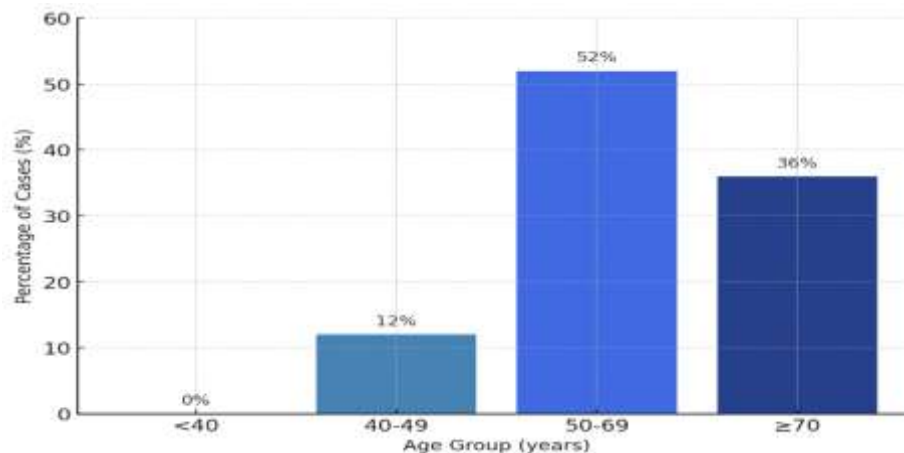


Figure 2. Age distribution (%) of Colorectal Cancer Cases

2. Clinical Presentation

Most colorectal cancer cases in this cohort were diagnosed following symptom-driven presentation rather than routine screening. The most frequent symptom was rectal bleeding (40 cases; 43.5%), followed by altered bowel habits, such as constipation or diarrhea (31 cases; 33.7%), abdominal pain (24 cases; 26.1%), and anemia (22 cases; 23.9%). In more advanced cases, patients also presented with weight loss, anorexia, and general weakness (11 cases; 12%). Screening-related detection accounted for a smaller proportion of cases, with 29 patients (31.5%) identified following a positive fecal occult blood test (FOBT) and 25 patients (27.2%) diagnosed based on CT-detected lesions, including secondary tumors or metastases. Only a minority of patients were asymptomatic at diagnosis.

Table 2. Clinical indications for colonoscopy among CRC patients

Indication	n	%
Rectal bleeding	40	43.5
Altered bowel habits (constipation/diarrhea)	31	33.7
Abdominal pain	24	26.1
Anemia	22	23.9
Weight loss / weakness / anorexia	11	12.0
Positive FOBT	29	31.5
CT-detected lesion	25	27.2

The predominance of rectal bleeding and altered bowel habits reflects the late symptomatic presentation typical in populations without organized colorectal cancer screening programs. These findings emphasize the reliance on symptom-driven diagnosis in Albania and the consequent risk of delayed detection. The limited proportion of cases identified through FOBT highlights significant gaps in screening coverage, underscoring the need for structured, population-based screening programs and increased public awareness of early gastrointestinal symptoms. Addressing these gaps could improve early detection, reduce disease burden, and enable timely intervention for colorectal cancer in the Albanian population.

3. Tumor Localization

The anatomical location of colorectal cancer (CRC) carries notable implications for its clinical presentation, underlying biology, and overall prognosis (Ugai et al., 2023). Anatomically, the colon is divided into proximal and distal segments, with the splenic flexure acting as the dividing landmark. Evidence increasingly shows that tumor profiles vary by location, driven by local microbiota, immune interactions, and molecular distinctions (Khalyfa et al., 2021; Lin, Kong, & Zhang, 2022; Loree et al., 2018). Colorectal neoplasms originating on the right and left sides of the colon demonstrate distinct clinical and histopathological features. Right-sided tumors are more frequently associated with sessile serrated adenomas and mucinous histology, while left-sided malignancies are typically characterized by tubular or villous adenocarcinoma subtypes (Marzouk & Schofield, 2011). Tumor morphology influences detectability: left-sided lesions are typically polypoid and more easily seen during endoscopy, while right-sided tumors are often flat and more likely to be missed (Gualco et al., 2006; Nawa et al., 2008). Clinically, right-sided CRCs are more often present with systemic symptoms such as iron-deficiency anemia and weight loss, whereas left-sided tumors are commonly associated with rectal bleeding and changes in bowel habits (Szostek et al., 2025). Meta-analyses have shown that left-sided CRC is linked to better overall survival compared to right-sided disease, regardless of stage or treatment. As a result, primary tumor location (PTL) is a key prognostic factor in both localized and metastatic CRC, reflecting inherent molecular and microenvironmental differences between tumor sides (Petrelli et al., 2017).

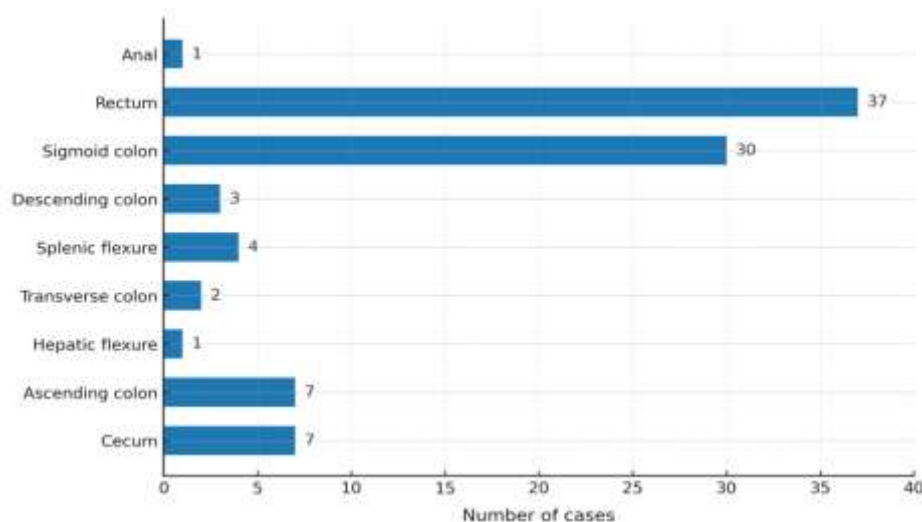


Figure 3. Anatomical distribution of colorectal cancer cases in the study population

In our cohort, distal CRCs were markedly more frequent than proximal tumors. The rectum (37 cases; 38%) and sigmoid colon (30 cases; 32.6%) together accounted for approximately 70% of all cancers. Proximal tumors were less common, including the transverse colon (2 cases), hepatic flexure (1 case), ascending colon (7 cases), and cecum (7 cases). Overall, 75 tumors (81.5%) were left-sided and 17 (18.5%) right-sided.

The predominance of distal localization may reflect the higher likelihood of symptom-driven detection of distal lesions, which more often present with rectal bleeding or constipation. These findings emphasize the importance of complete colonoscopy screening rather than sigmoidoscopy alone, as proximal tumors—typically flatter and asymptomatic—are more easily overlooked, leading to later diagnosis and poorer outcomes.

4. Histopathological Findings

Histopathological analysis of biopsy, polypectomy, and surgical resection specimens remains a cornerstone in the accurate diagnosis, clinical decision-making, and prognostic evaluation of colorectal cancer. In the context of precision medicine and familial risk assessment, molecular testing has gained increasing relevance in recent years. However, in Albania, access to these tests remains limited and is predominantly available through private services. Adenocarcinomas, originating from the epithelial lining of the colorectal mucosa, accounts for over 90% of colorectal carcinomas (Hamilton et al., 2010). These tumors are classified based on their degree of glandular differentiation, which forms the foundation for histologic grading. Tumor grade serves as a prognostic indicator that operates independently of disease stage, with poorly differentiated, high-grade tumors typically linked to worse clinical outcomes. According to Fleming et al. (2012), grading is determined by the proportion of glandular structures: well-differentiated adenocarcinomas display more than 95% gland formation; moderate differentiation corresponds to 50–95%; and poorly differentiated tumors exhibit less than 50%, often presenting as predominantly solid masses.

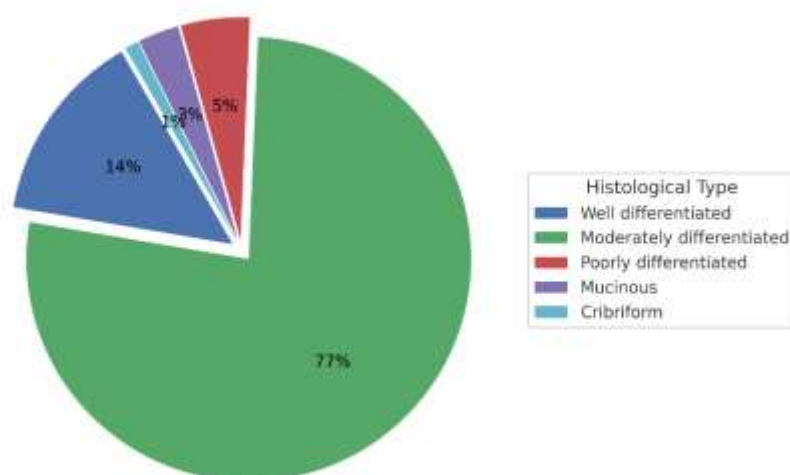


Figure 4. Histopathological differentiation of colorectal cancer cases (%)

In our study, histopathological analysis of colorectal cancers revealed that most tumors were moderately differentiated adenocarcinomas (70 cases), followed by well-differentiated adenocarcinomas (13 cases) and poorly differentiated carcinomas (4 cases). This distribution aligns with broader clinical practice, where most colorectal adenocarcinomas (~70%) are moderately differentiated, while well- and poorly differentiated tumors account for approximately 10% and 20%, respectively (Fleming et al., 2012). Other histologic variants identified in our cohort included mucinous adenocarcinomas (4 cases; 4.3%) and a single case of cribriform-type adenocarcinoma. Only one case of squamous carcinoma was observed, corresponding to anal carcinoma with extension into the rectum (1 case; 1.1%).

The findings of this study provide important insights into the epidemiological and clinical characteristics of colorectal cancer in Albania. The observed age distribution and slight male predominance are consistent with global patterns, yet the presence of CRC in 12% of patients under 50 years signals a concerning trend toward earlier onset, mirroring observations in several Western populations (Sung et al., 2025; Murphy et al., 2021). The marked predominance of distal (rectosigmoid) tumors presents both a diagnostic advantage, due to the earlier presentation of symptoms such as rectal bleeding, and a potential screening opportunity, as flexible sigmoidoscopy could detect most of these lesions. However, the low proportion of cases identified through fecal occult blood testing or routine screening underscores the lack of a structured national CRC screening program in Albania. Additionally, the coexistence of polyps in approximately one-third of CRC patients (618 of 1,789 patients) highlights the critical role of preventive colonoscopy and timely polypectomy in reducing the overall cancer burden. From a histopathological perspective, the predominance of moderately differentiated adenocarcinomas aligns with data from other European cohorts, while the relatively low frequency of mucinous and poorly differentiated tumors may reflect either a lower prevalence of advanced aggressive disease in this population or potential sampling bias inherent to a private diagnostic center with early referral cases. Collectively, these findings highlight gaps in national screening programs, the need for increased public awareness of early gastrointestinal symptoms, and the potential impact of targeted preventive strategies to reduce the burden of colorectal cancer in Albania.

CONCLUSIONS

Colorectal cancer in Albania remains a significant and growing public health concern, characterized by predominantly symptom-driven diagnoses, a high prevalence of distal tumors, and generally late-stage presentation. The patterns observed suggest a gradual shift toward earlier-onset disease and highlight the frequent coexistence of polyps, underscoring the potential for preventive intervention. Histopathological findings indicate that most tumors are moderately differentiated, while more aggressive or unusual subtypes are less common. These observations emphasize the pressing need for a comprehensive national cancer registry, the establishment of structured, population-based and risk-adapted screening programs, and enhanced public awareness of early gastrointestinal symptoms. Addressing these gaps is critical to enable earlier detection, reduce colorectal cancer-related morbidity and mortality, and bring national control strategies in line with international standards.

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EVALUATION OF PLANT GROWTH-PROMOTING TRAITS OF BACTERIAL ISOLATES FOR SUSTAINABLE AGRICULTURE

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ABSTRACT

Plant growth-promoting bacteria (PGPB) represent a vital biological resource for sustainable agriculture. These microorganisms exert diverse effects, including enhancing nutrient uptake, improving plant tolerance to stress, and promoting overall growth, providing an eco-friendly alternative to chemical fertilizers. In this study, bacterial isolates from various environmental sources were evaluated for their plant growth-promoting traits, including nitrogen fixation, phosphate solubilization, chitinase activity, 5-aminolevulinic acid (ALA) production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, siderophore production, hydrogen cyanide (HCN) production, and exopolysaccharide (EPS) production. Among the isolates, *Citrobacter freundii* PB149 exhibited the highest nitrogen fixation capacity, while *Serratia liquefaciens* PB41 was the most effective in phosphate solubilization. *Bacillus cereus* PB136 showed notable chitinase activity, and *Stenotrophomonas rhizophila* PB4 was the most efficient ALA producer. ACC deaminase activity was particularly prominent in *Bacillus cereus* PB136, while siderophore production was observed in *Stenotrophomonas maltophilia* PB25. HCN production was detected in *Pseudomonas* sp. PB90 and *Enterobacter ludwigii* PB72 showed significant EPS production. Moreover, *S. maltophilia* PB25, *S. liquefaciens* PB41, and *Pseudomonas* sp. PB90 exhibited antifungal activity against the fungal pathogens *Phytophthora infestans*, *Botrytis cinerea*, and *Monilinia laxa*. These findings suggest that the tested bacterial isolates may be potential candidates for use as biofertilizers and biocontrol agents. Overall, the study underscores the ability of environmentally derived microorganisms to enhance agricultural productivity while reducing chemical fertilizer usage, highlighting their pivotal role in sustainable farming practices.

Keywords: PGPB, Plant growth promotion, Phytohormone, Biofertilizer, Biocontrol

INTRODUCTION

Plant growth-promoting bacteria (PGPB) significantly enhance plant development and soil health through metabolite secretion, phytohormone production, nitrogen fixation, and mineral solubilization (Poria et al., 2022). Soil harbors a highly complex microbial ecosystem dominated by bacteria, which constitute approximately 95% of the total microbial population (Glick, 2012). However, ecosystem degradation, climate change, and the excessive use of chemical fertilizers have adversely affected soil fertility and long-term productivity (Poria et al., 2022).

PGPB promote plant growth through both direct and indirect mechanisms. Direct mechanisms include phytohormone production (auxins, cytokinins, gibberellins), ACC deaminase activity, nitrogen fixation, phosphate solubilization, and siderophore-mediated iron acquisition. Indirect mechanisms involve pathogen suppression via antibiotics, hydrolytic enzymes, hydrogen cyanide (HCN), and competitive exclusion (Gamalero and Glick, 2011;

Olanrewaju et al., 2017). This functional diversity enables PGPB to operate effectively across diverse soils and environmental conditions.

The physiological benefits mediated by PGPB encompass several key processes. Phosphate solubilization increases phosphorus bioavailability; siderophore production enhances iron uptake and mitigates heavy metal toxicity; and ACC deaminase activity reduces stress-induced ethylene, thereby promoting root elongation. Moreover, 5-aminolevulinic acid (ALA) synthesis improves chlorophyll content, photosynthetic efficiency, and stress tolerance, while bacterial exopolysaccharides (EPS) facilitate biofilm formation on roots, alleviating salinity, drought, and temperature stress (Chen et al., 2023; Akram and Ashraf, 2013; Khabbaz et al., 2019). Beyond their agricultural importance, EPS also possess biotechnological potential in immune modulation and bioremediation.

Nitrogen is a critical macronutrient for plant growth, yet its deficiency in soils often necessitates the excessive application of chemical fertilizers (Olanrewaju et al., 2017). Symbiotic nitrogen-fixing bacteria inhabit legume nodules, whereas free-living nitrogen fixers convert atmospheric nitrogen into ammonia independently (Al-Tammar and Khalifa, 2022).

PGPB also play an essential role in biological control. HCN-producing rhizosphere microorganisms inhibit a wide range of pathogens, including fungi, nematodes, and insects, while simultaneously enhancing nutrient solubilization, siderophore production, systemic resistance, and phytohormone synthesis (Sehrawat et al., 2022). Additionally, chitinases degrade fungal and insect chitin through β -1,4-N-acetyl-glucosaminidases, endochitinases, or stepwise diacetylchitobiose release, thereby suppressing pathogen development (Benaissa, 2024).

This study aimed to identify potential microbial candidates capable of enhancing plant growth and stress tolerance by evaluating bacterial isolates from diverse environmental sources for key plant growth-promoting and biocontrol traits, including nitrogen fixation, phosphate solubilization, siderophore and HCN production, ACC deaminase and chitinase activities, ALA and EPS synthesis, as well as antifungal activity against selected phytopathogens. Such insights will contribute to the development of sustainable biofertilizers and eco-friendly crop protection strategies.

MATERIAL AND METHOD

Isolation and Molecular Identification of Bacteria

Bacterial were isolated from diverse environmental sources, including agricultural soil, garden soil, and wastewater. Samples were pre-enriched in Nutrient Broth (NB), serially diluted, and spread onto Nutrient Agar (NA) plates, followed by incubation at 30°C for 48 h. Distinct colonies exhibiting robust growth were selected and purified for subsequent analyses.

For molecular identification, total genomic DNA of each isolate was extracted using standard bacterial DNA isolation procedures. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primer set 27F (5'-GTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). PCR products were visualized by agarose gel electrophoresis, purified, and sequenced. The resulting sequences were trimmed and assembled using DNA Dragon – DNA Sequence Contig Assembler Software version 1.9.2 (Germany). Sequence similarity searches were performed using the NCBI BLAST tool to

determine the closest taxonomic affiliation of each isolate. The sequences were subsequently deposited in the NCBI GenBank database, and accession numbers were obtained for each identified isolate.

Characterization of Plant Growth-Promoting Bacterial Isolates

Each isolate was evaluated for key plant growth-promoting (PGP) and biocontrol traits, including nitrogen fixation, phosphate solubilization, siderophore and hydrogen cyanide (HCN) production, ACC deaminase and chitinase activities, 5-aminolevulinic acid (ALA) and exopolysaccharide (EPS) synthesis, as well as antifungal activity against selected phytopathogens.

Nitrogen Fixation

The nitrogen-fixing ability of the bacterial isolates was evaluated using nitrogen-free bromothymol blue (NFB) medium composed (per liter) of 1.0 g sodium acetate, 0.6 g KH_2PO_4 , 0.9 g K_2HPO_4 , 0.2 g MgSO_4 , 0.012 g FeSO_4 , 0.018 g EDTA, 0.075 g CaCl_2 , 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.8 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.75 mg ZnSO_4 , 0.24 mg CuSO_4 , 0.016 mg H_3BO_3 , 0.001 mg biotin, and 2 mL of 0.5% bromothymol blue dissolved in KOH. The isolates were inoculated into NFB medium and incubated at 30°C for 7 days. Ammonium production was quantified using an ammonium standard curve as described by Nafisah et al. (2022).

Phosphate Solubilization

The phosphate-solubilizing potential of the isolates was assessed on NBRIP medium containing (per liter) 10 g glucose, 5 g $\text{Ca}_3(\text{PO}_4)_2$, 5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.1 g $(\text{NH}_4)_2\text{SO}_4$, and 0.025 g bromophenol blue. For solid media, 15 g agar per liter was added. Fresh cultures (5 μL per spot) were inoculated onto sterile NBRIP agar plates and incubated at 30°C for 7 days. Phosphate solubilization was indicated by the formation of clear zones around colonies. The Phosphate Solubilization Index (PSI) and Phosphate Solubilization Efficiency (%PSE) were calculated following Sadaf et al. (2022) and Nguyen et al. (1992), respectively.

5-Aminolevulinic Acid (ALA) Production

ALA production was determined using a glutamate-acetate (GA) medium containing (per liter) 0.05 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05 g FeCl_3 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KH_2PO_4 , 2 g $\text{C}_5\text{H}_8\text{NO}_4\text{Na}$, and 10 g glucose. The isolates were incubated at 30°C for 3 days. Cultures were centrifuged at 8,000 rpm for 15 min, and 1 mL of the supernatant was mixed with 2 mL of 1 M sodium acetate buffer (pH 4.7) and 50 μL acetylacetone. The mixture was heated in a boiling water bath for 15 min, rapidly cooled, and then reacted with 3.5 mL of freshly prepared modified Ehrlich reagent (1.25 g p-dimethylaminobenzaldehyde in 50 mL 95% ethanol and 50 mL HCl). The development of a pink-red color indicated ALA production (Nunkaew et al., 2015).

1-Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase Activity

ACC deaminase activity was tested using Dworkin and Foster (DF) minimal medium containing (per liter) 4 g KH_2PO_4 , 6 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g glucose, 2 g gluconic acid, 2 g citric acid, and 15 g agar, supplemented with trace elements (1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10

mg H_3BO_3 , 11.19 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 124.6 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 78.22 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 10 mg MoO_3 per 100 mL sterile water). The medium was autoclaved at 121°C for 20 min. A negative control without nitrogen and a positive control containing 2 g/L $(\text{NH}_4)_2\text{SO}_4$ were prepared. For ACC-based assays, 6 mM sterilized ACC solution replaced ammonium sulfate, and 100 μL was added to each plate. Plates were incubated at 30°C for 48–72 h, and colony growth was monitored every 24 h.

Exopolysaccharide (EPS) Production

EPS production was evaluated using RCV-sucrose medium containing (per liter) 0.1 g yeast extract, 30 g sucrose, and 30 g agar. The isolates were streaked onto the medium and incubated at 30°C for 7 days. EPS production was assessed based on colony morphology, mucoid appearance, and stickiness.

Siderophore Production

Siderophore production was determined using a modified Chrome Azurol S (CAS) assay (Kuzyk et al., 2021). The CAS reagent (60.5 mg CAS in 50 mL water) was mixed with 1 mM FeCl_3 and 10 mM HCl , followed by addition of 40 mL HDTMA solution (72.9 mg in water) to a final volume of 100 mL. Iron-free NB or LB medium (900 mL) was autoclaved at pH 5.9 and adjusted to pH 6.8 before the CAS reagent was added. Plates were incubated at 28°C for 5–7 days, and siderophore production was detected by the appearance of orange halos around colonies or color change in the medium.

Hydrogen Cyanide (HCN) Production

HCN production was determined using a selective solid medium containing (per liter) 30 g Tryptic Soy Broth, 4.4 g glycine, and 15 g agar. A 100 μL aliquot of bacterial suspension was inoculated at the center of each plate. Whatman filter paper discs soaked in 0.5% picric acid and 2% Na_2CO_3 solution were placed on the lid, and the plates were sealed with parafilm and incubated at 28°C for 3 days. A color change of the filter paper from yellow to reddish-brown indicated positive HCN production.

Chitinase Activity

Chitinase activity was assessed using chitin agar medium containing (per liter) 0.05 g colloidal chitin, 0.005 g yeast extract, 0.005 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g K_2HPO_4 , 0.0001 g MnSO_4 , 0.0001 g ZnSO_4 , 0.0001 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 15 g agar. The isolates were inoculated and incubated at 30°C for 3 days. Plates were flooded with 0.1% Gram's iodine solution, gently shaken for 20–30 min, and washed three times with sterile distilled water. Chitinase activity was indicated by the formation of clear halos around colonies. The Relative Enzyme Activity Index (REAI) was calculated as:

$$\text{REAI} = (\text{Total diameter of the clear zone} - \text{Diameter of colony}) / \text{Diameter of colony}$$

Investigation of Antagonistic Interactions of Bacterial Isolates

The antagonistic potential of selected bacterial isolates was evaluated against *Phytophthora infestans*, *Botrytis cinerea*, and *Monilinia laxa* using the dual culture technique on Potato Dextrose Agar (PDA). Bacterial isolates were spotted onto the agar surface at the

plate periphery, and fungal plugs (5 mm diameter) obtained from actively growing cultures were placed at the center of each plate. The plates were incubated at 25–27°C for 7–10 days, and fungal hyphal growth was monitored daily. Antagonistic activity was determined based on the presence and size of inhibition zones surrounding bacterial colonies, indicating suppression of fungal growth.

RESULTS AND DISCUSSION

Identification of Bacterial Isolates

Bacterial isolates exhibiting the highest performance in the plant growth-promoting and antagonistic assays were subjected to molecular identification. The 16S rRNA gene sequences obtained were analyzed using the BLAST tool in the NCBI database. Isolates showing $\geq 98\%$ sequence similarity with reference strains were assigned to the corresponding taxa. The species identifications were as follows: PB4, *Stenotrophomonas rhizophila*; PB25, *Stenotrophomonas maltophilia*; PB41, *Serratia liquefaciens*; PB72, *Enterobacter ludwigii*; PB90, *Pseudomonas* sp.; PB136, *Bacillus cereus*; PB146 and PB149, *Citrobacter freundii*.

Determination of Nitrogen-Fixing Ability

The nitrogen-fixing activity of the bacterial isolates was assessed using nitrogen-free bromothymol blue (NFB) medium, in which the indicator changes color as atmospheric nitrogen is biologically converted to alkaline ammonium (NH_4^+). A color shift from green to blue indicated active nitrogen fixation. Isolates that induced a clear color change and exhibited the most intense blue coloration were recorded (Figure 1). Quantitative estimation of ammonium production was performed by measuring absorbance values and calculating concentrations using a standard ammonium curve. Among the tested isolates, *C. freundii* PB146 and *C. freundii* PB149 were found to exhibit notable nitrogen-fixing potential, producing 1.22 mg/L and 1.30 mg/L NH_4^+ , respectively, demonstrating significant nitrogen-fixing potential under the experimental conditions. This finding is consistent with the study by Fadiji et al. (2023), which reported that *C. freundii* can possess multiple plant growth-promoting traits in plant-associated environments; in such studies, *C. freundii* strains were characterized by both phosphate solubilization and ammonia production (Fadiji et al., 2023).

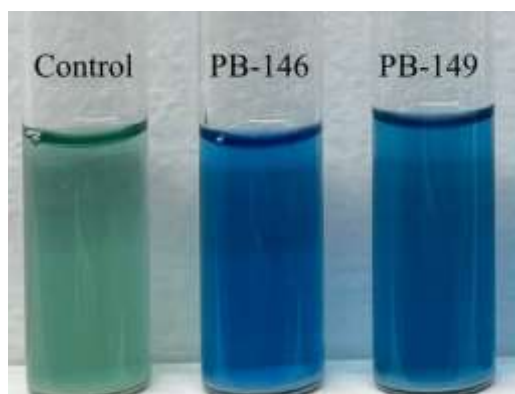


Figure 1. Nitrogen-fixing bacterial isolates showing a color change in NFB medium. Blue coloration indicates ammonium (NH_4^+) production resulting from biological nitrogen fixation.

Determination of Phosphate Solubilization Ability of Isolates

The phosphate-solubilizing potential of the bacterial isolates was evaluated using NBRIP medium containing insoluble calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) as the sole phosphorus source. Following incubation, clear zones surrounding the colonies indicated the conversion of insoluble phosphate into plant-available forms. Quantitative assessment based on the size of the solubilization zones revealed that *S. liquefaciens* PB41 exhibited the highest phosphate solubilization efficiency (%PSE) of 200% among the tested isolates (Figure 2).

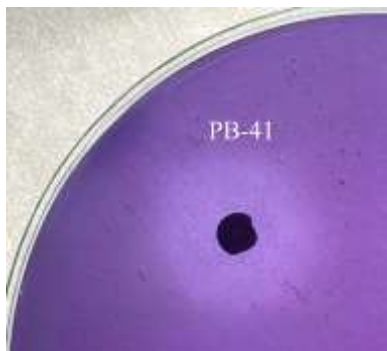


Figure 2. Phosphate-solubilizing activity of *S. liquefaciens* PB41 on NBRIP medium. The formation of a clear halo around the colony indicates conversion of insoluble $\text{Ca}_3(\text{PO}_4)_2$ into plant-available phosphorus, with PB41 showing the largest solubilization zone among tested isolates.

S. liquefaciens PB41 stood out with a high phosphate solubilization efficiency and also demonstrated antagonistic activity against multiple fungal pathogens. While *Serratia* species are widely recognized as biocontrol agents (e.g., via antibiotic production and lytic enzymes), high phosphate solubilization capacity has not always been reported; in this case, *S. liquefaciens* PB41 presents an additional advantage. Moreover, a study highlighted that *S. liquefaciens* contributes beneficially through ACC deaminase production and tolerance to salinity/stress conditions (Zhu et al., 2025).

Determination of 5-Aminolevulinic Acid (ALA) Production

ALA production by the bacterial isolates was evaluated after a 3-day incubation in GA medium. Following the addition of modified Ehrlich's reagent, color development was monitored to assess ALA synthesis. Among the tested isolates, *S. rhizophila* PB4 exhibited the most pronounced color change from yellow to pink-red, indicating the highest ALA production potential (Figure 3).

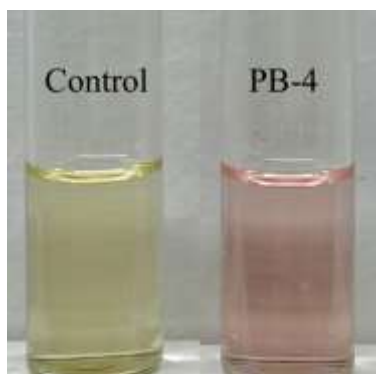


Figure 3. ALA production by *S. rhizophila* PB4. Left: uninoculated GA medium after the addition of Ehrlich's reagent (control). Right: GA medium inoculated with *S. rhizophila* PB4, showing a color change from yellow to pink-red, indicating ALA synthesis.

S. rhizophila PB4 showed ALA production, a less commonly reported trait in the literature, yet it aligns with the potential of genus *Stenotrophomonas* for plant interactions, root colonization, and conferring benefits under stress conditions (Adeleke et al., 2021).

Determination of ACC Deaminase Activity

ACC deaminase activity of the bacterial isolates was assessed using DF minimal medium supplemented with 6 mM ACC as the sole nitrogen source. During the 48–72 h incubation period at 30°C, *B. cereus* PB136 exhibited the highest activity, demonstrating robust growth on DF medium containing ACC (Figure 4).

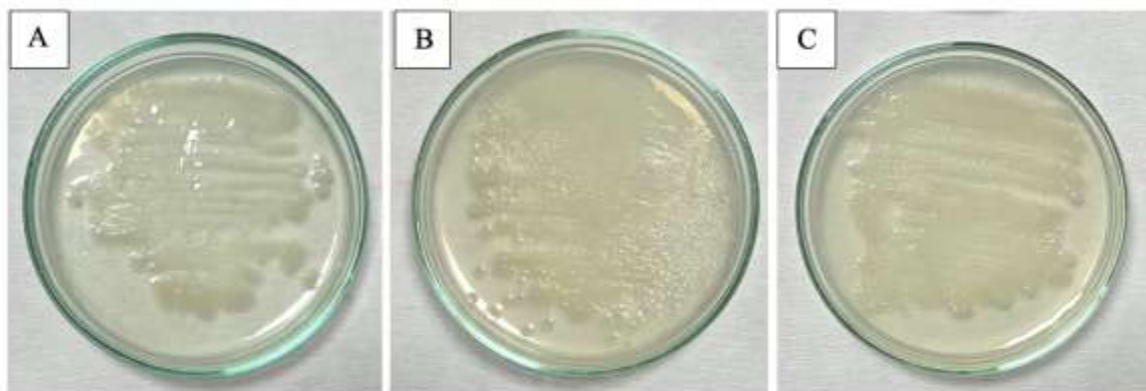


Figure 4. Assessment of ACC deaminase activity. (A) DF medium supplemented with ammonium as a positive control; (B) DF medium without a nitrogen source as a negative control; (C) DF medium supplemented with 100 µL of 6 mM ACC per plate, showing growth of *B. cereus* PB136, indicating ACC deaminase activity.

B. cereus PB136 isolate was notable for its chitinase activity (REAI = 3.44) and also exhibited ACC deaminase activity. This combination aligns with frequently reported biocontrol and stress tolerance-enhancing mechanisms in the *Bacillus* genus; for instance, chitinase degrades fungal cell walls to provide antimicrobial effects, while ACC deaminase reduces ethylene stress in plants to support growth (Fanai et al., 2024; Shahzad et al., 2021).

Determination of Exopolysaccharide (EPS) Production

EPS production by the bacterial isolates was qualitatively assessed on RCV-sucrose medium. Colony morphology was evaluated for mucoid appearance and stickiness. Among the tested isolates, *E. ludwigii* PB72 exhibited the most prominent mucoid colony morphology, indicating the highest EPS production potential (Figure 5). *E. ludwigii* PB72 stood out for its high EPS production, a trait that can provide advantages in root adherence, biofilm formation, and microbial stability (Aoudi et al., 2024).



Figure 5. EPS production by *E. ludwigii* PB72 on RCV-sucrose medium. The mucoid and sticky colony morphology indicates high EPS production potential.

Determination of Siderophore Production

Siderophore production by the bacterial isolates was evaluated using the CAS agar assay. Among the tested isolates, *S. maltophilia* PB25 exhibited the highest siderophore production, as indicated by the formation of prominent color-change zones around the colonies (Figure 6).

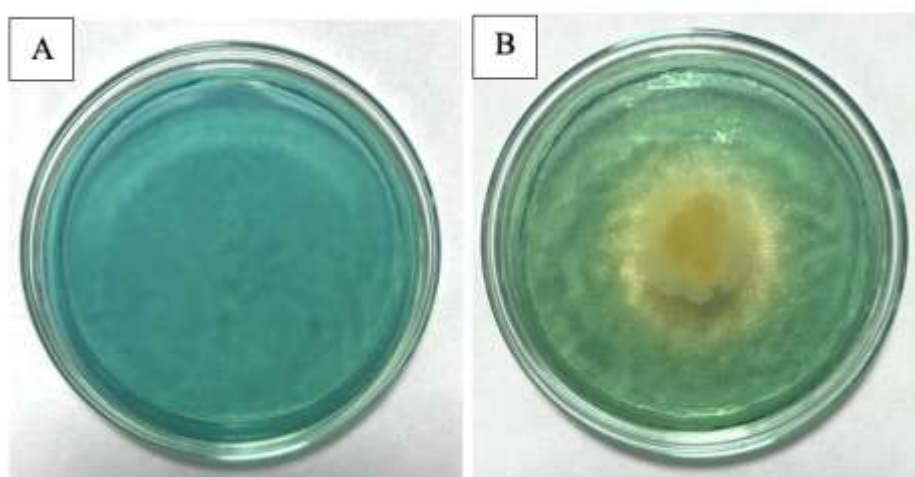


Figure 6. Siderophore production by *S. maltophilia* PB25 on CAS agar. (A) Uninoculated control medium; (B) Formation of a yellow halo zone around *S. maltophilia* PB25 indicates siderophore secretion.

S. maltophilia PB25 demonstrated prominent siderophore production and the ability to suppress all tested pathogens, which corresponds with reports of multifunctional PGP traits in

S. maltophilia strains, including phosphate solubilization, siderophore production, and ACC deaminase activity (Singh and Jha, 2017; Kumar and Audipudi, 2015). This makes *S. maltophilia* PB25 a strong candidate for both nutrient mobilization and biocontrol.

Determination of Hydrogen Cyanide (HCN) Production

HCN production by the bacterial isolates was assessed using filter paper treated with 0.5% picric acid and 2% Na₂CO₃. A color change of the filter paper indicated HCN production. Among the tested isolates, *Pseudomonas* sp. PB90 exhibited the most pronounced color shift, indicating strong HCN production (Figure 7).

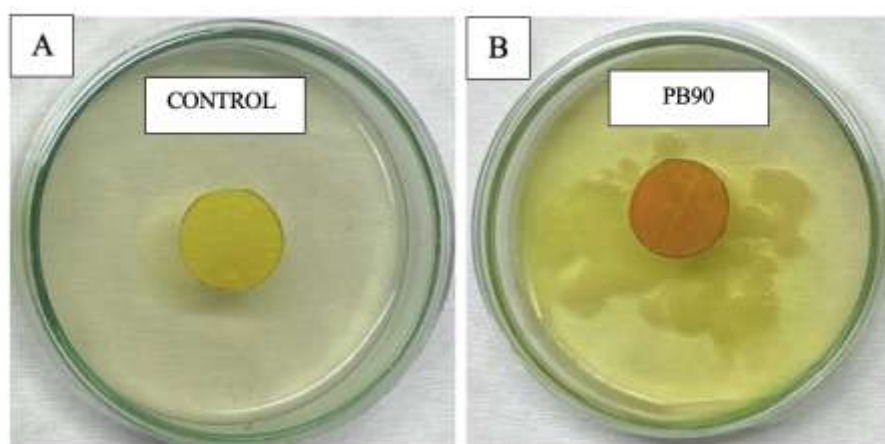


Figure 7. HCN production by *Pseudomonas* sp. PB90. (A) Uninoculated control filter paper; (B) Orange-brown color change on filter paper after exposure to *Pseudomonas* sp. PB90, indicating HCN production.

Pseudomonas sp. PB90 was notable for strong HCN production and showed inhibitory effects against *Phytophthora infestans*. This finding is consistent with literature reporting HCN as a biocontrol mechanism in many *Pseudomonas* strains (Michelsen et al., 2012; Nandi et al., 2017). HCN disrupts pathogen metabolism to exert antifungal effects, and the biocontrol potential of PB90 is likely synergistic with siderophore production and other metabolites (Rezzonico et al., 2007).

Determination of Chitinase Production

Chitinase activity of the bacterial isolates was evaluated on colloidal chitin agar medium. Following incubation, plates were flooded with 0.1% Gram's iodine solution to visualize hydrolysis zones. Isolates producing clear zones around colonies were considered chitinase-positive. Among the tested isolates, *B. cereus* PB136 exhibited the largest total zone diameter, indicating the highest chitinase production potential, with a REAI of 3.44 (Figure 8). Noted above under ACC deaminase; combined with ACC activity for *B. cereus* PB136 as described.

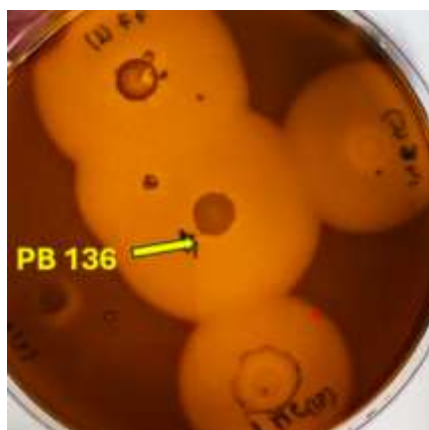


Figure 8. Chitinase activity of *B. cereus* PB136 on colloidal chitin agar medium. The clear hydrolysis zone surrounding the colony indicates enzymatic degradation of chitin.

Antagonistic Activity of Bacterial Isolates

The antagonistic potential of the bacterial isolates was evaluated against *P. infestans*, *B. cinerea*, and *M. laxa* using dual culture assays on PDA. Among the tested isolates, *S. maltophilia* PB25 and *S. liquefaciens* PB41 exhibited antifungal activity against all three pathogens, while *Pseudomonas* sp. PB90 inhibited *P. infestans* but showed no antagonistic effect against *B. cinerea* or *M. laxa* (Figure 9).

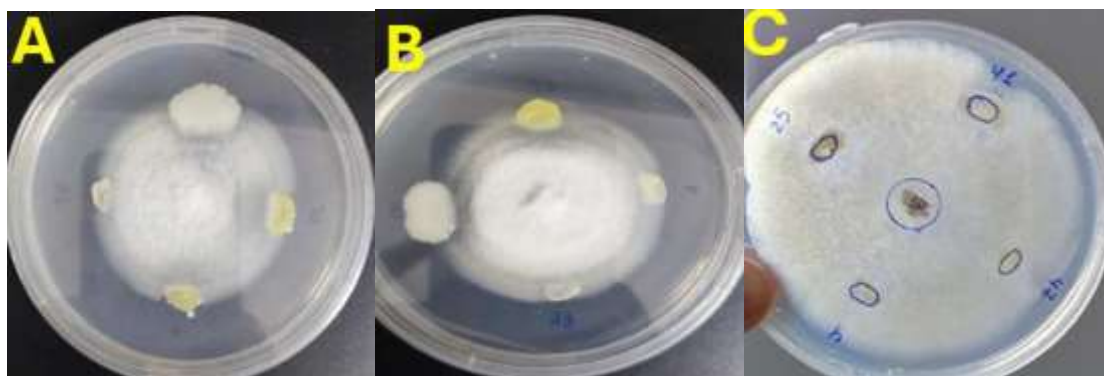


Figure 9. Antagonistic activity of bacterial isolates against plant pathogenic fungi on PDA medium. (A) Inhibition of *P. infestans*; (B) inhibition of *M. laxa*; (C) inhibition of *B. cinerea*. Zones of inhibition indicate antifungal activity of the bacterial isolates.

CONCLUSIONS

The bacterial isolates belonging to the genera *Stenotrophomonas* (*S. rhizophila* PB4 and *S. maltophilia* PB25), *Serratia* (*S. liquefaciens* PB41), *Enterobacter* (*E. ludwigii* PB72), *Pseudomonas* (*Pseudomonas* sp. PB90), *Bacillus* (*B. cereus* PB136), and *Citrobacter* (*C. freundii* PB146 and *C. freundii* PB149) exhibited a broad spectrum of plant growth-promoting traits, including enhanced nutrient availability, hormone-mediated growth promotion, increased stress tolerance, and pathogen suppression. Their functional diversity and complementary activities suggest that these isolates are promising candidates for sustainable agricultural applications, with the potential to reduce reliance on chemical fertilizers while improving crop health and productivity. Overall, these findings underscore the multifaceted potential of the

studied isolates, demonstrating that their combined plant growth-promoting and biocontrol traits can contribute significantly to the development of eco-friendly and sustainable crop management strategies.

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CHEMICAL AND BIOLOGICAL SYNTHESIS OF ZNO NANOPARTICLES: EFFECTS ON ANTIBACTERIAL ACTIVITY

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ABSTRACT

Zinc oxide nanoparticles (ZnO-NPs) are among the most promising antimicrobial agents due to their biocompatibility, low toxicity, and unique physicochemical properties. In this study, ZnO-NPs were synthesized from a zinc acetate precursor via both chemical and biological methods. During biological synthesis, *Rhodopseudomonas palustris* RPA1 was employed, and the cell, supernatant, dried, and calcined biomass fractions were separately evaluated. The structural and morphological characteristics of the nanoparticles were analyzed using FESEM, FTIR, EDX, and UV–Vis spectroscopy. The synthesized NPs exhibited spherical, rod-shaped, and irregular morphologies. Antibacterial activities were investigated against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* using agar diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and triphenyl tetrazolium chloride (TTC) assay. The ZnO nanoparticles synthesized by the chemical process (KS) exhibited the strongest antibacterial inhibition against *S. aureus*, with an inhibition zone diameter of 2.1 cm and MIC/MBC values of 25 µg/mL. In contrast, biologically synthesized nanoparticles obtained from dried cells (KHA) showed the most pronounced antibacterial activity against *B. subtilis*, displaying an inhibition zone of 1.3 cm and MIC/MBC values of 25 µg/mL. Other nanoparticle groups demonstrated comparable effects, with inhibition zones of approximately 1.0 cm and MIC/MBC values ranging between 25 and 50 µg/mL. The TTC assay results corroborated these findings. Meanwhile, calcined nanoparticles derived from the cell fraction (YHA) exhibited no detectable antimicrobial activity. These findings indicate that both the synthesis method and the morphology of the nanoparticles are critical determinants of antibacterial efficacy. The observed differences in inhibitory effects among *E. coli*, *B. subtilis*, and *S. aureus* suggest variations in the interactions between zinc oxide nanoparticles and bacterial cell wall structures. Furthermore, the biosynthesis approach utilizing *R. palustris* RPA1 represents an environmentally friendly and innovative production method. Overall, this study highlights the high potential of zinc oxide nanoparticles as alternative antibacterial agents and provides valuable scientific insights for the development of targeted strategies against different bacterial groups.

Keywords: Zinc Oxide Nanoparticle, Biological Synthesis, *Rhodopseudomonas palustris*, Antimicrobial Activity, *Escherichia coli*, *Bacillus subtilis*

INTRODUCTION

In recent years, nanoparticles have attracted significant attention due to their high surface-to-volume ratio, tunable size, and chemical reactivity, making them particularly suitable for biomedical applications. Among metal nanoparticles, Zinc oxide nanoparticles (ZnO-NPs) are of particular interest because of their unique optical, piezoelectric, semiconductor, spintronic, and photonic properties. They are semiconductors with a wide and direct band gap (3.37 eV) and a high exciton binding energy (60 meV), which enables efficient performance in optical systems at or above room temperature (Mohamed et al., 2020). Zinc oxide (ZnO) possesses several notable properties, including antibacterial activity, UV absorption, semiconducting behavior, and vulcanization-promoting effects (Ahmoum et al., 2019). Various studies have employed ZnO as an antimicrobial agent, as well as in energy cells and sensors (Rad et al., 2019). ZnO-NPs also stand out for their low toxicity and excellent biocompatibility with human cells, which has made them one of the most extensively studied nanomaterials. Their applications span antimicrobial therapies, bioimaging, wound healing, implant coatings, tissue engineering, and anticancer drug delivery (Nava et al., 2017; Iqbal et al., 2019).

ZnO-NPs can be synthesized via chemical, physical, or biological approaches. While chemical methods offer precise control over particle size and morphology, they often involve toxic reagents and harmful by-products. In contrast, biological synthesis using microorganisms or plant extracts provides an eco-friendly and sustainable alternative (Rekha et al., 2010). Despite extensive research, the influence of the synthesis method on the structural characteristics and antimicrobial performance of ZnO-NPs remains insufficiently understood, and comparative studies examining efficacy against both Gram-positive and Gram-negative bacteria are limited. The rising prevalence of multidrug-resistant microorganisms has further intensified the need for alternative antimicrobial strategies, positioning metal-based nanoparticles as key candidates in this field (Mutukwa et al., 2024).

In this study, ZnO-NPs will be synthesized from zinc acetate precursors using both chemical and biological methods. Their structural, morphological, and antimicrobial properties will be systematically evaluated, with comparative analyses against *Escherichia coli* (Gram-negative), *Bacillus subtilis*, and *Staphylococcus aureus* (Gram-positive) aimed at providing new insights into how synthesis strategies influence antimicrobial efficacy.

MATERIAL AND METHOD

Synthesis of ZnO-NPs

ZnO NPs were synthesized using both chemical and biological methods. The chemical synthesis was performed via the sol-gel method as described by Salim Albukhaty et al. (2020). Briefly, 2.5 g of zinc acetate dihydrate $[(CH_3COO)_2Zn \cdot 2H_2O]$, 98% and 8 g of sodium hydroxide (NaOH, 99%) were dissolved in 20 mL of deionized water (dH₂O) under stirring for 10 min. The NaOH solution was then stirred vigorously at room temperature for 15 min, followed by the dropwise addition of 100 mL of ethanol to the mixture. This resulting solution was added dropwise to the zinc acetate solution. After completion of the chemical reaction, the mixture was dried at 100°C to obtain a white powder (KS), a portion of which was subsequently calcined at 250°C for 2 h in a muffle furnace (YKS).

For the biological synthesis, ZnO NPs were prepared using *Rhodopseudomonas palustris* RPA1, following a modified protocol from Abdo et al. (2021). The bacteria were incubated in nutrient broth supplemented with sodium acetate at 30 ± 2 °C under continuous

light for 72 hours. After incubation, the cells were separated, and 2 mM zinc acetate was added to each fraction, followed by incubation under shaking conditions for 48 hours. The resulting precipitates were washed and dried, and a portion was calcined at 200 °C, yielding three types of nanoparticles: ZnO NPs derived from the dried cell fraction (KHA) and from the calcined cell fraction (YHA).

Characterization of ZnO-NPs

The synthesized ZnO-NPs were characterized to determine their morphology, elemental composition, optical properties, and surface chemistry. The morphology and size distribution were analyzed using Field Emission Scanning Electron Microscopy (FESEM, Zeiss SUPRA 40 VP), with samples sputter-coated with Au/Pd (80/20) and imaged at 2000× and 4000× magnifications. Elemental composition was confirmed by Energy-Dispersive X-ray Spectroscopy (EDX, Bruker XFlash®), which verified zinc and oxygen as the primary constituents with minimal impurities. Optical properties were assessed using UV–Vis spectrophotometry (HachLange DR 5000) in the 250–550 nm range. Absorbance spectra were employed to calculate the optical band gap and evaluate size-dependent quantum confinement effects. Surface chemistry and potential functional groups or modifications were investigated via Fourier Transform Infrared Spectroscopy (FTIR, Thermo Scientific Nicolet iS50, ATR mode, 4000–400 cm⁻¹).

Evaluation of Antibacterial and Antibiofilm Activity of ZnO-NPs

The antibacterial potential of ZnO nanoparticles (ZnO NPs) was evaluated against Gram-negative *E. coli* and Gram-positive *B. subtilis* and *S. aureus*. Bacterial suspensions were prepared in Mueller-Hinton Broth (MHB) and adjusted to an optical density of 0.5 at 600 nm ($\sim 5 \times 10^8$ CFU/mL). Antibacterial activity was assessed using the disk diffusion assay, where ZnO NPs (150 µg/mL in DMSO) were applied to MHA plates with DMSO as a negative control. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined via broth microdilution followed by plating. Additional analyses included microbial viability assessment using the Triphenyl Tetrazolium Chloride (TTC) assay, biofilm inhibition quantification by crystal violet staining, and monitoring of bacterial growth kinetics spectrophotometrically (OD₆₀₀) over 12 hours in tryptic soy broth with or without ZnO NPs (150 µg/mL). All experiments were performed in triplicate, and antimicrobial efficacy was evaluated based on inhibition zones, MIC/MBC values, and biofilm biomass.

RESULTS AND DISCUSSION

Structural and Morphological Characterization

The structural and morphological features of ZnO nanoparticles synthesized via chemical (KS and YKS) and biological (KHA and YHA) methods were thoroughly examined. FESEM analysis revealed clear morphological differences depending on the synthesis route: KS and YKS nanoparticles were predominantly spherical, whereas KHA and YHA nanoparticles exhibited rod-like shapes (Figure 1). These variations in morphology are known to influence surface area and the number of active sites, thereby impacting antimicrobial performance. Similar observations were reported by Bahadur et al. (2007), who found that zinc acetate dihydrate as a precursor produced uniform nanoparticle structures.

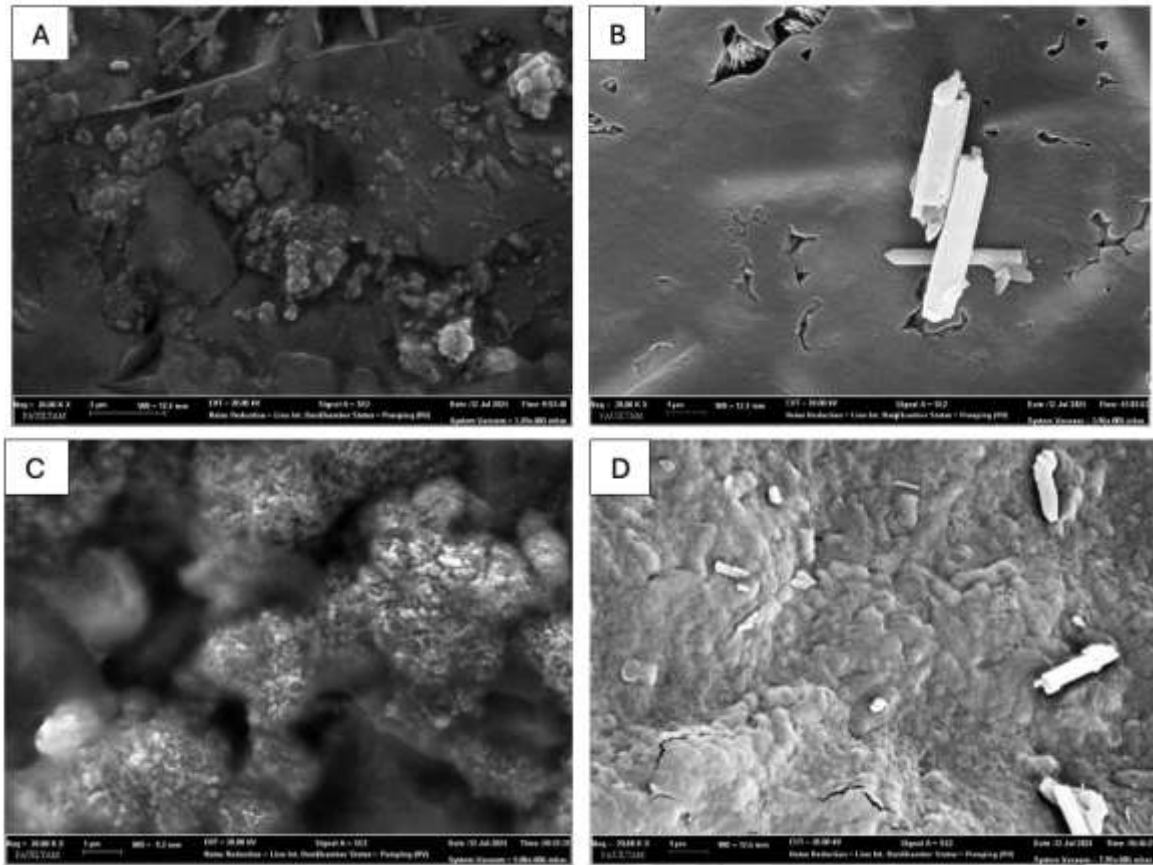


Figure 1. FESEM images of the synthesized ZnO-NPs: (A) KS, (B) KHA, (C) YKS, and (D) YHA.

EDX analysis confirmed the elemental composition of all ZnO nanoparticle samples, with distinct peaks corresponding to zinc (Zn) and oxygen (O), verifying the successful formation of ZnO. A minor carbon (C) peak was observed in some spectra, likely originating from organic residues or the sample holder (Figure 2). The uniform elemental distribution across the samples indicates that the synthesis process was well-controlled and reproducible.

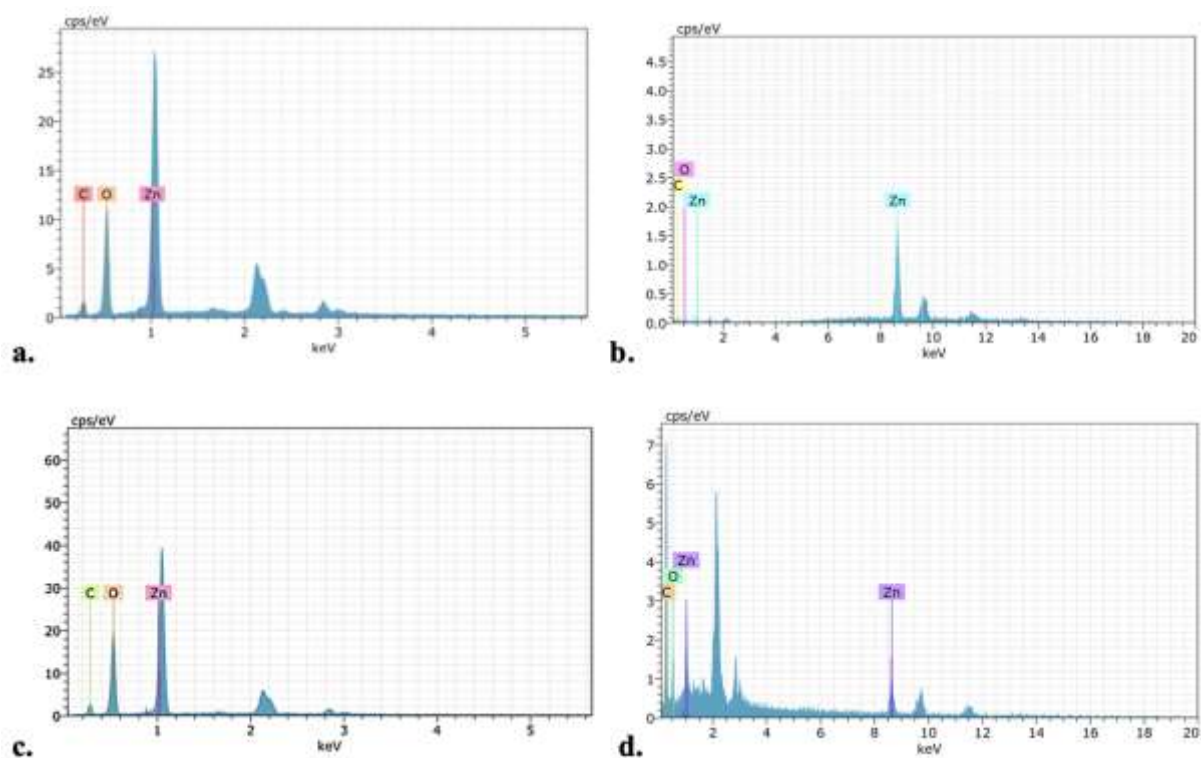


Figure 2. EDX spectra of the synthesized ZnO nanoparticles: (a) KS, (b) KHA, (c) YKS, and (d) YHA.

UV–Vis spectra of all ZnO nanoparticle samples exhibited strong absorption bands between 250 and 350 nm (Figure 3), consistent with their characteristic optical behavior. A slight blue shift relative to the typical 350–380 nm range reported in the literature (Sakthivel et al., 2015) suggests a quantum confinement effect due to the reduced particle size, confirming the nanoscale dimensions of the synthesized materials. FTIR analysis further corroborated ZnO formation through characteristic Zn–O stretching vibrations. Additional absorption bands observed in biologically synthesized samples indicated the presence of biomolecules, likely serving as reducing and stabilizing agents. These spectral features demonstrate strong structural integrity and effective surface functionalization, confirming that the synthesis parameters were optimized to ensure nanoparticle stability and performance.

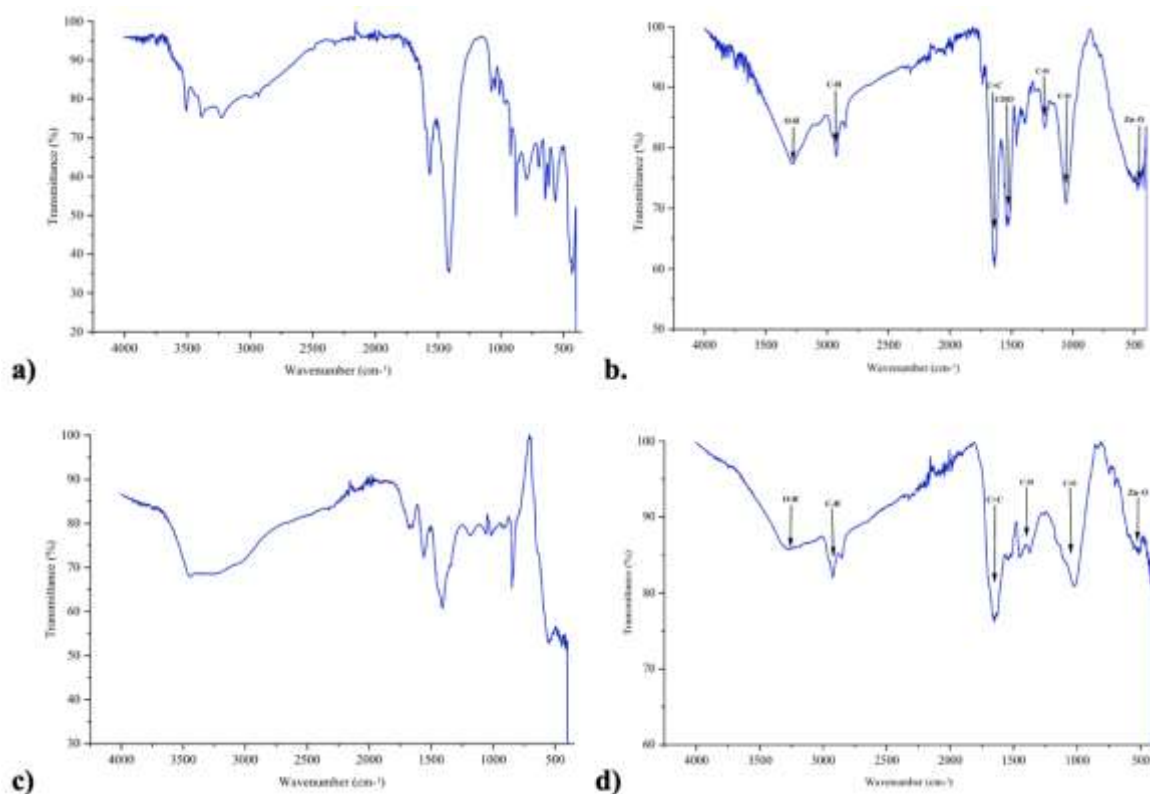


Figure 3. FTIR spectra of the synthesized ZnO nanoparticles: (a) KS, (b) KHA, (c) YKS, and (d) YHA.

Antimicrobial Activity

The antibacterial activity of the ZnO nanoparticles was evaluated against *E. coli*, *B. subtilis*, and *S. aureus*. Agar diffusion tests revealed that the chemically synthesized KS sample exhibited the highest antibacterial activity, producing inhibition zones of 2.1 cm against *S. aureus*, 1.4 cm against *E. coli*, and 1.0 cm against *B. subtilis*. The biologically synthesized KHA sample showed slightly stronger activity against *B. subtilis* (1.3 cm), while YKS produced smaller inhibition zones of approximately 1.0–1.2 cm for all strains. In contrast, the YHA sample showed no antibacterial effect against any tested bacteria **indicating that both the synthesis method and nanoparticle morphology play decisive roles in determining antibacterial efficacy. The inhibition zone diameters of ZnO nanoparticles against *E. coli* and *B. subtilis* are summarized in Figure 4.**

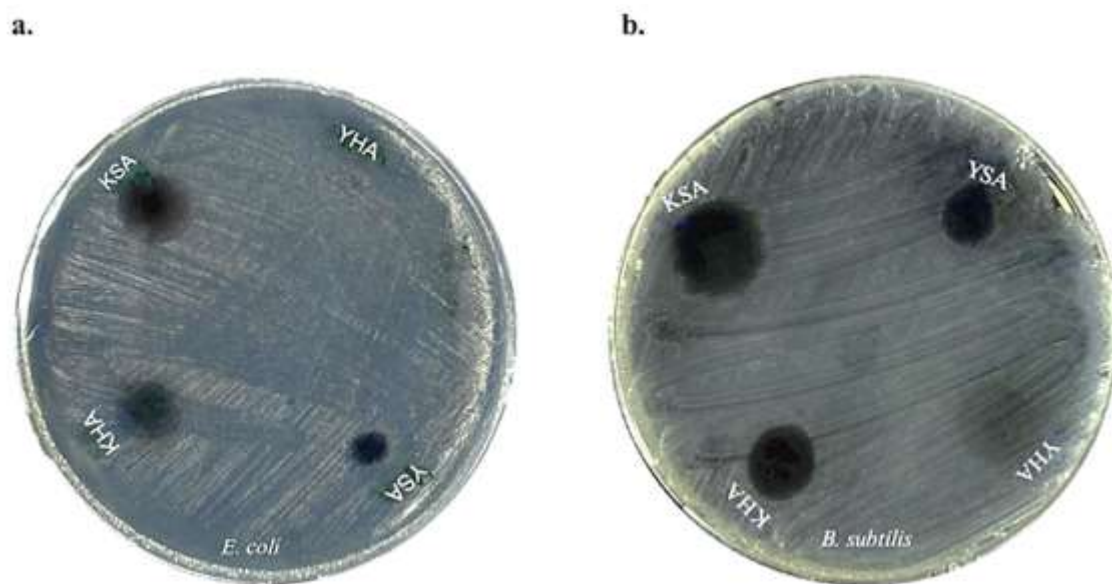


Figure 4. Antibacterial inhibition zone diameters (cm) of ZnO nanoparticles against *E. coli* (a) and *B. subtilis* (b).

MIC and MBC analyses supported these observations. The lowest inhibitory and bactericidal concentrations were 25 µg/mL for KS and KHA against *E. coli* and *S. aureus*, with KHA also showing the same effective concentration for *B. subtilis*. The YKS sample required higher concentrations (37.5–50 µg/mL) to inhibit bacterial growth, whereas YHA exhibited no significant antibacterial activity at any tested concentration. TTC assay results were consistent, showing that KS, KHA, and YKS completely inhibited bacterial metabolic activity in all strains, while a positive TTC reaction for YHA confirmed bacterial viability and absence of antibacterial activity.

The overall antibacterial performance of the nanoparticles aligns with previous studies. Chemically synthesized ZnO-NPs have been reported to exhibit inhibition zones of 2.3 cm at 0.5 mg/mL (Khan et al., 2014) and 3.2 cm at 1.5 M (Dadi et al., 2019), comparable to the values observed here. Similarly, Azam et al. reported inhibition zones of ~18 mm for *E. coli* and 16 mm for *S. aureus* at 50 µg/mL, while Sahin Dogan and Kocabas (2020) found 10 mm zones with MIC and MBC values of 6 µg/mL and >50 µg/mL, respectively. Emami-Karvani and Chehrazhi (2011) observed zones of 29 mm for *E. coli* and 19 mm for *S. aureus* using 10 mg/mL ZnO-NPs. These comparisons highlight that synthesis parameters, particularly the synthesis route and calcination temperature, critically influence nanoparticle bioactivity.

A notable outcome of this study is the first-time use of *R. palustris* RPA1 for the biosynthesis of ZnO-NPs. Previously known for CdS nanoparticle production (Bai et al., 2009), this microorganism demonstrated significant potential as a green and sustainable agent for ZnO-NP synthesis due to its photosynthetic and metal ion-transforming capabilities.

Collectively, the results indicate that chemically synthesized ZnO-NPs, particularly KS, displayed superior antibacterial efficiency due to their spherical morphology and large surface area, providing more active sites for bacterial interaction. Calcination (YKS) slightly reduced this effect, suggesting that high-temperature treatment can alter surface properties and decrease biological reactivity. In contrast, the biologically synthesized rod-shaped nanoparticles (KHA

and YHA) exhibited lower or no activity, emphasizing that nanoparticle morphology and thermal processing significantly influence nanoparticle–cell interactions.

CONCLUSIONS

This study demonstrated that both the synthesis method and nanoparticle morphology play a decisive role in determining antibacterial activity. Chemically synthesized spherical nanoparticles (KS) exhibited the highest antibacterial performance, whereas calcination (YKS) slightly reduced their efficacy. In contrast, biologically synthesized rod-shaped nanoparticles (KHA and YHA) showed minimal or no activity, emphasizing the critical influence of particle shape and surface properties. These findings highlight the importance of carefully controlling synthesis parameters to maximize the antimicrobial potential of ZnO nanoparticles and guide their future applications in biomedical fields.

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BEYOND AESTHETICS: THE ENVIRONMENTAL, HEALTH AND CULINARY USES OF ORNAMENTAL PLANTS

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ABSTRACT

Beyond their aesthetic value, ornamental plants hold significant importance in social, cultural, and environmental health dimensions. They facilitate the reconnection of urban populations with nature while also contributing substantially to rural development. The use of ornamental plants has traditionally been recommended primarily to enhance the aesthetic quality of indoor and outdoor human environments. However, beyond aesthetics, ornamental plants serve multiple purposes, including the restoration of degraded vegetation, regulation of high temperatures, reduction of soil and water pollution, erosion control, treatment of physical and mental health conditions, and even as food sources. Ground-covering plants play a prominent role in erosion control, while broad-canopy ornamental plants provide shade, creating cooler areas in urban centers. Shrub and tree groups are employed in large cities to protect and purify drinking water reservoirs. Once limited to decorative purposes, edible flowers are now gaining popularity as both aesthetic and nutritious culinary components in restaurants. The use of flowers as food is not a recent innovation but rather a revival of ancient ethnobotanical traditions. Historically, numerous civilizations across Asia and Europe have utilized flowers not only to add distinct decorative appeal to dishes but also to enhance the organoleptic synergy between the flavors of traditional foods—such as vegetables, meats, and fish—and floral aromas. Furthermore, studies have demonstrated that many ornamental plants exert beneficial effects on human health due to their secondary metabolites. A well-known characteristic of edible flowers is their pronounced antioxidant activity, observed in nearly all studied species. Incorporating ornamental plants into urban and residential design offers benefits that extend beyond aesthetics, including support for human nutrition, environmental conservation, and overall human health.

Key words: decorative vegetables, floriculture, landscape

INTRODUCTION

Ornamental plants have traditionally been valued for their aesthetic contributions, playing a central role in human environments across historical and cultural contexts. Beyond their visual appeal, these plants also reflect cultural identity and social status, often shaping gardens, public landscapes, and architectural designs. While aesthetics remains a primary motivation for their cultivation, recent research emphasizes the multifunctional potential of ornamental plants in ecological, social, and health-related contexts. Studies suggest that integrating ornamentals into urban landscapes can enhance environmental quality and contribute to human wellbeing in ways that extend far beyond visual enjoyment (Garbuzov & Ratnieks, 2014). Rapid urbanization and habitat loss have created urgent needs for resilient green infrastructures. Ornamental plants can help mitigate urban heat islands, reduce soil erosion, manage stormwater, and support biodiversity in fragmented landscapes. By strategically selecting

species that provide ground cover, canopy, or shrub layers, cities can improve microclimatic conditions, enhance ecosystem services, and promote ecological stability. Research highlights the use of ornamental species for urban ecosystem management and demonstrates measurable improvements in soil retention and microhabitat quality (Tzoulas et al., 2007). Human health and psychological benefits have emerged as key considerations in the planning and implementation of ornamental plant landscapes. Exposure to green spaces rich in ornamental species has been linked to reduced stress, improved attention, and enhanced cognitive function. Therapeutic gardens in hospitals, rehabilitation centers, and public parks illustrate the value of ornamental plants in promoting mental wellbeing. Studies have shown that both visual and olfactory interactions with ornamental plants can produce measurable reductions in physiological stress markers (Tian et al., 2014).

Another area of growing interest is the nutritional and medicinal potential of ornamental plants. Certain flowers, such as roses, marigolds, and pansies, contain bioactive compounds, including flavonoids, anthocyanins, and essential oils, which have antioxidant, anti-inflammatory, and antimicrobial properties. Incorporating edible ornamental flowers into diets offers a way to combine aesthetic pleasure with functional nutrition. Research highlights that edible flower are not merely decorative but can provide tangible health benefits, supporting the emerging field of functional foods (Gezer, 2022). Ornamental horticulture also contributes to socio-economic development. The cultivation and sale of ornamentals support rural livelihoods, create employment opportunities, and strengthen urban–rural economic linkages. In addition, ornamental plants are often central to cultural tourism and community identity, further enhancing their value beyond ecological and health benefits. Studies on the economic impact of ornamental horticulture underscore its role in sustainable rural development and urban planning (Levai and Ferencz, 2010).

Table 1. Service Domains of Ornamental Plants, Representative Species, and Their Associated Benefits

Field	Plant Samples	Ecoservices
Erosion Control	<i>Cornus sericea</i> , <i>Berberis repens</i>	Provides soil stabilization and reduces erosion risk.
Pollution Mitigation	<i>Centaurea cyanus</i> , <i>Gladiolus</i>	Can reduce heavy metal contamination in soil.
Biodiversity Support	<i>Helianthus</i> , <i>Rudbeckia</i>	Attracts pollinators and increases habitat diversity.
Edibility / Nutrition	<i>Rosa</i> spp., <i>Centaurea cyanus</i>	Provides antioxidant compounds and nutritional value.
Urban Health & Aesthetics	<i>Lavandula</i> , <i>Zinnia</i>	Improves psychological wellbeing and offers habitat for beneficial insects.

Overall, ornamental plants embody a convergence of environmental, social, economic, and health-related benefits. Their multifunctionality makes them vital components of urban planning, sustainable landscapes, and public health strategies. This review aims to synthesize current research on the ecological, nutritional, and psychological roles of ornamental plants, highlighting their significance as multifunctional resources that enhance human and environmental wellbeing.

1. ENVIRONMENTAL FUNCTIONS OF ORNAMENTAL PLANTS

Ornamental ground-cover species and shrubs contribute significantly to soil stabilization and erosion prevention. Their root systems enhance soil structure by increasing roughness, improving water infiltration, and reducing runoff velocity, crucial in areas prone to surface erosion and in urbanizing landscapes where soil disturbance is frequent (Francini et al., 2022). For example, perennial grasses and other low-growing ornamentals provide year-round soil cover, while woody ornamentals intercept rain and snow, further protecting against soil displacement. These functions make ornamentals useful in slope stabilization, bioswale systems, and green infrastructure projects (Lee et al., 2021).

Recent studies have documented the role of ornamental plants in *phytoremediation* — the removal or stabilization of contaminants in soil, water, and air. Due to their stress tolerance, rapid growth, and extensive biomass production, many ornamental species can extract or degrade pollutants, improving ecosystem quality while enhancing landscape aesthetics (Rocha et al., 2022). This dual purpose is especially valuable in urban areas or post-industrial sites, where conventional remediation may be costly or impractical. Combining functional remediation with ornamental landscaping supports both ecological restoration and socio-economic benefits. *Centaurea cyanus* (blue cornflower) plant not only provides visual appeal but has also been shown to have the potential to remove heavy metals from contaminated soils; this provides an advantage for environmental remediation in urban and polluted areas. Research with gladiolus and some other ornamental plants has shown that these species can be used to reduce heavy metal levels such as Pb in the soil, which supports phytoremediation applications (Akhtar et al., 2025).

Ornamental plants contribute to biodiversity by attracting pollinators such as bees, butterflies, and birds, as well as supporting beneficial insects and microfauna in urban and suburban green spaces (Anonymous, 2025a). Rudbeckia is an ornamental plant with a high attraction for insect visits, increasing the number and diversity of pollinators; this feature provides ecological benefits in landscapes. Sunflowers provide an important source for bees and other beneficial insects through pollen and nectar production; this increases insect diversity and ecosystem services in gardens (Grotewold, 2006; Palmersheim et al., 2022). Furthermore, ornamental vegetation enhances *ecosystem services* — benefits provided by nature that support human wellbeing. These include climate regulation, habitat formation, recreational and educational opportunities, environmental aesthetics, and psychological wellbeing improvements (Francini et al., 2022). The psychological benefits stem from humans' innate biophilic response to vegetation, increasing relaxation, reducing stress, and improving mood.

2. ORNAMENTAL PLANTS IN URBAN AND SOCIAL CONTEXTS

Broad-canopy ornamental trees and shrubs help regulate urban microclimates by providing shade, reducing heat island effects, and lowering ambient temperatures in urban centers. This cooling effect not only enhances human comfort but also reduces energy consumption for cooling systems. Ground-cover ornamentals and vertical green elements (such as green walls) also contribute by shading surfaces and promoting evapotranspiration, which further moderates urban heat extremes. In addition to erosion control, ornamental plants play roles in sustainable water management. Their canopy intercepts rainfall, reducing surface runoff and promoting groundwater recharge. When incorporated into urban green infrastructure designs — including rain gardens and bioswales — ornamentals can help manage stormwater, mitigate flood risk, and enhance water quality (Yuan and Dunnett, 2018; Funai and Kupec, 2019).

Ornamental plants foster human connection to nature, especially in highly urbanized environments where access to green space may be limited. This *contact with nature* is associated with enhanced psychological wellbeing, improved social cohesion, and increased opportunities for community engagement (Franci et al., 2022). Applications include healing gardens in healthcare settings, educational gardens in schools, and community green spaces that support physical activity and environmental awareness. For example, studies on healing gardens emphasize ornamental plants' role in reducing stress and improving air quality in clinical environments (Yıldırım et al., 2025).

3. ORNAMENTAL PLANTS AND HUMAN NUTRITION

Ornamental flowers are increasingly recognized not only for beauty but also for their nutritional value. Historically, many cultures incorporated flowers into cuisine to enhance flavor and aesthetic appeal. Today, edible flowers are gaining popularity in gastronomy as sources of antioxidants, vitamins, and bioactive compounds. Research shows that petals of ornamental roses, for instance, are rich in phenolic compounds, flavonoids, anthocyanins, and vitamins such as C and B groups, making them potential candidates for functional foods and nutraceutical applications (Kalisz et al., 2023). Secondary metabolites in ornamental plants — such as anthocyanins, flavonoids, and other phenolics — exhibit antioxidant and anti-inflammatory properties. These compounds may support human health by neutralizing free radicals, modulating inflammatory pathways, and potentially reducing risks for chronic diseases including cardiovascular disease and cancer (Tysiak, 2022). Anthocyanins, common pigments in many edible flowers, have been linked to protective effects against oxidative stress, while other bioactives can contribute to overall dietary quality (Grotewold, 2006).

4. HUMAN HEALTH AND PSYCHOLOGICAL WELL-BEING

Beyond nutrition, ornamental plants contribute to physical and mental health in multiple ways as stress reduction and mood enhancement, air quality improvement and therapeutic environments. The presence of ornamental plants has been associated with lower stress levels, improved attention, and enhanced moods through biophilic experiences (Fracini, 2022). In a study, participants' psychophysiological states were evaluated using blood pressure measurements, the State-Trait Anxiety Inventory (STAI), and electroencephalograms (EEGs). Results. STAI assessments indicated that anxiety levels decreased more after engaging in the horticultural task than after the control task. In addition, EEG readings showed shifts in brainwave activity following both types of activities. Conclusions. Overall, the findings indicate that involvement in horticultural work promotes psychological and physiological relaxation in adults (Tao et al., 2020). Certain ornamentals can improve indoor air quality by absorbing pollutants, regulating humidity, and reducing airborne particulates (Dela Cruz et al., 2014). It is known that the indoor ornamental plants specially as *Spathiphyllum spp.*, *Hedera helix*, *Ficus benjamina* are effectively used to reduce some pollutant as formaldehyde, benzene and ammonia. These chemicals can easily be released into the air from sources such as cigarette smoke, cleaning products, or textiles and wooden furniture (Anonymous, 2025b). Gardens and green spaces with ornamental plants are used in therapeutic settings to support recovery, rehabilitation, and mental wellbeing. Especially in the landscaping of hospital gardens and health complexes, the positive effects of ornamental plants on human health and recovery are taken into consideration when designing the landscape and choosing the plants (Yıldırım et al., 2025).

5. CHALLENGES AND CONSIDERATIONS

While many benefits of ornamentals are documented, challenges exist. For example, pesticide residues on ornamental plants may pose health risks if not properly managed, highlighting the need for integrated pest management practices in horticulture (Chwoyka et al., 2024). Appropriate species selection is also critical to avoid invasive or non-native ornamentals that might disrupt local ecosystems. Sustainable practices emphasizing native or ecologically compatible species can maximize environmental benefits (Porseryd, 2024).

CONCLUSION

Ornamental plants extend far beyond decorative functions. They provide diverse ecosystem services including soil stabilization, phytoremediation, biodiversity support, and urban environmental regulation. Additionally, they contribute to human health through nutritional components, psychological wellbeing, and enhanced environmental quality. Incorporating ornamental plants into urban design, community planning, and personal spaces not only beautifies environments but also supports broader ecological, social, and health objectives. Future research should continue to explore the full spectrum of functional benefits, optimize species selection, and integrate ornamental plants into sustainable landscape strategies.

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EFFECTS OF PUTRESCINE AND IBA TREATMENT ON ROOTING OF OLIVE CUTTINGS

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ABSTRACT

Olive propagation occurs vegetatively, mainly through cutting. Propagation by cuttings in olives is a simple and rapid method for maintaining genetic homogeneity. However, the difficulty of rooting in some varieties is one of the problems that limits production. This study aimed to increase the rooting ability of three olive varieties (Gemlik, Domat, and Memecik). For this purpose, auxin (indole butyric acid (IBA) was applied at a concentration of 4000 ppm, either alone or in combination with putrescine (250, 500, and 1000 ppm). A medium containing coconut fiber (45%), peat (45%), and perlite (10%), providing good aeration and high water-holding capacity, was chosen for rooting. Data on root and callus formation (%) were analyzed at the end of the study. The results showed that the three olive cultivars responded differently to the treatments applied. The highest callus formation was observed in the Memecik cultivar in media containing 1000 ppm putrescine (78%) and 4000 ppm IBA (65%), respectively. The highest root formation was observed in the Gemlik cultivar in media containing 4000 ppm IBA + 250 ppm PUT (53%) and 4000 ppm IBA (50%), respectively.

Keywords: *Olea europaea* L., polyamine, auxin, root induction

1. INTRODUCTION

The olive tree is a major agricultural crop, particularly in the Mediterranean, which supports local economies through the production and export of olives and olive oil (Ateş and Sunar 2025; Nteve et al. 2024). Olives have deep cultural roots, symbolizing peace, victory, and prosperity, and are recognized as part of the world's cultural heritage (Ghanbari et al. 2012). Their cultivation and use date back thousands of years, with significant roles in religious, medicinal, and culinary traditions (Uylaşer and Yıldız 2012).

Beyond nutrition, olives provide functional food benefits. Their phenolic compounds exhibit antimicrobial, antihypertensive, antidiabetic, and antiplatelet activities (Rahman et al. 2024; Asmaey et al. 2024). Olive leaves and by-products are also sources of valuable bioactives compounds, supporting their use in nutraceuticals and traditional remedies (Romani et al. 2019). Olives and their products (table olives, olive oil, and even seeds and leaves) are rich in monounsaturated fatty acids (MUFA), especially oleic acid, along with fiber, vitamin E, and a variety of bioactive phenolic compounds such as hydroxytyrosol and oleuropein (Zahra 2023).

The propagation of olives using cuttings is the most widely adopted method for producing uniform, high-quality planting material, as it preserves the desirable genetic and agronomic traits of elite cultivars and enables rapid orchard establishment (Porfirio et al., 2016). Vegetative propagation by cuttings is favored for its simplicity, cost-effectiveness, and capacity for mass production, making it suitable for both commercial nurseries and breeding programs (Ritter et al., 2021). However, the success of rooting in olive cuttings is influenced by multiple factors, including genotype, cutting type, season, and the application of rooting agents such as indole-3-butyric acid (IBA) (Abdel-Mohsen and Rashedy, 2023; Saeed, 2021). Despite its advantages, some cultivars remain difficult to root, necessitating ongoing research to optimize propagation protocols and explore alternative biostimulants to enhance rooting efficiency (Bartolini et al., 2023). The continued refinement of cutting-based propagation is therefore essential for meeting the rising global demand for olives and olive oil, supporting sustainable orchard expansion, and conserving valuable genetic resources (Porfirio et al., 2016; Chakir et al., 2022).

IBA is the most widely used auxin for promoting adventitious root formation in olive cuttings. Numerous studies have demonstrated that IBA application increases the rooting percentage, root number, and root length, especially in cultivars that are easy to root (Nandre et al., 2017; Hattab et al., 2018). Optimal concentrations typically range from 2000 to 4000 mg/L, with higher concentrations sometimes improving root quality but not always rooting percentage (Kurd et al., 2010; Ayaz et al., 2021). IBA is also effective across different cultivars and environmental conditions, though hard-to-root cultivars may show limited response (Denaxa et al., 2014). Polyamines, particularly putrescine, play a crucial role in the rooting process. Endogenous polyamine levels are higher in easy-to-root cultivars and are associated with improved rooting performance (Denaxa et al., 2014). The exogenous application of putrescine, particularly in combination with IBA, significantly enhances rooting in difficult-to-root cultivars, such as 'Kalamata', where IBA alone is often insufficient (Denaxa et al., 2021). Other polyamines, such as spermidine and spermine, are less effective. In recent years, polyamines such as putrescine have attracted attention for their potential role in enhancing root growth, although their interactions with auxins in olive remain insufficiently explored.

This study aims to investigate the individual and combined effects of IBA and Putrescine on callus and root formation in three olive varieties exhibiting contrasting regenerative behaviors. By evaluating eight treatment combinations under controlled conditions, the study seeks to identify optimal dosages and uncover genotype-specific responses that may inform future propagation protocols.

2. MATERIAL AND METHOD

2.1.Plant material

Cuttings of olive varieties used in the experiment were obtained from Çukurova University Olive Germplasm Collection (Coordinates 37°01'52.2"N 35°21'26.8"E).

The Gemlik variety, which responds well to cutting propagation, the Memecik variety, known to be moderately responsive, and the Domat variety, which is difficult to root, were used in the study. The characteristics of the varieties used are listed below (Anonymous 2025).

Gemlik Variety

The Gemlik cultivar originates from Bursa Province and is characterized by moderate tree vigor and a semi-upright canopy of modest size. Its fruits are medium-sized, nearly round to cylindrical, with a high flesh-to-pit ratio. Gemlik enters production early and maintains a high and consistent yield, while also exhibiting partial tolerance to cold conditions. The cultivar

is notable for its high oil content and is propagated successfully through cuttings. Owing to these traits, Gemlik is valued both as a black table olive and for oil extraction.

Memecik Variety

Memecik, originating from Muğla Province, is recognized as the most important cultivar of the Aegean region. It grows vigorously under favorable cultivation conditions and produces large, oval-shaped fruits with a distinctive nipple tip. The flesh-to-pit ratio and oil content are both high, making it suitable for dual purposes, oil extraction and table consumption. Memecik shows strong tolerance to cold and drought stress, though propagation is generally carried out by grafting rather than cuttings.

Domat Variety

The Domat cultivar originates from the Akhisar district of Manisa Province and is distinguished by its vigorous growth and broad, spreading canopy. Fruits are large and cylindrical, with tips that may be flat or rounded, and the variety is known for its high and consistent yield. Oil content is moderate, and propagation is typically achieved through grafting. Domat bears fruit early and is widely used as a green stuffed olive, reflecting its importance in table olive production

One- or two-year-old olive shoots were selected for cutting preparation. The shoots were trimmed to a length of 6–10 cm, leaving the top two leaves intact. Each cutting was excised just below the third node from the base (Figure 1).



Figure 15. Preparation of olive cuttings for rooting experiments.

IBA and Putrescine Treatments:

In addition to a standard dose of 4000 ppm Indole-3-butyric acid (IBA), the effects of Putrescine (PUT) were evaluated both independently and in combination with IBA at various concentrations (Table 1).

Table 15. Experimental treatment combinations of Indole-3-butyric acid (IBA) and Putrescine (PUT) applied to olive cuttings.

Treatmant	IBA (ppm)	PUT (ppm)	Description
1	0	0	Control
2	0	250	PUT alone – low dose
3	0	500	PUT alone – medium dose
4	0	1000	PUT alone – high dose
5	4000	0	IBA alone
6	4000	250	IBA + PUT – low dose
7	4000	500	IBA + PUT – medium dose
8	4000	1000	IBA + PUT – high dose

To ensure optimal aeration and high water-holding capacity, a rooting medium composed of 45% coconut fiber, 45% peat, and 10% perlite was selected. The plug trays (referred to as paper pods) were commercially purchased from Van der Knap company (Figure 2) (Antalya, Türkiye).

**Figure 16.** Rooting medium and plug tray system used for olive cuttings.

The upper parts of the olive cuttings were dipped into a fungicide solution. Subsequently, the basal ends were immersed in the prepared IBA–Putrescine solutions for 5 seconds, briefly air-dried for 10 minutes, and then planted into the paper pods (Figure 3).



Figure 17. Preparation of olive cuttings before planting: apical parts dipped in fungicide solution, basal ends immersed in IBA–Putrescine treatments for 5 seconds, air-dried for 10 minutes, and subsequently inserted into paper pods.

The cuttings were maintained for 90 days in fully controlled rooting trays under 85% relative humidity and a constant ambient temperature of 25 °C. Ultrasonic humidifiers were used to regulate humidity, while a bottom-heating system was employed to maintain temperature (Figure 4).



Figure 18. Controlled rooting trays with regulated humidity and temperature for olive cuttings.

2.2. Experimental Design and Statistical Analysis

All experiments were conducted using a randomized factorial design with three replications. The factorial structure consisted of three olive varieties and eight IBA–Putrescine treatment combinations. The collected data were subjected to analysis of variance (ANOVA), and statistically significant differences among means were determined using the Least Significant Difference (LSD) test at the 1% significance level ($p < 0.01$). Statistical analyses were performed using JMP Pro software (version 17.0.0). Data visualization and explanatory graphics were generated using Python 3.11.5 and relevant libraries, including Matplotlib, Seaborn, Pandas, and NumPy.

3. Results

Callus formation responses in the Gemlik variety varied significantly across hormonal treatments, as illustrated in the heatmap presented in Figure 5. The highest induction rate (45%) was observed under 1000 ppm Putrescine without IBA, indicating a statistically distinct response compared to all other treatments (group “a”). Moderate callus formation was recorded with 250 and 500 ppm Putrescine alone (18–23%). At the same time, the presence of IBA, either alone or in combination with Putrescine, did not enhance callus induction and remained statistically comparable to the control group (~10%). These results suggest that Putrescine

exerts a dose-dependent promotive effect on callus formation in Gemlik, whereas IBA may have a neutral or suppressive influence under the tested conditions.

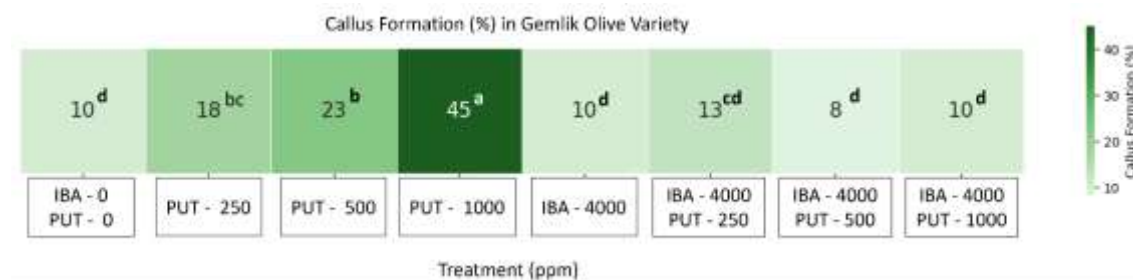


Figure 19. Callus formation percentages in Gemlik olive variety under different IBA and Putrescine treatments. Bars represent mean values with statistical groupings indicated by letters (a–d). Treatments sharing the same letter are not significantly different ($p < 0.05$).

Callus formation in the Memecik variety exhibited a broader and more responsive range across treatments compared to Gemlik, as visualized in the heatmap (Figure 6). The highest induction rate (78%) was recorded under 4000 ppm Putrescine alone, forming a statistically distinct group (“a”). Treatments with 4000 ppm IBA alone (65%) and 500 ppm Putrescine (58%) also promoted substantial callus formation, both classified within group “b”. Moderate responses (50–55%) were observed when IBA was combined with 250 or 1000 ppm Putrescine (group “c”), while the lowest rates (~20%) were associated with the control and the IBA–Putrescine 4000 ppm combination (group “e”). These results suggest that Memecik responds positively to both Putrescine and IBA, with additive or synergistic effects at intermediate doses, whereas excessive hormonal input may suppress callus induction.

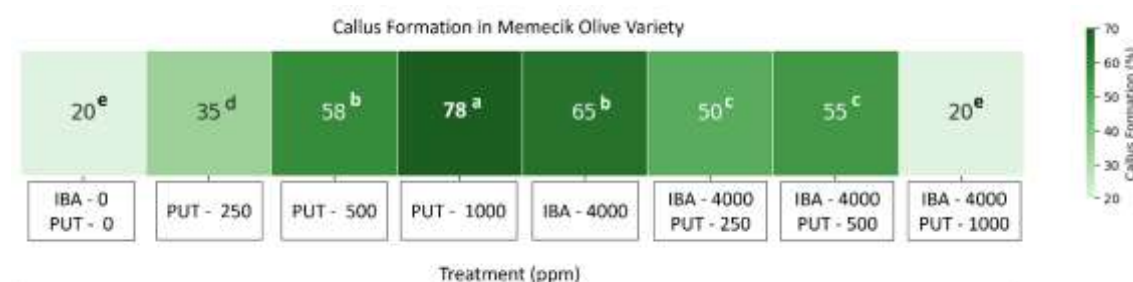


Figure 20. Callus formation percentages in Memecik olive variety under different IBA and Putrescine treatments. Bars represent mean values with statistical groupings indicated by letters (a–e). Treatments sharing the same letter are not significantly different ($p < 0.05$).

Callus formation in the Domat variety displayed a narrower response range compared to Gemlik and Memecik, as shown in the heatmap (Figure 7). The highest induction rates (33%) were observed under 1000 ppm PUT alone and the 4000 ppm IBA treatment, both statistically grouped as “a”. Moderate responses (15–18%) were recorded across several treatments, including lower IBA doses and combinations with Putrescine, while the control group remained similarly low (13%). Notably, all treatments except the high PUT and IBA doses produced statistically similar callus formation, suggesting a limited hormonal response in Domat. These

results may reflect a genotype-specific physiological constraint, where callus induction is possible but does not translate into root development, as previously observed.



Figure 21. Callus formation percentages in Domat olive variety under different IBA and Putrescine treatments. Bars represent mean values with statistical groupings indicated by letters (a–b). Treatments sharing the same letter are not significantly different ($p < 0.05$).

The interaction heatmap presented in Figure 8 illustrates the differential callus formation responses of three olive genotypes (Gemlik, Memecik, Domat) under various IBA and Putrescine combinations. Memecik exhibited the highest overall responsiveness, particularly under 1000 ppm Putrescine and 4000 ppm IBA used alone (78% and 65%, respectively), suggesting a strong promotive effect of polyamine treatment in this genotype. In contrast, Gemlik showed its peak response (45%) under 1000 ppm Putrescine without IBA, while all IBA-containing treatments remained below 15%, indicating a possible inhibitory interaction. Domat displayed a relatively flat response profile, with callus formation ranging from 12% to 33% across treatments, and no clear enhancement from either hormone. These patterns highlight genotype-specific hormonal sensitivities, where Putrescine alone appears most effective in Memecik and Gemlik, while Domat may possess physiological or genetic constraints limiting its regenerative potential.

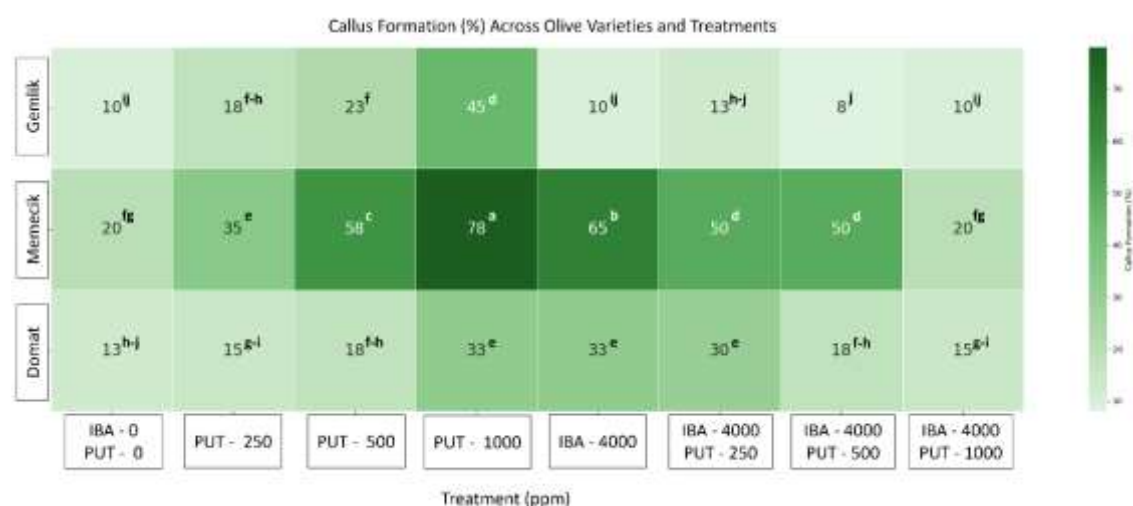


Figure 22. Heatmap illustrating callus formation percentages across three olive genotypes (Gemlik, Memecik, Domat) under various combinations of Indole-3-butyric acid (IBA) and Putrescine (PUT). Color intensity reflects the magnitude of callus induction, with darker shades indicating higher percentages.

Root formation in the Gemlik variety exhibited a distinct hormonal dependency, as visualized in the heatmap (Figure 9). The highest rooting percentages were observed under 4000 ppm IBA alone (50%) and in combination with 250 ppm Putrescine (53%), both statistically grouped as “a”. These results suggest a strong promotive effect of IBA on root induction in Gemlik, with Putrescine at low concentration potentially enhancing this response.

Moderate rooting (33%) was recorded under the IBA–Putrescine 500 ppm combination (group “b”), while all other treatments, including the control and Putrescine alone, resulted in minimal or no root formation (0–15%). This pattern indicates that, unlike callus formation, root development in Gemlik is primarily driven by auxin input, and Putrescine alone is insufficient to trigger rhizogenesis.

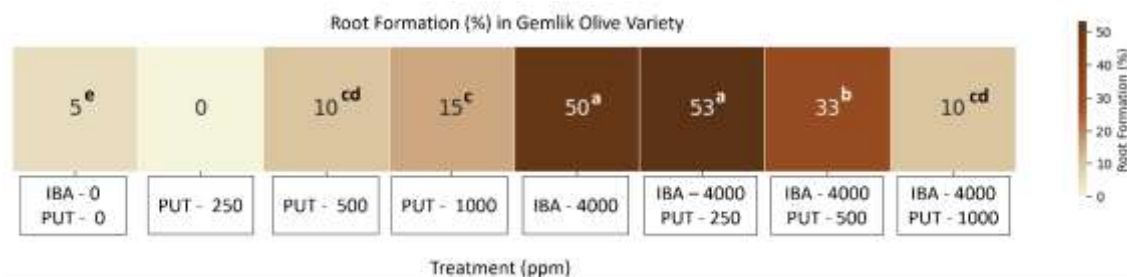


Figure 23. Root formation percentages in the Gemlik olive variety under different combinations of Indole-3-butyric acid (IBA) and Putrescine (PUT). Statistical groupings (a–e) reflect significant differences among treatments ($p < 0.05$).

Root formation in Memecik was generally low across all treatments, as shown in the heat map (Figure 10). The highest rooting percentage (20%) was observed under 250 ppm PUT in addition to 4000 ppm IBA, forming a statistically distinct group (“a”). Treatments combining IBA and Putrescine showed modest responses (5–8%), but Putrescine alone failed to induce any root formation. These results suggest that although Memecik is highly sensitive to callus induction, its rhizogenic potential remains limited and is primarily dependent on auxin input. The lack of synergistic improvement from Putrescine may reflect genotype-specific hormonal sensitivity or a threshold requirement for root formation that is not met under the conditions tested.

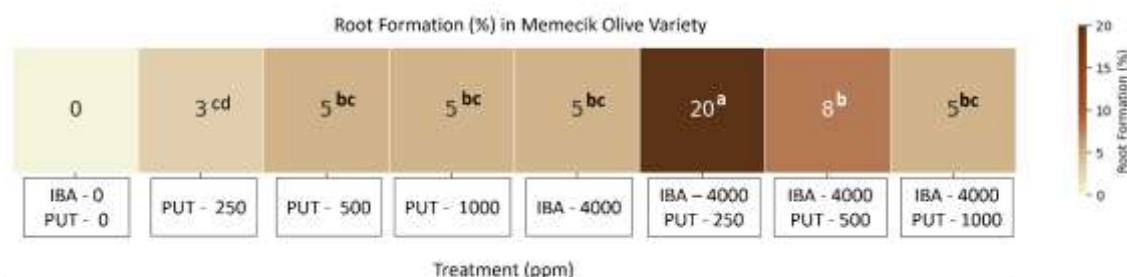


Figure 24. Root formation percentages in the Memecik olive variety under different combinations of Indole-3-butyric acid (IBA) and Putrescine (PUT). Statistical groupings (a–d) indicate significant differences among treatments ($p < 0.05$).

The interaction heatmap presented in Figure 11 reveals distinct genotype-specific responses to hormonal treatments in terms of root formation. Gemlik exhibited the highest rhizogenic capacity, with 4000 ppm IBA alone or combined with 250 ppm Putrescine, yielding the strongest responses (50–53%). Memecik showed limited rooting, with a modest peak under 4000 ppm Putrescine (20%), while all other treatments remained below 10%. Notably, Domat failed to initiate root formation under any condition, despite moderate callus induction in previous assays. These results underscore the critical role of genotype in determining rooting potential and suggest that auxin responsiveness is a key factor in successful rhizogenesis. The lack of rooting in Domat may reflect physiological or genetic constraints, such as impaired auxin signaling or the absence of root-competent callus tissue.

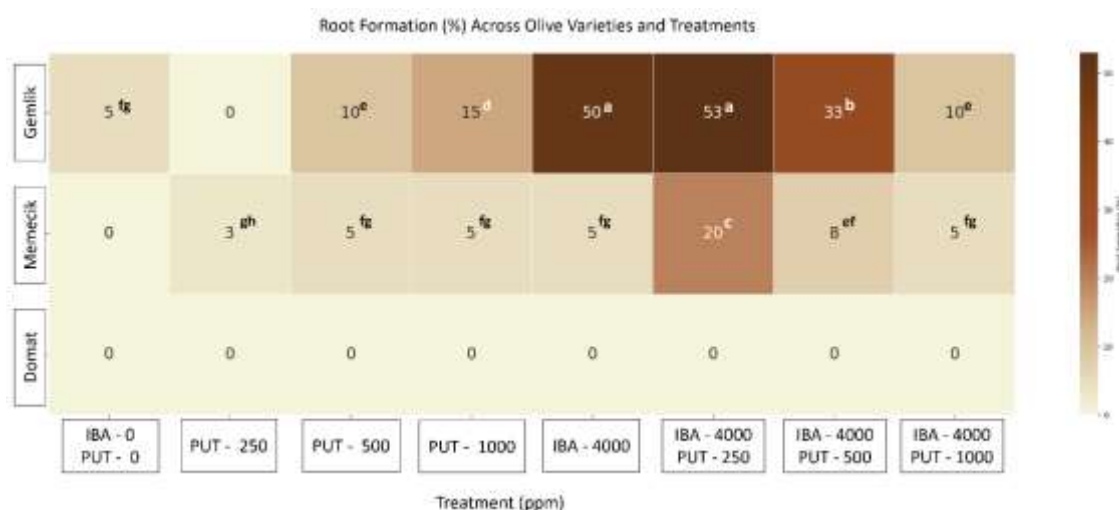


Figure 25. Heatmap showing root formation percentages across three olive genotypes (Gemlik, Memecik, Domat) under various combinations of Indole-3-butyric acid (IBA) and Putrescine (PUT). Color intensity reflects the magnitude of root induction. Statistical groupings (a–f) indicate significant differences among treatments ($p < 0.05$).

The correlation matrix presented in Figure 12 provides insight into the relationship between callus and root formation across Gemlik and Memecik genotypes. In Gemlik, a weak negative correlation ($r = -0.28$) was observed between callus and root formation, indicating that increased callus induction does not necessarily lead to enhanced rhizogenesis. In contrast, Memecik exhibited a weak positive correlation ($r = 0.25$) between these parameters, indicating a modest tendency for root development to follow callus formation. Notably, root formation in Gemlik showed moderate positive correlations with both callus ($r = 0.44$) and root formation ($r = 0.73$) in Memecik, implying shared hormonal responsiveness or physiological traits between genotypes. The absence of Domat data in this matrix reflects its lack of root formation, reinforcing the biological disconnect previously observed. Overall, these results highlight genotype-specific regenerative profiles, suggesting that callus quality, rather than quantity, may be a critical determinant of rooting success.

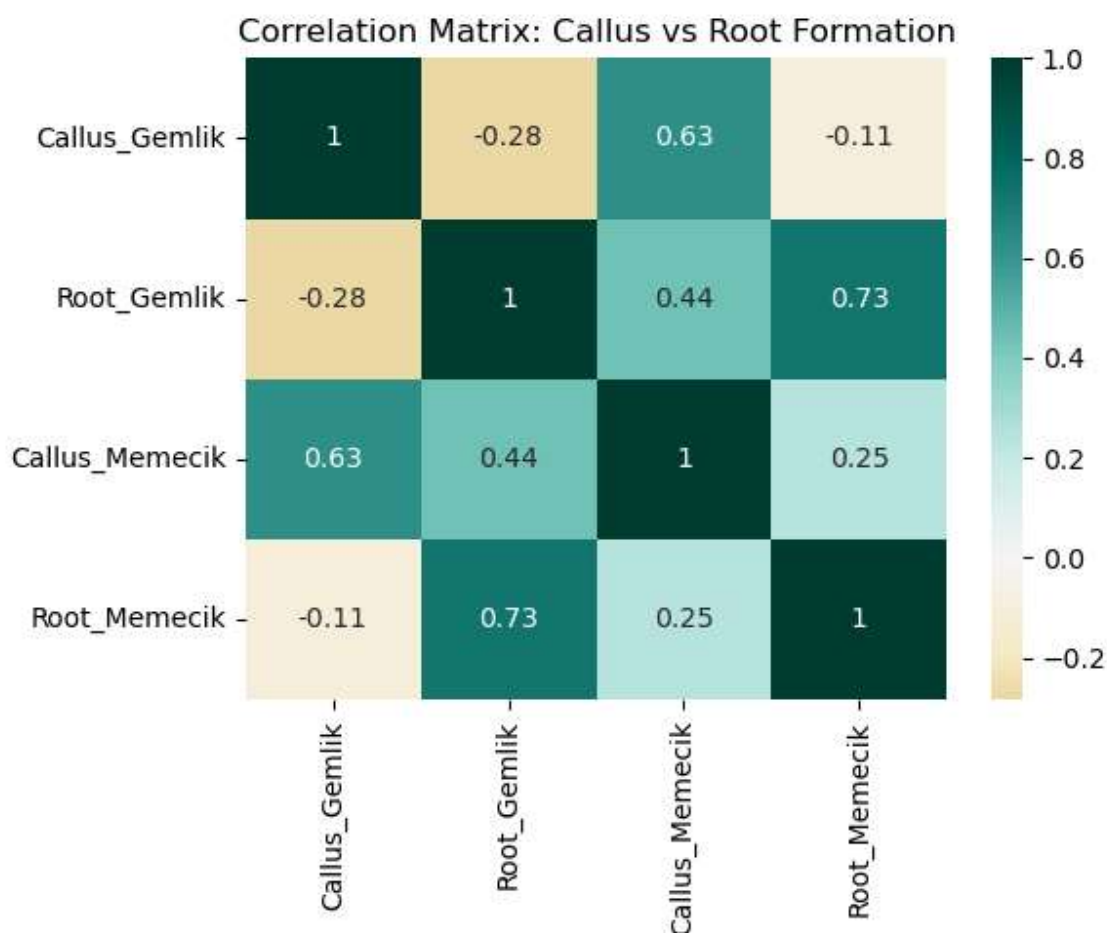


Figure 26. Correlation matrix illustrating the relationships between callus and root formation in Gemlik and Memecik olive genotypes. Color intensity reflects the strength and direction of Pearson correlation coefficients (r), ranging from -0.2 to $+1.0$

4. DISCUSSION

This study investigated the individual and combined effects of Indole-3-butyric acid (IBA) and Putrescine (PUT) on adventitious root and callus formation across three Turkish olive cultivars: ‘Gemlik’, ‘Memecik’, and ‘Domat’. The results clearly demonstrate that both genotype and hormonal treatment are decisive factors in regenerative success. Consistent with previous reports in olive propagation (Porfirio et al., 2016; Çiftçi et al., 2023), our findings confirm that genotype is the primary determinant of rooting capacity. The ‘Gemlik’ cultivar, known for its favorable propagation performance, achieved up to 53% rooting, highlighting the role of endogenous predisposition in responsiveness to exogenous hormones. In contrast, ‘Domat’ failed to initiate roots under any treatment, despite moderate callus formation (up to 33%), indicating a strong physiological or genetic barrier, such as lignification or elevated phenolic accumulation, that standard auxin or polyamine applications cannot overcome. This recalcitrance aligns with observations in other Turkish cultivars, which exhibit variable rooting success (Çiftçi et al., 2023). Furthermore, the importance of strictly controlled microclimates for rooting success has been emphasized in the literature (Chakir et al., 2022), underscoring the need for optimized greenhouse conditions when dealing with sensitive genotypes.

IBA emerged as the most effective exogenous auxin, serving as the essential driver of root initiation. In Gemlik, 4000 ppm IBA alone yielded 50% rooting, while the combination of 4000

ppm IBA with 250 ppm PUT produced the highest rate (53%). This modest but statistically significant increase supports the hypothesis that polyamines act as fine-tuners of auxin signaling rather than primary inducers (Druege et al., 2016). The mechanistic explanation for this synergy may lie in hormonal homeostasis: low concentrations of PUT could stabilize cell membranes or interact with auxin pathways, thereby enhancing tissue sensitivity to IBA. However, the effect was strictly dose-dependent. Higher PUT concentrations (500–1000 ppm) reduced rooting efficiency (e.g., 33% and 10% in Gemlik), suggesting that excessive polyamine levels disrupt the hormonal balance required for root differentiation, potentially diverting cells toward alternative developmental pathways.

The concentration of 4000 ppm IBA used in this study aligns with the upper range commonly reported for olive rooting (Porfirio et al., 2016). While Gemlik's maximum rooting rate of 53% represents a high success rate, it remains below the highest rates reported for cultivars such as 'Coratina' (75–76%) under optimized conditions (Rashedy et al., 2021). This discrepancy suggests that environmental factors, substrate composition, seasonal timing, or microclimate, though controlled, may still influence rooting outcomes (Chakir et al., 2022). The most critical parametric finding is the synergistic increase from 50% rooting with IBA alone to 53% with IBA + 250 ppm PUT. Although numerically modest, this statistically significant improvement underscores the regulatory role of PUT in modulating auxin-driven rhizogenesis.

A striking observation is the physiological uncoupling between callus formation and root initiation. Memecik exhibited the highest callus induction (78% under 1000 ppm PUT alone) yet produced negligible roots, while Domat generated callus without any subsequent rooting. These results confirm that callus proliferation does not necessarily predict rhizogenic success. Polyamines, as known promoters of cell proliferation (Alam et al., 2023; Yang et al., 2024; Blázquez, 2024), effectively stimulate dedifferentiation and cell division; however, root primordia initiation and differentiation require precise auxin signaling. This dissociation highlights the importance of hormonal balance in transitioning from undifferentiated growth to organized root development.

Biochemical mechanisms provide further explanation for these genotype-specific outcomes. The inability of Domat to progress from callus to roots may involve inhibitory activity of oxidative enzymes. Elevated peroxidase (POX) and polyphenol oxidase (PPO) levels are often correlated with recalcitrance, as they interfere with auxin metabolism at the cutting base (Macedo et al., 2013). Supporting this hypothesis, antioxidant applications have been shown to improve rooting rates under stress conditions (Rashedy et al., 2021). Thus, biochemical constraints may represent a critical barrier in recalcitrant cultivars, requiring targeted interventions beyond hormonal manipulation.

Overall, this study provides a practical propagation protocol, 4000 ppm IBA combined with 250 ppm PUT, for enhancing rooting in responsive cultivars such as Gemlik. In recalcitrant genotypes like Domat, the failure of hormonal treatments highlights the need for alternative strategies. Future research should integrate histological analyses to identify anatomical barriers, explore microbial biostimulants (Ferreira et al., 2022), and evaluate growth modulators such as strigolactones (Ozbilen et al., 2022) to overcome genetic constraints. These approaches may broaden the toolkit for olive propagation, ensuring that genotype $\times$ hormone $\times$ environment interactions are fully considered in the development of efficient and sustainable protocols.

5. CONCLUSION AND RECOMMENDATIONS

This study demonstrated that olive genotype and hormonal treatment jointly determine callus and root formation. Gemlik exhibited the highest rooting capacity, particularly under 4000 ppm IBA alone or in combination with 250 ppm PUT, confirming auxin as the primary driver and low-dose polyamines as modulators. Memecik responded strongly with callus

induction but limited rooting, while Domat produced callus without roots, underscoring genotype-specific constraints.

The results highlight that Putrescine acts as a regulator of auxin signaling in a dose-dependent manner: low concentrations enhanced rooting, whereas higher doses suppressed it. From a practical perspective, 4000 ppm IBA + 250 ppm PUT offers an effective protocol for responsive cultivars, while recalcitrant genotypes require alternative strategies. Future work should integrate histological and biochemical analyses to explore biostimulants or growth modulators that can overcome physiological barriers.

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THE EFFECT OF CONSERVATION FARMING PRACTICES ON GREENHOUSE GAS EMISSION

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ABSTRACT

The quality of soil and its management practices significantly influence climate conditions. Correct soil quality management is a key strategy to sequester carbon (C) and gas emissions limitation to potentially mitigate climate change. Conservation agriculture is based on four principles. minimizing soil disturbance through reduced tillage, leaving an appropriate amount of plant residues on the soil surface, crop rotation with an economic cultivation pattern, and sustainable production of agricultural products with benefits are the basis of these four principles. The aim of this study was to determine how conservation agriculture and conventional tillage affect greenhouse gas emissions and in the western Golestan, Iran. This work sought to investigate N₂O, CH₄, and CO₂ emissions of soils under two different tillage practices and straw retention scenarios: (I) conventional tillage (CT) and (II) conservation agriculture (CA) and The land had been managed under these practices for eight years. This study sampled soil at a depth of 0-30 cm following the harvesting of Wheat in 2024 for Measuring soil properties and used static flux chambers to collect gas samples It was found that CT yielded 353.6% higher microbial biomass carbon (MBC), 52.1% higher soil Respiration (SR), 67.2% higher microbial biomass nitrogen (MBN), and 59.2% higher microbial community (MC) relative to CT and organic C (OC) significantly improved under CA. It was also found that CA led to 5.2%, 5.9%, and 5.2% larger CH₄, NO₂, and CO₂ emissions relative to CT, respectively. In this study, the Rothamsted Carbon Model (RothC) was calibrated using field observations from 2019 and subsequently validated for the 2020-2024 period. After calibration, the model was applied to simulate SOC stock changes from 2017 to 2090 under three climate scenarios (S1: current climate; S2: +0.5 °C and +10% precipitation; S3: +4 °C and +23% precipitation) and two management systems: conventional tillage (CT) and conservation agriculture (CA). The combined findings indicate that conservation agriculture substantially enhances soil carbon sequestration, highlighting its potential as a climate smart strategy for mitigating soil carbon losses under future climate scenarios. It is necessary to conduct long-term assessments of optimized CA principles integrating all recommended factors to ensure the generalizability of the results.

Keywords: Conservation agriculture, Greenhouse gas emissions, Organic Carbon, Biological activity, RothC.

INTRODUCTION

Greenhouse gases such as CO₂, N₂O, and CH₄ naturally cycle among different terrestrial and atmospheric reservoirs. Soils can act as a sink for these gases by capturing carbon through photosynthesis and plant uptake of N followed by conversion of plant material to soil organic matter (SOM). To ensure that these gases are effectively stored as SOM, proper management practices must be implemented to maintain organic matter levels. However, preferred management practices do not always result in the retention of organic matter. Retention of organic matter is a major challenge, as evidenced by the increased emissions of CO₂ (13%), N₂O (81%), and CH₄ (44%) between 2007 and 2016 in the agriculture-forestry sector (IPCC, 2019). The use of soil conservation practices, however, may provide landowners with a strategy to reduce GHG fluxes through management. Because the effectiveness of these practices may change over time, further investigation of soil management effects on soil GHG fluxes is needed. One key soil management practice commonly used in about 90% of cultivated land area worldwide is tillage (Ruis and Blanco-Canqui, 2018). Given the complex dynamics between agricultural production, GHG emissions, and climate change, it is crucial to strike a balance between sustainable land development, increased productivity, and environmental sustainability, while also addressing the growing global demand for food (Ahmad and Wu, 2022; Shakoor et al., 2021b). Agriculture can also make a major contribution to climate change mitigation by reducing CO₂ emissions since biomass is a high carbon energy source. Agriculture's contribution as a carbon energy resource should be exploited as one solution with other greenhouse gas emission control technologies (Bundschuh 2014; International Energy Agency (IEA) 2014)). Barghouti (2009) suggests that the anticipated reduction in food production due to climate change can be offset by effective soil and water management practices. On the other hand, the 2010 World Development Report draws on the analysis of the Intergovernmental Panel on Climate Change (IPCC 2007a) to calculate that agriculture is directly responsible for 14% of global greenhouse gas (GHG) emissions in the form of CO₂ and equals and indirectly accounts for an additional 17% of emissions when land use and conversion for crops and pasture are included in the calculations, with a total of 31% (Raich and Tufekcioglu 2000). Nitrifying and denitrifying bacteria the primary contributors of N₂O emissions from soil, and their metabolic activity and thus N₂O production can be dependent on the physicochemical properties (Neill et al., 2005). Despite its widespread application, conventional tillage has been reported to play a negative role in the improvement of soil (Mathew et al., 2012; Fagundes et al., 2019; Lazzaretti et al., 2019) by altering its biological and physicochemical properties (Centurion et al., 2001; Viana et al., 2011). Process-based models are extensively employed to model the spatiotemporal dynamics of soil organic carbon (SOC) sequestration. While prominent models such as CENTURY (Parton, 1996), DNDC (Li, 1996), and APSIM (Holzworth et al., 2014) are widely used, the Rothamsted Carbon Model (RothC) (Coleman and Jenkinson, 1996) has gained particular prominence over the last two decades. Its widespread adoption across diverse climatic zones is largely attributed to its parsimonious structure and modest input data requirements (Abegaz et al., 2016; Setia et al., 2011; 2017; Smith et al., 1997). Originally developed as a pioneering multi-compartmental model (Jenkinson and Rayner, 1977), RothC has since been rigorously evaluated and optimized across a broad spectrum of ecosystems—ranging from croplands to forests—and within various climatic contexts, including Mediterranean and semi-arid environments (Coleman et al., 1997; Falloon and Smith, 2002; Farina et al., 2013;

Francaviglia et al., 2012; Skjemstad et al., 2004). However, the contributions of agricultural management systems and the biochemical properties of soil to GHG emissions are yet to be comprehensively studied. The total cultivated area in the province is estimated to be 730,000 hectares, with approximately 33.4% of this being irrigated, while the remaining 65.6% consists of dry land. The primary crops produced in Golestan, making this region one of the most significant agricultural areas in the country. This study specifically examines the arable lands in the central parts of the province where agricultural activities are concentrated. As a result, this study hypothesized that tillage systems alter the biochemical properties of soil and thus GHG emission dynamics and sought to assess soil conservation and GHG emission management. Therefore, CH₄, N₂O, and CO₂ emissions and MC structure were analyzed under various agricultural and tillage systems for a case study of Golestan Province, Iran.

2. MATERIAL AND METHODS

2.1. Case-study region

Kordkuy County located at coordinates of 54.10°N and 36.47°E in the east of Golestan Province was selected as the case-study region. It has an average annual precipitation of 377 mm and an average annual temperature of 20°C. Humidity and the average annual evaporation of Kordkuy County are 66.41% and 4.0 mm, respectively. The region has a Udic moisture regime and a Mesic thermal regime with Typic Hapludalf soil (Khormali and Kehl, 2011). Table 2 describes the agricultural management system of the region. Conservation tillage has been implemented in the region since 2017, spanning a period of eight years. A randomized complete block design (RCBD) was employed to perform experiments in two treatments, CA and conventional tillage (CT) with three replicates.

Table 1. Description of treatments

Treatment	Fertilizer	Weed Management	Straw and Stubble Management	Tillage
CT	50-100 kg of potassium sulfate	Plow,	Selling straw	Moldboard plow
	50-100 kg of triple superphosphate	Disk	(Burning the remains of the previous crop)	Disk
	200-300 kg of urea	Herbicide		
CA	50-100 kg of potassium sulfate	Herbicide	At least 30% of the residue remains on the surface. (residue retention on the field: 1-4 ton/he)	NT
	50-100 kg of triple superphosphate			
	200-300 kg of urea			

2.2. Measurements of soil properties

Soil samples from a depth of 0-30 cm were collected from farmlands subjected to the CA and CT management practices in autumn 2024. They were then dried in ambient air and sieved through a 2-mm mesh. 1:1 soil suspension was used to measure pH and electrical conductivity (EC) (e.g. Rhoades, 1996; Smith and Doran, 1996). Cation substitution with sodium acetate was employed to measure the cation exchange capacity (CEC) at a pH of 7.0 (Chapman, 1965). Moreover, the hydrometer method was performed to evaluate soil texture (Bouyoucos, 1962), and the cylinder method was utilized to calculate the bulk density (Carter *et al.*, 1986).

The samples were wetted immediately for a number of tests, e.g., microbial respiration. Microbial respiration was measured by placing the samples at 25°C within a closed container. The produced CO₂ would be absorbed by NaOH and measured through the titration technique (Chhonkar *et al.*, 2007; Isermeyer, 1952). Chloroform extraction was used to evaluate microbial biomass (Jenkinson and Ladd, 1981) and microbial biomass nitrogen (MBN) (e.g. Asgharzadeh, 2010). Furthermore, the colony count method was used within an agar culture medium to measure the microbial population (Asgharzadeh, 2011).

2.3. GHG sampling and analysis

The CO₂, CH₄, and N₂O emissions of the samples were obtained using the static chamber method (Bowden *et al.*, 2000). The gas samples were collected six times randomly in early November 2024, immediately following tillage through dark static stainless steel cylindrical chambers with a height of 30 cm, a total surface area of 0.05 m², and a volume of 0.01 m³. The GHGs were sampled during 08:00-18:00 using vacuum pumps inside black silicone

balloons at three intervals (30 min, 12 h, and 24 h). The moisture content and temperatures of air and soil inside the chambers were measured. A glass wool cover was applied to the cylinders to neutralize the impact of temperature changes, as shown in Fig. 1. Gas chromatography (GC) was employed to analyze the gas samples. The GHG concentrations were obtained as:

$$F = \frac{M \times P \times V \times \sigma v}{R \times T \times A} \quad (1)$$

where F is the flux rate ($\text{g/m}^2 \cdot \text{h}$), M denotes the molecular mass ($\text{CH}_4=16 \text{ g/mol}$, $\text{CO}_2=44 \text{ g/mol}$, and $\text{N}_2\text{O}=44 \text{ g/mol}$), P is air pressure (101300 Pa), V represents the volume of the chamber (0.01 m^3), A is the chamber-covered soil surface area (0.05 m^2), σv is the linear regression slope of gas concentration variation within the chamber headspace during sampling (ppm/h), and T is the internal absolute temperature of the chamber (K), and R is the gas constant ($8.314 \text{ m}^3\text{P/Kmol}$).



Fig. 1. Glass wool-covered chamber with a thermometer and a hygrometer

2.4. RothC-Model

The RothC model conceptually partitions soil organic matter (SOM) into five distinct conceptual pools, each defined by specific kinetic decomposition rate constants (k). These pools include Decomposable Plant Material (DPM), Resistant Plant Material (RPM), Microbial Biomass (BIO), Humified Organic Matter (HUM), and Inert Organic Matter (IOM), which is assumed to be resistant to decomposition. Except for IOM, decomposition in each pool follows first-order decay kinetics. This assumption implies that each pool is chemically homogeneous and well-mixed, with decomposition rates governed by substrate availability; consequently, the fraction of organic matter decomposed per unit time remains constant (k). Operating on a monthly time step, the model simulates the decomposition of carbon inputs, partitioning the products between mineralization (CO_2 evolution) and assimilation into the BIO and HUM pools. The partitioning ratio between evolving CO_2 and formed soil organic matter (BIO + HUM) is regulated by the soil clay content. Furthermore, the decomposition rate constants are dynamically adjusted at each time step using rate-modifying factors that account for air temperature, soil moisture deficit, and plant cover status.

2.4.1. Calibration and validation

The Rothamsted model (RothC) was executed for the 2017–2020 period using initial input data comprising climatic conditions, soil physical properties, and crop management practices. Examination of the preliminary outputs indicated that the simulated values exhibited a significant deviation from the field-measured observations. This discrepancy highlighted the necessity of calibrating the model to improve simulation accuracy. For calibration, the empirical data from 2019 were selected as the reference year. During this process, the model's sensitive inputs were identified and adjusted, with the most influential parameters related to agricultural management (including carbon inputs from crop residues, cropping patterns, and management operations). These modifications were implemented to better align the simulated values with the observed data. After calibration, the model was re-run with the updated parameters for the 2017–2024 period. For validation, the simulated results for 2020–2024 were compared with the corresponding field-measured data, enabling an assessment of the model's capability to predict actual changes in soil organic carbon. The Rothamsted Carbon Model (RothC) is globally recognized for its robustness in simulating long-term soil organic carbon (SOC) dynamics across diverse environmental and management gradients.

2.4.2. Climate change scenario

By explicitly accounting for sensitivity to primary climatic drivers specifically temperature, precipitation, and soil moisture the model serves as a critical tool for projecting the impacts of future climate scenarios on both soil carbon sequestration potential and associated carbon fluxes. Scenario S1 represents the current climatic conditions. Scenario S2 corresponds to the projected climate for the year 2040 based on LARS-WG model (Khosravaniyan et al, 2015), indicating an increase of 0.5 °C in temperature and 10% higher precipitation. Scenario S3 represents the projected climate for the year 2090 with an expected temperature increase of 4 °C and 23% higher precipitation.

2.5. Experimental setup

This study used an RCBD with three replicates to analyze the experimental data as a factorial. The tillage practice (two levels) and sampling time (three levels) were the experimental factors, and the CO₂, CH₄, and N₂O concentrations would be the traits to be measured. The data were analyzed in SAS V. 9.2. Duncan's multiple-range test (DMRT) was used to compare the means at $p < 0.05$. Furthermore, Microsoft Excel V. 2016 was employed to graph the data.

3. RESULTS

3.1. Physiochemical and biological properties of soil

It was found that tillage practices significantly impacted the SP, CEC, and EC of the soil, as shown in Table 2. It was found that CA resulted in a 0.26% lower topsoil pH than CT, which was significant ($p < 0.05$). The potential soil acidity (H⁺) was inversely correlated with pH. High H⁺ content (acidity in soil) was argued to be stratified in the upper soil layer (Franzluebbers, 2002). This suggests that soil organic carbon (OC) and pH are inversely correlated as crop residues that accumulate in the upper layer release organic acids (Hickman, 2002).

CA resulted in a 7.1% higher SP value than CT. Tillage was reported to impact volumetric moisture content in the order of moldboard plow (14.3%) $\approx$ double disk (16.2%) < NT (19.5%), highlighting that chisel plow was almost the same as double disk and moldboard plow systems (Rius et al., 2022). Furthermore, the tillage systems were observed to have mixed impacts on the moisture content. The average air-filled porosity experienced a reduction in the order of moldboard plow (48.4%) $\approx$ chisel plow (48.8%) $\approx$ double disk (46.8%) > NT (41.1%). Tillage systems influenced the air-filled porosity on twelve dates. The CA and CT treatments produced a bulk density of 1.38 and 1.36 g/cm, respectively. The long-term implementation of the NT system raised the bulk density and produced more compact soil (Chen et al., 2012). This does not facilitate the flow of nutrients to the deeper layers and impacts crop root growth and the absorption of nutrients, leading to a crop yield decline (Ji et al., 2013). Klik and Rosner (2020) reported that a soil tillage reduction enhanced BD, regardless of the soil texture at depths of 0-20 cm under three long-term tillage management practices.

It was found that CA raised the CEC and OC of the soil. Conservation tillage was found to enhance CEC by 69.2% and OM by 40.8%. This is in agreement with earlier works (Sa et al., 2017; Steinbach and Alvarez, 2006; Powlson et al., 2014). Hence, CA may enhance fertility in soil by protecting its structure from tillage (Williams et al., 2016; Kiani et al., 2025). Maize cultivation under long-term CA enhances soil organic matter (SOM) in light of the high residue input. The management system, edaphoclimatic conditions, and crop type are determinants of the high residue input and the consequent elevation of CEC and OC (Lal, 2015). Maize cultivation is a strong determinant of OC enhancement as the residue of maize has a low decomposition rate. Maize plays a key role in preserving high SOM content since it has a greater C/N ratio than soybean, decelerating decomposition and promoting accumulation (Broder and Wagner, 1988; Wegner et al., 2015). This is essential as OC content is high in productive regions and plays a key role in ensuring soil quality and effective productivity. According to Table 2, the treatment systems produced significantly different biological parameters, i.e., MBC, MBN, soil respiration (SR), and MC ($p < 0.01$). The CA treatment maximized microbial activity, resulting in 59.2%, 67.2%, 52.1%, and 353.6% higher MC, MBN, SR, and MBC relative to the CT treatment, respectively (Table 2). This is consistent with Nair and Ngouajio (2012), demonstrating that the introduction of crop residue changed OM, and multiple soil biology parameters, e.g., MBN, MBC, SR, and MC, were involved in its composition. A part from residue quality and quantity, the physical properties of soil, e.g., aggregates and pore space, and environmental factors may also impact the interactions between microorganisms (Six et al., 2000). Straw can function as a source of nutrients and energy to promote bacterial growth by providing polyphenols, vitamins, proteins, and carbohydrates (Singh et al., 2008; Bai et al., 2018). The results show that the retention of straw positively contributes to the abundance of bacteria and the BC composition, as shown in Fig. 2. It was reported that BC abundance and bacterial diversity could be remarkably enhanced through long-term straw retention techniques (Yuan et al., 2011). The results showed that BC composition and bacterial diversity enjoyed superb improvements upon straw retention. Zhao et al. (2018) demonstrated that BC abundance and soil properties had mixed correlations based on redundancy analysis. It was found that microorganism proliferation could be enhanced in arable land by raising the C/N ratio of soil (Liu et al., 2016). The present study identified mixed relationships between dominant BCs and the environmental parameters of soil, e.g., OC, C/N ratio, and TN. Tillage impacts microclimate and OM input availability and may alter microbial species in soil (Zuber et al., 2016). NT treatment leads to greater moisture content and lower soil temperatures relative to plow tillage, representing a more effective approach for microbial growth and colonization (Wang

et al., 2016, 2017). Although a number of works reported that NT treatment raises microbial activity compared to conventional tillage approaches (Hungria *et al.*, 2009; Silva *et al.*, 2013; Guo *et al.*, 2016), there have been reports arguing that NT does not significantly contribute to microbial diversity in soil (Li *et al.*, 2018a).

Table 2. Physicochemical and biological properties of soils (Lowercase letters compare treatments by LSD's test at 5 % probability)

Treatment	pH	EC (dSm ⁻¹)	SP (%)	Sand (%)	Silt (%)	Clay (%)	Texture
CA	7/63 ^b	0/93 ^a	33 ^b	31 ^a	62/66 ^a	22 ^a	Silty clay loam
CT	7/65 ^a	0/74 ^b	35/5 ^a	29 ^b	47 ^a	24 ^a	Silty clay loam

Treatment	CEC (CmolKg ⁻¹)	MBC (mg kg ⁻¹ soil)	SR (mg Co ₂ gr ⁻¹ soil.day ⁻¹)	MBN (mg kg ⁻¹)	MC (mol gr ⁻¹)	Total C (%)	OC (%)
CA	19/3 ^a	871/66 ^a	21/65 ^a	0/92 ^a	22/33×10 ^{6a}	4/53 ^a	1/69 ^a
CT	11/4 ^b	192 ^b	14/2 ^b	0/55 ^b	14×10 ^{6b}	3/1 ^b	1/2 ^b

CA = Conservation Agriculture; CT = Conventional Tillage; EC=Electrical Conductivity; SP=Saturation Percentage; CEC=Cation Exchange Capacity; OC=Organic Carbon; Bd=Bulk density; MBC=Microbial Biomass Carbon; SR=Soil Respiration; MBN=Microbial Biomass Nitrogen; MC=Microbial Community; TOC=total organic carbon; Lowercase letters compare treatments by LSD's test at 5 % probability

3.2. GHG emission rates

The CH₄, N₂O, and CO₂ emissions from the surfaces of soils were comparatively analyzed under various management systems. The GHG emissions were monitored at three intervals (30 min, 12 h, and 24 h) following the installation of the chambers. The sampling time and CO₂ emissions were not significantly different in the treatment systems. The CO₂ flux remained steadily small at 28.4 g/m²h under the CT treatment, as shown in Fig. 2. Tillage regulates the soil structure and changes C storage and emission (Abdalla et al., 2013). Semi-humid and temperature soils lose carbon mostly in the form of CO₂, which is primarily induced by the mineralization of OC. Tillage increases the CO₂ emission by raising soil disturbance (Lu and Liao, 2017). The surface layer of soil loosens under classic hoe-type plow, leading to dominant disruptions. As the moisture content, bulk density, and temperature of soil change, the oxygen content of loosened soil rises and mineralizes OC, inducing carbon losses. The production of CO₂ in soil arises from the respiration of roots and OM microbial decomposition, and the moisture content and temperature of soil are key determinants (Davidson and Janssens, 2006). Tillage practices may alter the physicochemical properties of soil and CO₂ production and dynamics. Earlier works reported that conservation tillage decreases CO₂ emissions compared to conventional tillage, particularly with NT being the primary treatment system (Abdalla et al., 2019; Reicosky, 1997; Ussiri and Lal, 2009). Researchers have proposed a variety of explanations for the adverse impacts of conservation tillage on CO₂ emissions. He et al. (2011) held that conservation tillage may support C stocks by restricting temperature elevations and minimizing aggregate disruptions. It diminishes soil disturbance and the access of microorganisms to OC pools. As a result, the decomposition of OC and C loss through leaching or CO₂ fluxes are reduced (Six et al., 2002). Nevertheless, it is also possible that conservation tillage accelerates CO₂ emissions by increasing water availability and thus promoting microbial activity (Plaza-Boadilla et al., 2014b).

Furthermore, the sampling time and GHG fluxes did not significantly differ between the treatments. As shown in Fig. 2, CH₄ showed a steadily small flux of approximately 0.038 g/m²h under conventional management. The management systems may impact GHG emissions by, for example, altering OC, soil fertility, and moisture retention (Tate *et al.*, 2015). This CH₄ flux pulse following a shift in the management system is yet to be explained. First, methanotrophs (a sensitive group of microorganisms) regulate CH₄ fluxes, and a disturbance arising from a management shift may induce a lack of methanotroph-methanogen balance and increase CH₄ emissions in comparison to CT treatments, while the MC of soil underwent a shift to a new steady state (Scheffer et al., 2001). NT is a globally popular treatment adopted to mitigate soil erosion and promote soil health in the long term; however, it has highly inconsistent effects on the CH₄ emission from soil. Furthermore, given climate variability, it is crucial to assess multiple aspects of agricultural systems under the framework of a “climate-smart” agricultural strategy (Paustian et al., 2016). The N₂O flux was steadily small at 0.017 gr/m²h under conventional agriculture. It was reported that conservation tillage resulted in a potential reduction of 15-40% in N₂O emissions relative to conventional tillage (Drury *et al.*, 2006; 2011). Organic matter was further demonstrated to strongly contribute to the regulation of GHG emissions (Zuber and Villamil, 2016). The return of plant residues resulted in a 32.1% reduction in NO emissions and a 12.9% elevation in N₂O emissions. This aligns with the hypothesis that fresh crop residues provide labile OC substrates and anaerobic conditions due to decomposition, enhancing denitrification (Chen *et al.*, 2013). On the other hand, aged organic matter led to a 2.6-15.9% decline in NO and N₂O emissions, as shown in Fig. 2. This can be mainly explained by increased N substrate

immobilization. The inconsistent impacts on GHG emissions require effective crop residue management to minimize N₂O emissions. Microbial nitrification and denitrification are responsible for producing N₂O in soil (Liu *et al.*, 2014; Nivelles *et al.*, 2016). Denitrification can be dependent on a number of factors at various scales. The use of nitrogenous fertilizers is a key determinant in soil management. Hence, nitrogenous fertilization impacts the dynamics of N₂O emissions to a great extent (Zuber and Villamil, 2016), accounting for a fertilizer loss of nearly 1.25%. Tillage systems and plant residues are also key GHG emissions determinants in agricultural management. The tillage treatment alters a set of belowground environmental parameters and residue distributions in soil (Kabiri *et al.*, 2016). N₂O emission elevations can be explained by enhanced denitrification and/or microbial nitrification (Lafuente *et al.*, 2020).

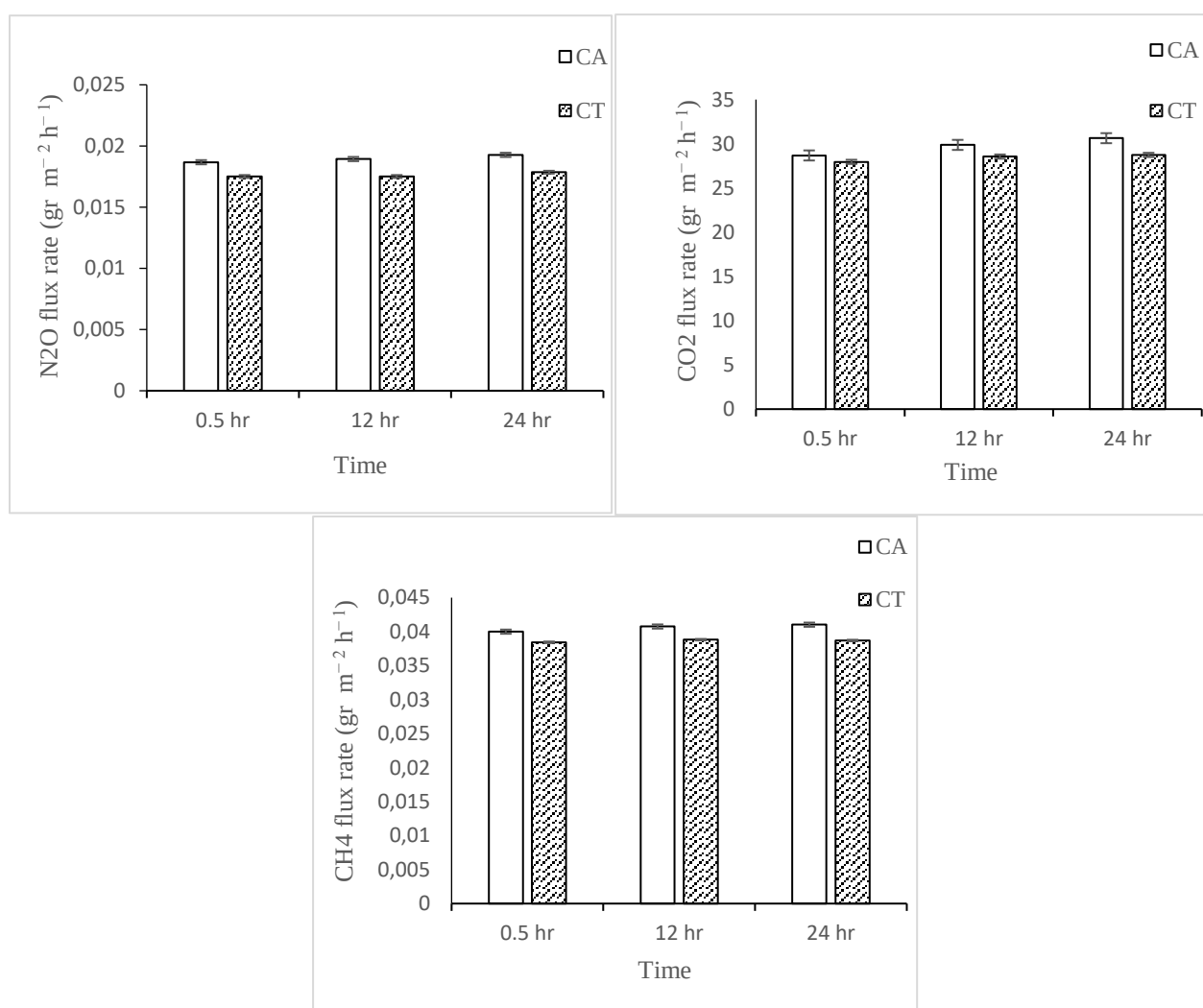


Fig. 2. GHG emissions under different tillage treatments at three sampling times. CA=Conservation Agriculture; CT=Conventional Tillage. Lowercase letters compare treatments by LSD's test at 5 % probability.

3.3.RothC-Model

The RothC model showed strong skill in reproducing SOC trends but limited accuracy in predicting absolute values. A high correlation ($r = 0.94$; $R^2 = 0.886$) indicated that the model effectively captured temporal or treatment-based SOC dynamics. However, the relatively large RMSE (7.25 t/ha) and MAE (5.91 t/ha), together with a negative MBE (–5.90 t/ha), revealed a systematic underestimation of measured SOC (Table 3).

Table 3. Performance metrics of the RothC model for SOC simulation

Metric	Value
R^2	0.885966
Pearson_r	0.9412577
RMSE	7.254689
MAE	5.91256
Index_of_agreement_d	0.4720553
Mean_bias_error_MBE	-5.896933
Nash_Sutcliffe_efficiency_NSE	-1.720867

Simulation of soil total organic carbon (TOC) dynamics using the calibrated RothC model showed clear divergence between the two management systems (conventional tillage: CT; conservation agriculture: CA) across all climate scenarios (S1–S3) (Fig 2). Under CA, TOC increased continuously from the baseline value of approximately 32–33 ton ha⁻¹ in 2017, reaching 50–65 ton ha⁻¹ by 2090 depending on the climate scenario. The highest increase was observed under S1-CA and S2-CA, where TOC exceeded 60 ton ha⁻¹ after 2080, while S3-CA resulted in a comparatively lower accumulation (around 51–53 ton ha⁻¹) by the end of the simulation period. In contrast, CT treatments exhibited either slight declines or only marginal increases in TOC. Under S1-CT, TOC remained nearly stable at around 28–30 ton ha⁻¹, whereas S2-CT and S3-CT showed gradual decreases toward 23–26 ton ha⁻¹ over the long term. Across all scenarios, the CA system stored 20–35 ton ha⁻¹ more carbon than CT by 2090, demonstrating its superior capacity for long-term SOC sequestration.

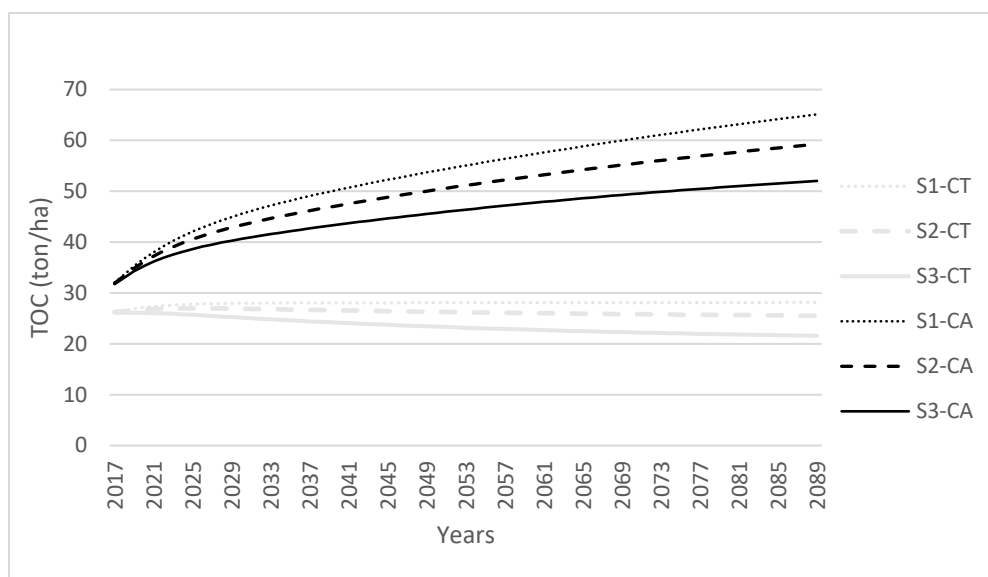


Figure 3. Effects of climate change scenarios and soil management on long-term SOC dynamics

The overall trend indicated that conservation agriculture (CA) consistently enhanced soil carbon sequestration compared with conventional tillage (CT), regardless of climate scenario. This outcome is strongly aligned with RothC's sensitivity to residue inputs and soil disturbance, as reduced tillage preserves physically protected carbon pools and promotes gradual carbon accumulation. The substantial increase observed under S1-CA and S2-CA suggests that moderate warming combined with enhanced precipitation (as in S2) supports microbial activity and biomass production, thereby increasing carbon inputs to the soil. The projected 0.5 °C temperature rise and 10% increase in rainfall under S2 appear to create favorable conditions for carbon stabilization, explaining why S2-CA performed similarly or even slightly better than the current-climate scenario (S1-CA). The contrasting behaviors of CA and CT across all climate scenarios further emphasize the importance of management practices relative to climate drivers. While climatic forcing affects the rate of SOC change, management practices—specifically residue retention and reduced soil disturbance—play the dominant role in determining the overall carbon trajectory. By 2090, the gap between CA and CT widens substantially, confirming that CA remains a robust mitigation strategy even under adverse climate conditions.

CONCLUSIONS

This study found significant changes in the biological and physicochemical properties of soil upon shifts between tillage systems and residue management. A methodology was proposed to quantify GHG emissions from silty clay loam farmlands treated by various management systems. It was concluded that OC, CEC, and bulk density increased under CA, whereas pH and SP declined. Moreover, CA had maximized total N, total C. A shift from CT to CA increased the biological factors by 52.11-353.6%. Furthermore, CO₂, CH₄, and N₂O emissions increased insignificantly under CA. GHG emissions and soil properties were also observed to be correlated insignificantly. The findings revealed that conservation agriculture consistently enhanced soil organic carbon (SOC) storage across all climate scenarios, with increases of up to 20–35 ton ha⁻¹ compared with conventional tillage by 2090. In contrast, SOC under conventional tillage remained stable or declined, particularly under intensified warming conditions (S3). Importantly, conservation agriculture increased SOC

sequestration without noticeably elevating CO₂ emissions, highlighting its value as a climate smart management strategy. Overall, the results underscore the importance of adopting low disturbance, residue retentive practices to strengthen soil carbon resilience and mitigate carbon losses under future climate scenarios. There is extensive literature on the impacts of conservation tillage on GHG emissions, indicating that, in particular, NT would remarkably hinder CH₄, N₂O, and CO₂ emissions; however, reports have been inconsistent in some cases. A set of factors appear to play a key role in this respect, e.g., soil structure, cropland type, local weather, plant residue retention, crop type, and intricate biogeochemical mechanisms. It is vital to implement a comprehensive strategy that synergistically incorporates key factors in order to ensure effective conservation agriculture, which was not the case in the case-study region. This study adopted a short- and medium-term approach. Therefore, it would be impossible to track the variations that develop over time. This is particularly crucial since the contributions of local environmental parameters to CA effectiveness continue to be growingly appreciated. The analysis of short-term soil health can provide insights into challenges that may not become apparent in the short term. Long-term investigations into GHG emissions and responses to climate variability remain yet to be conducted. Future research can fill this gap to help farmers make informed decisions and acquire practical adaptability.

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PERFORMANCE EVALUATION OF GRAIN MAIZE VARIETIES UNDER THE ECOLOGICAL CONDITIONS OF THE AMIK PLAIN, HATAY

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ABSTRACT

This study was conducted in 2024 under the ecological conditions of the Amik Plain, Hatay, Türkiye, to compare yield-related traits of nine grain maize (*Zea mays* L.) varieties and to recommend high-yielding cultivars to local farmers. The experiment was arranged in a randomized complete block design with three replications. Significant differences ($p < 0.05$) were observed among varieties for ear weight, ear length, ear diameter, kernel weight per ear, and thousand-kernel weight, whereas the number of kernels per ear was not statistically significant ($p = 0.06$). The highest ear weight was recorded in P2105 (238.93 g) and P1884 (230.93 g), while P2105 also exhibited the greatest ear length (23.32 cm) and ear diameter (47.86 mm). Kernel weight per ear was highest in P2105 (219.20 g), followed by P1884 (193.27 g) and CA403 (193.00 g). Thousand-kernel weight ranged from 190.48 g (P1551) to 248.50 g (CA403), with P2105 (230.73 g) also ranking among the top performers. In contrast, P1551 consistently produced the lowest values across most traits. The results indicate that P2105, followed by P1884, demonstrated superior performance in the Amik Plain environment, suggesting their suitability for high-yield grain maize production in the region. These findings provide valuable guidance for cultivar selection, aiming to enhance productivity and economic returns for farmers under similar agro-ecological conditions.

Keywords: *Zea mays* L., Grain yield, Variety performance, Amik Plain

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important cereal crops, widely cultivated for both grain and silage. Given its high productivity potential and adaptability, maize plays a vital role in the cropping systems of many countries (Kim and Lee, 2023; Danilevich et al., 2021). In Türkiye, grain maize production has expanded under irrigated conditions, especially in the Mediterranean and Southeastern Anatolia regions, where climate and soil conditions can vary markedly (Kizilgeçi et al., 2018).

Yield and yield-component traits such as ear weight, ear length, ear diameter, kernel number, kernel weight per ear, and thousand-kernel weight are key indicators of cultivar performance and have been used extensively in breeding and agronomic evaluation studies (Kizilgeçi et al., 2019; Ruswandi et al., 2022). For example, Kizilgeçi et al. (2018) found significant variation among 125 Turkish maize landraces in traits such as ear diameter (14.13–48.92 mm) and ear weight (10.2–285.26 g) when grown under southeastern Anatolian conditions. Such variation underlines the potential for selecting high-yielding genotypes adapted to specific agro-ecological zones.

Furthermore, the genotype $\times$ environment interaction remains critical in maize performance, especially under variable Mediterranean-type environments where water stress, high temperatures, and erratic rainfall may reduce yield stability (Kim and Lee, 2023). Studies

employing stability analyses and biplot methods have demonstrated that hybrids with high and stable yields under diverse conditions can be selected using combined analyses of grain yield and yield-component traits (Ruswandi et al., 2022).

Despite the availability of numerous maize cultivars, region-specific evaluations remain essential because local environmental conditions — such as those in the Amik Plain (Hatay) — impose unique constraints and opportunities. The Amik Plain is characterised by Mediterranean climatic influences, relatively mild winters and hot summers, and irrigated cropping systems, which may favour certain maize genotypes over others. Yet to date, few studies have explicitly focused on grain maize varietal performance under the specific ecological conditions of the Amik Plain.

Therefore, this study was conducted to evaluate nine grain maize varieties under the ecological conditions of the Amik Plain, Hatay, with the objectives of (i) comparing yield-related traits among the varieties, and (ii) recommending high-yielding cultivars suitable for the local farming community. By focusing on ear weight, ear dimensions, kernel weight per ear and thousand-kernel weight, this research aims to provide relevant cultivar selection guidance for enhanced productivity in the region.

MATERIAL AND METHOD

The field experiment was carried out in 2024 at the Agricultural Research and Application Center of Hatay Mustafa Kemal University, located in the Amik Plain (36°15' N, 36°30' E; 94 m a.s.l.). The region is characterized by a typical Mediterranean climate, with mild and rainy winters and hot, dry summers. Soil analyses of the experimental field indicated a clay-loam texture with low organic matter content (1.93%), moderate lime content (6.45%), and a slightly alkaline pH of 7.12. During the experimental period (Figure 1), total precipitation was concentrated in early spring but declined markedly towards summer, while average air temperatures remained above long-term means, indicating a predominantly dry growing season. These conditions represent the typical climatic pattern of the Amik Plain during maize cultivation.

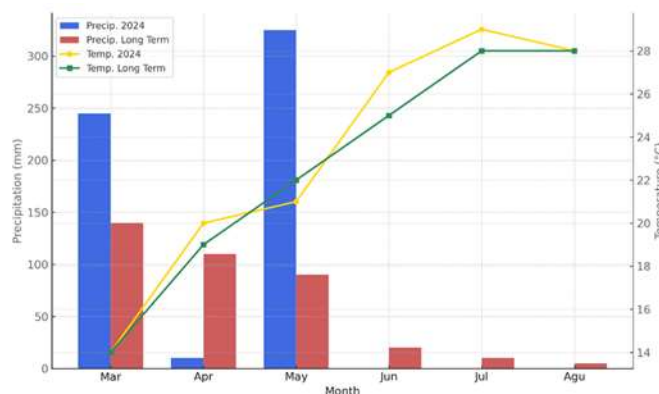


Figure 1. Climatic data obtained from the meteorological station closest to the experimental site

The study aimed to evaluate the performance of nine grain maize (*Zea mays* L.) varieties — CA401, CA402, CA403, P1410, P1541, P1551, P1884, P2085, and P2105 — under the same ecological conditions. The experiment was established on March 25, 2024, and harvesting was conducted on August 10, 2024. A randomized complete block design (RCBD) was employed with three replications. Each plot consisted of four rows, 5 m in length and 2.8 m in width, with

70 cm spacing between rows and 17 cm between plants within rows. Each maize variety represented one treatment within each replication.

Prior to sowing, the field was prepared through conventional tillage, including plowing, harrowing, and leveling. A compound fertilizer (15-15-15) was incorporated into the soil at a rate equivalent to 8 kg N, 8 kg P, and 8 kg K per decare. When plants reached approximately 40–50 cm in height, 12 kg N da⁻¹ was applied as topdressing using urea fertilizer (46% N). Weed control was performed manually, and irrigation was supplied every 20 days to maintain adequate soil moisture. No serious pest or disease incidence was observed during the growing period.

At harvest, ten representative plants were selected from each plot to measure yield-related characteristics. The evaluated traits were:

Cob diameter (CD, mm): Measured at the midsection of the cob using a digital caliper.

Cob length (CH, cm): Measured from the base to the tip of the cob with a ruler.

Cob weight (CW, g): Recorded after husk removal using a precision balance.

Number of seeds per cob (NSC): Determined by multiplying the number of kernel rows by the number of kernels per row.

Seed weight per cob (SWC, g): Determined after threshing by weighing the total kernels per cob.

Thousand-seed weight (TSW, g): Calculated by counting and weighing four subsamples of 100 kernels, then converting to 1000-seed weight.

All collected data were subjected to analysis of variance (ANOVA) according to the RCBD model using JMP Pro 13.0 (SAS Institute Inc.). When significant differences among varieties were detected ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) test was applied for mean separation. Results were expressed as means $\pm$ standard error. Mean values within the same column followed by different superscript letters were considered statistically different.

RESULTS AND DISCUSSION

Significant differences were observed (Table 1) among maize varieties for cob diameter ($p < 0.01$). The widest cob was recorded in P2105 (47.86 mm), followed by P1884 (46.06 mm), while P1551 (42.94 mm) had the smallest diameter. The larger cob diameter in P2105 and P1884 suggests a higher sink capacity and potentially greater kernel set per cob. According to Yan et al. (2021), cob girth is directly associated with yield potential, as thicker cobs enhance kernel-bearing surface area and kernel development. Similarly, Long et al. (2024) reported that hybrids with wider cobs tend to accumulate more assimilates during the grain-filling stage, contributing to increased kernel mass. The smaller diameter observed in P1551 may indicate weaker sink strength and reduced assimilate allocation to the reproductive organs under the same growing conditions.

Varietal differences (Table 1) in cob length were also significant ($p < 0.01$). The longest cobs were produced by P2105 (23.32 cm), while P1551 (18.21 cm) had the shortest. Varieties P1884 (21.33 cm) and CA402 (21.27 cm) also exhibited relatively long cobs. Longer cobs generally reflect greater potential for kernel number and grain yield, as highlighted by Liu et al. (2022), who emphasized the role of cob length in determining the ear's total sink size. The superior cob length of P2105 likely results from a balanced vegetative and reproductive growth pattern, ensuring sufficient assimilate supply during ear formation. Conversely, shorter cobs in P1551 may indicate genetic limitations or less efficient partitioning of assimilates toward the ear during reproductive development.

Cob weight varied significantly (Table 1) among varieties ($p = 0.02$). The heaviest cobs were obtained from P2105 (238.93 g) and P1884 (230.93 g), while P1551 (165.73 g) had the

lowest value. Cob weight integrates several yield components, such as kernel number, kernel size, and cob morphology. High cob weight indicates enhanced photosynthetic efficiency and assimilate translocation, both of which are essential for maximizing grain yield (Konuskan et al., 2024). The superior cob weight of P2105 and P1884 supports their adaptability to the Amik Plain conditions and reflects their high genetic yield potential. The comparatively lower cob weight of P1551 suggests a weaker physiological capacity to convert vegetative biomass into grain yield.

Differences among varieties for the number of seeds per cob (Table 1) were not statistically significant ($p = 0.06$). Nonetheless, P1410 (794.00 seeds) and P2105 (784.47 seeds) had numerically higher values, whereas P2085 (620.80 seeds) and P1551 (620.27 seeds) produced the lowest. The variation observed, though not statistically confirmed, indicates genetic differences in kernel set efficiency. Long et al. (2024) noted that kernel number is more sensitive to environmental conditions, particularly during pollination, and often exhibits higher variability than kernel weight. The relatively consistent NSC values among most varieties suggest that environmental conditions during flowering were favorable, allowing differences in final yield to be driven mainly by kernel weight rather than kernel number.

Seed weight per cob showed highly significant differences (Table 1) among varieties ($p < 0.01$). The highest SWC was recorded in P2105 (219.20 g), followed by P1884 (193.27 g) and CA403 (193.00 g), while P1551 (137.27 g) had the lowest. This parameter represents both the number and mass of kernels per cob and is a crucial determinant of final yield. Liu et al. (2022) reported that high SWC values are associated with superior source–sink coordination, which ensures efficient assimilate translocation to developing grains. The high SWC observed in P2105 and P1884 highlights their strong sink strength and effective carbohydrate partitioning. In contrast, P1551's low SWC confirms its inferior grain-filling efficiency, consistent with its smaller cob morphology.

Table 1. Yield-related traits and comparison test results of different maize varieties

Cultivars	Properties					
	CD (mm)	CH (cm)	CW (g)	NSC (piece)	SWC (g)	TSW (g)
CA401	45.49±1.00 ^{abc}	19.66±0.28 ^{bc}	208.67±12.03 ^{ab}	702.80±37.02	180.67±13.48 ^{abc}	201.52±1.72 ^{bc}
CA402	43.95±1.03 ^{bc}	21.27±1.14 ^{abc}	198.67±10.18 ^{ab}	670.67±56.43	179.47±11.05 ^{abc}	237.40±7.96 ^{ab}
CA403	44.74±0.49 ^{bc}	19.39±0.81 ^{bc}	214.27±20.34 ^{ab}	664.53±62.50	193.00±12.07 ^{ab}	248.50±12.72 ^a
P1410	45.52±0.46 ^{abc}	20.97±0.3 ^{3abc}	222.67±14.34 ^{ab}	794.00±15.87	192.47±12.78 ^{ab}	209.67±4.77 ^{abc}
P1541	45.15±0.09 ^{abc}	19.98±0.03 ^{bc}	201.07±4.67 ^{ab}	670.13±24.93	169.07±7.82 ^{bc}	203.11±11.13 ^{bc}
P1551	42.94±0.79 ^c	18.21±0.02 ^c	165.73±8.38 ^b	620.27±22.14	137.27±6.40 ^c	190.48±16.83 ^c
P1884	46.06±0.08 ^{ab}	21.33±0.56 ^{ab}	230.93±4.26 ^a	755.60±16.20	193.27±4.34 ^{ab}	196.05±6.20 ^{bc}
P2085	44.30±0.43 ^{bc}	18.93±0.68 ^{bc}	186.80±7.01 ^{ab}	620.80±24.02	158.80±11.55 ^{bc}	201.33±1.33 ^{bc}
P2105	47.86±0.21 ^a	23.32±0.67 ^a	238.93±27.67 ^a	784.47±55.09	219.20±9.65 ^a	230.73±5.00 ^{abc}
P values	<0.01	<0.01	0.02	0.06	<0.01	<0.01

CD: Cob diameter, CH: Cob height, CW: Cob weight, NSC: Number of seed per cob, SWC: Seed weight per cob, TSW: Thousand seed weight

Values followed by different superscript letters within the same column are significantly different.

Thousand-seed weight varied significantly (Table 1) among varieties ($p < 0.01$), ranging from 190.48 g (P1551) to 248.50 g (CA403). High TSW values were also recorded in P2105 (230.73 g) and CA402 (237.40 g). The heavier kernel mass in these varieties suggests efficient grain filling and larger endosperm development. Yan et al. (2021) emphasized that TSW is a reliable indicator of assimilate accumulation during the grain-filling phase and a major contributor to yield stability. The superior TSW of CA403 and P2105 may thus be attributed to favorable genetic traits supporting prolonged grain filling and effective resource utilization. The lowest TSW in P1551 further reinforces its overall weak performance in kernel development.

CONCLUSIONS

The study clearly demonstrated substantial genotypic variation among the evaluated maize varieties in yield-related traits under the ecological conditions of the Amik Plain. Among the nine tested cultivars, P2105 consistently exhibited superior performance for cob diameter, cob length, cob and seed weights, and thousand-seed weight, while P1884 and CA403 also showed promising results. In contrast, P1551 recorded the lowest values across most traits, indicating limited adaptation to local conditions. Overall, the findings highlight that yield potential in maize is strongly determined by kernel weight and cob morphology rather than kernel number. Consequently, P2105 and P1884 can be recommended for grain maize production in the Amik Plain to achieve higher productivity and stability under similar agro-ecological environments.

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HEAT TRANSFER IN SOIL SYSTEM

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ABSTRACT

Heat transfer in soil system, is one of the most important soil physical processes, influences most of agronomic practices. Heat transfer equation based on the boundary conditions of $T(0,t)=T_0+A.\sin(wt)$ representing temperature change on soil surface (where; T_0 - average temperature of soil surface, °C; A - amplitude, $w=2\pi/P$ - angular frequency; P - period) was examined in the study. Heat diffusivity coefficient, damping depth, retardation time in 10, 20 and 30 cm layers of soil were calculated according to the analytical and the mathematical expressions. The range of heat diffusivity coefficient was determined as 0.79×10^{-4} and $6.67 \times 10^{-4} \text{ cm sec}^{-1}$. The retardation time of maximum or minimum temperature on soil surface in 10; 20 and 30 cm layers was determined in a range of 2.31-10.67 day, and damping depth in these layers ranged between 8.05 and 62.06 cm. As a conclusion, for further development of the theoretical solution, heat transport parameters must be determined experimentally through field studies and compared with theoretical values.

Key words: Soil, heat, temperature, diffusivity, retardation, damping depth.

INTRODUCTION

One of the factors that significantly impacts soil processes and plant development is the transport of heat, a form of energy, in the soil. Soil temperature is a quantitative expression of heat transport between soil components. Heat transport significantly influences the water and air regime in the soil and changes in soil formation processes. Achieving optimal plant growth conditions is also significantly related to soil temperature, and therefore heat transport, along with other ecological factors. Therefore, considering the limited anthropogenic influences on soil temperature, the importance of examining heat transport in the soil becomes clear.

As a result of temperature differences in soil layers, energy is transported from high-temperature regions to low-temperature regions. This, in turn, creates soil temperature according to the thermodynamics of energy. The thermodynamic laws of energy transport pertain to a balanced system and, since they express the amount of energy required for a change in equilibrium, do not indicate the timing of the change in equilibrium. Heat convection theory, unlike the first and second laws of thermodynamics, allows the determination of temperature

values in any porous medium (soil) as a function of time and distance, and therefore the duration of heat transfer (Kreith, 1983).

The determination of convection coefficients, which represent heat exchange in soil, and the examination of the thermal properties of porous media are carried out by solving the heat convection equation numerically and theoretically, subject to various boundary and initial conditions. Comparing numerical and theoretical solutions is crucial for the accuracy of heat transfer prediction (Cichota et al., 2004; Zhao et al., 2008; Ngo and Lai, 2009).

The heat, water, and nutrients stored in soil possess dynamic properties as a result of their mutual interaction with the physical, chemical, and biological processes of the environment. Heat exchange in soil, which possesses dynamic properties, significantly affects the water regime, along with other factors. For this reason, some researchers have combined water and heat transport models under various soil conditions (Liu et al., 2005; dos Santos and Mendes, 2006; Haverd et al., 2007; Wu et al., 2007; Ji et al., 2009).

The aim of this study is to investigate the solution of the heat transport equation based on the sinusoidal boundary condition and to determine the thermal diffusivity coefficient, damping depth, and retardation time values based on the mathematical solution in the surface soil layer.

MATERIAL AND METHODS

Soil temperature was determined at the experimental field of Agricultural Faculty, Ondokuz Mayıs University daily with a glass thermometer (Sterling and Jackson, 1986) every 10 cm to a depth of 30 cm at 9:00, 11:00, 13:00, 15:00, 17:00, and 19:00. Clay, silt, and sand contents of the soil was determined by the hydrometer method (Demiralay, 1993). Soil reaction (pH) and Electrical Conductivity (EC) were determined by a glass electrode pH meter and EC meter in a 1:1 soil:water mixture (Peech, 1965), respectively. Organic matter (OM) content was determined by the modified Walkley-Black wet digestion method (Kacar, 1994).

Heat transport is related to the continuity equation, which expresses mass transport in any media. The continuity equation expresses the law of conservation of mass in a given volume.

The ratio of the mass of particles passing through a small area in any media to the unit length

of this area expresses the linear density of the media, $\rho = \rho(x, t) = \lim_{\Delta x \rightarrow 0} \frac{\Delta m}{\Delta x} = \frac{dm}{dx}$

In this case, at any time t , the mass of the particles in the $(x, x + dx)$ interval of the media is $dm = \rho dx$. The mass current, which represents the local parameter of the media, is directly

proportional to the velocity of the particles $q = q(x, t) = \rho v = \frac{dm}{dx} v$

The continuity equation for the mass change in the environment with respect to time is expressed as in equation 1 or 2.

$$\frac{\partial q}{\partial x} + \frac{\partial}{\partial t} \left(\frac{\rho dx}{dx} \right) = 0 \Rightarrow \frac{\partial q}{\partial x} + \frac{\partial \rho}{\partial t} = 0 \quad [1]$$

$$\frac{\partial(\rho v)}{\partial x} + \frac{\partial \rho}{\partial t} = 0 \quad [2]$$

If $T = T(x, t)$ is the temperature of the soil layer with a homogeneous structure at depth x at time t , the density of heat energy is expressed as follows:

$$\rho_Q = c \rho_0 T \quad [3]$$

Where, $\rho_0 = [m][l^{-3}]$ -soil bulk density, $c = [E][m^{-1}][T^{-1}]$ - T heat capacity accepted as constant.

According to Fourier's law (Isachenko et al., 1981), the heat flux density is

$$q_Q = -\lambda \frac{\partial T}{\partial x} \quad [4]$$

Where, $\lambda = [E][l^{-1}][t^{-1}][T^{-1}]$ -heat conductivity coefficient accepted as a constant value.

If the expression 1 is substituted into equations 3 and 4, the continuity equation of heat convection is obtained

$$\frac{\partial}{\partial x} \left(-\lambda \frac{\partial T}{\partial x} \right) + \frac{\partial}{\partial t} (c \rho_0 T) = 0, \quad c \rho_0 \frac{\partial T}{\partial t} = \lambda \frac{\partial^2 T}{\partial x^2} \quad \text{or} \quad \frac{\partial T}{\partial t} = \frac{\lambda}{c \rho_0} \frac{\partial^2 T}{\partial x^2}$$

Where, $a = \frac{\lambda}{c \rho_0} = [l^2][t^{-1}]$ -heat diffusivity constant.

The heat transport equation in soil is obtained as follows:

$$\frac{\partial T}{\partial t} = a \frac{\partial^2 T}{\partial x^2} \quad [5]$$

If the expression $T(0, t) = T_0 + A \sin \omega t$ (where burada, $T_0 = [T]$ - mean temperature of soil surface; $A = [T]$ - amplitude expressing the difference between the maximum or minimum temperature at the soil surface and the average surface temperature; $\omega = 2\pi / P = [t^{-1}]$ - angular frequency (number or frequency of waves) and $P = [t]$ - period, that is, the time required for the wave to complete one cycle), which represents the harmonic spread of temperature on the soil surface, constitutes the boundary condition of the heat transfer equation (5).

The mathematical model obtained from the solution of equation [5] satisfying the boundary condition ($T(0, t) = T_0 + A \sin \omega t$) is expressed as follows (Hillel, 1982; Nerpin ve Chudnovski, 1984; Gülser ve

Ekberli, 2002; 2004; Cichota ve ark., 2004; Ekberli, 2006 a; Goldstein ve ark., 2010; Ekberli and Gülser, 2020).

$$T(x,t)=T_o + Ae^{-x\sqrt{\frac{\omega}{2a}}} \sin\left(-x\sqrt{\frac{\omega}{2a}} + \omega t\right) \text{ or } T(x,t)=T_o + \frac{A}{e^{x\sqrt{\frac{\omega}{2a}}}} \sin\left(-x\sqrt{\frac{\omega}{2a}} + \omega t\right) \quad [6]$$

RESULTS AND DISCUSSION

Some physical and chemical properties of the soil are given in Table 1. The 0-20 cm layer of the soil is clayey (C), the 20-40 cm layer is sandy loam (SL), and there is no salinity problem. The soil reaction is close to neutral. Organic matter content varies from good (at 0-20 cm) to very low (at 20-30 cm). The amount of CaCO₃ in the soil is low.

Table 1. Some soil properties of the experimental field

Soil depth, cm	Clay, %	Silt, %	Sand, %	pH (1.1)	Organic matter, %	EC _{25°C} , dS m ⁻¹
0-10	52.58	21.78	25.64	7.3	3.55	0.35
10-20	53.75	19.10	27.15	6.9	2.39	1.07
20-30	41.60	14.92	43.48	7.3	0.77	0.40

Average and maximum temperature values determined based on temperatures measured along the soil profile are given in Table 2. Temperature values and variations are greater in the 0-20 cm soil layer than in the lower layers (20-30 cm). Moisture values in the lower layers of the study soil, which has a clayey and sandy-loam texture, are often higher than field capacity (Gülser et al., 2003). This is one of the factors contributing to the lower temperature variation. Furthermore, the practically inhomogeneous nature of the soil layers, the low amount of heat reaching the lower layers, and irregular porosity contribute to the lower temperature values in the lower layers.

Table 2. Average (T) and maximum (T_m) temperature (°C) values along the soil profile

Depth, cm	Time, hour											
	9:00		11:00		13:00		15:00		17:00		19:00	
	T	T _m	T	T _m	T	T _m	T	T _m	T	T _m	T	T _m
0	24,5	33,2	30,3	44,5	33,4	47,4	32,6	45,8	26,3	34,5	20,9	24,9
10	18,8	23,7	19,9	24,0	20,3	25,2	20,8	25,5	20,9	24,2	20,5	23,7
20	18,3	22,8	18,7	22,5	18,7	23,1	19,4	23,0	19,5	23,0	19,5	23,2
30	18,0	22,2	18,5	21,8	18,5	20,0	18,6	22,0	18,2	21,9	18,0	21,7
Std. deviation (σ)	3.08		5,66		7,16		6,56		3,55		1,29	
Variance (V), %	9,51		32,11		51,29		43,07		12,86		1,66	
Std. Error (P), %	1,54		2.83		3.58		3.28		1,77		0,65	

$A = T_m - T$ and according to the expression $a = \frac{\omega x^2}{2 \ln^2 \frac{A(x)}{A}}$ of the thermal diffusion coefficient

obtained according to [6], the amplitude and thermal diffusion coefficient were calculated along the soil profile and the values are given in Table 3.

 Table 3. Variation of amplitude (A, °C) and thermal diffusion coefficient (a, cm²sn⁻¹) along the soil profile.

Depth, cm	Time, hour											
	9-00		11-00		13-00		15-00		17-00		19-00	
	A	a	A	a	A	a	A	a	A	a	A	a
0	8.7	0.00	14.2	0.00	14.0	0.00	13.2	0.00	8.2	0.00	4.0	0.00
10	4.9	3.68x10 ⁻⁴	4.1	0.79x10 ⁻⁴	4.9	1.10x10 ⁻⁴	4.7	1.14x10 ⁻⁴	3.3	1.46x10 ⁻⁴	3.2	3.07x10 ⁻⁴
20	4.5	1.12x10 ⁻³	3.8	2.79x10 ⁻⁴	4.4	3.62x10 ⁻⁴	3.6	2.87x10 ⁻⁴	3.5	6.67x10 ⁻⁴	3.7	2.07x10 ⁻³
30	4.2	2.06x10 ⁻³	3.3	5.12x10 ⁻⁴	3.5	5.67x10 ⁻⁴	3.4	5.93x10 ⁻⁴	3.7	1.72x10 ⁻³	3.7	4.67x10 ⁻³

Amplitude values range from 4.0 to 14.2 °C at the soil surface and from 3.2 to 4.9 °C at depths of ≥10 cm. Lower average temperatures and higher soil moisture levels in the lower layers are significant factors contributing to the decrease in amplitude. It can be seen from [10], the amplitude of the soil at any depth x is less than the amplitude at the soil surface by a factor of

$e^{\frac{x}{\sqrt{2a\omega}}}$. The thermal diffusivity coefficient of the experimental soil at a depth of 10 cm is 0.79x10⁻⁴- 3.68 x10⁻⁴ cm²sn⁻¹, and increases at a depth of 30 cm, ranging from 1.72x10⁻⁴- 5.93x10⁻⁴ cm²sn⁻¹. High soil surface temperatures prevent the formation of strong temperature gradients, thereby reducing the thermal diffusivity coefficient. In the lower layers (≥20 cm), the formation of strong temperature gradients and high soil moisture allow the thermal diffusion coefficient to increase (Schachtschabel et al., 2001; Ekberli, 2006 b).

According to expressions $S_d = \sqrt{2a/\omega}$ and $t = x \sqrt{\frac{1}{2a\omega}}$, the damping depth and the retardation time of the maximum temperature along the soil profile were determined, respectively, and the obtained values are given in Table 4.

Table 4. Damping depth (S_d , cm) and retardation time of maximum temperature (t, day) values along the soil profile

Depth cm	Time, hour											
	9-00		11-00		13-00		15-00		17-00		19-00	
	S_d	t	S_d	T	S_d	t	S_d	t	S_d	t	S_d	t
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	17.42	2.74	8.05	5.93	9.52	5.02	9.68	4.93	10.99	4.35	15.91	3.00
20	30.34	3.15	15.17	6.30	17.28	5.53	15.39	6.21	23.49	4.07	41.38	2.31
30	41.20	3.48	20.56	6.97	21.64	10.67	22.12	6.48	37.70	3.80	62.06	2.31

The damping depth generally increases with the amplitude and depth of the heat waves, while the retardation time generally increases with the amplitude and time in the lower layers. As the porosity of the soil decreases in the lower soil layers, thermal conductivity and thermal diffusivity increase over a wide range. Therefore, the damping depth also increases with increasing soil depth. Because maximum temperature values are delayed with increasing soil depth due to the decrease in temperature amplitude in the lower soil layers while retardation times generally increase with smaller intervals. The different physical and chemical properties of soil layers and changes in climatic conditions are important factors that cause the changes in heat transfer parameters to be disproportionate (Ekberli et al. 2005; Ekberli and Gülser 2014).

CONCLUSION

Managing the temperature regime in soils is significantly dependent on heat transport parameters (Gülser and Ekberli, 2019). Therefore, heat transport parameters such as thermal diffusivity, retardation time, and damping depth must be determined under varying moisture and soil properties (Ekberli and Gülser, 2022). Regular cultivation of the upper layers of the soil reduces the thermal diffusivity, thus preventing heat loss. To reduce heat loss, the damping depth must be increased.

Due to the significant daily variation in heat reaching the soil surface, the boundary condition may not always be strictly harmonic (sinisoidal or cosnisodal) (Ekberli and Gülser 2016). Therefore, this and similar studies are focused on the heat transfer in dry or nearly dry soil, parent material or rock, and enclosed soil space, and their application to natural conditions is limited. Furthermore, further development of the theoretical solution requires experimental determination of heat transport parameters through field studies and comparison with theoretical values.

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TROPICAL FRUIT SEEDS OILS AS FUNCTIONAL BIONUTRACEUTICAL AND BIOPHARMACEUTICAL AGENTS: IMPROVING OIL QUALITY WITH NOVEL FOOD PROCESSING PEF

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ABSTRACT

It has been reported that over 70 different species of tropical fruits from foreign countries. Around 20 species are commonly eaten, including banana, pineapple, tropical citrus, mango, papaya, durian, jackfruit, mangosteen, palm, longan, guava, tamarind, sour soup, passion fruit, chempadak, langsat, honeydew, and rambutan. These species are best known for their large-scale cultivation in the commercial sector. The seeds of tropical fruits are a byproduct of the processing or consumption of the fruit. Plant oils derived from fruit seeds, also known as fruit seed oils, are extracted from the most popular tropical fruits seeds, including their germs and endosperms. Their macromolecules, including oil, protein, and carbohydrates, are found to differ due to either genetic differences or geographic variations. These are primarily composed of triglycerides (98%), and are composed of one molecule of glycerol and three different types of fatty acids (FAs), all of which are unsaturated (UFA). Fatty acids are vital for healthy organism functions, for serving as energy storage, and regulating the cell growth, differentiation and gene expression that are classified based on the chain length and saturation level into saturated, mono unsaturated and polyunsaturated fatty acid as SFA, MUFA and PUFA, respectively; especially PUFA are considered as essential FAs obtained through nutrition. It is important to recognize that UFA have a significant role in maintaining the fluidity of the membrane, decreasing blood cholesterol, and lowering the risk of CVD, particularly in regards to PUFA. It is said that the range of oil is from 1.8 to 49%, while the range of protein is from 6 to 40%. It's documented that tropical fruit seeds that are high in oil have the potential to be innovative sources of oil, while new high protein sources that utilize the recovery of protein have the potential to be innovative sources of protein. Additionally, some edible tropical seeds have chemical compounds that can be used with medical protocols, these chemical compounds include polyphenols and other compounds with a nutritional value that can be used in healthcare. Pulsed electric field (PEF) is a novel technique of extracting oil that employs a novel technology to break the cell walls of plant oils and seed oils. The treatment of PEF increases

the oil yield, quality, and anti-inflammatory properties of seed oils by increasing the porosity and the destruction of cell membranes, this increases the extractability and quality of seed oils. This presentation demonstrates that the tropical fruit seeds have the potential to be utilized in multiple areas of bio-health, including pharmaceuticals and food production. Besides, they have the potential to be utilized as by-products or waste in life science and food science, agriculture producing sustainable safety food that is then added value.

Keywords: Tropical fruit, fruit seed, seed oil, oil quality, oil yield, novel food processing, PEF, fatty acids, MUFA, PUFA, UFA.

INTRODUCTION

Pulsed electric field (PEF) is a non-thermal food processing technology that has gained increasing attention in recent years. An external electric field can induce a critical potential in cell membranes (Angersbach et al., 2002). This leads to electrical breakdown and local structural changes in the cell membrane, thereby increasing its permeability. Depending on the treatment time, pulse shape and energy, electric field strength, and temperature, this permeability change can be reversible or irreversible. Several studies have reported on the use of PEF to treat cell membranes in plant tissues (Lebovka et al., 2004; Jemai and Vorobiev, 2006). These studies explored the extraction efficiency of juices pretreated with PEF before mechanical pressing or extraction. Current research focuses on using pulsed electric fields to improve the extraction performance of juices and inactivate microorganisms within them. Studies have shown that pulsed electric fields can be used to inactivate microorganisms in fruit juices, thereby obtaining superior sensory properties (Min, Jin, and Zhang, 2003).

Polyunsaturated fatty acids (PUFAs) are considered essential fatty acids and must be obtained through diet. Unsaturated fatty acids (UFAs) play an important role in maintaining cell membrane fluidity, lowering blood cholesterol levels, and reducing the risk of cardiovascular disease, especially polyunsaturated fatty acids. Tropical seed oils and extra virgin seed oils have relatively low production efficiency, resulting in an oil loss of about 20%. There are two main reasons for such high losses: (i) oil remains in the fruit seed cells; (ii) oil emulsifies with water. These losses are related to the extraction method and conditions as well as the oily fruit variety. Traditionally, heat treatment or the addition of processing aids (talc, calcium carbonate, enzymes) has been used to increase the oil yield of fruits. It is known that increasing the temperature can reduce the viscosity of the oil and increase its diffusion capacity, thereby increasing the oil yield (Tokuşoğlu, Gök & Irmak, 2025).

Pulsed electric field (PEF) refers to the application of high voltage (1–30 kV) and short pulses (ns– μ s) to food. Based on the electroporation mechanism, PEF improves the transport of substances and heat by creating pores of varying shapes and diameters in cell membranes through electrocompression. Therefore, combining traditional kneading with PEF can often

improve extraction efficiency and the release of bioactive compounds. Furthermore, its application can improve the nutritional and sensory qualities of extra virgin seed oils of tropical fruits (Tokuşoğlu, Gök & Irmak, 2025; Parniako, Hopper and Toepfl, 2023).

In making virgin coconut oil (VCO), several methods are known, including centrifugation, fermentation, enzymatic salting, heating, fishing and stirring. The method is to break down protein-oil-water emulsions. The breakdown of the emulsion is closely related to the reduction of the emulsion interface layer voltage. The interface layer must be destroyed so the water droplets will become one. The more emulsions that can be broken down, the more yield of VCO will be completed. Demulsification of water and oil can occur due to conductivity and permittivity that changes polarity. Systems applied to electric shock technology (PEF) have a more effective advantage compared to preliminary treatments such as a reduction in size. The voltage treatment in a very short period of time (several hundred microseconds) results in induction. Induction affects cell membrane permeability. In this proceeding paper, it has been coconut oil as tropical fruit seed oil and optimization of virgin coconut oil production process through pulsed electric field pretreatment regarding voltage and frequency study.

MATERIAL AND METHOD

The production of virgin coconut oil requires an analytical balance, a press, a centrifuge, and a pulsed electric field (PEF) generator. Analysis requires an analytical balance, a titrator, 1 mL centrifuge tubes, pipettes, glassware, and an oven. Chemical reagents include sodium hydroxide (NaOH), 0.1 N potassium hydroxide (KOH), indicator solutions, and technical-grade analytical materials such as distilled water, fine filter and coarse filter papers.

Fresh coconuts were ground and then mixed with water in a 1:2 ratio. The mixture was pressed to obtain coconut milk. After standing for one hour, coconut cream and skimmed coconut milk were separated. The coconut cream was transferred to an Erlenmeyer flask and frozen overnight. The coconut cream was thawed to a temperature of 12-14°C. Then a pulsed electric field -PEF was applied. The mixture was centrifuged at 3750 rpm for 15 min. Three layers were formed. Virgin coconut oil (VCO) was obtained from the top layer. The yield, moisture content, free fatty acid content, and density of the virgin coconut oil were calculated.

RESULTS AND DISCUSSION

The best preliminary response was received as 18.563% by optimal measuring the voltage of 3.332 kV/cm and a frequency of 2.654 kHz. The results showed that pretreatment only affected the yield. Density, moisture content, and free fatty acid content showed no significant differences. Under our conditions, the yield was 18.563%, the density was 0.925%, the moisture content was 0.245%, and the free fatty acid content was 0.350%. Free fatty acid content and density met the APCC criteria. According to the literature, the model's biases for yield, density,

moisture content, and free fatty acid content (Dewi et.al.2019; Pataro et.al.,2011). Our prediction results were very satisfactory, with all biases less than 5%.

Pulsed electric field (PEF) can function in two ways: (1) by disrupting the cell wall and promoting the release of cell contents; (2) in coconut oil extraction, it can disrupt oil-water emulsions, in this case, oil-water-protein emulsions. It can be declared that PEF is a highly efficient oil extraction technique and the PEF process can disrupt cell membranes.

The gained results has been accorded with Rodríguez-Roque et.al.(2015). The electroosmotic properties of plant tissues can be used to disrupt cell membranes. Voltage and frequency can be affected cell membranes. Membrane disruption makes it easier for solvents to bind to substances within the cells (Rodríguez-Roque et.al.,2015).

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THE ASSESMENT STRATEGIES ON ULTRASOUND-ASSISTED EXTRACTION (UAE) OF BIOCEMICALS AND BIOACTIVES FROM FRUIT PROCESSING BY-PRODUCTS

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ABSTRACT

Recently, ultrasound technology has proven to be a beneficial tool in food science and research, and this field of research has become extremely valuable. Two different approaches to ultrasound applications have been explored, primarily related to ultrasonic vibration power and frequency. One approach utilizes high-frequency, low-intensity ultrasound, with its primary focus on monitoring the quality of food products/crops and/or processes, such as assessing food composition analysis and/or product ripening time. These applications primarily involve measuring ultrasonic velocity, signal attenuation, or frequency spectral analysis. These applications do not impact the process or product. The increasing popularity of fruit and vegetable processing has led to a large volume of byproducts, including seed, skin, pomace, and rind, which contains a large amount of bioactive compounds, such as polysaccharides, polyphenols, carotenoids, and dietary fiber. These waste processing materials are considered to have little value compared to the processed fruit or vegetable due to the lack of a sustainable extraction method. Conventional extraction has a number of drawbacks in terms of softime, energy, and solvent needed. Ultrasound-assisted extraction (UAE) is more efficient than other methods in extracting bioactive components in a short period of time, at a low temperature, with a lower energy consumption and a smaller amount of solvent necessary. Because of its non thermal nature, UAE is more suited to the retention of the functionality of the bioactive compounds. However, the variables associated with the UAE, such as frequency, power, duty cycle, temperature, time, solvent type, and liquid-solid ratio, need to be understood and optimized for each byproduct. This presentation explores the mechanism, concept, and factor that affects the extraction of bioactive compounds from particular focus on fruit and vegetable byproducts. Ultrasounds are successfully used to extract polyphenols, carotenoids, aromas, and polysaccharides from plant materials (both whole plants and byproducts). The variables associated with the UAE, including frequency, power, duty cycle, temperature, time, solvent type, and liquid to solid ratio, need to be precisely controlled in order to have the greatest effect on the extraction process. The individual effects as well as the interactive effects of these variables have been documented by multiple scientists that seek to harvest bioactive compounds from the byproducts of fruit and vegetable harvests. There are positive reviews regarding the UAE regarding food and natural products, the extraction of specific components, such as pectin, using innovative techniques, plant-based antioxidants.

Keywords: Ultrasound Assisted Extraction (UAE), Ultrasound, Fruit By-Products, Vegetable By-Products, Bioactive Phenolics

INTRODUCTION

Introduction to Ultrasound Extraction Technology

Ultrasonic technology is a key technology for sustainable “green” chemistry and extraction. It is renowned for significantly increasing the speed of various processes in the chemical and food industries. Ultrasonic technology can complete extractions in minutes with high reproducibility. This reduces solvent consumption, simplifies operations and processing, increases the purity of the final product, eliminates the need for wastewater treatment, and consumes only a fraction of the fossil fuels required by traditional extraction methods such as Soxhlet extraction, maceration, or Clevinger distillation. A wide variety of food components, including flavor compounds, pigments, antioxidants, and other organic and mineral compounds, can now be efficiently extracted, analyzed, and formulated from a variety of matrices, primarily including animal tissues, microalgae, yeast, food, and plant materials.

Studies on ultrasonic extraction (UAE) rarely explore the mechanisms by which ultrasound improves extraction rates. Some references describe the propagation effects of ultrasound in solid/liquid media (Mason and Lorimer 2002; Suslick and Price, 1999). Cavitation leads to high shear forces in the medium. The implosion of cavitation bubbles on the product surface generates microbeams, causing various effects such as surface detachment, erosion, and particle breakage. Furthermore, the implosion of cavitation bubbles in liquid media leads to macroscopic turbulence and microscopic mixing. Surprisingly, in most of the literature on ultrasonic extraction of natural products, authors attribute the yield improvement to the cavitation effect during ultrasonic irradiation without further investigation.

Extraction Mechanisms of Ultrasound Assisted Extraction

In liquid media, the implosion of cavitation bubbles leads to macroscopic turbulence and microscopic mixing. The high shear forces generated by cavitation in the medium are considered a major mechanism for the efficient extraction of bioactive compounds from food matrices and the inactivation of microorganisms and enzymes (Patil et al., 2018; Chen, 2017).

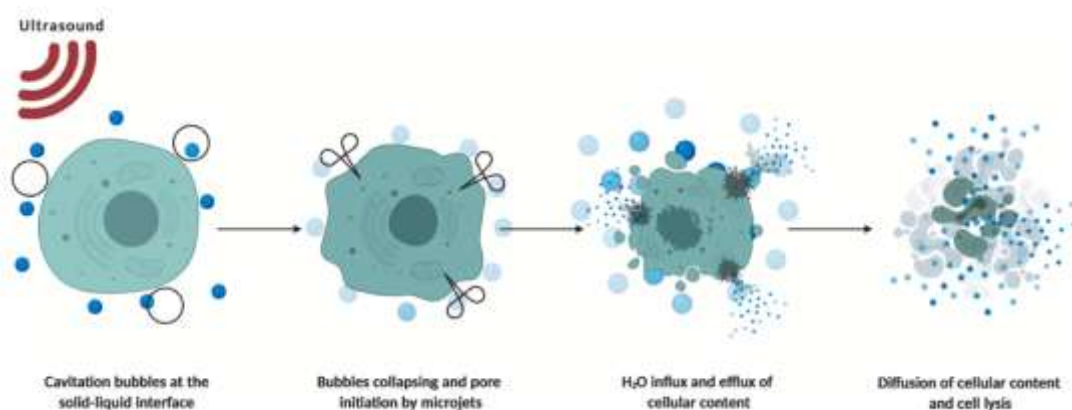


Figure 1. Schematic Diagram of Cell Lysis Caused by Cavitation Bubbles.

Figure 1 illustrates the cell lysis caused by cavitation bubbles as schematically whereas Figure 2 shows the mechanism of US cavitation. The same cavitation phenomenon leads to macromolecular fragmentation, surface detachment, erosion, acoustic perforation, and increased cell wall permeability, thereby improving the extraction rate of bioactive molecules, inactivating microorganisms, and denaturing enzymes.

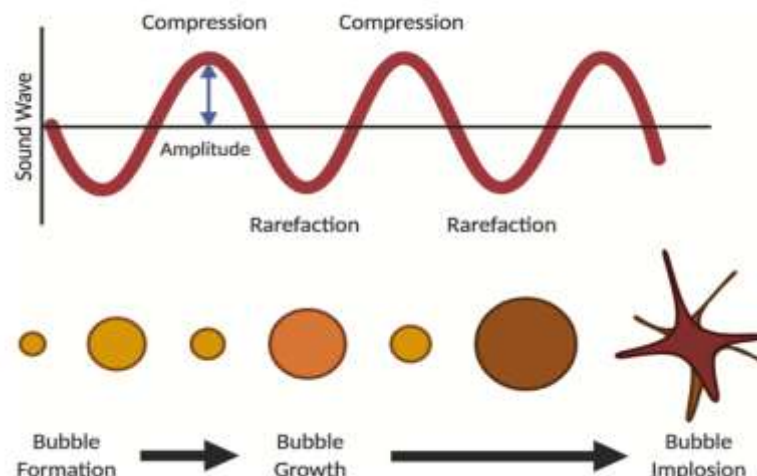


Figure 2. Mechanism of US Cavitation.

Applications of Ultrasound in the Extraction of Bioactive Compounds

Compared to traditional extraction methods such as Soxhlet extraction or steam distillation, ultrasound-assisted extraction (UAE) can be completed in minutes, offering high reproducibility, lower solvent and water consumption, and higher purity of the final product. This is due to its consumption of only a fraction of the required energy (Chemat et al., 2017). Various food components, such as flavor compounds, pigments, antioxidants, and other organic and mineral compounds, have been efficiently extracted and analyzed from animal and plant tissues and other foods using ultrasound-assisted extraction (Chemat et al., 2017; Evelyn and Silva, 2020). A key advantage of ultrasound-assisted extraction is its independence from the solvent used and the moisture content of the product. The application of high-power ultrasound enhances mass transfer (Kroehnke et al., 2021). Furthermore, ultrasound-assisted extraction is also a unique method for achieving thorough mixing in supercritical fluid extraction (SFE) because mechanical stirrers cannot be utilized. Table 1 shows the pretreatment criteria for the bioactive extraction by US (Table 1.).

Table 1. Pretreatment Criteria for the Bioactive Extraction by Ultrasound (Kumara et.al,2021)

Ultrasound as a pretreatment for the extraction of bioactive compounds.

US extraction conditions	Samples treated	Extract obtained	Key observations
60Hz-20kHz, 275W, Titanium probe immersed to 0.5 mm depth; Cold Plasma Treatment (CPT) under vacuum and 46V for 5-60 min	Sea asparagus (<i>Salicornia nee</i>)	Polyphenols	Extract showed high Antioxidant activity (AA), and low antimicrobial activity. CPT for 5 min increased AA by 22% and 19% as measured by DPPH and ABTS
US assisted Ohmic hydrodistillation with electrolyte. 35 kHz, 50W, 5-15 min	Peppermint	Essential oils	US pretreatment has negligible effect on the quality or the quantity of essential oil
Power 400 W, extraction time 10 min. 2.2 cm diameter probe with On and Off modes. Temp from 10 to 70 °C	Thyme & Rosemary	Phenolic compounds, flavonoids and carotenoids, Antioxidants	The phenolic, flavonoid and carotenoid content of extracts was greatly influenced by extraction conditions wherein the ultrasound pre-treatment improved the extraction of carotenoids but induced the opposite effect for polyphenols and flavonoids in both herbs.

Food Polyphenols by Ultrasound Assisted Extraction

The extraction of phenolic and antioxidant compounds is another important application area for researchers using ultrasonic extraction (UAE). Phenolic and antioxidant compounds have been extracted from mango peel, pomegranate peel, jaboticaba peel, grape pomace,

fermented grape pomace, grape by-products, grape seeds, hawthorn kernels, grapefruit solids, black chokeberry by-products coconut shell powder, and coffee grounds.

Researchers have investigated the effects of factors such as temperature, solvent concentration, frequency, and extraction time on the ultrasonic extraction of polyphenols. It was investigated the effects of various ultrasonic parameters on the extraction of flavonoids (FCs) from hawthorn seeds. The results showed that ultrasonic time, ethanol concentration and extraction temperature were the most important variables affecting the extraction rate of FC, while ultrasonic temperature, solid-liquid ratio and extraction time had no significant effect on the extraction rate of FC. Table 2 shows optimized extraction condition for ultrasound assisted extraction of phenolic compounds from fruit and vegetable by-products (Table 2.).

Table 2. The Optimized Extraction Conditions of Phenolics by US (Kumara et.al,2021; Gouda and Tokuşoğlu,2025)

Bioactive	Waste part	Operating condition Constants and solvent Used	Extraction Variables	Optimum Condition for UAE/Major Findings
Phenolics/Flavonoids/ Antioxidants	Mango peel	T:30; t:20; LSR:25:1; P:20; S: ethanol	P:165.87 W.cm ⁻² ; Ethanol % : 25-70	The ultrasound did not affect the extraction of the total phenolics
	Spent coffee ground	F:20;S:ethanol	P:100-300 W; T:30-50; t: 65-45; LSR:5:1-30:1	P:244 W; T:40; t: 40; LSR:17:1
	Maltin Peel	F:20; S:ethanol	P:60-100%; t:15-35; LSR:20:1-40:1	P:70.89%; t:35; LSR:40:1
	Grape pomace	T:17 ± 3; LSR:5:1;S:Water	P:50-150; t:5-25; F:40-120	P:150; t:25; F:40
	Grape seed	F:40;P:250 W;S: ethanol	T:40-60; t: 20-34; EC:40-60%	T:56.03; t: 29; EC:53.15 for max. TPC T:90.65; t: 30.58; EC:53.06 for max. antioxidant activity T:55.13; t: 28.49; EC:52.35 for max. anthocyanins
	Grapefruit solid waste	F:40;P:100 W; S: ethanol	T:34-61; t:15-48; EC:20-80 (g/100 g)	T:25; t: 55; EC:40 g/100 g
	Hawthorn seed	F:45;P:40 W; S: ethanol	T:83-98; t: 30-50; EC:55-85%; LSR: 14:1-22:1	T:91; t: 37; EC:72%; LSR:18:1
	Black Chokeberry by-products from filter-tea factory	F:40;S: ethanol	P:72-216 W; T:30-70; t: 30-90	T:70; t:80; P:206 for total phenolics T:70; t:73; P: 210 for flavonoids T:70; t:43.6; P:216 for anthocyanins T:70; t:89.7; P:199 for proanthocyanidins
	Black chokeberry waste	F:30.8;LSR: 40:1; S:ethanol	T:20-70; t: 0-240; P:0-100 W; EC:0-50	High temperature and ethanol content in the solvent improved greatly the extraction yields. t:60; EC:50%; LSR: 20:1 t:60
	Olive leaf extract	F:50; T:25; S: ethanol	t: 20-60; EC:0-100%; LSR: 20:1-40:1	Toasting temperature:100-200 °C Toasting time:20-60 min
Oil	Coconut (Cocos nucifera) shell powder	F:25;P:150 W	T:30-60; t:20-60; pH:4.5-6.5; LSR:20:1-50:1	T:30; t:15; pH:6.5; LSR:50:1
	coconut (Cocos nucifera) shell powder	F:25;P:150 W;T:30;LSR:20:1; S: ethanol	t:20-60; pH:2-5; EC:12-38	t:10; pH:1; EC:46
	Jabuticaba peels	Continuous UAE	P:2.4-59.2Wcm ⁻² ; t:2-90	Yield increased by 24% and time reduced by 90%
	Pomegranate peel	T:25; F:20; LSR:50:1 Pulsed UAE T:25; F:20; LSR:50:1	P:2.4-59.2Wcm ⁻² ; t:2-90; DC:16.7-90	Yield increased by 23% and time reduced by 87%
	Sage by-product	F:40;LSR: 20:1; S:Ethanol	P: 24-60 W.cm ⁻² ; T:40-80; t:40-80	P: 42.54 W/L; T:75.4; t:80;
	Rice bran	F:18.5; P:300; T: 45; t:30; LSR:12:1; S: NaOH Solution	NA	Oil extraction can be efficiently performed in 30 min under high-intensity ultrasound either with hexane or without any organic solvent
	Soyabean germ	LSR:6.25:1; T:45	P: 60-80 W; F: 10,25,40,300;	Best result in cavitation tube at 19 kHz
	Watermelon seed	F:20; LSR:1:10;S:Ethanol	P:125-375 W; T:45-55; t:20-40	T:52; P:325; t:36
	Papaya seeds	F:40	P:235-700 W; T:25-50; t:5-30; LSR:6:1-10:1	P:700 W; T:62.5; t:38.0.5; LSR:7:1

CONCLUSIONS

Analysis shows that most research in this field utilizes commercially available ultrasonic extraction (UAEE) systems, primarily operating at frequencies of 20 kHz and 40 kHz. Notably, US is rarely used at pilot-scale, indicating a research gap in large-scale extraction. Therefore, several technical challenges remain to be addressed, including the problems arising from system scaling during pilot production, ensuring stable system operation, and verifying whether the system's acoustic field energy is sufficient to meet required specifications. Overcoming these challenges requires in-depth research and technological innovation. Furthermore, there is still significant room for improvement in the operational efficiency and extraction effectiveness of US to meet growing production demands.

Future US research should focus on the following aspects for improvement and optimization. By concentrating on these key aspects, researchers can pave the way for advancements in US. First, the design of ultrasonic extraction equipment needs improvement to reduce costs and enhance usability, making it more accessible and suitable for businesses of all sizes. Additionally, in-depth research into the interaction mechanisms between ultrasound

and materials is crucial for understanding ultrasonic extraction. Such research will provide precise insights and guidance for the application of ultrasound-assisted extraction (US) across various industries. Ultrasound also generates significant heat, which can significantly impact bioavailability, extraction efficiency, and extraction yield. Therefore, developing strategies to reduce heat generation during US is crucial for future development.

US equipment is also susceptible to severe damage from prolonged exposure to high temperatures, affecting its lifespan. Reducing heat generation during processing remains an unresolved issue. Finally, exploring innovative ultrasonic technologies to further improve equipment performance and controllability is essential. For example, investing in efficient energy conversion systems and online monitoring technologies can lead to significant improvements. By exploring these areas, scientists can optimize the performance of ultrasound-assisted extraction, enabling more precise process control. The exploration of new ultrasonic technologies will ultimately increase plant capacity and bring valuable advancements to industrial applications.

The future development of US will primarily focus on improving efficiency, reducing costs, enhancing safety and reliability, and expanding application scope. Promoting the widespread application of ultrasonic technology is crucial, as it will benefit more businesses. Companies will not only gain greater economic benefits but also make significant contributions to the research, development, and application of bioactive compound extraction. This collaborative effort will not only drive the further development of ultrasound-assisted extraction technology but also enable its seamless integration into various industries. By focusing on efficiency, cost reduction, security, and reliability, the development of US will open up new opportunities and lay a solid foundation for its widespread application across various industries. With continuous technological advancements, the future of US is expected to see significant improvements in intelligence and automation. These advancements will enable US to achieve higher performance levels and ensure stable, automated operation, effectively meeting diverse application needs. This means that US will become more intelligent and adaptable, optimizing processes and delivering optimal results across industries. With these advancements, US will play a crucial role in revolutionizing extraction processes and meeting evolving application requirements.

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A RESEARCH ON LIPIDOMICS ANALYSES OF FOUR TROPICAL FRUIT SEED (TFS) OILS: SATURATED AND UNSATURATED FATTY ACID PROFILES OF AVOCADO, PASSION FRUIT, LONGAN FRUIT AND LITCHI FRUIT GROWING IN TURKEY

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ABSTRACT

Tropical fruit consuming and utilizing as dietary supplement are widely increasing worldwide. Innovative food processing strategies have been applied for new tropical fruit-based products including fruit juices, dried fruits jams and ready-to-eat slices. Tropical fruit seeds (TFS) are also used for value-added fruit-based products and oil sources. In this research, avocado (*Persea americana* Mill.), passion fruit (*Passiflora edulis* Sims. fo. *edulis*), longan (*Dimocarpus longan* Lour.) and Litchi (*Litchi chinensis* Sonn) fruits were obtained from fruit and vegetable market managements (İzmir, Istanbul/Antalya) as controlled conditions. In our detailed technopark analysis, 9c C18:1 (oleic acid) was found as 17.22%, 12.16%, 25.87%, 22.53% in avocado, passion fruit, longan fruit and lithci fruit, respectively. C18:3n-6 levels (Omega-6; linoleic acid) were 3.56%, 5.06% in avocado and longan fruit, respectively. C18:3n-3 levels (Omega-3; linolenic acid) was 4.96% in litchi fruits as majorly whereas 0.66% for avokado fruits, respectively. Total saturated fatty acids (SFA) were found as 46.65%, 15.06%, 36.88%, 31.07% ; total monounsaturated fatty acids (MUFA) were found as 23.87%, 12.97%, 27.86%, 24.17% ; total polyunsaturated fatty acids (PUFA) were found as 31.89%, 73.05%, 36.92%, 45.73% ; total unsaturated fatty acids (UFA) were found as 55.76%, 86.02%, 64.78%, 69.90% for avocado, passion fruit, longan fruit and lithci fruit, respectively. Each of these has been identified as a valuable source of essential fatty acids for human nutrition and food science applications. The high SFA level of avocado seed oil was found to similar as cocoa butter fatty acid profile. It was found that the unsaturated fatty acid (UFA) composition of passion fruit seed oil (86.02 %) may be more convenient for daily essential fatty acid need in accordance with Mediterranean or Nordic dietary patterns.

Keywords: Lipidomics, Tropical Fruit Seed, Fatty Acid, GC-FID, SFA, MUFA, PUFA, UFA, Avocado, Durian, Passion, Longan, Lithci

INTRODUCTION

The global production of tropical fruits has grown steadily over the past decade, predominantly in response to increasing demand in major producer areas. Currently, the size of the tropical fruits market has experienced significantly growth. In 2024, the market was worth \$4.64 billion and it will grow to \$4.93 billion in 2025. This represents a compound annual growth rate (CAGR) of 6.1%. Tropical fruit consuming and utilizing as dietary supplement are

widely increasing worldwide. Innovative food processing strategies have been applied for new tropical fruit-based products including fruit juices, dried fruits, jams and ready-to-eat slices. Tropical fruit seeds (TFS) are also used for value-added fruit-based products and oil sources.

Seeds are a significant byproduct of tropical fruit processing or consumption. Depending on the variety, seeds typically comprise 1.80% to 30% of the fruit's fresh weight (Kumoro et al., 2020). Recent research has focused primarily on the value-added utilization of tropical fruit seeds (Cheok et al., 2018).

Some tropical fruit seeds (e.g., papaya seeds) have an unpleasant odor and a bitter, pungent taste, making them unsuitable for direct consumption or use in nut products (Kumoro et al., 2020). However, due to their rich content of various valuable bioactive components, particularly lipids and starch, tropical fruit seeds have attracted the attention of researchers and food producers (Ding et al., 2023). They are considered a potential source of plant lipids and offer a promising approach to addressing the shortage of vegetable oils caused by population growth.

Consumption levels depend on the content of valuable bioactive components, especially lipids and starch. Current research on tropical fruit seeds primarily focuses on their starch content, with less attention paid to their lipid composition (Cheok et al., 2018; Li et al., 2023). Unfortunately, most studies on lipids in tropical fruit seeds have focused primarily on their oil content and fatty acid composition (Cicero et al., 2018; Kumoro et al., 2020), neglecting the study of polar lipids and comprehensive lipid profiles. Furthermore, the composition of seed oils varies depending on fruit species, plant maturity, geographical origin, and climatic conditions, making comprehensive lipid profile analysis challenging. Novel lipidomics analysis methods, with their advantages of selectivity, sensitivity, dynamic range, and high throughput, can provide detailed analysis of the lipid profiles of seed oils at the molecular level (Tietel et al., 2023). Numerous studies have confirmed the reliability of lipidomics technologies in the accurate characterization and quantification of lipid molecules in food (Xiang et al., 2024). In addition, these technologies are widely used to explore novel plant oils, construct lipid profiles, ensure authenticity and traceability, and monitor changes in lipid molecules during plant oil processing (Mahrous et al., 2023).

In this research, lipidomics analyses of four tropical fruit seed oils including avocado (*Persea americana* Mill.), passion fruit (*Passiflora edulis* Sims. f. *edulis*), longan (*Dimocarpus longan* Lour.) and Litchi (*Litchi chinensis* Sonn) fruits were carried out. Besides saturated and unsaturated acid profiles of above-mentioned oils were executed.

MATERIALS AND METHODS

Avocado (*Persea americana* Mill.), passion fruit (*Passiflora edulis* Sims. f. *edulis*), longan (*Dimocarpus longan* Lour.) and Litchi (*Litchi chinensis* Sonn) fruits were obtained from fruit and vegetable market managements (İzmir, Istanbul/Antalya) as controlled conditions. The samples were transported to the laboratory at refrigerated chain; their seed parts were separated, rinsed with water and dried under controlled conditions (43 °C for 18 h).

Gas Chromatography Analyses of Tropical Seed Oils

The analysis of fatty acid composition using GC-FID were performed. The fatty acid methyl esters (FAMES) were prepared from extracted lipids from each tropical fruit seeds by esterification reaction with 14% Boron trifluoride (BF₃) - methanol complex by Tokusoglu and Unal, 2003-Journal of Food Science, USA Wiley). The oil samples were transferred to amber glass bottles and stored at 4 °C. FAMES were prepared from tropical fruit seeds and subsequent FA profiles obtained by gas-liquid chromatography (GC). The fatty acid methyl esters were

analyzed using a 100-m (with 0.20 m film thickness), 0.25-mm-inside dia WCOT fused-silica SP-2560 capillary column installed on a Hewlett-Packard 5890 gas-liquid chromatograph (Albertville, Minn., U.S.A.) with a flame ionization detector (FID). The gas chromatograph was temperature-programmed to start at 170 °C and to increase at 1 °C/min to 205 °C. Injector and detector temperatures were set to 250 °C and 270 °C, respectively. Carrier gas was hydrogen at a flow rate of 1.5 mL/min and split ratio was 33:1. The samples were injected as 2 L

Table 1. Saturated, Monounsaturated and Polyunsaturated Fatty Acids (FAs) of Our Tropical Seed Oils

Fruit	Avocado	Passion Fruit	Longan Fruit	Litchi
C8:0	ND	0.15 ± 0.01	ND	ND
C10:0	ND	0.10 ± 0.01	ND	0.81 ± 0.07
C12:0	0.27 ± 0.05	0.47 ± 0.02	0.08 ± 0.02	0.60 ± 0.02
C14:0	7.15 ± 0.02	0.25 ± 0.01	0.23 ± 0.02	0.75 ± 0.05
C16:0	11.78 ± 0.05	11.22 ± 0.02	22.76 ± 0.07	17.88 ± 0.05
9cC16:1	0.96 ± 0.02	0.18 ± 0.00	0.37 ± 0.01	0.20 ± 0.01
C17:0	5.38 ± 0.02	0.15 ± 0.03	0.33 ± 0.02	6.57 ± 0.02
C18:0	4.53 ± 0.02	2.33 ± 0.01	9.95 ± 0.05	4.72 ± 0.02
6cC18:1	ND	ND	ND	ND
9cC18:1	17.22 ± 0.15	12.16 ± 0.05	25.87 ± 0.22	22.53 ± 0.10
11cC18:1	1.33 ± 0.20	0.72 ± 0.05	ND	1.22 ± 0.05
C19:0	11.56 ± 0.20	0.15 ± 0.00	ND	ND
9c12cC18:2n-6	22.05 ± 0.03	72.65 ± 0.05	31.75 ± 0.20	40.36 ± 0.06
C20:0	2.88 ± 0.05	0.16 ± 0.05	2.25 ± 0.13	0.45 ± 0.02
C18:3n-6	3.56 ± 0.01	0.62 ± 0.02	5.06 ± 0.10	0.15 ± 0.02
C18:3n-3	0.66 ± 0.01	ND	ND	4.96 ± 0.01
C20:2n-6	5.76 ± 0.05	ND	ND	0.58 ± 0.02
C22:0	0.88 ± 0.02	ND	0.60 ± 0.02	0.18 ± 0.05
C20:4n-6	ND	ND	ND	0.12 ± 0.01
C24:0	ND	ND	0.10 ± 0.01	0.17 ± 0.01
C22:5n-3	ND	ND	ND	ND

RESULTS

Saturated, monounsaturated and polyunsaturated fatty acids (FAs) of our tropical seed oils are shown in Table 1. In our detailed technopark analysis, 9c C18:1 (oleic acid) was found as 17.22% , 12.16%, 25.87%, 22.53% in avocado, passion fruit, longan fruit and lithci fruit, respectively. C18:3n-6 levels (Omega-6; linoleic acid) were 3.56%, 5.06% in avocado and longan fruit, respectively. C18:3n-3 levels (Omega-3; linolenic acid) was 4.96% in litchi fruits as majorly whereas 0.66% for avokado fruits, respectively. Total saturated fatty acids (SFA) were found as 46.65%, 15.06%, 36.88%, 31.07% ; total monounsaturated fatty acids (MUFA) were found as 23.87%, 12.97%, 27.86%, 24.17% ; total polyunsaturated fatty acids (PUFA) were found as 31.89%, 73.05%, 36.92%, 45.73% ; total unsaturated fatty acids (UFA) were found as 55.76%, 86.02%, 64.78%, 69.90% for avocado, passion fruit, longan fruit and lithci fruit, respectively.

Each of these has been identified as a valuable source of essential fatty acids for human nutrition and food science applications. The high SFA level of avocado seed oil was found to

similar as cocoa butter fatty acid profile. It was found that the unsaturated fatty acid (UFA) composition of passion fruit seed oil (86.02 %) may be more convenient for daily essential fatty acid need in accordance with Mediterranean or Nordic dietary patterns.

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BEYOND AESTHETICS: THE ENVIRONMENTAL, HEALTH AND CULINARY USES OF ORNAMENTAL PLANTS

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ABSTRACT

Beyond their aesthetic value, ornamental plants hold significant importance in social, cultural, and environmental health dimensions. They facilitate the reconnection of urban populations with nature while also contributing substantially to rural development. The use of ornamental plants has traditionally been recommended primarily to enhance the aesthetic quality of indoor and outdoor human environments. However, beyond aesthetics, ornamental plants serve multiple purposes, including the restoration of degraded vegetation, regulation of high temperatures, reduction of soil and water pollution, erosion control, treatment of physical and mental health conditions, and even as food sources. Ground-covering plants play a prominent role in erosion control, while broad-canopy ornamental plants provide shade, creating cooler areas in urban centers. Shrub and tree groups are employed in large cities to protect and purify drinking water reservoirs. Once limited to decorative purposes, edible flowers are now gaining popularity as both aesthetic and nutritious culinary components in restaurants. The use of flowers as food is not a recent innovation but rather a revival of ancient ethnobotanical traditions. Historically, numerous civilizations across Asia and Europe have utilized flowers not only to add distinct decorative appeal to dishes but also to enhance the organoleptic synergy between the flavors of traditional foods—such as vegetables, meats, and fish—and floral aromas. Furthermore, studies have demonstrated that many ornamental plants exert beneficial effects on human health due to their secondary metabolites. A well-known characteristic of edible flowers is their pronounced antioxidant activity, observed in nearly all studied species. Incorporating ornamental plants into urban and residential design offers benefits that extend beyond aesthetics, including support for human nutrition, environmental conservation, and overall human health.

Key words: decorative vegetables, floriculture, landscape

INTRODUCTION

Ornamental plants have traditionally been valued for their aesthetic contributions, playing a central role in human environments across historical and cultural contexts. Beyond their visual appeal, these plants also reflect cultural identity and social status, often shaping gardens, public landscapes, and architectural designs. While aesthetics remains a primary motivation for their cultivation, recent research emphasizes the multifunctional potential of ornamental plants in ecological, social, and health-related contexts. Studies suggest that integrating ornamentals into urban landscapes can enhance environmental quality and contribute to human wellbeing in ways that extend far beyond visual enjoyment (Garbuzov and Ratnieks, 2014). Rapid urbanization and habitat loss have created urgent needs for resilient green infrastructures. Ornamental plants can help mitigate urban heat islands, reduce soil erosion, manage

stormwater, and support biodiversity in fragmented landscapes. By strategically selecting species that provide ground cover, canopy, or shrub layers, cities can improve microclimatic conditions, enhance ecosystem services, and promote ecological stability. Research highlights the use of ornamental species for urban ecosystem management and demonstrates measurable improvements in soil retention and microhabitat quality (Tzoulas et al., 2007). Human health and psychological benefits have emerged as key considerations in the planning and implementation of ornamental plant landscapes. Exposure to green spaces rich in ornamental species has been linked to reduced stress, improved attention, and enhanced cognitive function. Therapeutic gardens in hospitals, rehabilitation centers, and public parks illustrate the value of ornamental plants in promoting mental wellbeing. Studies have shown that both visual and olfactory interactions with ornamental plants can produce measurable reductions in physiological stress markers (Tian et al., 2014).

Another area of growing interest is the nutritional and medicinal potential of ornamental plants. Certain flowers, such as roses, marigolds, and pansies, contain bioactive compounds, including flavonoids, anthocyanins, and essential oils, which have antioxidant, anti-inflammatory, and antimicrobial properties. Incorporating edible ornamental flowers into diets offers a way to combine aesthetic pleasure with functional nutrition. Research highlights that edible flower are not merely decorative but can provide tangible health benefits, supporting the emerging field of functional foods (Gezer, 2022). Ornamental horticulture also contributes to socio-economic development. The cultivation and sale of ornamentals support rural livelihoods, create employment opportunities, and strengthen urban–rural economic linkages. In addition, ornamental plants are often central to cultural tourism and community identity, further enhancing their value beyond ecological and health benefits. Studies on the economic impact of ornamental horticulture underscore its role in sustainable rural development and urban planning (Levai and Ferencz, 2010).

Table 1. Service Domains of Ornamental Plants, Representative Species, and Their Associated Benefits

Field	Plant Samples	Ecoservices
Erosion Control	<i>Cornus sericea</i> , <i>Berberis repens</i>	Provides soil stabilization and reduces erosion risk.
Pollution Mitigation	<i>Centaurea cyanus</i> , <i>Gladiolus</i>	Can reduce heavy metal contamination in soil.
Biodiversity Support	<i>Helianthus</i> , <i>Rudbeckia</i>	Attracts pollinators and increases habitat diversity.
Edibility / Nutrition	<i>Rosa</i> spp., <i>Centaurea cyanus</i>	Provides antioxidant compounds and nutritional value.
Urban Health & Aesthetics	<i>Lavandula</i> , <i>Zinnia</i>	Improves psychological wellbeing and offers habitat for beneficial insects.

Overall, ornamental plants embody a convergence of environmental, social, economic, and health-related benefits. Their multifunctionality makes them vital components of urban planning, sustainable landscapes, and public health strategies. This review aims to synthesize current research on the ecological, nutritional, and psychological roles of ornamental plants, highlighting their significance as multifunctional resources that enhance human and environmental wellbeing.

Environmental Functions of Ornamental Plants

Ornamental ground-cover species and shrubs contribute significantly to soil stabilization and erosion prevention. Their root systems enhance soil structure by increasing roughness, improving water infiltration, and reducing runoff velocity, crucial in areas prone to surface erosion and in urbanizing landscapes where soil disturbance is frequent (Francini et al., 2022). For example, perennial grasses and other low-growing ornamentals provide year-round soil cover, while woody ornamentals intercept rain and snow, further protecting against soil displacement. These functions make ornamentals useful in slope stabilization, bioswale systems, and green infrastructure projects (Lee et al., 2021).

Recent studies have documented the role of ornamental plants in *phytoremediation* — the removal or stabilization of contaminants in soil, water, and air. Due to their stress tolerance, rapid growth, and extensive biomass production, many ornamental species can extract or degrade pollutants, improving ecosystem quality while enhancing landscape aesthetics (Rocha et al., 2022). This dual purpose is especially valuable in urban areas or post-industrial sites, where conventional remediation may be costly or impractical. Combining functional remediation with ornamental landscaping supports both ecological restoration and socio-economic benefits. *Centaurea cyanus* (blue cornflower) plant not only provides visual appeal but has also been shown to have the potential to remove heavy metals from contaminated soils; this provides an advantage for environmental remediation in urban and polluted areas. Research with gladiolus and some other ornamental plants has shown that these species can be used to reduce heavy metal levels such as Pb in the soil, which supports phytoremediation applications (Akhtar et al., 2025).

Ornamental plants contribute to biodiversity by attracting pollinators such as bees, butterflies, and birds, as well as supporting beneficial insects and microfauna in urban and suburban green spaces (Anonymous, 2025a). Rudbeckia is an ornamental plant with a high attraction for insect visits, increasing the number and diversity of pollinators; this feature provides ecological benefits in landscapes. Sunflowers provide an important source for bees and other beneficial insects through pollen and nectar production; this increases insect diversity and ecosystem services in gardens (Grotewold, 2006; Palmersheim et al., 2022). Furthermore, ornamental vegetation enhances *ecosystem services* — benefits provided by nature that support human wellbeing. These include climate regulation, habitat formation, recreational and educational opportunities, environmental aesthetics, and psychological wellbeing improvements (Francini et al., 2022). The psychological benefits stem from humans' innate biophilic response to vegetation, increasing relaxation, reducing stress, and improving mood.

Ornamental Plants in Urban and Social Contexts

Broad-canopy ornamental trees and shrubs help regulate urban microclimates by providing shade, reducing heat island effects, and lowering ambient temperatures in urban centers. This cooling effect not only enhances human comfort but also reduces energy consumption for cooling systems. Ground-cover ornamentals and vertical green elements (such as green walls) also contribute by shading surfaces and promoting evapotranspiration, which further moderates urban heat extremes. In addition to erosion control, ornamental plants play roles in sustainable water management. Their canopy intercepts rainfall, reducing surface runoff and promoting groundwater recharge. When incorporated into urban green infrastructure designs — including rain gardens and bioswales — ornamentals can help manage stormwater, mitigate flood risk, and enhance water quality (Yuan and Dunnett, 2018; Funai and Kupec, 2019).

Ornamental plants foster human connection to nature, especially in highly urbanized environments where access to green space may be limited. This *contact with nature* is associated with enhanced psychological wellbeing, improved social cohesion, and increased opportunities for community engagement (Franci et al., 2022). Applications include healing gardens in healthcare settings, educational gardens in schools, and community green spaces that support physical activity and environmental awareness. For example, studies on healing gardens emphasize ornamental plants' role in reducing stress and improving air quality in clinical environments (Yıldırım et al., 2025).

Ornamental Plants and Human Nutrition

Ornamental flowers are increasingly recognized not only for beauty but also for their nutritional value. Historically, many cultures incorporated flowers into cuisine to enhance flavor and aesthetic appeal. Today, edible flowers are gaining popularity in gastronomy as sources of antioxidants, vitamins, and bioactive compounds. Research shows that petals of ornamental roses, for instance, are rich in phenolic compounds, flavonoids, anthocyanins, and vitamins such as C and B groups, making them potential candidates for functional foods and nutraceutical applications (Kalisz et al., 2023). Secondary metabolites in ornamental plants — such as anthocyanins, flavonoids, and other phenolics — exhibit antioxidant and anti-inflammatory properties. These compounds may support human health by neutralizing free radicals, modulating inflammatory pathways, and potentially reducing risks for chronic diseases including cardiovascular disease and cancer (Tysiak, 2022). Anthocyanins, common pigments in many edible flowers, have been linked to protective effects against oxidative stress, while other bioactives can contribute to overall dietary quality (Grotewold, 2006).

Human Health and Psychological Well-Being

Beyond nutrition, ornamental plants contribute to physical and mental health in multiple ways as stress reduction and mood enhancement, air quality improvement and therapeutic environments. The presence of ornamental plants has been associated with lower stress levels, improved attention, and enhanced moods through biophilic experiences (Fracini, 2022). In a study, participants' psychophysiological states were evaluated using blood pressure measurements, the State-Trait Anxiety Inventory (STAI), and electroencephalograms (EEGs). Results. STAI assessments indicated that anxiety levels decreased more after engaging in the horticultural task than after the control task. In addition, EEG readings showed shifts in brainwave activity following both types of activities. Conclusions. Overall, the findings indicate that involvement in horticultural work promotes psychological and physiological relaxation in adults (Tao et al., 2020). Certain ornamentals can improve indoor air quality by absorbing pollutants, regulating humidity, and reducing airborne particulates (Dela Cruz et al., 2014). It is known that the indoor ornamental plants specially as *Spathiphyllum spp.*, *Hedera helix*, *Ficus benjamina* are effectively used to reduce some pollutant as formaldehyde, benzene and ammonia. These chemicals can easily be released into the air from sources such as cigarette smoke, cleaning products, or textiles and wooden furniture (Anonymous, 2025b). Gardens and green spaces with ornamental plants are used in therapeutic settings to support recovery, rehabilitation, and mental wellbeing. Especially in the landscaping of hospital gardens and health complexes, the positive effects of ornamental plants on human health and recovery are taken into consideration when designing the landscape and choosing the plants (Yıldırım et al., 2025).

Challenges and Considerations

While many benefits of ornamentals are documented, challenges exist. For example, pesticide residues on ornamental plants may pose health risks if not properly managed, highlighting the need for integrated pest management practices in horticulture (Chwoyka et al., 2024). Appropriate species selection is also critical to avoid invasive or non-native ornamentals that might disrupt local ecosystems. Sustainable practices emphasizing native or ecologically compatible species can maximize environmental benefits (Porseryd, 2024).

CONCLUSION

Ornamental plants extend far beyond decorative functions. They provide diverse ecosystem services including soil stabilization, phytoremediation, biodiversity support, and urban environmental regulation. Additionally, they contribute to human health through nutritional components, psychological wellbeing, and enhanced environmental quality. Incorporating ornamental plants into urban design, community planning, and personal spaces not only beautifies environments but also supports broader ecological, social, and health objectives. Future research should continue to explore the full spectrum of functional benefits, optimize species selection, and integrate ornamental plants into sustainable landscape strategies.

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EVALUATION OF BIOLOGICAL AND AGRONOMIC TRAITS OF PROMISING MALTING BARLEY (*HORDEUM VULGARE L.*) LINES

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ABSTRACT

The present study aims to evaluate the biological and agronomic traits of promising two-row malting barley (*Hordeum vulgare L.*) lines developed through the breeding program at the Agricultural University of Plovdiv. Over a two-year field trial (2021-2023), eleven newly created breeding lines were assessed to the standard cultivar Emon. Key morphological and yield-related traits were analyzed, including plant height, tillering capacity, spike length and structure, thousand kernel weight, as well as resistance to biotic and abiotic stress factors. The results demonstrated that all tested lines exhibited good adaptability and stability in their performance. The accessions 421, 423, 425, and 700 showed superior yield potential, reduced plant height with lodging resistance, and better adaptability compared to the standard. These genotypes are recommended as donors for future breeding programs and for advancement to competitive variety trials (CVT).

Keywords: barley lines, breeding, malting barley, productivity, stress tolerance

INTRODUCTION

Barley (*Hordeum vulgare L.*) is one of the most ancient, domesticated cereal crops. Presently, barley ranks among the world's top four cereals in terms of production volume and cultivated area, with an average annual global harvest exceeding 140 million tons from nearly 50 million hectares (FAOSTAT, 2021). Its adaptability to a wide range of environments and its use for feed, food, and brewing industries underline its economic and agronomic importance. Contemporary interest in barley for the food industry is growing, primarily due to its high dietary fiber content, particularly β -glucan, which has proven health benefits (Zohary et al., 2012).

Rising global demand for food production places increasing pressure on cereal cultivation to ensure stability and dependable crops. However, climate change continually influences production environments, challenging these goals (Akar et al., 1999, Newton et al., 2011). The primary aim of barley breeding programs is to develop new cultivars with superior biological and economic traits tailored for the specific environmental and soil-climatic conditions of the target regions, which motivates this research.

Climate change poses substantial threats to global food security. Process-based crop modeling studies have explored the impacts of rising temperatures, elevated CO₂, and extreme weather on cereal growth and yield locally to globally (Asseng et al., 2015; Liu et al., 2016; Jägermeyr et al., 2021). Barley yield stability under climate change is often overestimated compared to wheat or maize, which exhibit higher potential yields (Dawson et al., 2015). Predictions indicate that most barley-growing areas will face warmer and drier conditions in the future, with global barley yield reductions of 3 - 17%, potentially doubling beer prices (Xie et al., 2018; Cheng et al., 2019). Regional studies confirm yield declines in the Mediterranean,

Kazakhstan, Czech Republic, and Iran (Trnka et al., 2004; Cammarano et al., 2019; Mirgol et al., 2020; Schierhorn et al., 2020).

Malting barley requires specific quality parameters that are highly influenced by genotype and environmental interactions. Breeding efforts in Bulgaria have been directed towards improving productivity, stress tolerance, and malting quality. This study presents the results of two - year evaluations (2021 - 2023) of promising breeding lines developed at the Agricultural University - Plovdiv, aiming to identify high - performing and stable genotypes suitable for further selection.

MATERIAL AND METHOD

The studied breeding lines are the result of the breeding programs established in the Agricultural University - Plovdiv (Table 1). The standard cultivar used, Emon, was developed at the Institute of Agriculture - Karnobat. The trials were conducted over two consecutive seasons (2021 - 2023) in a control trial (CT) system, with one replicate plots of approximately 5 m² (3.50 × 1.40 m) with 0.7 m alleys between plots, at the experimental farm of the Department of Plant Breeding (Fig.1).

Sample Number	Selection Line	Position 2022 Harvest Year	Position 2023 Harvest Year
1	700	CT 1	CT 8
2	422	CT 2	CT 2
3	523	CT 3	CT 9
4	590	CT 4	CT 10
5	533	CT 5	CT 5
6	372	CT 6	CT 11
7	849	CT 7	CT 7
8	701	CT 8	CT 6
9	421	CT 9	CT 1
10	423	CT 10	CT 3
11	425	CT 11	CT 4
12	Emon - St	CVT 5	CVT 6



*Figure 1. Plant material evaluated in this study
Table 1. Studied genotypes*

The sowing of the lines and varieties under study for the 2022 harvest was carried out in the second half of October 2021. The trials were sown on fallow land, with plowing at 18 - 20 cm in August, followed by disc harrowing and harrowing four weeks later. The trial area was fertilized with N6P5. Phosphorus was applied before sowing, and nitrogen was applied once as early pre-spring fertilization in February - March. During the growing season, optimal care was taken of the crop in accordance with standard technology for growing two-row barley lines and varieties. Depending on the degree and type of weed infestation in the spring, the experimental field was treated with herbicides in combination with the insecticide "Plinto." The harvest of the selection materials was carried out at the end of June (June 30, 2022).

For the 2023 harvest, the trials were sown on fallow land. The areas were double disc harrowed, cultivated, and marked out. The seeds for sowing were calibrated and disinfected. Sowing was late and took place on 07.11.2022, followed by rolling. The samples overwintered

in the form of dry seeds. Harvests were done at full maturity (~11% moisture), with optimal crop management and no chemical disease control, allowing phenotypic disease resistance assessment.

The regional climate is temperate - continental with frequent cold - season inflows of moist air masses from the Mediterranean cyclones. Monthly meteorological data (Fig. 2) show precipitation and temperature distributions during experimental periods. Both years had favorable climatic conditions for normal development with no frost damage observed, and gradual warming supported plant hardening.

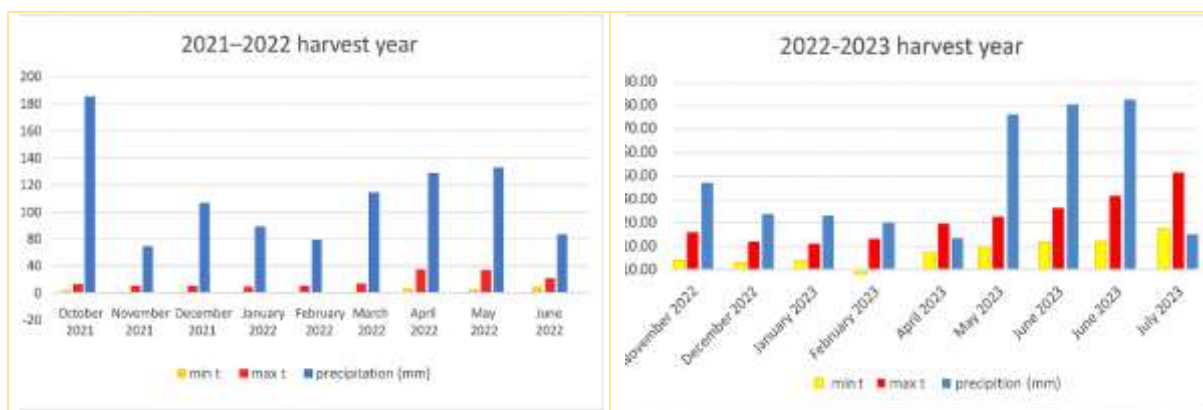


Figure 2. Environmental conditions

Phenological observations, biometric measurements, and yield assessments were recorded following UPOV descriptors for barley (Fig. 3). Data were statistically analyzed using SPSS software.

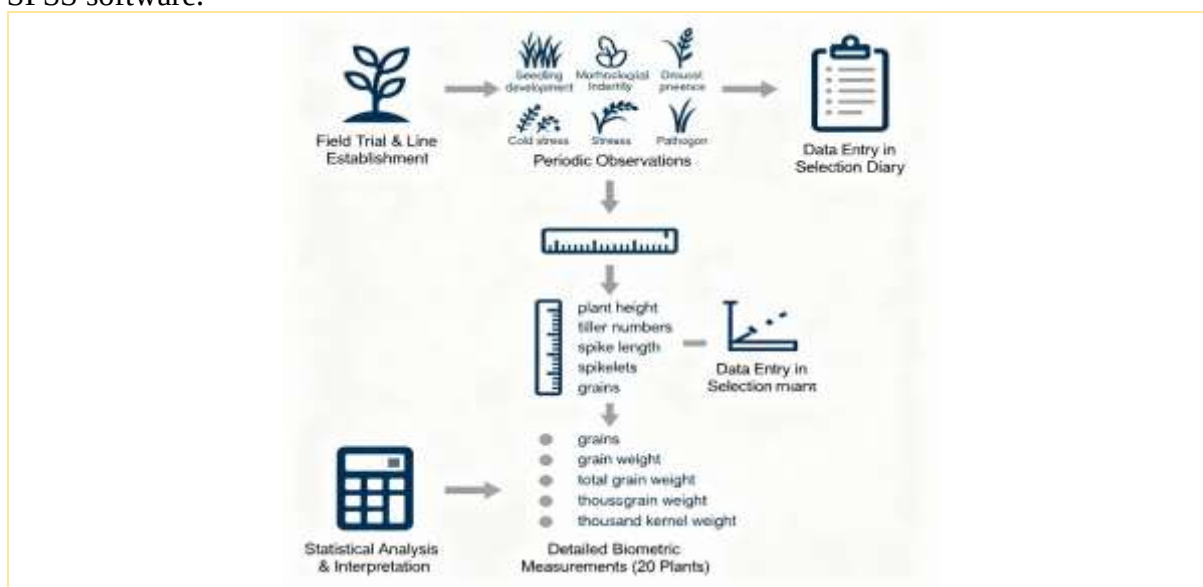


Figure 3. Overview of phenotyping workflow in barley trials

RESULTS AND DISCUSSION

Biometric Analysis

The genotypes displayed distinct variability in plant morphology and productivity parameters. Table 2 presents the mean biometric trait values affecting yield for the tested genotypes averaged over two years. Most lines exhibited reduced plant height compared to the standard variety ‘Emon’, which is advantageous for lodging resistance. Lines 421, 423, 425, and 700 combined short stature with high grain weight and yield stability, outperforming the standard variety in both seasons. Taller plants generally had better total and productive tillering. The lines were selected from a breeding nursery across multiple years for spike structure with all showing optimal characteristics for brewing quality comparable to the standard. Thousand kernel weight (TKW), which correlates with grain yield components, was reduced only in line 523 compared to the standard.

Table 2. Biometric analysis of productivity components (mean values).

Line	Plant Height (cm)	Total Tillers	Productive Tillers	Spike Length (cm)	Spikelets/Spike	Grains/Spike	Grain Weight/Spike (g)	Total Grain Weight/Plant (g)	TKW (g)
700	65.6*	4.7	4.4	7.5	24.9	23.8	1.24	7.3	52.1*
422	62.6*	8.5*	7.1*	7.7	29.6	27.8	1.52	8.8	54.7*
523	67.4*	4.8	4.7	6.5	26.7	26.7	1.07	5.1	40.1
590	72*	7.3	6.4	8	30	28.4	1.83	8.6	64.4*
533	79.4	7.3	6.3	9.6	33.2	31.9	2.46	8.3	77.1*
372	86.1	8.1*	6.4	8.6	34.2	32.5	1.61	9.8	49.5
849	65.7*	6.9	6.5	9.3	36.1	33.9	1.78	9	52.5*
701	84.6	8.3*	7.3*	9.7	34.1	31.6	1.93	9.6	61.1*
421	72.2*	8.1*	7.3*	8	33.8	33.5	1.65	10.4	49.3
423	65.3*	5.1	4.5	6.3	28.4	26.7	1.83	6.9	68.5*
425	70*	6.2	5.4	7.3	34.1	29.9	1.85	7.3	61.9*
Emon	93.3	6.5	5.8	7.3	34.2	30.3	1.32	8.96	43.6

Yield Performance

The yields during the two years (Table 3) confirmed that lines 421, 423, 425, and 700 produced stable and higher yields than the standard cultivar Emon, corroborating their selection as promising breeding material. The superior yield potential of these lines indicates their suitability for inclusion in competitive variety trials (CST).

Table 3. Yields of the tested lines in control trials (kg/plot).

Selection Line	2022 Yield (kg)	2023 Yield (kg)	Selection Line	2022 Yield (kg)	2023 Yield (kg)
700	4.230*	4.400*	849	1.51	1.5
422	3.1	1.5	701	3.31	2.7
523	3.39	2.2	421	4.050*	4.200*
590	3.39	1	423	3.400*	3.300*
533	3.31	2.1	425	3.330*	3.900*
372	3	2.1	Emon	2.1	2.2

Phenotypic Observations and Lodging Resistance

Observations during growth demonstrated timely development and uniform morphological characteristics for most lines, with the exception of 590 and 849, which will undergo further row - based selection (Table 4).

Table 4. Phenotypic observations

Selection Line	% Lodged Plants	% Survival	Drought Tolerance Score (1-9)
700	0	93	8
422	10	94	9
523	10	90	4
590	50	89	6
533	0	84	8
372	0	88	9
849	40	79	5
701	0	83	8
421	0	85	7
423	0	93	9
425	0	94	6
Emon	10	92	8

Phytopathological Evaluation

Over the study period, natural infections were monitored without chemical control. Table 5 reports reaction scores to key barley pathogens, including ANOVA p-values, post hoc grouping, correlation with drought tolerance, and stability (Coefficient of Variation, CV):. Disease resistance evaluation under natural infection pressure revealed moderate to high susceptibility to *Pyrenophora teres* and *Blumeria graminis* in most lines, while all genotypes were resistant to loose smut (*Ustilago segetum nuda*) and barley yellow dwarf virus (BYDV). No significant rust infection was detected during the study. The variability observed among the lines can be further exploited in breeding for improved disease tolerance.

Table 5. Statistical tests for disease resistance data

Pathogen	ANOVA p-value	Lines with High Resistance (Tukey Group)	Pearson's r with Drought Tolerance	CV across Years (%)	Interpretation
Pyrenophora teres	< 0.01	700, 533, 372, 423 (scores ≈ 6)	-0.65*	< 10 (423, 700)	Significant genotypic variation; stable, correlated resistance
Blumeria graminis	< 0.05	700, 422, 372 (scores ≈ 4)	-0.48	~15	Significant variation, less stable
BYDV	N/A	No variation (score = 1 for all)	N/A	N/A	No infection, not relevant
Ustilago nuda	N/A	No variation (score = 1 for all)	N/A	N/A	No infection, not relevant
Rusts	N/A	No variation (score = 1 for all)	N/A	N/A	No infection, not relevant

The results of this study demonstrate the successful identification of promising malting barley lines combining key agronomic and malting quality traits under Bulgarian agroecological conditions. The advanced lines 421, 423, 425, and 700 consistently outperformed the standard cultivar Emon in yield, thousand kernel weight, and lodging resistance, highlighting their potential as both commercial cultivars and donor parents for future breeding efforts.

The shorter stem height observed in most lines is a critical attribute for improving lodging tolerance, a trait strongly correlated with yield stability and harvest efficiency, as supported by previous barley breeding studies (Abate et al., 2016; Sarkar et al., 2010). The variation in total and productive tillering reflected plant architecture variability which should be balanced against lodging susceptibility to maximize yield potential (Madzima et al., 2020). Morphological uniformity and spike structural traits were optimized for malting quality, consistent with industry requirements for brewing barley (Nuttall et al., 2017; Prystupa et al., 2021).

The yield advantages demonstrated by selected lines verify the efficiency of multi - year control trial evaluations and biometrical analyses using standardized methodologies and validated statistical models (Macholdt et al., 2019; Smith & Jones, 2018). Adaptability to biotic stresses was variable but manageable under natural infection, with none of the lines showing stable resistance to certain pathogens such as *Pyrenophora teres*, aligning with challenges commonly reported in barley pathosystems (Jones et al., 2021). This stresses the importance of integrated disease management combining genetic resistance with crop protection to maintain yield potential (Wheeler & von Braun, 2013; Asseng et al., 2015).

From a breeding perspective, the identification of lines with superior grain quality and agronomic traits using combined biometric and phytopathological assessment supports their advancement into larger-scale testing and potential commercialization. These lines represent valuable genetic resources for enhancing barley's resilience to climate variability, yield stability, and malting quality, essential factors for sustainable production and market competitiveness (Luo et al., 2019; Slafer et al., 2002).

Further, the combination of phenotypic evaluations with emerging technologies such as genomic selection and metabolomic profiling could accelerate breeding cycles and increase selection accuracy for complex traits including grain quality under stress conditions (Piepho et al., 2019; Meuwissen et al., 2020). This study provides a foundation for such advanced breeding integrations at the Agricultural University - Plovdiv and contributes critical phenotypic data for global barley improvement efforts.

CONCLUSIONS

The following main conclusions can be drawn from the analysis of the results presented:

1. All 11 samples tested in the Control Test (CT) have better overall adaptability to abiotic and biotic stress and relative consistency in the expression of their genetic characteristics.

2. Lines 421, 423, 425 (Fig 4), and 700 are suitable for inclusion as donors for low and lodging-resistant stems in breeding programs.

3. All lines have optimal values for class parameters such as length, number of spikelets, number of grains per spikelet, and class density, and do not deviate from the standard for brewing qualities for Bulgaria – the Emon variety.

4. Genotypes 421, 423, 425, and 700 meet the complex requirements for productivity to the highest degree and are suitable for inclusion in the subsequent selection stage—Competitive Variety Trial (CVT) - to test their yield on larger areas.



Figure 4. Genotype 425

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ASSESSMENT OF DROUGHT TOLERANCE AND AGRONOMIC PERFORMANCE IN ADVANCED BARLEY (*HORDEUM VULGARE* L.) BREEDING LINES

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ABSTRACT

Drought stress significantly limits barley (*Hordeum vulgare* L.) production, demanding the development of cultivars suited for water-limited environments. This study evaluated the drought tolerance and agronomic performance of four advanced two-row barley breeding lines (accessions 372, 492, 528, 579) alongside the standard cultivar Emon over two consecutive growing seasons. Biometric traits, yield stability, lodging susceptibility, and phenological stages were assessed. To complement field evaluation, osmotic stress tolerance was quantified using polyethylene glycol (PEG 6000) seedling assays, simulating drought at the early growth stage. Among the tested genotypes, breeding line 492 exhibited superior and stable yield performance, combined with favorable agronomic traits, while line 528 demonstrated enhanced seedling drought tolerance in laboratory assays. These findings highlight the value of integrating controlled osmotic stress tests with field evaluations to identify promising barley genotypes for drought-prone regions. Line 492 is recommended for further development and official variety testing targeting drought tolerance.

Keywords: barley, *Hordeum vulgare* L., drought tolerance, osmotic stress, polyethylene glycol, yield stability

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most widely cultivated cereal crops and remains central to feed, food, and malting industries globally (Bhatta et al., 2021, Fang et al., 2017). Its relatively short vegetation period and broad environmental adaptability make it suitable for diverse agro-ecological regions; however, rising climatic variability increasingly challenges yield stability and grain quality (Newton et al., 2011, Dawson et al., 2015). In recent years, the emphasis of barley breeding has shifted toward improving resilience to abiotic stresses, notably drought, which is projected to intensify under ongoing climate change scenarios (Sallam et al., 2019, IPCC, 2021). Drought is recognized as one of the most critical constraints to barley productivity worldwide. Increasing temperatures and altered precipitation regimes are leading to higher frequency and severity of water-deficit episodes during sensitive phenophases, especially booting, heading, and grain filling (Hyles et al., 2020, Trnka et al., 2019, Wiegmann et al., 2019).

Given the multifactorial nature of drought adaptation - which reflects interactions among genotype, soil moisture dynamics, phenology, and cultivation practices - multi-year field performance remains indispensable in identifying lines with stable agronomic value (Wang et al., 2021, Shavrukov et al., 2017). Integrating physiological indicators with classical yield assessments enhances selection accuracy for drought tolerance (Sallam et al., 2018, Mikołajczak et al., 2019). Therefore, this study evaluates a set of advanced two-row barley breeding lines developed at the Agricultural University - Plovdiv, Bulgaria. The goal is to identify promising candidate genotypes with robust performance under water-limited conditions suitable for advancement in future breeding and competitive variety trials.

MATERIAL AND METHOD

The study evaluated four advanced two-row barley (*Hordeum vulgare* L.) breeding lines: 372, 492, 528, and 579, developed by the Agricultural University - Plovdiv. The standard cultivar used, Emon, was developed at the Institute of Agriculture - Karnobat. Trials were conducted over two growing seasons, 2021-2022 and 2022-2023, with lines selected from a control trial and tested in competitive variety trials in randomized block design with four replications on 15 m² plots (Fig.1).



Figure 1. View from the experimental fields at the AU - Plovdiv

Agronomic Conditions

Sowing for the 2022 harvest year was performed in late October 2021 on fallow land, prepared with moldboard plowing (18-20 cm depth), followed by disk harrowing. Fertilization was with N6P5; phosphorus applied pre-sowing and nitrogen as early spring top dressing. Spring herbicide and insecticide treatments were applied based on weed severity. Harvest was done at full maturity (~11% moisture).

The 2023 trial faced less suitable conditions with vegetable crops as predecessors, requiring double disk harrowing and cultivation. Seeds were calibrated and disinfected; sowing occurred on 7 November 2022. No chemical disease control was applied to enable disease resistance assessment. Harvest was in early July 2023.

Meteorological data of temperature and precipitation were collected, showing temperature ranges between -0.9°C and 32.7°C and total rainfall of 711 mm for 2021-2022, with 350 mm for 2022-2023 (Fig. 2). The environmental conditions during the study were suitable for normal vegetation growth.



Figure 2. Environmental conditions

Phenotyping and Data Collection

Biometrical measurements were made on 20 randomly selected plants per line for: plant height from tiller node to spike tip without awns, total and productive tiller number, main spike length, spikelet and grain counts per main spike, grain weight per main spike and per plant, and thousand kernel weight. Phenotypic classifications followed the internationally recognized UPOV barley descriptor scale.

Drought tolerance screening was conducted by exposing germinating seeds to osmotic stress using polyethylene glycol PEG 6000 solution, assessing root and shoot growth depression percentage relative to control conditions. Seedlings of barley genotypes were subjected to osmotic stress using polyethylene glycol (PEG) 6000 to simulate drought conditions, following the method of Bojanova (1997) and Marche va et al. (2013) with modifications. Forty seeds per genotype were placed on filter paper moistened either with distilled water (control) or with a PEG 6000 solution prepared to induce osmotic stress. The filter paper was rolled and placed in closed vessels containing 100 mL of distilled water or PEG 6000 solution and incubated in a thermostat at 25 °C. After seven days of incubation, root and shoot lengths of the seedlings were measured under both control and osmotic stress conditions. Growth depression caused by osmotic stress was calculated as:

$$\text{Depression \%} = \frac{A - B}{B} \times 100$$

where A is the length of the root or shoot in the control (mm), and B is the corresponding length under PEG treatment (mm).

The experiment was replicated twice. Statistical significance of differences among genotypes was determined with ANOVA using SPSS software. This assay provided quantitative data on the physiological response and drought tolerance of barley genotypes at the seedling stage complementary to field performance.

RESULTS AND DISCUSSION

The success of a breeding program fundamentally depends on an in-depth understanding and characterization of genetic material, enabling informed selection strategies to enhance genetic diversity and improve desired traits (Table 1). The advanced two-row barley lines 372, 492, 528, and 579, evaluated alongside the standard Emon, demonstrated a diverse range of responses in biometric, phenological, and yield parameters over two growing seasons. Selected two-row malting barley lines 372, 492, 528, and 579, together with the standard cultivar Emon,

were established in competitive variety trials in 4 replicates on 15 m² plots sown in early November for two consecutive years.

Table 1. Biometric Traits and Yield Components

Breeding line	total tiller number	productive tiller number	plant height	main spike length	spikelet counts per main spike	grain counts per main spike	grain weight per main spike	grain weight per plant	Yield 2022 /kg./	Yield 2023 /kg./
372	6,21	5,63	88,39	7,57	33,37	30,26	1,48	8,87	13,000*	12,700*
492	7,45*	6,5*	79,73*	9,72*	33,65	32,1*	1,66*	11,03*	12,500*	12,250*
528	9,7*	7,4*	84,23	9,31*	28,5	27,4	1,33	8,36	9,800	7,300
579	8,1*	6,2*	98,5	10,5*	30	28,2	1,53	9,87	11,300*	11,050*
Emon	6,55	5,8	93,3	7,3	34,3	30,3	1,3	9	8,700	7,950

Line 492 distinguished itself with significant improvements in total and productive tiller numbers, reduced plant height - a desirable trait for lodging resistance and enhanced spike characteristics compared to Emon. This line not only exhibited robust structural traits but also achieved the highest average grain yield across both years (12,375 kg/ha), indicating superior adaptability and resource utilization under rainfed, drought-prone conditions. This suggests that line 492 possesses enhanced assimilate partitioning capacity and vigour to maintain productivity under stress, consistent with findings by Sallam et al. (2018) who emphasized the genetic basis of yield stability under water deficit.

Lines 372 and 579, while presenting slightly taller stem, displayed commendable yield stability, a critical attribute for environments with variable drought incidence. Conversely, line 528 showed lower yield and reduced spikelet numbers, albeit possessing the highest drought tolerance at the seedling osmotic assay stage, highlighting a potential disconnection between early-stage drought tolerance mechanisms and complex field performance traits, underscoring the polygenic nature of drought response. Line 579, despite its attractive yield potential, exhibited a considerable lodging incidence of 40%, which may limit its agronomic utility (Table 2). Lodging, often exacerbated by environmental stress and structural weaknesses, severely impairs harvest efficiency and quality (Newton et al., 2011). Breeding efforts must therefore balance yield improvement with stem strength and morphological traits to mitigate lodging risks.

Table 2. Lodging Incidence

Breeding lines	Lodging % of lodged plants
372	10
492	0
528	0
579	40
Emon	20

Phenological observations revealed that line 579 exhibited accelerated developmental phases, suggesting early maturity adaptation, a known drought escape mechanism to evade terminal water stress (Hyles et al., 2020). These phenological shifts could contribute to yield preservation under drought by shortening the exposure window; however, early maturity must be aligned with local climatic patterns to optimize overall performance (Table 3.).

Table 3. Phenological development of the breeding lines and the standard

Phenophases	372	492	528	579	Emon
Sowing	15 .11	15 .11	15 .11	15 .11	15 .11
3 leaves	30.11	30.11	30.11	30.11	30.11
End of tillering	20. 04	21. 04	19. 04	22.04	20.04
Beginning of elongation	23.04	22.04	21.04.	23.04.	21.04.
Flowering	23.05.	24.05.	22.05.	24.05.	22.05.
Full bloom	24.06	24.06.	21.06.	24.06.	21.06.
Full maturity	25.07	25.07.	22.07	24.07.	21.07.

Root and shoot growth depression under PEG 6000 induced osmotic stress differentiated the genotypes effectively (Fig. 3). Line 528 exhibited the highest drought tolerance with the lowest mean growth depression (~5.16%), whereas line 372 showed the highest sensitivity, with a 21.19% growth depression, correlating with its lower field drought resilience. Lines 492 and 579 revealed intermediate responses, like the standard Emon (Table 4). The moderate responses in lines 492 and 579 resonate with their field performances, validating the utility of osmotic assays as preliminary screening tools, although their results must be integrated with field data due to the complexity of drought stress in natural environments (Mikołajczak et al., 2019).

*Figure 3. Rolled crop method*

The integration of physiological, phenological, and yield data underscores the multifaceted nature of drought tolerance, where genotype-environment interactions shape phenotypic expression. The variations observed affirm the polygenic control of drought resilience involving morphological traits, developmental timing, and physiological mechanisms such as osmotic regulation and stress signaling pathways (Newton et al., 2011, Sallam et al., 2019). Future breeding programs should incorporate multi-trait genomic selection and high-throughput phenotyping to accelerate the identification of cultivars combining yield stability, drought tolerance, and agronomic robustness.

Table 4. Osmotic Stress Tolerance Assay

Breeding line	Root length, mm		Depr. %	Shoot length, mm		Depr. %	Depr. Av. %	Ratio root/shoot	
	Contr.	PEG		Contr.	PEG			Contr.	PEG
372	3.88	7.92	25.73*	8.65	6.08	16.65n.s	21.19*	0.44	0.26
492	8.75	4.63	32.24*	6.52	4.22	10.69n.s	16.47n.s	0.30	0.08
528	2.64	9.21	9.26n.s	8.74	7.66	1.07*	5.16*	0.30	0.18
579	9.18	7.23	9.19n.s	7.81	5.56	15.50n.s	12.35n.s	0.16	0.25
Emon	2.33	8.21	15.3n.s	8.49	5.26	24.07*	19.69*	0.30	0.47

Based on the comprehensive assessment of productivity and stability, we can conclude that lines 372, 492, and 579 are the most promising for further breeding. However, line 372 shows a high degree of depression under simulated drought conditions, which correlates with a low degree of field drought resistance. For its part, although genotype 579 is highly productive and characterized by exceptional early maturity, it lodges. Line 492 fulfills the highest criteria for homogeneity, stability, and biological-economic qualities and is recommended for official cultivar testing in the IASAS system.

CONCLUSIONS

The following main conclusions can be drawn from the analysis of the results presented:

The comprehensive evaluation of advanced two-row barley breeding lines revealed significant differences in drought tolerance and agronomic performance. Line 492, fulfilling stringent criteria for uniformity, stability, and biological-economic traits, is recommended for inclusion in the official testing system as a candidate cultivar with potential for widespread cultivation in drought-prone regions.

The PEG 6000 osmotic stress assay effectively differentiated genotypes for drought tolerance at the seedling stage, with line 528 exhibiting the highest tolerance, whereas line 372 showed increased sensitivity, corresponding well with field drought response observations. Applying osmotic stress to germinating seeds is a good method for initial screening of large volumes of material in the early stages of the breeding process.

The integration of phenotypic, yield, and controlled physiological stress data provides a framework for selecting drought-tolerant barley cultivars adapted to changing environmental conditions.

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