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CONTENT

Tasbası, H., Asan-Ozusaglam, M. (2025). Investigation of biological effects of edible films developed with Citrus medica L. var. *sarcodactylis ethanol extract and lactic acid bacteria lysate and potential use in food and aquaculture. Agriculture and Forestry, 71 (4): 7-15. https://doi:10.17707/AgricultForest.71.4.01*

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INVESTIGATION OF BIOLOGICAL EFFECTS OF EDIBLE FILMS DEVELOPED WITH *Citrus medica* L. var. *sarcodactylis* ETHANOL EXTRACT AND LACTIC ACID BACTERIA LYSATE AND POTENTIAL USE IN FOOD AND AQUACULTURE

SUMMARY

Edible films (EF) have gained significant attention as a sustainable alternative to synthetic packaging, offering potential benefits in food preservation, environmental impact reduction, and increased recyclability. The aim of the study was to investigate the antimicrobial activity of EF containing *Citrus medica* L. var. *sarcodactylis* (fingered citron) ethanol (CE) extract and lactic acid bacteria (LAB) lysate. In this context, the inhibitory effect of CE extract on some food-borne and fish-borne microbial strains was evaluated by disc diffusion and microdilution assay. Additionally, the biological activities of EF prepared using natural components (CE extract and LAB lysate) were determined by well diffusion test. Disc diffusion assay results indicated that the highest inhibition zone diameter was recorded as 10.63 mm against *Escherichia coli* O157:H7, while the lowest value was determined as 7.91 mm against *Yersinia ruckeri*. The lowest value for minimum inhibitory (MIC) and bactericidal concentration (MBC) was determined against *Streptococcus agalactiae* Pas. Ins. 55118 (5 mg/ml), and the highest value was determined on *Vibrio anguillarum* M1 (40 mg/ml). In addition, the antimicrobial activity tests of the developed EF formulation stated that the highest inhibition zone diameter was measured as 11.37 mm against *E. coli* O157:H7, while the lowest value was determined as 4.49 mm against *V. anguillarum* M1. As a result, it was revealed that EF developed with CE extract and LAB lysate with good antimicrobial activity may have potential use as natural preservatives in the food and aquaculture industries.

Keywords: Fingered citron, Probiotic, Bioactive component, Pathogen, Natural preservative

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INTRODUCTION

Edible films (EF) are thin sheets that are applied to foods as a protective layer and can also be consumed (Galus *et al*., 2020). EF has attracted great attention with their potential to be used as an alternative to synthetic films. This can make a significant contribution to reducing environmental pollution. It can also simplify the structure of packaging materials by acting as a barrier. EF can increase recyclability compared to traditional environmentally unfriendly packaging (Bourtoom, 2008). Nowadays, solutions have been developed to prevent food spoilage and increase the nutritional value of food products by adding bioactive compounds and microorganisms (e.g. probiotics) to coating materials. These applications stand out as important techniques affecting inhibition of pathogens, product quality, ripening, thickening effect and shelf life (Díaz-Montes and Castro-Muñoz, 2021).

Plants are a rich source of antimicrobial substances that are effective against many bacteria. Some of the important components found in plants, polyphenols, are the most potent antimicrobial compounds. These components tend to have synergistic or antagonistic effects on pathogens (Hochma *et al*., 2021). Plant-based antimicrobials are found in plant parts such as roots, stems, leaves, flowers, fruits and seeds, providing a powerful alternative to antibiotics (Li *et al*., 2024). In this context, *C. medica* L. var. *sarcodactylis*, which attracts attention with its appearance, is a member of the Rutaceae family. It is mostly spread in Taiwan, China, Malaysia, India, Vietnam. In recent years, its cultivation has also begun in Türkiye. Its bark, leaves and root have been used as a folk medicine in the treatment of edema, hepatitis, headache, stomachache, arthritis and rheumatism (Chan *et al*., 2010). *C. medica* L. var. *sarcodactylis* leaves have been reported to have anti-inflammatory, antifungal, antioxidant and antimicrobial activities (Chan *et al*., 2010; El Hawary *et al*., 2022; Wu *et al*., 2021). However, most of the by-products of the plant, such as flowers and leaves, are discarded, leading to waste of valuable bioactive resources. It has been reported that Limonene is high among the main components of *C. medica* L. var. *sarcodactylis* leaves (Wu *et al*., 2021). It has been stated that the basic mechanism of antibacterial activity of limonene is to change the permeability of the membrane by causing damage to the cell membrane. Additionally, studies have shown that limonene not only inhibits pathogenic microbes that cause disease, but also protects various food products from potential contaminants (Gupta *et al*., 2021).

Probiotics, especially lactic acid bacteria (LAB), have an important place in the food industry (Abedin *et al*., 2024). LAB can be used to control microorganisms to prevent food spoilage. Some of these LAB strains, primarily from the Lactobacillus and Bifidobacterium genera, can promote health, known as probiotics (Mokoena *et al*., 2021). Also, the advantages provided by LAB in preventing or controlling infectious diseases in cultured fish are also taken into consideration. LAB are important members of the fish microbiota and have an antagonistic effect against opportunistic pathogens, fungi and viruses that pollute the aquatic ecosystem and cause diseases in aquatic organisms (Chizhayeva *et al*., 2022). For these reasons, LAB is therapeutically useful against many foodborne illnesses and is a highly recommended alternative for extending product shelf life (Zapaśnik *et al*., 2022). *Limosilactobacillus fermentum* strains produce various potent antimicrobial components that can be applied as food preservatives or alternatives to antibiotics (Naghmouchi *et al*., 2020). Therefore, it has been reported that edible coatings containing *L. fermentum* can increase the health value while increasing the shelf life of food (Najabi *et al*., 2024). In this study, the effects of EF developed using CE extract and LAB lysate on food quality and safety were investigated. In the first stage, the antibacterial activity of CE extract on some food-borne and fish originated pathogens was evaluated. Then, the antimicrobial effects of EF obtained with carrageenan and natural components (CE extract and LAB lysate) were investigated and its potential for use as a natural preservative for food and aquatic products was determined.

MATERIAL AND METHODS

Sample Collection and Extraction Method with Ethanol Solvent

Fresh *C. medica* L. var. *sarcodactylis* leaves were collected from the Muğla Köyceğiz Plant World located in Türkiye, in October 2022. The leaves were then air-dried in a dark environment. After drying, the leaf samples were ground into a fine powder. Extraction was carried out using ethanol as the solvent. A homogeneous mixture, consisting of 10 grams of leaf powder and 30 milliliters of water, was prepared and subjected to extraction in a water bath at 70°C for 24 hours. Following extraction, the remaining solvent was evaporated to yield the final extract. The prepared CE extract was stored at 4°C (Figure 1.).

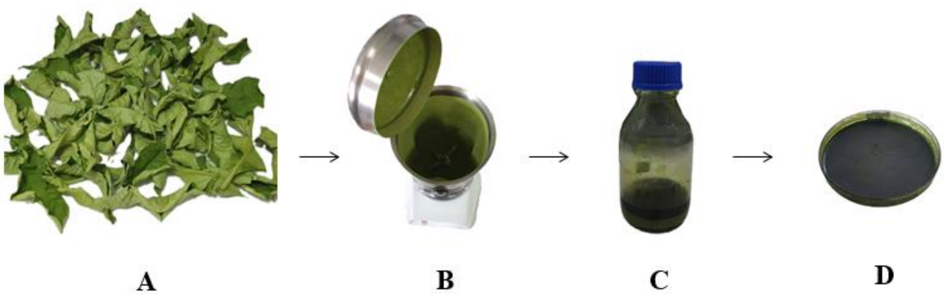

Figure 1. Extraction steps of *Citrus medica* L. var. *sarcodactylis* leaves: Dried leaf samples (A), grinding process (B), ethanol extraction (C), and concentrated extract (D).

Culture Conditions of Test Microorganisms

The biological activity of the CE extract on some pathogenic bacteria was determined. *Escherichia coli* O157:H7 (food-borne) and *Aeromonas hydrophila* ATCC 19570 (fish pathogen) were grown in Nutrient Broth at 37˚C. *Streptococcus agalactiae* Pas. Ins. 55118 (fish pathogen), *Yersinia ruckeri* (fish pathogen) were incubated in Tryptic Soy Broth at 37˚C. Fish pathogens *Vibrio anguillarum* A4 and *V. anguillarum* M1 were cultured in Tryptic Soy Broth-NaCl

(2%) medium at 25˚C. All bacterial cultures were maintained for 24 h incubation under appropriate conditions to ensure optimum growth.

Antibacterial Assay

The antimicrobial properties of the CE extract were evaluated using the disc diffusion assay (Tasbası and Asan-Ozusaglam, 2024). A microbial suspension standardized to a density of 0.5 McFarland was spread onto the solid medium. Sterilized 6 mm discs were placed in the medium three times and the extract solution (20 µl) prepared at a concentration of 100 mg/ml (2 µg/disc) was applied to each disc. The plates were then incubated for 24 hours under the conditions stated above. Positive control antibiotic disc contained Kanamycin (30 µg). Negative control used was DMSO.

Micro-Dilution Method

The micro-dilution assay was used to determine the minimum inhibition and bactericidal concentrations (MIC and MBC) of the CE extract on the above-mentioned bacteria (Tasbası and Asan-Ozusaglam, 2024). Firstly, solution (medium, bacterial suspension and CE extract) was prepared in tubes for the MIC value. The tubes were then incubated for 24 hours at the appropriate temperature specified above. As a result of incubation, the concentration in the tube with no microbial growth was determined as MIC. For the MBC value, 5 µl samples were taken from tubes containing various concentration of the extract (2.5, 5.0, 10.0, 20.0, 40.0, 80.0 mg/ml) and applied to solid media. Then, microbial growth was observed by incubating 24 hours. At the end of this period, the concentration at which microbial growth stopped completely was defined as MBC value.

Determination of Antibacterial Activity of Edible Film Containing Extract and Lactic Acid Bacteria

EF developed with CE extract and LAB components was tested against the bacteria by modifying the procedure described by Venkatachalam *et al*. (2023). In the formulation where EF were prepared, CE extract (10% w/v), LAB lysate isolated from human milk (*L. fermentum* MA-7), carrageenan gum and glycerol (1% and 3% w/v) were used. The present study includes four experimental groups: Gum (G/Control), Gum-Lysate (GL), Gum- CE Extract (GCE), and Gum- CE Extract-Lysate (GCEL). In the determined groups, the final volume of the groups without LAB was completed with water, while the final volume of the other groups was completed with LAB lysate. The film mixtures were mixed with a magnetic stirrer at 90˚C for 30 minutes. The biological activity of the homogenized EF was first determined. Then, drying was performed in a Pasteur oven until constant weight was reached for the analysis of mechanical properties. For the biological activity of the film mixtures, sterilization was performed with a filter (0.45 µm). The culture adjusted to 0.5 McFarland (100 µl) was added to each petri dish and the film mixture (100 µl) was added to the wells with a diameter of 6 mm. The petri dishes were incubated and kept in the environment and temperature conditions suitable for pathogens. Then, the diameters of the inhibition areas were determined using calipers.

Statistical analysis

One-way ANOVA test was performed with the SPSS program (GNU) to determine significant differences between the values of antimicrobial activity. The statistical significance of the change between the determined mean values was assessed using the Tukey test ($p < 0.05$).

RESULTS AND DISCUSSION

Pathogenic microorganisms have been a threat since the beginning of human history and have been one of the leading causes of disease and death in humans (Górniak *et al.*, 2019). In this context, the antimicrobial activity of CE extract containing biologically active components against pathogens was evaluated. The results obtained against various microbial strains important for the safety of the food and aquaculture industries are given in Table 1.

Table 1. Antibacterial activity and micro-dilution assay of *C. medica* L. var. *sarcodactylis* leaf extract

Microorganisms	**Inhibition Zone Diameters (SD±mm)**		**MIC (mg/ml)**	**MBC (mg/ml)**
	CE Extract	**Kanamycin (30 µg)**		
E. coli O157:H7	10.63 ± 0.39^{a}	15.85 ± 0.58^{a}	10	20
S. agalactiae Pas. Ins. 55118	$9.69 \pm 0.51^{a,b}$	19.21 ± 3.06^{a}	5	5
V. anguillarum A4	8.85 ± 0.55^{b}	6.28 ± 0.17^{b}	20	20
V. anguillarum M1	10.11 ± 0.21^{a}	$-^{c}$	40	40
A. hydrophila 19570 ATCC	$8.33 \pm 0.41^{c,b}$	10.58 ± 0.24^{d}	5	20
Y. ruckeri	$7.91 \pm 0.20^{c,b}$	$12.50 \pm 1.43^{a,d,e}$	10	40

*CE: *C. medica* L. var. *sarcodactylis* ethanol, MIC: Minimal inhibition, MBC: Minimal bactericidal concentrations, CE Extract F(*Sig*): 20.962(0.000), Kanamycin F(*Sig*): 71.541(0.000).

According to the Table 1, *E. coli* O157:H7 (10.63 mm) and *V. anguillarum* M1 (10.11 mm) stand out among microbial strains with similar and high inhibition zone diameters. No statistically significant difference was found between these food-borne and fish pathogens ($p > 0.05$). However, a significant difference was determined when compared with other microbial strains ($p < 0.05$). The lowest inhibition zone diameter was determined as 7.91 mm against *Y. ruckeri*. It was obtained that CE extract was more effective against *V. anguillarum* A4 than the traditional antibiotic Kanamycin (30 µg, positive control). In fact, although *V. anguillarum* M1 was resistant to Kanamycin, the CE extract showed antimicrobial activity on this bacterium with an inhibition zone diameter of 10.11 mm, which was an important result. On the other hand, although the inhibition zone diameters of the CE extract are low compared to Kanamycin, the effect can be increased with appropriate concentration adjustments. CE extract, which is not a pure compound, shows good potential for antimicrobial activity with good inhibition zone diameters. It was determined that DMSO solvent (negative control) had no effect on the tested microorganisms.

One of the most dangerous foodborne pathogens, *E. coli* O157:H7, poses a major threat to human health, public safety, and economic property worldwide (Bai *et al.*, 2022). *V. anguillarum* is a pathogenic bacterium that poses a significant risk to the seafood industry, can lead to foodborne diseases, and can cause infections known as "vibriosis". Vibriosis causes serious problems both from a food safety and economic point of view, and prevention and treatment methods are usually quite costly (Hickey and Lee, 2018). Although the highest two inhibition zones were obtained against these pathogenic bacteria, the lowest MIC and MBC values of the CE extract was identified on *S. agalactiae* Pas. Ins. 55118. According to the MIC and MBC values, CE extract exhibited the highest antimicrobial activity on *S. agalactiae*, as compared to the other bacteria that were tested. These findings indicate that CE extract can be used as an effective antimicrobial substance especially against certain food-borne and fish pathogens.

The studies conducted with *C. medica* L. var. *sarcodactylis* leaves are limited. In addition, it has been reported that the inhibition zone diameters of dry and wet lemon grass ethanol extracts against *E. coli* at concentrations of 250, 500 and 1000 ppm varied between 3.10-5.40 mm (Nyamath and Karthikeyan, 2018). The MIC values of *C. hystrix* leaf and *C. maxima* methanol red leaf extracts against *E. coli* MTCC 40 were determined as 50 and 75 mg/ml. The antimicrobial activity of the leaf extract was determined to be directly related to the bioactive compounds they contain (Abirami *et al*., 2013). In this study, it was determined that CE extract with lower MIC and MBC values (10 and 20 mg/ml) had stronger antimicrobial activity on *E. coli* O157:H7. In a study conducted by Asan-Ozusaglam *et al*. (2021), the inhibition zone diameters of methanol extract prepared from kumquat leaves against *V. anguillarum* A4 and M1 were reported as 24.41 and 24.80 mm. MIC and MBC values on microbial strains were also determined as 25 mg/ml. Comparing this study with our current study, it was determined that CE extract had lower inhibition zone diameter against *Vibrio anguillarum* M1 and A4 strains, but showed higher antimicrobial activity on *Vibrio anguillarum* A4 with lower MIC and MBC values (20 mg/ml).

The antibacterial activity of EF formulations was determined using the well diffusion method and the results are given in Table 2. Gum (G/control) group did not show inhibitory activity against the tested microorganisms. In the Gum-Lysate (GL) group, inhibition zone diameters were determined against other test microorganisms except *Y. ruckeri*. The highest inhibition zone diameter was determined as 10.11 mm against *A. hydrophila* 19570 ATCC. In the Gum-Extract (GE) group, 1.63 mm of inhibition zone diameter was detected against *E. coli* O157:H7, while no activity was observed against other test microorganisms. Statistical analysis showed significant differences between all tested EF groups for *E. coli* O157:H7 ($p<0.05$). Also, a significant difference was found between the GL and GCEL groups ($p<0.05$). In addition, it was observed that the inhibition zone diameters increased significantly between the GL and the GCEL groups. In GCEL group, the inhibition zone diameters of CE extract on the tested bacteria except for *Y. ruckeri* were higher than GL group. While there was no inhibitory effect against *Y. ruckeri* in G, GL and GCE groups, antimicrobial activity was observed in GCEL group with an inhibition zone diameter of 6.09 mm. When evaluated for all tested bacteria, antibacterial activity or higher antimicrobial activity was detected in the GCEL group compared to the other EF groups. This may be due to the synergetic effect of *L. fermentum* MA-7 lysate and CE extract. These results indicate that CE extract and LAB lysate may provide a natural antimicrobial solution against food and fish pathogens with their rich bioactive compound contents. In particular, the combination of CE extract and LAB lysate is promising for functional applications in food, pharmaceutical and aquaculture industries.

Table 2. Antibacterial activity of EF groups prepared with CE extract and/or LAB lysate

Microorganisms	**Inhibition Zone Diameters (SD±mm)**				
	Gum (G/Control)	**Gum-Lysate (GL)**	**Gum-CE Extract (GCE)**	**Gum-CE Extract-Lysate (GCEL)**	**F (*Sig*)**
E. coli O157:H7	-[a]	3.27±0.10[b]	1.63±0.30[c]	11.37±0.36[d]	1328.951 (0.000)
S. agalactiae Pas. Ins. 55118	-[a]	2.36±0.12[b]	-[a]	5.59±0.49[c]	328.084 (0.000)
V. anguillarum A4	-[a]	2.73±0.11[b]	-[a]	4.49±0.16[c]	1570.882 (0.000)
V. anguillarum M1	-[a]	4.24±0.33[b]	-[a]	6.30±0.55[c]	294.746 (0.000)
A. hydrophila 19570 ATCC	-[a]	10.11± 0.14[b]	-[a]	10.25±0.18[b]	7701.243 (0.000)
Y. ruckeri	-[a]	-[a]	-[a]	6.09±0.07[b]	22578.270 (0.000)

* CE: *C. medica* L. var. *sarcodactylis* ethanol, -: no inhibition zone diameter

Febriana *et al*. (2021) reported that edible coatings protect fillets from spoilage by reducing water vapor, aroma, and lipid transmission. In their study, they examined the effects of adding *C. hystrix* leaf essential oil to the gelatin coating on the shelf life of red sea bream fish, which deteriorates rapidly due to its high protein and water content. According to the research results, the edible coating method was determined to be an effective approach in extending the shelf life of red sea bream fillets at room temperature and preventing bacterial growth. In addition, Azizah *et al*. (2023) evaluated the effects of adding pectin and lemongrass essential oil to gelatin film on its properties, chicken breast quality preservation potential and antimicrobial activity. It was revealed that the edible film had an inhibitory effect on *Salmonella* and its application to chicken breast could maintain its quality during storage. In another study, antimicrobial film was obtained from *C. aurantifolia* leaf extract. The addition of leaf extract to the film was found to have effective activity in growth inhibition against *Staphylococcus aureus* (Asria and Elizarni, 2015). In our study, it was determined that edible film obtained from CE extract and/or LAB lysate could show significant inhibitory activity against pathogens that can be observed especially in the food and aquaculture industries.

CONCLUSIONS

The use of CE extract as a ‘natural’ antibacterial agent in the food and aquaculture industry and the potential application areas of the prepared EF were investigated. Consumer interest in natural preservatives that can replace chemical additives has increased significantly in recent years. Our study revealed that EF containing bioactive components (CE extract and LAB lysate) exhibited a strong antimicrobial effect against important pathogens in the food and aquaculture industry. This offers a solution for the development of films with natural and antimicrobial properties that can prevent spoilage by extending the shelf life of foods. The statistical significance of the CE extract's antimicrobial activities underscores its potential as a promising natural additive for food and aquaculture applications.

This effect may be due to limonene, a terpene found in high amounts in FC leaves. In addition, it has been determined that CE extract can lead to the development of new plant-based products as a safe option. The findings obtained from in-vitro studies of CE extract support the applicability of this extract in different areas such as food and aquaculture and show that it can form the basis for in-vivo research with cell culture. In this way, CE extract with antimicrobial activity offers potential application areas in various industries.

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URBAN TREE CARBON STOCK ASSESSMENT BASED ON REMOTE SENSING AND FIELD INVENTORY DATA

SUMMARY

The increasing concentration of CO_2 in the atmosphere is one of the causes that has lead to what we define as global warming with dramatic events on ecosystems, biodiversity and human life. An accurate prediction of aboveground biomass (AGB) and carbon stock is crucial for the assessment of forest and urban trees' contributions to climate game. The objective of this study was to establish a reliable way to estimate aboveground biomass and potential carbon stock by integrating remote sensing methods with ground-based measurements. The procedure consisted of the following three major steps: (1) calculation of NDVI, GNDVI and SAVI; (2) systematic sampling of field plots using species distribution by biomass and carbon quantification through allometric equations according to the species; and (3) exponential regression between biomass vs vegetation indices. Results indicated that GNDVI has the maximum correlation with biomass in comparison with NDVI, and SAVI. When extrapolated, GNDVI model predicted 2798.23 and 1315.17 tons of above ground biomass and carbon respectively. In the field, biomass and carbon was measured as 4,650.75 tons and 2,185.85 tons respectively. These results also highlight the significance of integrating remote sensing and field data for precise estimation of biomass and

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carbon, providing useful information even for resource management, urban green planning and global climate change countermeasures such as a carbon credit program.

Keywords: remote sensing, urban forest, aboveground biomass, carbon sequestration, allometric equations, carbon credit, net zero

INTRODUCTION

Global warming and climate change are now great international concerns. One of the major contributors to these issues, is the rise in greenhouse gases (GHG), specifically carbon dioxide (CO_2), released into the atmosphere from human activities like industrial processes, transportation and everyday energy use (Uttaruk and Laosuwan, 2020). The effects of the change in climate are now becoming increasingly evident across the globe (Angkahad *et al*., 2025a). Biodiversity is also affected by increasing global temperature, which has led to the adaption of many species into new areas (Muluneh, 2021). Others will go extinct if they don't evolve fast enough. Further, many ecosystems are deteriorating, causing imbalances in the natural elements that sustain life. These secondary effects reach outside of the natural environment and are beginning to impact human living standards from local issues up to a global level.

Farmers attempt to adapt their farming practices to changing climate conditions, such as unpredictable rainfall and droughts that reduce crop yields (Semosa, 2025). Public health is also under threat, with communities experiencing increases in heat-related illnesses and greater exposure to air pollution (Ofremu *et al*., 2025). In this regard, the ecosystem services (involving urban trees) have been referred to one of the key strategies for both climate change adaption and mitigation (Rahim *et al*., 2024). Atmospheric CO_2 is captured by trees and stored as organic carbon in their AGB (Laosuwan *et al*., 2025a; Uttaruk *et al.,* 2024). In addition to carbon sequestration, they serve as a critical mitigation against heat-related urban heat islands, air pollutions via filtration and therapeutic green spaces that benefit both physical and psychological health for citizens living in the city (Wolf *et al.,* 2020).

Aboveground biomass (AGB) is a principle parameter commonly used to estimate the carbon sequestered in vegetation because it is directly related to carbon stored in different components of a tree including tree trunks, branches, leaves and roots (Laosuwan *et al.,* 2025b; Saengpradit *et al.,* 2024; Vattanavongsiri *et al.,* 2023). The estimation of AGB in various zones is valuable tool for sustainable natural resource management, urban planning and environmental restoration. In general, the estimation of AGB is based on measurements of the physical characteristics of trees such as tree height, DBH and crown size (Bhebhe *et al.,* 2025; Liu *et al*., 2024a). These measurements can be employed in allometric equations to estimate aboveground biomass and carbon stocks precisely (Laosuwan *et al*., 2023).

Environmental data, in particular for biomass and carbon studies, can be effectively gathered and analyzed by remote sensing technology. Remote sensing allows for fast and reliable results since it minimizes fieldwork (Lamenkova, 2024) as well as time and labor demands, by making images coming from satellite or aerial available (Roy *et al*., 2016). One of their main advantages is the long-term monitoring of biomass changes by which researchers can assess the effects of land use, resource management practices and environmental policies (Dhiman and Kumar, 2024; Castillo *et al.,* 2017). As a result, remote sensing has become one of the pivotal tools for progressive and sustainable resources management and supporting scientific based decision-makings (Angkahad *et al.,* 2025b).

In urban environments, predicting AGB and carbon storage highlights the significant contributions of urban ecosystems to climate change abatement (Aabeyir *et al*., 2020). The expansion and maintenance of urban green spaces not only absorbs atmospheric CO_2 but also helps reduce the urban heat island effect, improve local air quality, and increase physical and psychological welfare for residents (Xiao *et al.,* 2022). Thus, bridging remote sensing approaches with urban forestry work provides a powerful avenue for greener and more climate-resilient city design (Wavrek *et al*., 2023). Because the carbon stored in trees is closely related to their biomass, an accurate prediction would have to consider structural parameters such as tree height, diameter at breast height and crown width. These parameters are frequently used in species-specific allometric equations, and are predictive of aboveground biomass and carbon estimates (e.g., Ngomanda *et al*., 2014; Van Breugel *et al*., 2011). The knowledge gained from such research is essential for the development of local and national climate policy, for CO_2 mitigation strategies, and adaptive planning with future climate scenarios (IPCC, 2022). Given these challenges and opportunities, there is a clear need for more reliable methods of assessment. Accordingly, the present study proposes an integrated approach that combines remote sensing data with field measurements to improve the accuracy of AGB and carbon stock estimation in urban trees.

MATERIAL AND METHODS

Study Area

The study of carbon storage from urban trees was conducted in the campus of Mahasarakham University (Figure 1), Talad Sub-district, Mueang Maha Sarakham District, Maha Sarakham Province, Thailand. The site has coordinates of E 316471.52 and N 1791741.78 and Maha Sarakham is in predominating flat plain with slight undulating hills, the elevation ranges from 130 to 230 meters above sea level. The climate of the area is tropical monsoon type, with strong influences from the southwest monsoon wind flow from Indian Ocean in summer season giving distinct warm wet and dry season. This region has an average annual temperature of 27.4 °C, and the mean monthly temperatures vary from around 22.4 °C during the coldest months to even 33.7 °C in the hottest months.

April is usually the full-swing summer and the hottest month of the year.

Other than the climate, the campus of Mahasarakham University is endowed with mixed land use such as academic buildings, open green fields and random tree clusters to enhance ecological stability of the campus. These downtown trees help provide cover for sidewalks, they reduce the temperature of the walking surfaces and they improve the quality of air that students and workers breathe. This green envelope of the campus is an ideal living laboratory for research in carbon sequestration, urban forestry and sustainable environmental management.

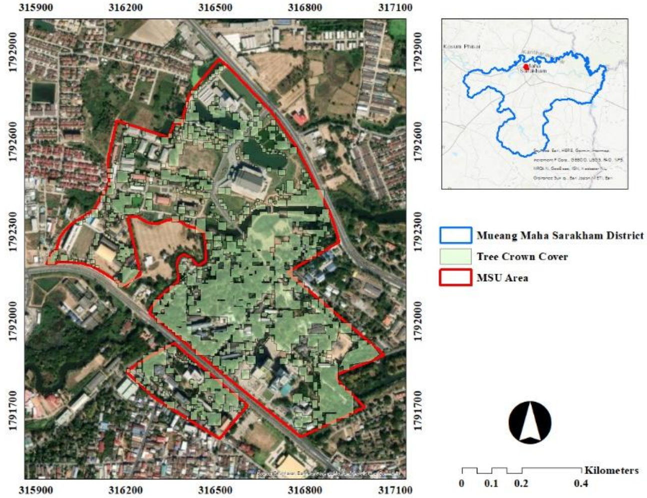


Figure 1. Urban trees within the campus of Mahasarakham University

Tree Physical Data Analysis

The field survey in this research was conducted to collect information on tree species and physical attributes of each individual specimens sample in the study site. This measurement was performed in 30 sample plots of 20 m x 20 m each. Within each plot, measurements were obtained from all trees >4.5 cm DBH. Tree diameter at breast height (DBH) was measured with a diameter type and tree height (H) was recorded with a laser rangefinder. These two variables, DBH and H, are the basic measures of tree growth and form.

The measurements obtained were subsequently used in an allometric equation to convert DBH and H into aboveground biomass (AGB) values, representing the amount of biomass stored in each tree (Equation 1) (Ogawa *et al*., 1965). Once AGB values were determined, they were applied to a carbon conversion equation (Equation 2) to estimate the quantity of carbon sequestered in the trees (Intergovernmental Panel on Climate Change, 2006). This approach, combining allometric equations for biomass and carbon estimation, is a key step in evaluating the environmental value of trees at the local scale. Moreover, the resulting data provide a scientific foundation for academic research and can inform effective planning and management of urban green spaces.

$$
\begin{aligned}
W_S &= 0.0396(D^2H)^{0.933} \\
W_B &= 0.00349(D^2H)^{1.030} \\
W_L &= \left(\frac{28}{W_S + W_B} + 0.025\right)^{-1} \\
W_T &= W_S + W_B + W_L
\end{aligned}
\qquad (1)
$$

D denotes diameter at breast height (cm), H represents tree height (m), WS indicates stem biomass (t ha^{-1}), WB represents branch biomass (t ha^{-1}), WL denotes leaf biomass (t ha^{-1}), and WT refers to total aboveground biomass (t ha^{-1}).

$$C_j = W_{tc} \times FC \tag{2}$$

C_j represents the carbon storage potential of an individual tree (kg), W_tc denotes its aboveground biomass (kg), and FC is the carbon fraction of biomass, which is assumed to be constant at 0.47.

Satellite Image Data Processing

Sentinel-2 satellite images were used as a primary source of data for the assessment of vegetation conditions within the study area. In this analysis, three commonly applied vegetation indices, namely NDVI (normalized difference vegetation index) (Tucker 1979), GNDVI (green normalized difference vegetation index) (Gitelson *et al*., 1996) and SAVI (soil adjusted vegetation index) (Huete 1988), were derived as described in Equation 3–5. When the vegetation index values were obtained, they were used to calculate FC (Fractional Green Vegetation Cover) in a second step according to Equation 6, to transform the index values into physical takes on surface fraction of green canopy. The selection of these indices is important due to the information they have embedded about vegetation cover, phenological stages and general condition of plants within our study area, which in combination provides a sound basis for conducting spatial analysis to estimate aboveground biomass and carbon stocks at the landscape level.

$$NDVI = \frac{NIR - RED}{NIR + RED} \tag{3}$$

The Normalized Difference Vegetation Index (NDVI) is a simple yet powerful indicator of green vegetation, comparing near-infrared reflectance with red-light absorption by chlorophyll. NDVI values range from −1 to 1, with negative values typically indicating water bodies, values near zero (−0.1 to 0.1) representing barren areas such as rock, sand, or snow, moderate positive values (≈0.2–0.4) indicating shrubs and grasslands, and values approaching 1 corresponding to dense temperate and tropical forests.

$$GNDVI = \frac{NIR - GREEN}{NIR + GREEN} \tag{4}$$

The Green Normalized Difference Vegetation Index (GNDVI) is widely used to assess plant greenness and photosynthetic activity, particularly for evaluating water and nitrogen uptake within the crop canopy. Its values range from −1 to 1, with negative values (−1 to 0) indicating water or bare soil. GNDVI is especially useful during the intermediate and late stages of crop growth.

$$SAVI = \frac{(NIR - RED) \times (1 + L)}{(NIR + RED + L)} \qquad (5)$$

NIR denotes the reflectance in the near-infrared band, Red represents the reflectance in the red band, and L is the soil brightness correction factor that reduces the influence of soil background on the vegetation signal. The value of L generally ranges from 0 for areas with very dense vegetation to 1 for bare soil, with 0.5 commonly applied in regions with moderate vegetation cover.

$$FC = \frac{Index - Index_{soil}}{Index_{forest} - Index_{soil}} \qquad (6)$$

Index refers to the vegetation index values used for analysis. Indexforest represents the vegetation index values of areas with the densest vegetation cover, while IndexSoil represents the vegetation index values of areas without vegetation (bare soil).

Correlation Analysis

The relationship between Fractional Green Vegetation Cover (FC) and aboveground biomass (AGB) density was evaluated using an exponential regression model, which has proven to be effective for estimating biomass from Sentinel-2 satellite data. The general form of the regression model is expressed in Equation 7.

$$AGB = a \times b^{e \times FC} \qquad (7)$$

a represents the intercept of the regression curve, and b is the regression coefficient that determines the rate of biomass increase as FC rises. The analysis revealed that AGB increases exponentially with FC, confirming a strong positive relationship between vegetation cover and biomass accumulation in the study area.

Model Accuracy Assessment Using Root Mean Square Error (RMSE)

The accuracy of the model in estimation of AGB density was examined by comparing predicted values with the field-measured observations. The model evaluation criterion was the Root Mean Square Error (RMSE), which is defined as in Equation 8. RMSE is measured from the observed and predicted values, where each difference (observed – predicted value) is squared; then an average of the squared differences is computed, and finally you square root to get back to the original range.

RMSE can be used to quantify the degree of prediction error, with lower

values indicating the model's performance and hence lower deviation between prediction and measurements. In the present study where satellite derived VIs were employed to estimate AGB, RMSE offered an important criterion for validating model robustness. Additionally, RMSE can be used as a diagnostic measure for analyzing potential error sources and model improvement, thus helping to secure biomass assessments in other landscape types.

$$RMSE = \sqrt{\Sigma\ (Pi\ -\ Oi)^2\ /\ n} \tag{8}$$

P_i represents the predicted biomass value derived from the vegetation index, O_i denotes the observed biomass value obtained from field measurements, and n indicates the total number of samples.

RESULTS AND DISCUSSION

This section presents the findings of the study, beginning with the results of field data collection and the calculation of aboveground biomass (AGB) and carbon stock, followed by the analysis of vegetation indices, model development, and accuracy assessment. The discussion integrates these results to highlight key patterns, interpret their ecological significance, and evaluate the potential of remote sensing for large-scale biomass and carbon estimation.

Results of Tree Physical Data Processing

Field surveys were conducted from 4 to 5 October 2024, coinciding with the end of the rainy season—an ideal period for data collection due to favorable weather that enabled efficient and uninterrupted measurements.

Table 1. Results of Tree Physical Data Processing

Plot	Mean DBH (cm)	Mean Height (m)	Branch Biomass (kg)	Stem Biomass (kg)	Leaf Biomass (kg)	Total AGB (kg)	Total AGB (t/400 m²)	Total AGB (t/ha)
01	21.23	7.23	235.08	1,156.81	49.72	1,441.61	1.44	36.04
02	21.69	5.43	1,079.90	3,949.66	179.64	5,209.20	5.21	130.23
03	22.36	8.23	271.52	1,338.01	57.49	1,667.02	1.67	41.68
04	19.78	7.94	310.43	1,501.53	64.73	1,876.69	1.88	46.92
05	38.48	12.00	1,439.85	5,754.56	256.95	7,451.36	7.45	186.28
06	35.65	10.67	476.37	2,043.86	90.01	2,610.25	2.61	65.26
07	37.40	14.13	1,104.23	4,421.06	197.34	5,722.63	5.72	143.07
08	28.46	10.60	489.38	2,206.36	96.29	2,792.02	2.79	69.80
09	67.48	21.75	2,032.66	7,474.00	339.53	9,846.19	9.85	246.15
10	65.49	17.50	2,023.25	7,208.00	329.69	9,560.95	9.56	239.02
11	58.09	19.50	2,958.65	11,109.71	502.45	14,570.81	14.57	364.27
12	31.19	10.14	600.32	2,424.50	108.04	3,132.86	3.13	78.32
13	7.79	5.74	48.15	271.45	11.43	331.03	0.33	8.28
14	22.18	8.89	456.61	1,939.73	85.59	2,481.94	2.48	62.05
15	35.57	10.25	870.19	3,120.51	142.53	4,133.22	4.13	103.33
16	30.74	13.00	1,181.44	5,036.95	222.10	6,440.48	6.44	161.01
17	14.26	7.21	291.33	1,356.14	58.86	1,706.33	1.71	42.66
18	29.33	10.43	1,116.56	4,031.71	183.87	5,332.14	5.33	133.30
19	27.25	12.13	2,817.58	9,878.23	453.44	13,149.25	13.15	328.73
20	28.03	10.50	905.27	3,824.41	168.93	4,898.61	4.90	122.47
21	87.54	21.00	1,842.89	6,350.37	292.62	8,485.88	8.49	212.15
22	17.54	6.91	113.09	595.72	25.32	734.13	0.73	18.35
23	16.64	7.00	104.51	554.43	23.54	682.49	0.68	17.06
24	54.37	13.20	1,442.02	5,300.96	240.83	6,983.80	6.98	174.60
25	32.20	11.50	700.38	2,761.89	123.66	3,585.93	3.59	89.65
26	43.99	9.80	587.15	2,422.83	107.50	3,117.48	3.12	77.94
27	127.32	15.00	1,231.16	4,195.18	193.80	5,620.14	5.62	140.50
28	25.46	9.77	876.49	3,972.19	173.19	5,021.87	5.02	125.55
29	32.07	8.13	359.79	1,643.35	71.55	2,074.69	2.07	51.87
30	20.51	7.29	213.80	1,085.46	46.41	1,345.68	1.35	33.64

In each sample plot, all trees were measured for diameter at breast height (DBH) and total height (H), and the geographic coordinates of each plot were recorded using a GPS device to ensure spatial accuracy. The information showed great mutation of tree features among the plots. AGB (aboveground biomass) was estimated employing species-specific allometric equations using DBH and height values, shown in Table 1.

The total AGB of the study area is estimated to be 4,650.75 tons which means that there are approximately 2,185.85 tons of carbon in stock. Positive linear relationship was found between biomass accumulator, mean DBH and tree height. For instance, Plots 11 and 27 with mean DBH of 58.09 cm and 127.32 cm had total biomass of 14,570.81 kg and 5620.14 kg respectively. Plots 21 and 19 also had high biomass values at 8,485.88 kg and 13,149.25 kg respectively. In tons per hectare (t/ha), Plot 11 had the highest biomass density with 364.27 t/ha, whereas plot 13 recorded the lowest with only 8.28 t/ha of biomass.

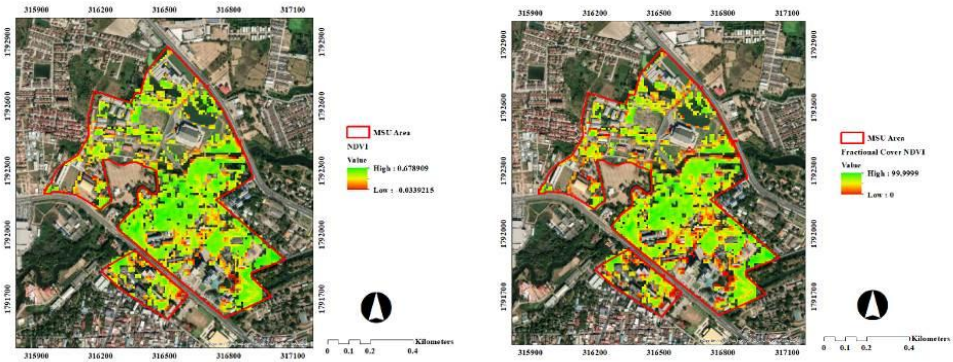
These findings may reflect the differences in the structure of forest stands of the study region, being mixed species and uneven aged forests where competition for light, nutrients and space could cause to vary growth patterns. The high positive correlation between DBH and biomass is consistent with studies of the Chave *et al*. (2014) and Saatchi *et al.* (2021), indicating that DBH is a good predictor of AGB.

Very high biomass density in Plot 11 likely indicates mature and highly stocked trees with high potential for carbon sequestration, while low biomass density in Plot13 may be due to younger stands or lower density of trees, or recent disturbances such as thinning, natural mortality. That variation really drive home the value of spatially explicit measurements and stratified sampling in landscape level biomass estimation. Management wise, conservation values of high biomass plots are important due to their significant impact on carbon sequestration and the low biomass areas may be targeted for rehabilitation or enrichment planting. Combined, this information forms the basis for informed decisions in forest management and climate mitigation plans.

Results of Satellite Image Analysis

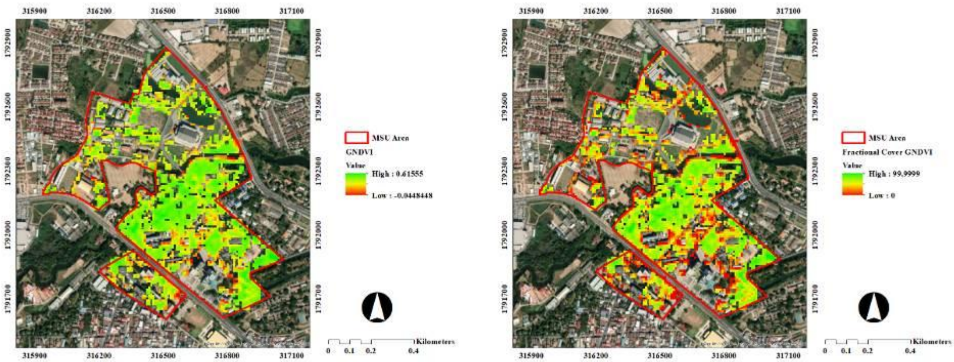
Satellite imagery from Sentinel-2 was utilized to evaluate vegetation indices across the Mahasarakham University area, providing a spatially explicit assessment of vegetation cover.

Three major indices were analyzed: NDVI (Normalized Difference Vegetation Index), GNDVI (Green Normalized Difference Vegetation Index), and SAVI (Soil-Adjusted Vegetation Index). The spatial patterns of these indices are illustrated in Figures 2 to 4, while their quantitative results are summarized in Table 2.



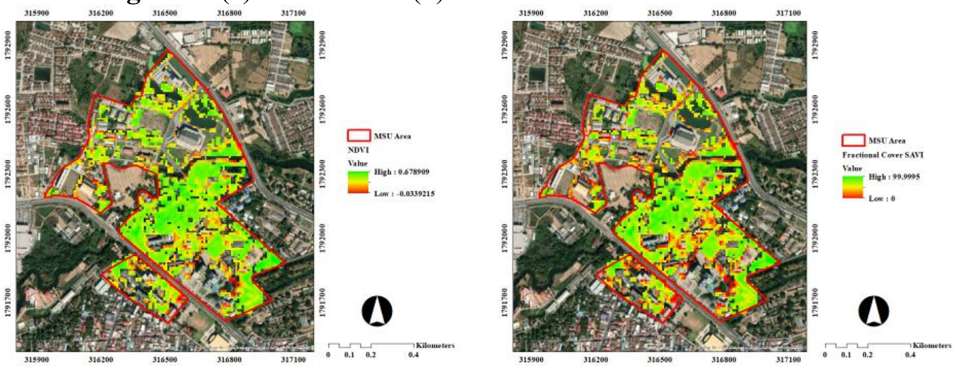
(a) (b)

Figure 2. (a) NDVI and (b) NDVI-derived Fractional Cover



(a) (b)

Figure 3. (a) GNDVI and (b) GNDVI-derived Fractional Cover



(a) (b)

Figure 4. (a) SAVI and (b) SAVI-derived Fractional Cover

Table 2. Fractional cover (%) derived from NDVI, GNDVI, and SAVI for the 30 sample plots

Plot	Fractional Cover (NDVI)	Fractional Cover (GNDVI)	Fractional Cover (SAVI)
1	61.15	48.62	61.15
2	69.17	58.15	69.17
3	75.91	65.02	75.91
4	73.43	64.29	73.43
5	67.17	55.91	67.17
6	75.20	66.05	75.20
7	64.97	51.54	64.97
8	57.75	45.84	57.75
9	77.01	69.48	77.00
10	73.56	64.39	73.56
11	58.19	46.17	58.19
12	72.68	63.83	72.68
13	84.75	77.76	84.75
14	75.60	68.10	75.59
15	76.37	67.60	76.37
16	41.81	32.78	41.81
17	82.75	74.08	82.74
18	79.07	71.33	79.07
19	83.27	76.89	83.27
20	80.99	73.68	80.99
21	42.54	32.93	42.53
22	84.97	74.92	84.96
23	87.21	83.20	87.21
24	66.64	56.92	66.64
25	45.91	37.92	45.91
26	69.82	61.19	69.82
27	82.61	72.73	82.61
28	74.54	67.04	74.54
29	63.15	49.50	63.15
30	74.29	67.07	74.28
Min	41.81	32.78	41.81
Max	87.21	83.20	87.21
Mean	70.75	61.50	70.75
SD	12.13	13.25	12.13

The NDVI values (Figure 2a) varied from −0.03 to 0.68, while the fractional green cover (Figure 2b) ranged between 0.00 % and 100.00 %. The GNDVI, in Figure 3a, was observed over the whole university surface area (in red) with similarly ranged (−0.03 to 0.68) values of fractional cover (Figure 3b) from 0.00 to 100.00 %. For SAVI, the numerical range was slightly wider (−0.05–1.02; Fig. 4a), though coded with values of fractional cover across an identical range from 0.00 to 100.000 %. Overall, these 3 indices provide a clear spatial delineation to green vegetation, representing the variability in canopy density across the landscape and forming an essential reference for further biomass and carbon sequestration investigations.

Fractional cover values are displayed for all 30 sample plots in Table 2 according to the three indices. NDVI varied from 41.81 % to 87.21 % with a mean of 70.75 % and a standard deviation (SD) of 12.13, signifying relatively dense vegetation in general condition for plots with moderate variability among the plots. GNDVI presented a mean of 61.50 % (range: 32.78–83.20 %, SD=13.25), slightly lower due to its strongest sensitivity to chlorophyll content. SAVI values were highly consistent with NDVI in minimum, maximum and mean values, which verified that SAVI was capable of adjusting the soil background effect and enhancing detection accuracy of vegetation in different soil environments.

At the plot-level, Plots 23, 19 and 22 showed the largest fractional cover across all indices reflect relatively vigorous growth of vegetation and a dense canopy cover; in contrast Plots 16 and 21 displayed the smallest values most likely due to site specific limitations i.e. drought stress, reduced stand density or soil infertility. The small differences between the indices illustrate the complementary nature of these: while NDVI gives an overall greenness measure, GNDVI picks up signals on chlorophyll concentration by leveraging information in the green band and SAVI accounts for soil influence - hence is particularly applicable in sparsely vegetated land surfaces. In general, simultaneous use of several vegetation indices leads to more thorough and reliable explanation of site properties. This integration will improve the empirical and spatial base of later modelling efforts to estimate aboveground biomass and carbon stock potential.

Analysis of the Relationship

This section analyzes the relationship between Fractional Vegetation Cover (FC) and Aboveground Biomass (AGB) using an exponential regression model, as presented in Figure 5. The results indicate that the GNDVI-based model provided the strongest relationship, achieving a coefficient of determination (R^2) of 0.7201. This finding demonstrates that variations in biomass can be reliably explained by FC values derived from GNDVI. The regression equation describing this relationship is expressed in Equation 9.

$$AGB=3.9972\times^{e0.0533\times FC}\ (R^2=0.7201) \qquad (9)$$

where AGB is the estimated aboveground biomass (kg per plot) and FC is the fractional vegetation cover (%) derived from GNDVI.

The strength of GNDVI lies in its sensitivity to both the near-infrared and green spectral bands, which are closely associated with chlorophyll concentration and vegetation density. This spectral responsiveness allows GNDVI to capture vegetation greenness more precisely, resulting in a stronger and more consistent

relationship between FC and AGB compared to other indices.

During the modeling process, the number of sample plots was reduced from 30 to 10 to enhance the robustness of the regression. This refinement was necessary because some plots exhibited irregular tree distribution patterns, particularly those with only a few large trees, which led to disproportionately high biomass values relative to their fractional cover. Such anomalies introduced bias and increased model error, thereby lowering the overall R^2.

By selecting plots with more homogeneous vegetation structure and clearer FC–AGB patterns, the reliability and predictive power of the model improved. Following this refinement, the R^2 value increased, confirming better model performance and reduced influence from outliers. Consequently, the final regression equation is more accurate and well-suited for predicting aboveground biomass in similar ecological settings.

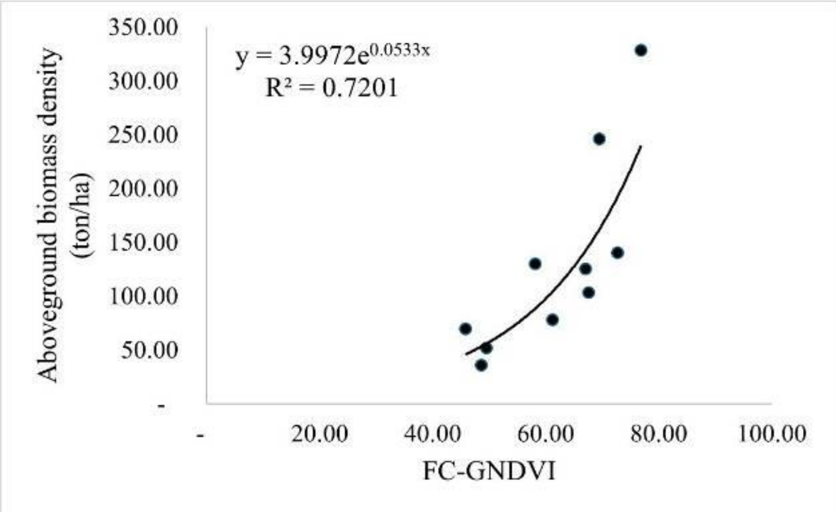
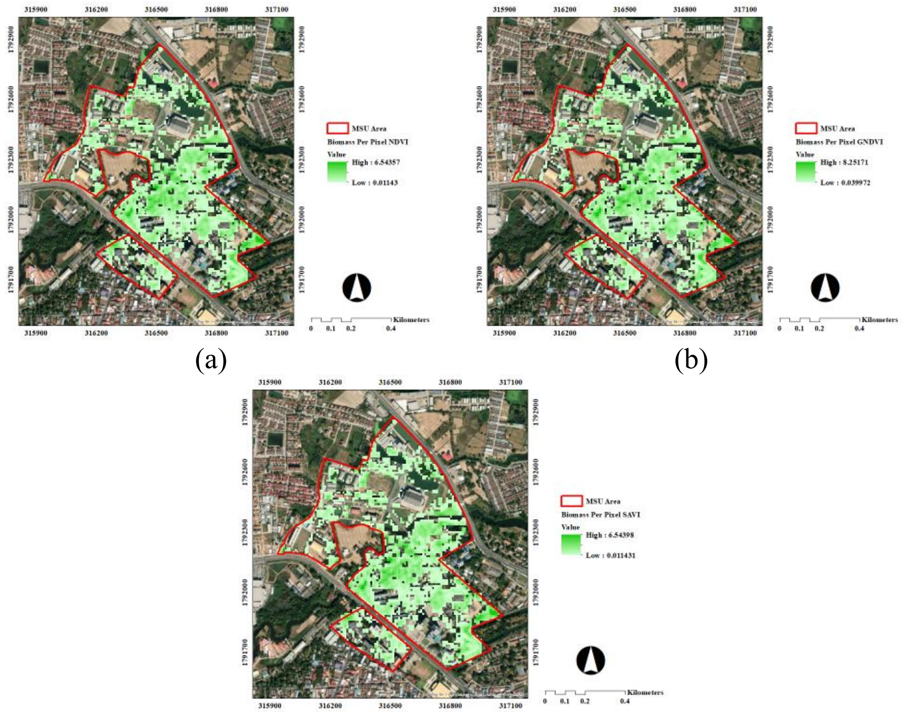


Figure 5. Relationship Between Fractional Cover and Aboveground Biomass

Spatial Assessment of Aboveground Biomass

The spatial estimation of aboveground biomass (AGB) and carbon storage across the study area was carried out using vegetation indices derived from Sentinel-2 imagery, applying the exponential regression model developed in the previous section. Biomass density (tons per hectare) was converted to biomass per pixel by multiplying the density values by the pixel area (100 m^2) and dividing by 10,000, thereby expressing the result in tons per pixel. The results of the spatial analysis were visualized in raster format, where each pixel represents an estimated AGB value, allowing a detailed examination of biomass distribution across the study area.

From NDVI (Figure 6a), biomass per pixel ranged from 0.01 to 6.54 tons, with a total AGB of 2,558.12 tons. GNDVI (Figure 6b) produced the highest biomass estimates, ranging from 0.04 to 8.25 tons per pixel and resulting in a total of 2,798.23 tons. SAVI (Figure 6c) resulted in values between 0.01 and 6.54 tons per pixel, corresponding to a total biomass of 2,558.02 tons. These findings provide a comprehensive picture of biomass accumulation, with GNDVI consistently yielding slightly higher estimates, indicating its greater sensitivity to vegetation density and chlorophyll concentration.



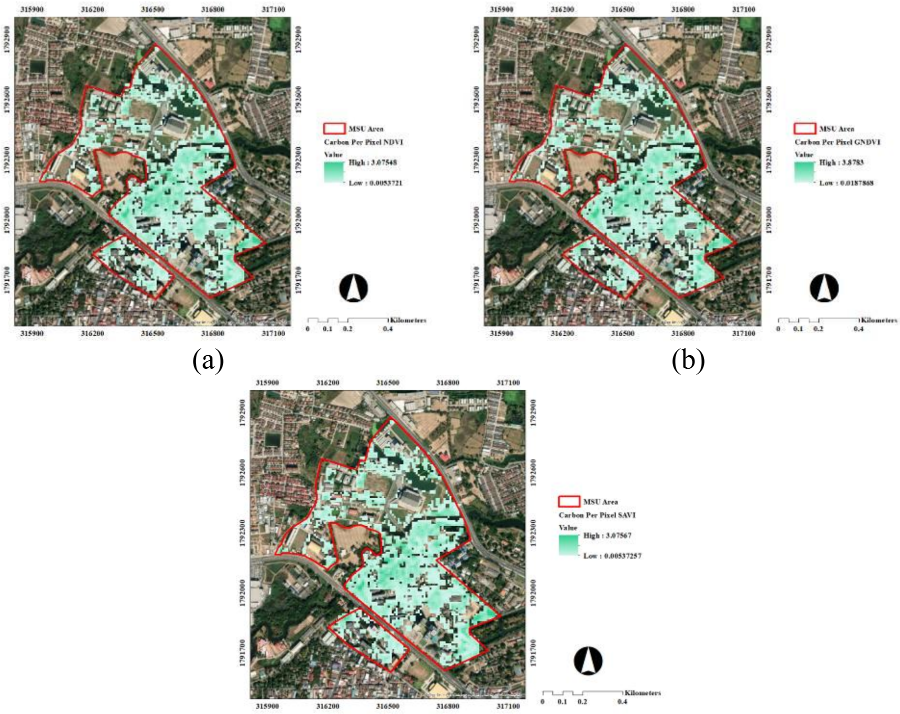
(a) (b)

(c)

Figure 6. Biomass per pixel (a) NDVI, (b) GNDVI, and (c) SAVI

From NDVI (Figure 7a), biomass per pixel ranged from 0.01 to 6.54 tons, with a total AGB of 2,558.12 tons. GNDVI (Figure 7b) produced the highest biomass estimates, ranging from 0.04 to 8.25 tons per pixel and yielding a total of 2,798.23 tons. SAVI (Figure 7c) resulted in values between 0.01 and 6.54 tons per pixel, corresponding to a total biomass of 2,558.02 tons. These findings provide a comprehensive picture of biomass accumulation, with GNDVI consistently yielding slightly higher estimates, indicating its greater sensitivity to vegetation density and chlorophyll concentration.

The corresponding carbon stock estimates, derived from the same vegetation indices, exhibited a similar spatial pattern and are shown in Figures 7 (a)–(c). NDVI-based carbon values ranged from 0.0053721 to 3.07548 tons of carbon per 100 m² pixel, producing a total carbon stock of 1,202.316209 tons. GNDVI yielded the highest carbon estimates, with values ranging from 0.0187868 to 3.8783 tons of carbon per pixel and a total of 1,315.166321 tons. SAVI showed carbon values between 0.00537257 and 3.07567 tons, with a total of 1,202.271152 tons. These results confirm the spatial heterogeneity of carbon storage within the study area and emphasize the reliability of vegetation indices—particularly GNDVI—as effective tools for landscape-scale carbon stock assessment and mapping, providing essential information for precision forest management and climate-mitigation planning.



(a) (b)

(c)

Figure 7. Carbon per pixel (a) NDVI, (b) GNDVI, and (c) SAVI

Statistical Accuracy Assessment

In order to assess the accuracy of biomass estimation provided by satellite images, NDVI, GNDVI and SAVI were compared with values measured in field conditions using Root Mean Square Error (RMSE) an d its percentage equivalent (R MSE %). The findings demonstrate a consistent pattern in model behavior. For NDVI, the estimated RMSE was 2.36 t ha^{-1} that means on average the satellite derived estimates are different from field measurement by around 2.36 t ha^{-1} and its relative percentage error was expressed in terms of %RMSE (44.99%). GNDVI conversely, provided the most optimal fit with RMSE value of 1.90 t ha^{-1} and corresponding to lowest RMSE% (36.21%), which means a reasonable approximation between predicted and observed biomass densities. SAVI produced intermediate results with an RMSE of 2.15 t ha^{-1} and an RMSE% of 41.11%, indicating a moderate level of accuracy. Taken together, these findings demonstrate that GNDVI provides the most accurate representation of aboveground biomass density in the study area, outperforming both NDVI and SAVI by reducing estimation error and offering a more reliable basis for biomass modeling. The consistently lower error metrics observed for GNDVI confirm its suitability as the preferred index for aboveground biomass assessment, particularly in heterogeneous landscapes where precision is essential for carbon stock estimation and land management planning.

The statistical accuracy assessment indicated that the GNDVI-based model provided the most optimal performance among all indices, with the lowest RMSE (1.90 t ha^{-1}) and RMSE% (36.21%). This suggests that GNDVI most effectively captures canopy density and chlorophyll characteristics, leading to more reliable aboveground biomass (AGB) predictions in heterogeneous urban landscapes. NDVI and SAVI showed moderate accuracy with RMSE values of 2.36 t ha^{-1} and 2.15 t ha^{-1}, respectively, confirming their usefulness but also their limitations in soil-influenced or mixed-vegetation environments. These findings are consistent with previous studies, indicating that green-band-based indices such as GNDVI perform better in complex canopies. The results reinforce the suitability of exponential regression for representing biomass–cover relationships, with an R^2 of 0.72 showing a strong correlation between fractional vegetation cover (FC) and AGB.

In comparison with other research, Faria *et al.* (2024) reported an RMSE of approximately 15.9% for biomass prediction at the Amazon–Cerrado transition, lower than the RMSE in this study. This difference may be due to their application of Artificial Neural Networks (ANNs), which can capture complex nonlinear relationships between spectral features and biomass. Similarly, Niu *et al.* (2024) achieved an improved coefficient of determination (R^2 = 0.74) by combining UAV and Sentinel-2 datasets, demonstrating the advantage of multi-source integration for improved model robustness. Furthermore, Liu *et al.* (2024b) found that including environmental parameters such as topography significantly enhanced AGB mapping in boreal forests of China (R^2 = 0.75 with reduced error). These comparative observations highlight that while regression-based models remain effective for practical estimation, integrating higher-resolution and multi-source datasets—such as UAV imagery, LiDAR, and environmental parameters—could further improve prediction accuracy. Such hybrid approaches would strengthen spatial–temporal representation of urban ecosystems, supporting precise carbon stock evaluation and sustainable green-space planning.

CONCLUSIONS

This study demonstrated that integrating field-based measurements with This study demonstrated that integrating field-based measurements with Sentinel-2 satellite imagery provides an effective framework for estimating aboveground biomass (AGB) and carbon stock in urban tree ecosystems. The combination of remote sensing data and allometric modeling produced accurate, spatially explicit estimates essential for sustainable urban forest management. The GNDVI-based model showed the strongest correlation with AGB (R^2=0.72) and yielded the lowest RMSE (1.90 t ha^{-1}), confirming its superior capability for representing vegetation density and chlorophyll content. These findings indicate that GNDVI is the most suitable vegetation index for biomass estimation in heterogeneous urban landscapes, and the developed model can be applied to monitor vegetation dynamics and assess carbon storage capacity in different urban contexts.

By linking remote sensing techniques with ground observations, this study presents a cost-effective and replicable approach for large-scale monitoring of carbon dynamics. The framework can support local authorities and policymakers in formulating urban greening strategies, climate adaptation measures, and the development of carbon credit initiatives aligned with Thailand's Net Zero goals. Furthermore, the approach can be utilized in verification and monitoring processes under carbon credit schemes such as T-VER or VERRA, creating pathways for measurable greenhouse gas reduction. Future research should integrate high-resolution UAV data, LiDAR measurements, and environmental parameters through advanced machine learning algorithms to enhance predictive accuracy and deepen understanding of spatial–temporal variability in urban ecosystems, thereby supporting sustainable urban development and long-term climate resilience.

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SPATIAL DISTRIBUTION OF COPPER (Cu) IN APPLE ORCHARDS IN THE RESEN REGION

SUMMARY

The soils in apple orchards in the Resen region were examined. The field research was carried out in 2024, during which 26 surface soil samples were taken. In the framework of this master's thesis, an examination of some physical and chemical properties of the soil, as well as the content of the total forms of heavy metals, was carried out. Several soil types are formed in the area covered by the Resen region: regosols, cinnamon forest soils, colluvial soils, and complexes thereof. Based on the obtained values for the mechanical composition, for the most part, the soils in the surface part are sandy clay loam (SCL) at 41%, sandy loam (SL) at 36%, and loamy (L) soils at 23%. The content of total forms of heavy metals in all samples was analysed using atomic emission spectrometry with inductively coupled plasma (AEICP). All statistical analyses were conducted in the R programming environment (version 4.3.2; R Core Team, 2024). The 'FactoMineR' and 'factoextra' packages were used for principal component analysis (PCA), while 'cluster' and 'ggdendro' packages were applied for hierarchical cluster analysis (HCA). Prior to PCA, all variables were standardized (z-score normalization) to eliminate the influence of different measurement scales and to ensure comparability among variables. The selection of principal components was based on eigenvalues >1 (Kaiser criterion) and cumulative explained variance. Cluster analysis was performed using Ward's method and Euclidean distance as the dissimilarity measure to identify similarities among soil parameters. Spatial interpolation of soil properties and total Cu content was performed using the Inverse Distance Weighting (IDW) method in ArcGIS 10.8. The IDW approach was selected because it provides reliable and smooth spatial predictions for datasets with a moderate number of sampling points (n = 26) and

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without a clearly defined spatial trend or anisotropy. The power parameter (p) was set to 2, which emphasizes the influence of closer points while maintaining interpolation stability. Kriging interpolation was tested but not applied, as the dataset size and semivariogram structure did not justify its use due to the lack of a spatially autocorrelated variogram model. The obtained results allow us to distinguish between possible anthropogenic influences on the presence of heavy metals in the soils of in the Resen region and their lithological origin.

Keywords: physical-mechanical properties, characteristics, heavy metals.

INTRODUCTION

Soil has numerous ecological functions that are essential for the environment, but also for the economy and the progress of society as a whole. As a dynamic polydisperse system with many functions, soil provides support for the entire ecosystem (Mitkova *et al.*, 2022). Soil contamination is usually caused by industrial and mining activities; in agriculture, by the improper use of pesticides, mineral fertilizers, and low-quality irrigation water; as well as by traffic in large urban areas (poor fuel quality, outdated technology), wastewater (municipal and industrial), and improper waste disposal (municipal and technogenic).

Soil contamination with heavy metals is one of the most complex and long-term environmental issues (Kabata-Pendias *et al.,* 2001; Artiola *et al.*, 2004; Kabata-Pendias *et al.,* 2007; Mitkova *et al.,* 2009; Prentovic *et al*., 2010; Markoski *et al*., 2011; Pelivanoska *et al*., 2011; Andreevski *et al*., 2012, Petek *et al*., 2022; Markoski *et al*., 2025). In particular, the potential soil contamination with copper in the Resen region known as a fruit-growing area in our country is the subject of research.

Recent research highlights that climatic conditions represent one of the most important determinants controlling the accumulation and mobility of copper (Cu) in vineyard and orchard soils. According to the global review by (Neaman *et al*., 2024), soil Cu concentrations are directly proportional to precipitation and inversely related to the aridity index (the ratio of potential evapotranspiration to precipitation). In humid regions, where diseases such as Plasmopara viticola (downy mildew) are more prevalent, the need for frequent applications of copper-based fungicides leads to higher Cu accumulation in the surface soil layer.

The study confirmed that climate, farm age, and soil organic matter content are the three key factors explaining the variability of Cu in agricultural soils worldwide. Moreover, increased rainfall enhances Cu losses via surface runoff, but at the same time increases the use of Cu fungicides, resulting in a net positive relationship between precipitation and soil Cu levels in humid regions.

Copper is the first metal that humans used for various purposes. Historical data show that ancient civilizations used it more than 10.000 years ago (Markoski *et al*., 2025). Its importance throughout human history is evident from the fact that an entire historical period the Bronze Agewas named after an alloy of copper. Today, after iron and aluminum, copper ranks as the third most used metal in the world. Copper is an essential microelement for living organisms and, in small amounts, is necessary for vital physiological processes. On the one hand, its deficiency in organisms leads to various disorders (Romić *et al*., 2014), while on the other hand, in larger amounts, it causes damage and disease. Whether copper is an essential microelement or a toxin depends on its concentration.

When discussing copper as a chemical soil pollutant, it should be noted that the sources of copper pollution can be diverse and are generally divided into natural and anthropogenic sources. Copper naturally occurs in the Earth's crust in concentrations between 25–75 mg kg^{-1} (Kabata-Pendias and Mukherjee 2007; Zejak *et al*., 2022). It is found in the highest concentrations in igneous rocks, somewhat less in sedimentary rocks, and in very small amounts in carbonate rocks. Depending on the nature of the parent material, copper concentrations in soils worldwide range from 1 to 140 mg kg^{-1} (Romić *et al*., 2014).

Anthropogenic activities also have a significant impact on copper concentrations in soil. The most important sources include the use of copper-based fungicides, the application of liquid fertilizers, the discharge of sewage sludge, industrial activities, mining, emissions of particles from car brakes, and atmospheric deposition of copper (Halamic *et al.,* 2001; Mitkova *et al.,* 2005; Prentovic *et al.,* 2009; Matse *et al.,* 2024).The accumulation of copper in soil depends on various parameters, primarily on the physicochemical properties of the soil (pH, soil structure, organic matter content, clay content, Fe content) (Kelepertzis *et al*., 2015; Jeon *et al.,* 2024). In addition to the above, copper accumulation in soil can also be influenced by vegetation, climate (precipitation), as well as by certain agrotechnical practices such as soil tillage and plant protection measures (Ballabio *et al.* 2018).

STUDY AREA

The Municipality of Resen is located in the Prespa Valley, in the southwestern part of the Republic of North Macedonia, and covers an area of 739 km^2. It is divided into land (562 km^2) and water (177 km^2) and represents a distinct spatial unit geographically situated around the intersection of 41°N latitude and 21°E longitude (Figure 1).

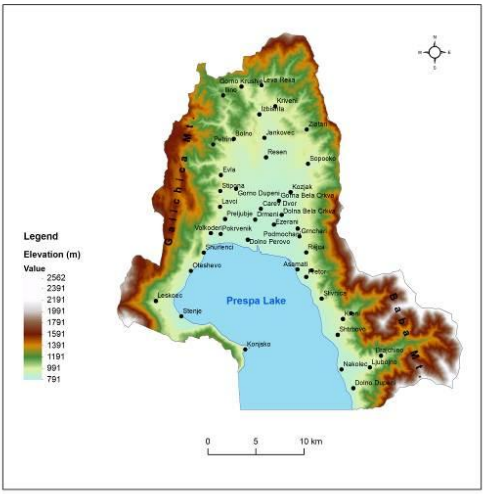


Figure 1. Location of the investigated area Prespa region Republic of North Macedonia

This region of North Macedonia is well known for its fruit production, which is closely linked to its climatic and soil conditions. The climate throughout the Prespa region is moderately continental with Mediterranean influence, while soil fertility also plays a major role.

The Prespa region is characterized by complex geological and tectonic structures, with rocks ranging in age from the oldest Paleozoic formations to the youngest Neogene and Quaternary sedimentary rocks. The Prespa valley and surrounding mountains are composed mainly of rocks of different ages, origins, and mineral compositions. Limestone rocks are the most dominant, although granodiorites and magmatic rocks are also present to a lesser extent. Syenites occur in areas of higher elevation, while Triassic carbonate rock masses are found in many parts of the region. Various types of Quaternary sediments such as proluvial, alluvial, organogenic marsh, deluvial, and fluvio-glacial sediments are dominant in the valleys along riverbeds.

The climatic conditions in the Prespa region determine the quality and uniqueness of apple fruits, as they have a significant influence on their successful cultivation. Most of the Prespa Valley lies within the warm continental zone, while the higher areas belong to the cold continental zone.

The climate can be characterized as continental or modified continental, since continental influence is dominant and Mediterranean influence is weaker. In the northern part, that is, in Upper Prespa, the continental influence is more pronounced, with colder winters, while in the southern part, especially in Lower Prespa, the Mediterranean influence is stronger, resulting in higher temperatures in both winter and summer.

The proximity of Lake Prespa has a major impact on the climate and moderates the extreme values of certain climatic elements that are characteristic of regions at this altitude. Despite the high elevation, the climate is relatively mild, which is due to the large water surface and the penetration of warm Mediterranean air currents from the southern part of the basin. In winter, relatively low temperatures are common, while in summer temperatures are moderately high. Spring is cooler than autumn, and May is cooler than September. Based on these characteristics, the climate can be described as moderately continental in the northern part, and moderately continental with Mediterranean influence in the southern part.

MATERIAL AND METHODS

Samples of surface soil (0–30 cm) were collected for analysis from 26 previously defined locations in the study area (Fig. 2).

All samples were collected in accordance with specific standards for collecting soil. Each sample must be representative, which means each sample should be a mixture of five samples collected in an area of 10 × 10 m. It is important to collect the sample at each defined coordinate.

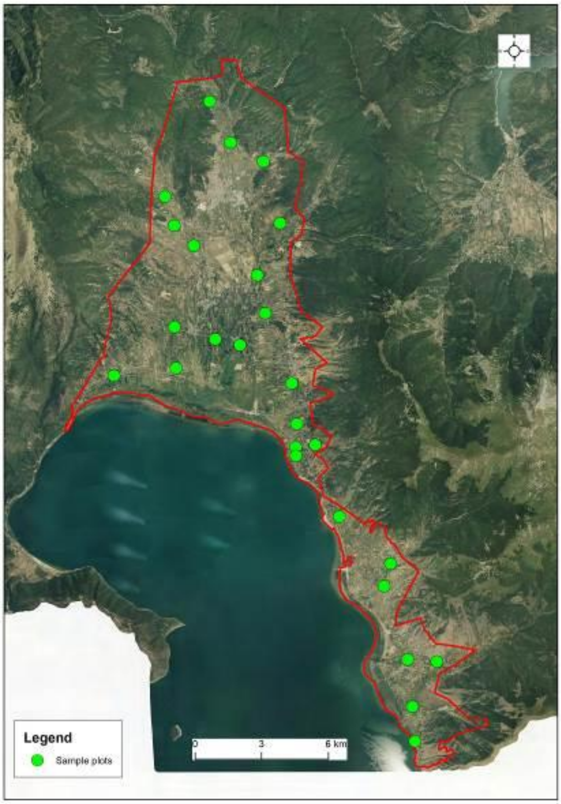


Figure 2. Map of the investigated area with locations, where the soil samples were taken

The soil samples were packed in polyethylene bags marked with a code and sample number. Samples pretreatment was done in accordance with ISO 11464:2006. First, they were air-dried, and after that crushed and sieved through a 2-mm sieve. Soil properties were determined, such as; mechanical composition (Mitkova and Markoski, 2022); (Markoski and Mitkova 2020), pH (10390:2005), total nitrogen (11261:1995), The content of organic carbon and humus - Spectrophotometric determination with the dichromatic method according to Walkley and Black, a validated method of the Faculty of Agricultural Sciences and Food in Skopje (Durdjević, 2014), and calcium carbonate equivalent volumetrically (ISO 10693). Additionally total organic carbon (TOC) was determined by dry combustion (according to ISO standard 10694), extractable phosphorus and potassium (ammonium lactate method) while the cation exchange capacity (CEC) was measured by the method described by (Sumner and Miller, 1996). The total form of Cu was analyzed. Soil samples were digested by two methods: Aqua Regia extraction method (ISO 11466) and digestion for determination the total element content with HF method (ISO 14869-1). Aqua regia (HCl and HNO3 3+1) extraction method was done after digestion at 180ºC for 2 h. Soil samples (3.0000 g) were digested directly in the relux digestion vessels where 21 ml HCl and 7 ml HNO3 were added. The solution was brought to boil and the relux was kept for 2 hours. The solution of each vessel was quantitatively transferred to 100 ml lasks. For total digestion, soil samples (0.2500 g) were placed in a Telon digestion vessel and were digested on a hot plate. In the first step, HNO3 was added to remove all organic matter, then a mixture of HF and HClO4 was added, followed by a third step where HCl and water were added to dissolve the residue. The solution was transferred quantitatively to the 25 ml volumetric lask. All reagents were of analytical grade (Merck, Germany). Analytical blanks were included in all extractions. Analysis of soil samples was performed using inductively coupled plasma-atomic emission spectrometry (ICP-AES). From the obtained results descriptive statistics were prepared and multivariate factor analyses by R-method were applied in order to identify the associations of the chemical elements (Reimann *et al.,* 2002). Spatial distribution maps were prepared for each factor using universal kriging method with a linear variogram interpolation. Factor analysis is the methodological basis of a set of statistical techniques used to analyse inter-relations of many variables. The approach includes processing information from a large number of original variables into smaller sets (called factors) with minimal loss of information from the original variables. Factor analysis as a set of statistical and mathematical procedures is useful in investigations whereby a number of variables that are mutually correlated and where is necessary to determine the basic source of covariance between the data (Reimann *et al.,* 2002). Multivariate cluster analysis was also applied to determine the significance of the factor analysis and the stability of the new synthetic variables, that is, associations of elements. The dendrogram of the distances among the individual elements is presented in Fig. 3. Identical results were achieved by application of multivariate factor analysis. All of the obtained clusters correspond to the four obtained factors.

RESULTS AND DISCUSSION

According to the obtained data, the soils in the surface part have a heterogeneous mechanical composition. The mechanical composition has an influence on the availability of heavy metals. At the same total content, the availability and mobility of heavy metals is higher in soils with a lighter mechanical composition (sandy) compared to heavier soils (clay). This means that the danger of translocation (moving) of heavy metals to groundwater and their pollution, with all the negative consequences for the ecosystem, is greater in soils with a lighter mechanical composition.

Several soil types have formed in the studied area: alluvial, colluvial, leached, brown forest soils, marshy gley soils, calcareous-dolomitic black soils (calcimelanosol), brown soils on limestone and dolomite, complexes of brown forest soils + regosol + leptosol, and leached + regosol, (Fig 3 Pedological map).

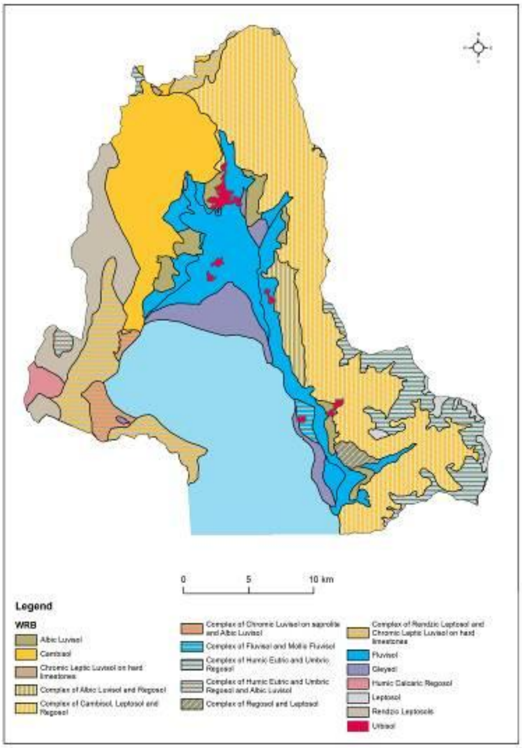


Figure 3. Pedological map of the Resen municipality

From the analyzed soil samples (Table 1), it can be seen that the fraction of total sand is dominated by the fraction of fine sand, on average 30.48% in relation to coarse sand 24.60%. The average value of the physical fraction of sand (fine + coarse sand) is 55.70% and is higher than the physical fraction of clay (clay + silt) which is 42.37%. The median for the total sand fraction is 58.1%. In the physical clay fraction, the clay fraction with the highest average value is 15.25% and ranges from 10.0 to 25.50% in relation to the powder fraction, which averages 27.94%. The median for the powder fraction is 26.60% higher than the median for the clay fraction - 15.7%. The soil samples according to the American classification triangle, with the obtained values for the mechanical composition, are classified into textural classes, (Mitkova and Markoski, 2022). The surface soils are classified as sandy clay loam (SCL), sandy loam (SL), and loam (L), each accounting for 30.77%, and as loamy sand (LS) and silty loam (SiL), each accounting for 3.84%.

The minimum and maximum values of soil bulk density in the surface layer range from 1.39 to 1.58 g/cm³, with an average value for all soil samples of 1.48 g/cm³ and a median of 1.48 g/cm³. The average value of hygroscopic moisture is 2.46%, with a minimum of 1.17%, a maximum of 4.17%, and a median of 2.23%.

Table 1. Descriptive statistics for the content of the analyzed elements in the soil samples

Parameters	Measurement values	n	min	max	X	SD	Md
pH H_2O	/	26	6.28	7.97	6.92	0.36	6.9
$CaCO_3$	[%]	26	0.00	15.82	0.59	3.04	0
Organic mater	[%]	26	0.97	7.25	4.13	1.45	4.24
P_2O_5	[mg/100 g]	26	9.25	61.19	20.34	11.24	19.76
K_2O	[mg/100 g]	26	8.74	75.29	27.53	15.24	24.03
CEC	[cmol(+) kg^{-1}]	26	9.53	27.88	17.67	4.27	17.72
Hygroscopic moisture	[%]	26	1.17	4.17	2.46	1.0	2.23
Coarse sand	[%]	26	6.87	74.17	24.60	14.93	24.39
Fine sand	[%]	26	12.37	61.01	30.48	12.57	30.36
Total sand	[%]	26	36.10	88.10	55.70	12.76	58.1
Silt	[%]	26	0.50	48.40	27.94	11.20	26.6
Clay	[%]	26	10.00	25.50	15.25	3.72	15.7
Clay + Silt	[%]	26	11.90	63.90	42.37	12.76	41.9
ρ_P	[g/cm^3]	26	1.39	1.58	1.48	0.05	1.48
Cu	[mg/kg]	26	14.90	53.50	32.13	12.23	30.85

Figures 4 and 5 show the spatial distribution of the physical clay fraction (clay + silt) and clay in the study area.

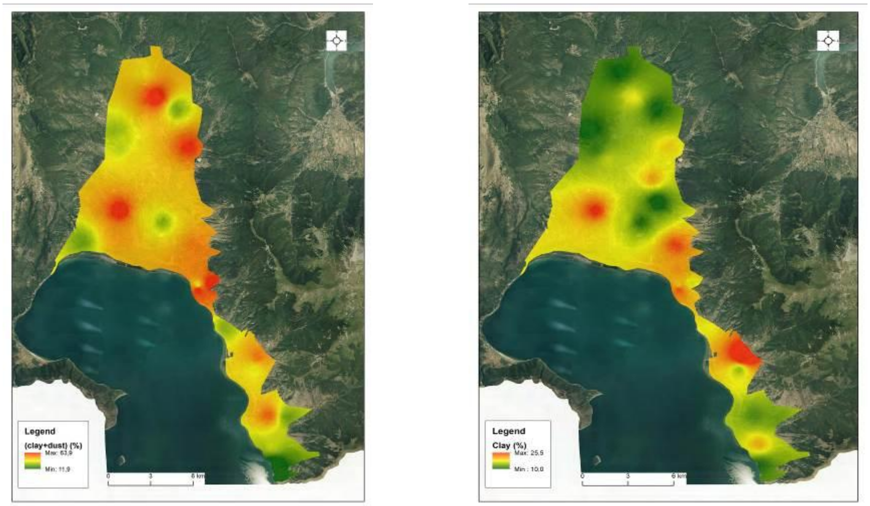


Figure 4 and 5. Spatial distribution of the physical clay fraction (clay+dust) and clay

In Table 1 and the PCA loading matrix, the statistically significant correlations ($p<0.05$) were marked to emphasize the most relevant relationships between soil parameters and copper accumulation. The strongest positive correlations were observed between Cu, organic matter (OM), cation exchange capacity (CEC), silt, and clay, indicating that higher Cu values are associated with soils richer in colloidal and organic fractions capable of binding metal ions. Conversely, a strong negative correlation was found with total sand content, confirming that lighter-textured soils with lower adsorption capacity tend to retain less copper.

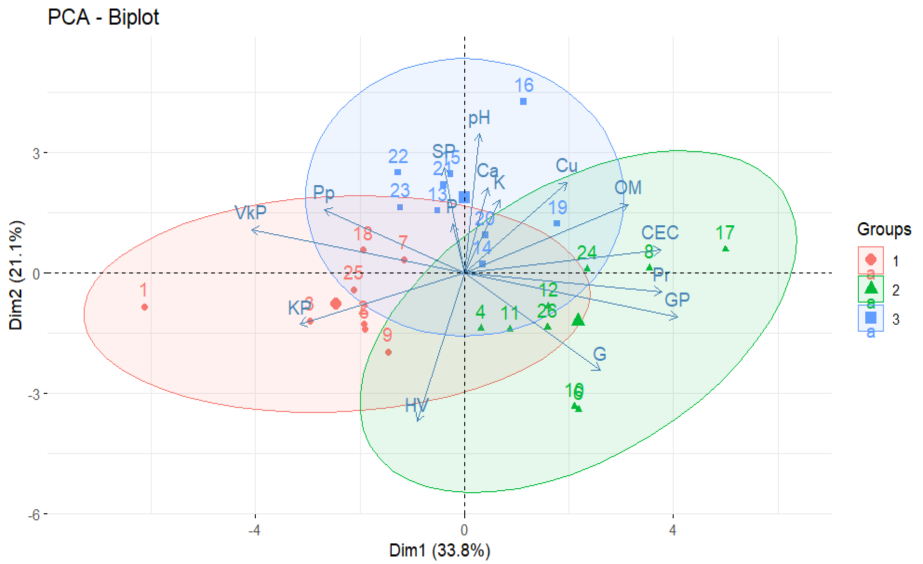
These results suggest that the sorption capacity of soils, governed by organic and fine mineral fractions, plays a dominant role in the retention and spatial distribution of Cu in the studied area. This is done by transforming the original data into a lower-dimensional space, where highly correlated variables are retained together, allowing for better visualization and understanding of the data.

Table 2. Principal Component Analysis

Parameters	PC1	PC2	PC3	PC4	PC5
pH H_2O	0.064	**0.780**	0.102	-0.307	0.300
$CaCO_3$	0.101	0.479	-0.465	-0.327	**0.548**
Organic mater	**0.704**	0.382	0.207	0.235	0.257
P_2O_5	-0.050	0.273	**0.736**	-0.020	0.021
K_2O	0.151	0.412	0.107	**0.620**	-0.282
CEC	**0.844**	0.131	0.063	0.217	0.267
Hygroscopic moisture	-0.201	**-0.829**	-0.175	-0.134	0.076
Coarse sand	**-0.706**	-0.285	0.348	0.238	0.441
Fine sand	-0.089	**0.587**	-0.560	-0.055	-0.417
Total sand	**-0.914**	0.244	-0.144	0.224	0.105
Silt	**0.847**	-0.103	0.262	-0.316	-0.226
Clay	**0.582**	-0.543	-0.279	0.193	0.301
Clay + Silt	**0.914**	-0.244	0.144	-0.224	-0.105
ρ_p	**-0.600**	0.356	0.364	-0.363	-0.063
Cu	0.440	**0.506**	-0.182	0.214	0.051
Eigen-value	5.07	3.17	1.65	1.18	1.15
% variability	33.83	21.12	10.97	7.88	7.68
% cumulative variance	33.83	54.95	65.92	73.80	81.48

Five principal components (PCs) were identified with eigenvalues > 1, which explain 81.48% of the total variability. PC1 explains 33.83% of the total variability, which is due to the high positive correlation of organic matter, CEC, silt, clay, and clay + silt with this component. The highest negative correlation with PC1 was observed with total sand. In the second principal component, pH has the greatest contribution, while P_2O_5 is positively correlated with PC3. The highest positive correlation with PC4 is observed for K_2O and with PC5 for $CaCO_3$.

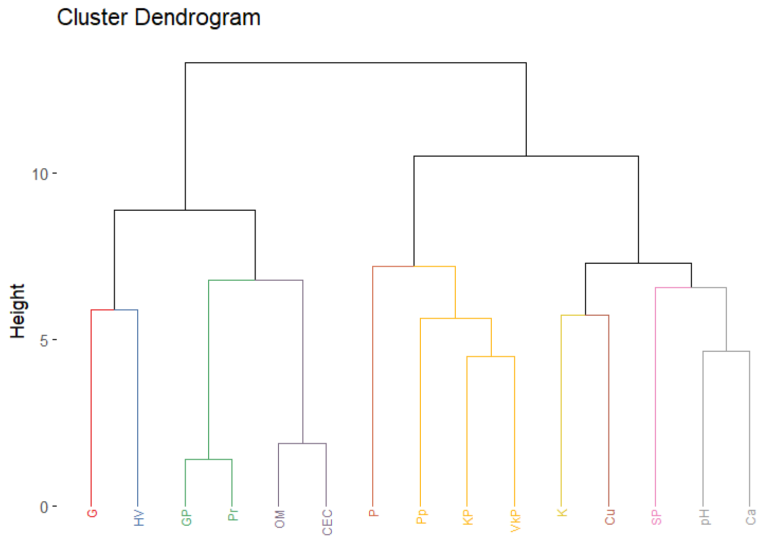
Based on the principal component analysis, a biplot was constructed showing the main components PC1 and PC2. From the graph, it can be observed that the analysed soil types are classified into three groups based on the analysed properties. In the first group (red color), the smallest number of soil samples are included; these are characterized by low contents of organic matter, CEC, silt, and clay + silt. In the second group (blue color), soil types with high values of pH, $CaCO_3$, P_2O_5, K_2O, and fine sand are distributed, while hygroscopic moisture is low. Nine soil samples are positioned in the third group (green color), which are characterized by high contents of organic matter, CEC, silt, clay, and clay + silt (Figure 6).



*Legend: pH; Ca-$CaCO_3$; P-P_2O_5; K-K_2O; O.M-organic matter; CEC-cation exchange capacity; Cu-total copper; KP-coarse sand; SP-fine sand; VkP-total sand; GP-clay + silt; G-clay; Pr-silt; Pp-bulk density; HV-hygroscopic moisture.

Figure 6. Biplot of PC1 and PC2 showing the distribution of the analysed soil samples based on the examined properties.

Based on the cluster analysis, the analysed parameters of the soil samples were divided into ten clusters. In six of them, only one soil property was classified. Clay + silt and silt were grouped in the third cluster. The cation exchange capacity (CEC) and organic matter belonged to the fourth cluster, while bulk density, coarse sand, and total sand were grouped in the sixth cluster. The properties pH and $CaCO_3$ were located in the tenth cluster (Figure 7).



*Legend: pH; Ca–$CaCO_3$; P–P_2O_5; K–K_2O; O.M – organic matter; CEC – cation exchange capacity; Cu – total copper; KP – coarse sand; SP – fine sand; VkP – total sand; GP – clay + silt; G – clay; Pr – silt; Pp – bulk density; HV – hygroscopic moisture.

Figure 7. Dendrogram of the cluster analysis of the analyzed characteristics in the soil samples.

In different soils, the content of organic matter varies widely and depends on the type of vegetation, that is, on the amount of organic residues left by the vegetation in the soil, on the conditions for their decomposition and humus formation (humification), on the rate of humus mineralization, as well as on the manner of soil use (Idrizi *et al*., 2023; Simić *et al*., 2023).

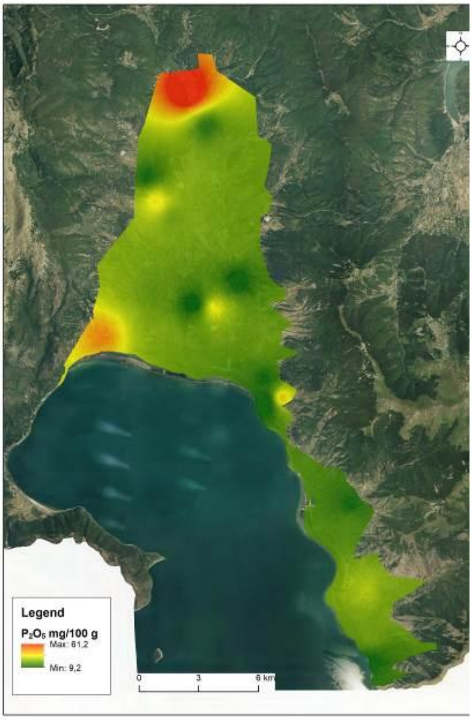
The results obtained from the examination of the chemical properties of the soil samples are presented in Table 6. The organic matter content ranges from 0.97% to 7.25%, with an average of 4.13%, and a median value of 4.24%.

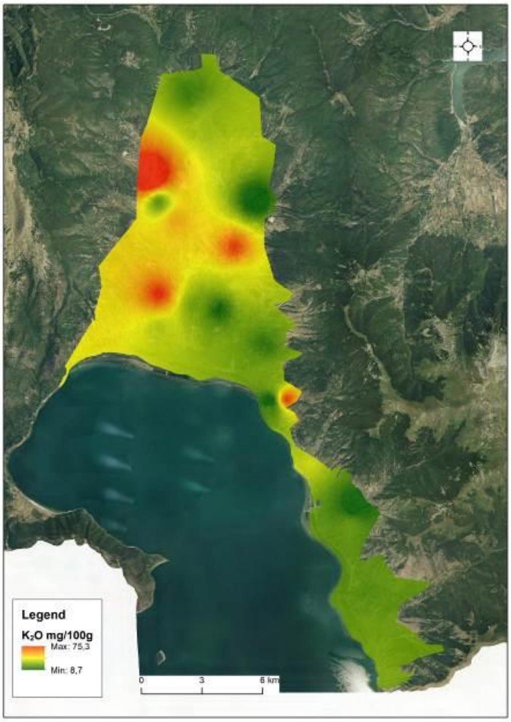
The cation exchange capacity (CEC) depends on the content of organic matter, humus, the mechanical composition and the composition of clay minerals, as well as on the reaction of the solution used to displace the adsorbed ions during their determination. In cultivated soils with an organic matter content of 2–3%, the cation exchange capacity in sandy soils ranges from 5 to 10 cmol/kg^{-1}, in loamy soils between 10 and 20 cmol/kg^{-1}, and in clay soils between 20 and 40 cmol/kg^{-1} (Mitkova and Markoski, 2022).

The values of the analyzed soil samples correlate with the content of organic matter and the mechanical composition of the soil, ranging from 9.53 to 27.88 cmol/kg^{-1}, with an average of 17.67 cmol/kg^{-1} and a median of 17.72 cmol/kg^{-1}.

According to the analyses, the soil samples are non-calcareous, with the exception of one sample that is calcareous (15.82%). In soils containing carbonates, copper solubility decreases, thus reducing its availability, and vice versa. The values of the soil solution reaction (pH) are very important, as they indicate the availability, unavailability, and potential toxicity of nutrients in the soil. According to Filipovski (2003), the uptake of many elements increases with increasing acidity. A strongly acidic reaction (pH below 5.5) can cause toxic levels of Fe, Mn, and Al, as well as Zn, Co, and Cu. In an alkaline reaction, these microelements (Fe, Mn, Zn, Co, Cu) occur in unavailable forms, leading to their deficiency in plant nutrition. The best solubility and availability of most biogenic elements occur at a pH of around 6.5, which is optimal both for plants and microorganisms. The solubility and availability of copper are highest within the pH range of 5 to 7 in the soil solution. In the analyzed soil samples, the pH (in water) ranged from 6.28 to 7.97, with an average value of 6.92 (slightly acidic to moderately alkaline according to the USDA classification) and a median of 6.90.

The content of easily available forms of P_2O_5 ranged from 9.25 mg/100 g soil to 61.19 mg/100 g soil, with a median of 19.76 mg/100 g soil. The content of easily available K_2O was higher, ranging from 8.74 to 75.29 mg/100 g soil, with a median of 24.03 mg/100 g soil. Figures 10 and 11 show the spatial distribution of available phosphorus (P_2O_5) and available potassium (K_2O) in the soil samples from the Prespa region.

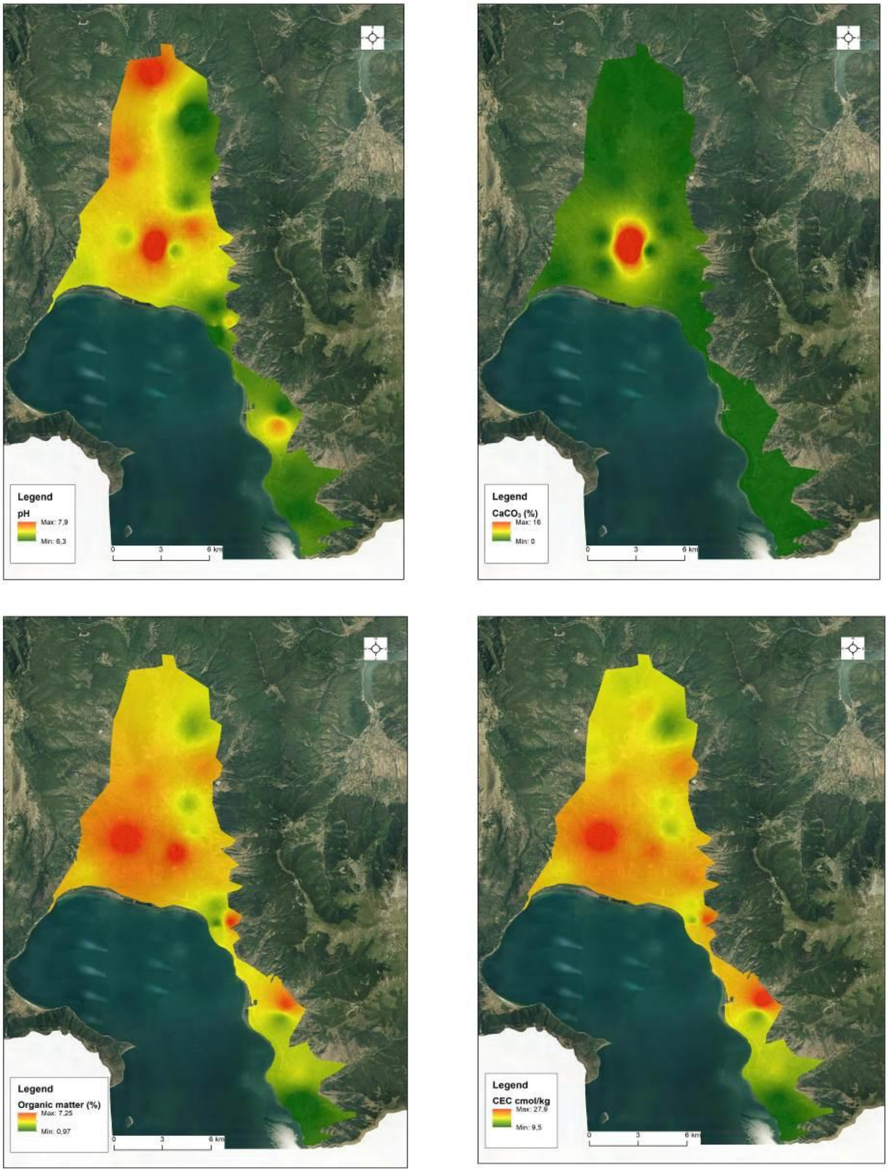




Figures 8 and 9. Spatial distribution of available phosphorus (P_2O_5) and available potassium (K_2O) in the soil samples from the Prespa region.

The soil samples are slightly, moderately, richly, to very richly supplied with P_2O_5 and K_2O. This indicates uncontrolled fertilization with mineral fertilizers without prior soil fertility testing.

Figures 10, 11, 12, and 13 show the spatial distribution of pH, $CaCO_3$, organic matter, and cation exchange capacity (CEC) in the studied area.



Figures 10, 11, 12, and 13. Spatial distribution of pH, $CaCO_3$, organic matter, and cation exchange capacity (CEC) in the studied area

Table 1 presents the total copper (Cu) content in the soil samples, while table 3 shows a comparison of the obtained results (median, minimum and maximum copper content values in the soil samples from the Prespa region) with those from the entire territory of the Republic of Macedonia (Stafilov *et al.,* 2016), the territory of Skopje (Stafilov *et al.,* 2017) and Europe (Salminen *et al.*, 2005).

Table 3. Comparative analysis of the median, minimum, and maximum copper (Cu) content values in the soil samples from the Prespa region, Skopje, the Republic of North Macedonia, and Europe

Element	**value**	**Prespa**		**Skopje (Stafilov *et al.*, 2017)**		**North Macedonia (Stafilov and Sajn, 2016)**		**Europa (Salminen *et al.*, 2005)**	
		Md	**min-max**	**Md**	**min-max**	**Md**	**min-max**	**Md**	**min-max**
Cu	**mg/kg**	30.85	14.90 – 53.50	33	7.3 – 59.0	16	1.7 – 73.0	13	0.81 – 256

The average copper content in the lithosphere is about 55 ppm. In cultivated soils, its total content varies widely from 1 to 390 ppm, most commonly between 5 and 50 ppm, with an average of approximately 26 ppm. However, most of the copper in the soil is not available to plants. The total copper content in soil depends on the chemical composition of the parent material. Acidic rocks contain the least copper (around 30 ppm), sedimentary rocks slightly more (around 57 ppm), and basic rocks the most (around 140 ppm). Copper also strongly binds to organic matter in the soil. Organic copper complexes play an important role in regulating its movement in the soil and availability to plants. Copper bound to organic matter is poorly available to plants. Therefore, soils with a high organic matter content (e.g., peat soils) may have a copper deficiency for plants. The content of available copper in the soil also depends on the soil's mechanical composition. Sandy soils are poor in copper. Copper deficiency also occurs in humus-rich sandy soils of deluvial and alluvial origin, as well as in some hydromorphic and peat soils. Copper deficiency in the soil does not always correlate with high organic matter content; it can also occur in soils formed under arid conditions with low organic matter content (Mitkova and Markoski 2022).

Excess copper (Cu), however, negatively affects plant growth and productivity. Numerous studies summarize the adverse effects of copper excess on germination, growth, photosynthesis, and antioxidant response in crops. Its inhibitory effect on mineral nutrition, chlorophyll biosynthesis, and antioxidant enzyme activity has been confirmed (Banas *et al.,* 2010). According to (Strumpf *et al.,* 2009), even in heavily contaminated locations, copper content in plants after harvest is low. Based on copper uptake and distribution in plants, for perennial crops (e.g., fruit trees, vineyards), the risk to consumers from the soil–plant connection can be excluded. So far, no references report that high copper content in soils results in high copper content in harvested products, indicating that restrictions on the use of copper-based fungicides are not necessary.

In Germany, estimates of copper pesticide use in 2008 showed that the expansion of organic farming, linked to location, increased copper use by 10 tons to 34.1 tons annually compared to 2005. This input is low compared to fertilizers (around 2.300 t/ha per year) or sewage sludge (around 450 t/ha per year). Continuous application of copper pesticides at the mentioned rates over the next the soil. Furthermore, the development of new low-concentration copper pesticides may reduce copper input in the coming years (Provenzano *et al.,* 2010).

The presence of at least three key factors influencing soil copper content in orchards and vineyards are: climate, age of plantations, and soil organic matter (Neaman *et al*., 2024). Their research showed that soil copper content in orchards and vineyards worldwide is directly proportional to rainfall and inversely affected by drought. Older plantations (orchards and vineyards) globally are characterized by increased copper content. Moreover, higher organic matter content shows an inverse effect on soil copper content. These soil property effects on copper content are also discussed regarding copper losses from the soil through surface runoff. No statistically significant differences were found between organic and conventional orchards and vineyards concerning soil copper content.

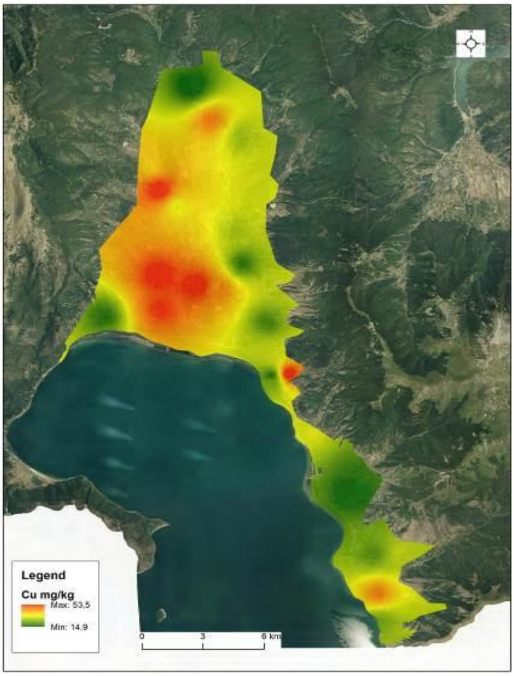


Figure 14. Spatial distribution of copper (Cu) in the soil samples from the Prespa region

The copper content in the soil samples ranged from 14.90 mg/kg to 53.50 mg/kg, with an average of 32.13 mg/kg and a median of 30.85 mg/kg. The minimum copper values in the Prespa region were higher than those reported for soils in the Skopje region (7.3 mg/kg), for the Republic of North Macedonia (1.7 mg/kg), and for European soils (0.81 mg/kg). The highest copper values in the Prespa region were lower than those in the Skopje region (59.0 mg/kg), North

Macedonia (73.0 mg/kg), and Europe (256.0 mg/kg). The median copper content in the analyzed soil samples was 30.85 mg/kg, higher than in soils of North Macedonia (16.00 mg/kg) and Europe (13 mg/kg), but lower than in the Skopje region (33.0 mg/kg). The higher median value in the Prespa region is likely due to greater use of plant protection products and mineral fertilizers containing this element. Similar values for total copper content in hydromorphic soils in the Resen field in surface horizons (13.22 to 33.60 mg/kg) were reported by Andreevski *et al.,* (2010), Savić *et al.,* (1970), in 12 alluvial soils of North Macedonia (0–20 and 20–40 cm), reported total copper content from 4.8 to 41.6 mg/kg, while for Skopje, Petkovski *et al.,* (2001) reported 15.5 to 74.6 mg/kg. When compared to earlier pedological surveys in the Prespa and Ohrid regions, the current study shows a slightly elevated median Cu content (30.85 mg/kg) relative to the values reported by Andreevski *et al*., (2010) for hydromorphic soils in Resen (13.22–33.60 mg/kg) and by Petkovski *et al*., (2001) for the Skopje area (15.5–74.6 mg/kg). In contrast, Cu levels remain well below the threshold values recorded in some European orchard soils (>100 mg/kg; Romić *et al.,* 2014). The observed variability may be attributed to differences in orchard age, intensity of fungicide use, and organic matter content across these regions. This comparison further confirms that, although the Prespa region exhibits moderately increased Cu accumulation, the concentrations remain within ecologically acceptable limits. Figure 16 shows the spatial distribution of copper (Cu) in the soil samples from the Prespa region in apple orchards.

CONCLUSIONS

Based on the obtained results for the spatial distribution of copper (Cu) in the soil samples from the Prespa region, the following conclusions can be drawn:

Several soil types have formed in the studied part of the Prespa region: alluvial, colluvial, leached, brown forest soils, marshy gley soils, calcareous-dolomitic black soils (calcimelanosol), brown soils on limestone and dolomite, complexes of brown forest soils + regosol + leptosol, leached + regosol.

Analyses of the mechanical composition of the samples showed that in the total sand fraction, fine sand dominates, averaging 30.48% compared to coarse sand at 24.60%. The average value of the physical sand fraction (fine + coarse sand) is 55.70%, which is higher than the physical clay fraction (clay + silt) at 42.37%. The median for the total sand fraction is 58.10%. In the physical clay fraction, clay has a lower average value of 15.25%, ranging from 10.00 to 25.50%, compared to silt, which averages 27.94%. The median for silt (26.60%) is higher than for clay (15.70%). Surface soils are sandy clay loam (SCL), sandy loam (SL), and loam (L) at 30.77% each, and loamy sand (LS) and silty loam (SiL) at 3.84% each.

The minimum and maximum soil density values in the surface layer range from 1.39 to 1.58 g/cm³, with an average and median of 1.48 g/cm³. The average hygroscopic moisture is 2.46%, with a minimum of 1.17%, a maximum of 4.17%, and a median of 2.23%.

Five principal components (PCs) with eigenvalues >1 were identified, explaining 81.48% of the total variability. PC1 explains 33.83% of the total variability, due to a strong positive correlation of organic matter, CEC, silt, clay, and clay + silt with this component. The strongest negative correlation with PC1 is observed with total sand. In the second principal component, pH has the largest contribution, while P_2O_5 is positively correlated with PC3. K_2O shows the highest positive correlation with PC4, and $CaCO_3$ with PC5.

Based on the principal component analysis, a biplot was constructed showing PCs 1 and 2. The graph shows that the analyzed soil types are classified into three groups based on the studied properties. In the first group (red), the smallest portion of soil samples is included, characterized by low organic matter, CEC, silt, and clay + silt. The second group (blue) includes soil types with high values of pH, $CaCO_3$, P_2O_5, K_2O, and fine sand, and low values of hygroscopic moisture.

Based on cluster analysis, the analyzed soil parameters were divided into ten clusters. Six of these include only one soil property. In the third cluster, clay + silt and silt are grouped. The fourth cluster includes CEC and organic matter, while bulk density, coarse sand, and total sand are grouped in the sixth cluster. The properties pH and $CaCO_3$ are located in the tenth cluster.

Organic matter content ranges from 0.97% to 7.25%, with an average of 4.13% and a median of 4.24%.

Soil samples are non-calcareous, except for one sample (15.82%).

The pH of the soil solution in water ranges from 6.28 to 7.97, with an average of 6.92 (slightly acidic to moderately alkaline according to the USDA classification) and a median of 6.90.

Cation exchange capacity ranges from 9.53 to 27.88 $cmol/kg^{-1}$, with an average of 17.67 $cmol/kg^{-1}$ and a median of 17.72 $cmol/kg^{-1}$.

Easily available P_2O_5 ranges from 9.25 mg/100 g soil to 61.19 mg/100 g soil, with a median of 19.76 mg/100 g soil. Easily available K_2O is higher, ranging from 8.74 to 75.29 mg/100 g soil, with a median of 24.03 mg/100 g soil.

Copper content in the soil samples ranges from 14.90 mg/kg to 53.50 mg/kg, with an average of 32.13 mg/kg and a median of 30.85 mg/kg. The minimum copper values in the Prespa region are higher than those reported for soils in the Skopje region (7.3 mg/kg), North Macedonia (1.7 mg/kg), and Europe (0.81 mg/kg). The maximum copper values in Prespa are lower than those in Skopje (590.0 mg/kg), North Macedonia (73.0 mg/kg), and Europe (256.0 mg/kg).

The results were statistically processed, and spatial distribution maps were prepared for all analyzed elements. The results were compared with international soil standards.

The studies generally indicate a correlation between the presence of copper and the geological formations in the studied area, but also suggest a certain link with insufficiently controlled use of plant protection products and mineral fertilizers containing this element.

Based on the obtained results, it is recommended to implement regular monitoring of Cu levels in orchard soils and to promote the rational and environmentally safe use of copper-based fungicides. Sustainable soil management practices should aim to prevent excessive accumulation of heavy metals while maintaining effective plant protection.

Future research should focus on the analysis of available (mobile) forms of copper and the assessment of Cu content in plant tissues, in order to better understand soil–plant transfer dynamics and potential ecological risks.

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ECONOMIC PRODUCTION AND COMPARATIVE EVALUATION OF BROAD BEAN ACCESSIONS UNDER ORGANIC CULTIVATION

SUMMARY

Vicia faba L. is determined as the main grain legume grown in Europe, with increasing in-terest from the organic sector. Cultivars suitable for organic cultivation conditions should meet a complex of traits and parameters. Based on a comparative evaluation of 11 faba bean accessions under organic cultivation conditions, it was found that they differed in vegetation duration, habitus, productivity, stability, biochemical composition and tolerance to *Botrytis fabae*. In herbaceous forage direction, BGP was most suitable accession. The same was characterized by a fast growth rate (0.94 cm/day), high values in terms of height and aboveground biomass (88.5 cm and 43.02 g/plant^{-1}, respectively), and the highest tolerance to *B. fabae* (I=11.4%). In grain direction, with suitable characteristics for organic production, Fb 04 (rank 2) was identified. It was distinguished by high seed productivity (22.71 g/plant^{-1}) and stability (YSi=8.00+), forage quality (crude protein - CP=235.60 g/kg^{-1} DM, dry matter) and moderate resistance to *B. fabae* (I=16.5%). Also, accession Fb 04 had good performances in the herbaceous-forage direction. The results of this study showed that the comparative evaluation of accessions under organic cultivation conditions gives promising outcomes, and BGP and Fb 04 may be recommended for organic faba bean production.

Keywords: *Vicia faba* L.; productivity; organic farming; *Botrytis fabae*.

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INTRODUCTION

Climate and economic changes on a global and national scale define the crucial importance of sustainable agriculture (Georgieva, 2017; Stupar *et al.,* 2023; Sekulić *et al.,* 2023). Building a sustainable farming system has agricultural, economic and environmental aspects. Stable production in sustainable agriculture is inextricably linked to the implementation of systems requiring less utilization application of pesticides and fertilizers or their complete elimination. The most demanding production system is organic farming defined by EU Directive 2092/91. It restores and maintains soil fertility, moreover it ensures ecosystem balance, reduces the negative impact of agriculture on climate change and implies production of quality food products in perfect harmony with nature (Niggli *et al*., 2009; Uhr *et al*., 2014; 2017).

Sustainable resource management can guarantee agricultural production's stability and quality. Health-safe products, economic advantage, conserving the environment and health can be attained by organic farming. Over 96 million hectares of agricultural land are handled organically by at least 4.5 million farmers in 188 countries. In 2022, sales of organic food and beverages were close to 135 billion euros worldwide. Oceania (36 mill. ha, or 47%) and Europe (17.8 mill. ha, or 23%) have the biggest organic agricultural land areas, followed by Latin America (9.9 mill. ha, or 13%), Asia (6.5 mill. ha, or 8.5%), Northern America (3.5 mill. ha, or 4.6%), and Africa (2.7 mill. ha, or 3.5%), (Popović *et al*., 2013; 2023).

Increased production of grain legumes in Europe will contribute to a greater supply of protein and have a positive effect by providing direct and indirect environmental benefits (De Notaris *et al.,* 2023). Incorporating the variety *Vicia faba* L. in an organic farming system improves soil fertility. The high efficiency in achieving symbiosis with the specific Rhizobium bacteria and the accompanying biological nitrogen fixation is associated with reduced need for fertilizers and increased biological activity of the soil. Further, incorporating plant residues from grain legumes into the soil as a green fertilization improves the growth and yield of subsequent crops in the crop rotation. Faba bean can fix up to 200 kg N/ha and incorporation of plant residues into the soil improve its properties, including organic matter content, bulk density and porosity (O'Donovan *et al*., 2014; Adekiya *et al*., 2017).

In addition to the positive characteristics of faba bean is its high productivity (Maalouf *et al*., 2015; 2021; Minguez *et al.,* 2021) and nutritional value (Avramidou *et al.,* 2023). The seeds contain up to 35% protein and are a good source of macro and micro nutrients (K, Ca, Mg, Fe, Zn) and other bioactive compounds such as polyphenols, carotenoids and carbohydrates. The protein content is approximately 29% of the dry weight, which is higher compared to other legumes (Knudsen *et al*., 2020; Warsame *et al.,* 2018). This makes it one of the main sources of protein for people in the Middle East, Latin America and Africa as well as for livestock feed in many developed countries (Burstin *et al*., 2011). According to De Notaris *et al.* (2023) *Vicia faba* is the main grain legume grown in Europe with increasing interest from the organic sector. Known as the primary source of dietary protein, the dry common bean

(*Phaseolus vulgaris* L.) is a legume that is enjoyed by people all over the world (Shellie-Dessert and Belis, 1991).

According to some authors (Lammerts *et al*., 2002; Popović *et al.,* 2013; 2022; Uhr *et al.,* 2014; Bozhanova *et al.,* 2014; Bozhanska *et al.,* 2024) the choice of cultivar is the crucial factor for a well-functioning system of organic farming. Overall, organic farmers need cultivars with stable and permanent yields which are tolerant to climate change (Uhr *et al.,* 2014; 2017), as well as cultivars with high quality, regardless of the reduced levels of investments (Konvalina *et al.,* 2009; Mihailović *et al.,* 2019).

However, modern varieties do not meet the needs and requirements of a system with reduced levels of production inputs. The majority of cultivars used in organic farming were developed for a conventional system with high inputs. Therefore, when developing "biological" cultivars, great attention should be paid to a complex of characteristics such as high stability, good productivity, favourable biochemical composition, tolerance to pests, competitiveness against weeds, etc. (Wolfe, 2002; Wolfe *et al*., 2008; Karkanis *et al*., 2018). Thus, one variety should combine as many of these traits and parameters as possible to ensure wider and stable production in specific environmental conditions (Flores *et al*., 2013; Asadova *et al.,* 2016).

The yields of *Vicia faba* L. are influenced by both biotic and abiotic influences. In order to determine the most promising faba bean varieties for use in organic farming as a significant source of protein for food and feed, we assessed the combined effects of faba bean types and climate on crop productivity and seed quality. Faba beans are important in animal feed and help in protein autonomy. There are logistical, technical, and sociological barriers to the promotion of faba beans (Segers *et al*., 2022).

The aim of this study was a comparative evaluation of faba bean accessions on a complex of characteristics and determine of suitable ones for organic farming conditions. Most impacting parameters yield were examined, these results show improvement of varietal resilience in European countries.

MATERIAL AND METHODS

Study Area

The field experiment was carried out at the Institute of Forage Crops-Pleven (43°24′28″N, 24°37′13″E) from February to June during the period 2019-2021. This region has a typical temperate continental climate with an average annual temperature of 12-13 °C and an annual precipitation of 548 mm. In agrometeorological terms, the conditions during the period 2019-2021 are characteristic of the region of Central Northern Bulgaria. The main climatic parameters amount of precipitation and temperature during the studied period are characterized by a strong fluctuation and uneven distribution during the vegetation period of the plants.

Figure 1 presents the data on the average monthly temperatures and the amount of precipitation by month during the growing season. The year 2019 was the most favorable with average monthly air temperatures for May 17.00 °C, June 22.3 °C and July 15.4 °C, and the rainfall was 67.8 mm, 72.1 mm and 66.0 mm, respectively. The second year (2020) is relatively similar to 2019 in terms of temperature regime (July of 14.6 °C), but with an amount of precipitation causing some dryness. The third year (2021) is characterized as cooler in the initial months of the crop vegetation and with a normalization of the temperature regime in the month of June, close to normal (20.45°C) and precipitation for May and June between 64.0 and 69.00 mm.

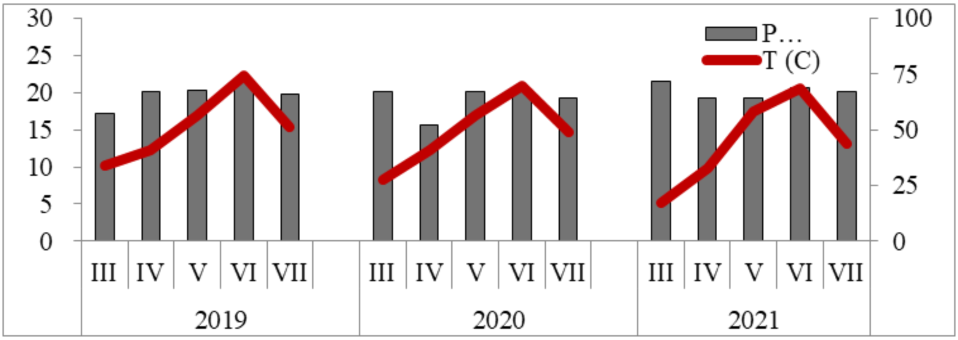

Figure 1. Climatic characterization of the experimental period, P-precipitation, mm, and T - temperature, °C, 2019-2021

In terms of geomorphology, the region is characterized by a flat-hilly relief. The soil type is leached chernozem with a slightly alkaline reaction (pH=7.2) with a sandy-clay composition. The soil reserves of hydrolyzable nitrogen are low, medium with phosphorus and very good with potassium. The content of the main nutrient elements in the soil is indicated in Table 1.

Table 1. Content of the main nutrient elements and humus in the soil

Depth (cm)	N_2 (mg/1000 g)	P_2O_5 (mg/100 g)	K_2 (mg/100g)	Humus (%)
0-20	15.60	8.63	39.90	2.24
20-40	14.28	6.79	38.70	1.95
40-60	8.86	4.48	34.10	1.36

Experimental Design

The objects of research were 11 faba bean (*Vicia faba*) accessions (Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP). The strip-plot design method with three replications was used. The sowing was carried out by hand at a rate of 40 seeds per m2. Plants were grown under organic farming conditions without the use of fertilizers and pesticides. The working plot of each genotype was 4m^2 in size.

Data Collection

During the growing season, weeds were removed manually and following indicators were recorded: plant height (PH, cm), average daily growth rate (ADGR, cm/day), aboveground biomass (AGB, g DM-dry matter/plant) (at full pod formation) and duration of vegetation period (days).

The biometric characteristics of plants at full maturity included: first pod height (FPH (cm), pods number/plant, seeds number/plant, 1000 seeds mass (g), seed weight/plant (g), pod length (cm) and pod width (cm). The seed chemical composition was determined as crude protein (CP) by Kjeldahl method and crude fiber (CF) by Weende system (AOAC, 2010). To assess the degree of attack by Botrytis fabae, a 5-point scale of EAVAS (Executive agency for variety testing, approbation and seed control) was used: 0 - healthy plants, without infestation (Immune); 1 - infested plants 0.01-5.00% (Resistance), 2 - from 5.01 to 20.00% (Moderately Resistance), 3 - 20.01-50.00% (Susceptible), 4 - above 50.00% (High Susceptible). To determine the disease atack the results were transformed by the McKinney (1923a; 2023b) formulas follows:

I - percentage index of atack (%);

E(nk) - sum of the works of the number of atacked plants or organs (n) in the corresponding attack ball (k);

N - the total number of plants examined (organs);

K – the highest grade in the corresponding scale;

$$I=(E(nk).100)/N.K, \quad (1)$$

Data Processing

The ecological stability of the studied accessions was performed using the following analyzes and parameters: regression analysis – according to Eberhart and Russell (1966), where the regression coefficient (bi) was calculated; variance analysis – through ecovalence (Wi2) Wricke (1962) and stability variance (σ^2) Shukla (1972) and non-parametric analysis – by the yield and stability index (YSi) of Kang (1993). The coefficient of variation (CVi, %) (Francis and Kannenberg, 1978) was used for adiitional evaluation. Also, a GGE biplot model that uses a single decomposition of the first two principal components (PC1 and PC2) was also applied Yan (2002). All experimental data were processed statistically using the computer software GENES for Windows XP (Cruz, 2009) and Statgraphics Plus (1995) for Windows Ver. 2.1.

RESULTS

Growing season

The growth and development of faba bean accessions during the three experimental years underwent under different environmental conditions, which is a prerequisite for their objective assessment. The vegetation period of 2019 and 2021 was characterized by favorable conditions for the crop development. Considerably less favorable were the conditions in 2020 (14.6 °C and 99 mm). The average duration of the growing season of faba bean accessions was 89 days. Most of them demonstrated a shorter vegetation of 88 days (Fb 02, Fb 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98), and the longest one (93 days) was the vegetation period in BGP, Figure 2.

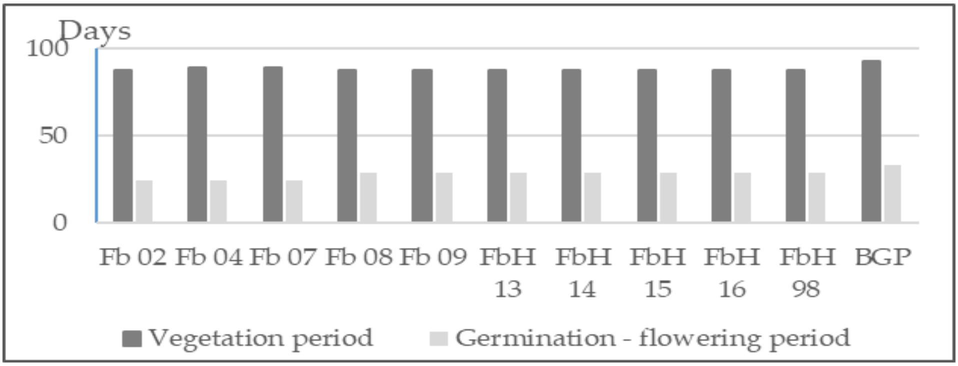

Figure 2. Duration of vegetation period in faba bean accessions (Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions)

Morphological characteristic

The data regarding the morphological characters PH, ADGR and AGB had variation coefficients from 14.2 to 15.9%, Table 2. On average, for the 3-year experimental period, PH and ADGR rate ranged from 49.1 to 85.5 cm and from 0.58 to 0.94 cm/day, respectively.

Table 2. Morphological parameters in faba bean accessions

Accessions	AGB		PH		ADGR		Average arithmetic rank
	g plant^{-1}	rank	cm	rank	cm/day	rank	
Fb 02	41.02 ^{ef}	4	60.3^{e}	4	0.71 ^{ef}	4	4
Fb 04	43.10 ^{f}	1	63.4 ^{f}	2	0.73 ^{fg}	3	2
Fb 07	34.67^{c}	9	54.8^{b}	9	0.64 ^{b}	8	9
Fb 08	41.72^{f}	3	53.8^{b}	10	0.63^{b}	9	7
Fb 09	37.48^{d}	6	56.8^{c}	8	0.67^{c}	7	7
FbH13	30.53^{b}	10	59.7^{de}	5	0.70 ^{de}	5	7
FbH 14	38.27^{de}	5	58.8^{de}	6	0.69 ^{cd}	6	6
FbH 15	36.42^{cd}	8	58.4^{cd}	7	0.69 ^{cd}	6	7
FbH 16	36.93^{cd}	7	62.7 ^{f}	3	0.74 ^{g}	2	4
FbH98	23.39 ^{a}	11	49.1 ^{a}	11	0.58 ^{a}	10	11
BGP	43.02 ^{f}	2	85.5 ^{g}	1	0.94 ^{h}	1	1
VC (%)	15.9		15.4		14.2		
min	23.39		49.1		0.58		
max	43.10		85.5		0.94		
average	36.39		61.37		0.71		
IV	19.71		36.4		0.36		

Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP-faba bean accessions; AGB-aboveground biomass, PH-plant height, ADGR-average daily growth rate; IV - Interval variation

Regarding the specified parameters, BGP significantly exceeded the average values for the group, respectively, by 41.8 and 34.8%. The same genotype was distinguished by in-creased biomass accumulation, in which case the difference compared to the average group value was 16.4%.

According to the analysis of variance, genotype, environment, and their interaction had a significant effect on the variation of all traits except pod length and width (Table 3).

For the first trait, the influence of environment factor was not significant, and for the second trait – the influence of the genotype and genotype × environment interaction. Variation of the traits of plant height, 1st pod height, pods and seeds/plant, and seed weight/plant was determined to the greatest extent by the environmental factor.

Although the effects of genotype and Genotype × environment - G × E interaction in this study were less pronounced than the environmental effect, they were statistically significant. G×E interaction is determinant for the manifestation of 1000 seeds mass. The existence of a significant influence of the three factors is a prerequisite for stability evaluation. The assessment of stability under changing environmental conditions enables a complex evaluation of the accessions and their further use for breeding purposes.

Table 3. ANOVA regarding main quantitative traits in faba bean accessions

Source of variation	**Deg.of Freed.**	**Mean squares**			
		PH	**FPH**	**PNP**	**SNP**
Environment, E	2	2671.71**	114.24**	161.44**	1821.44**
Genotype, G	10	260.16**	15.22**	17.75**	125.47**
G × E	20	89.738**	29.47**	7.91**	84.36**
		SWP	**TSM**	**PL**	**PW**
Environment, E	2	1436.51**	600.06**	1.41^{ns}	1.73**
Genotype, G	10	30.64**	1240.29**	3.93**	0.15^{ns}
G × E	20	122.59**	4961.23**	1.96**	0.61^{ns}

PH - plant height (cm), FPH - 1st pod height (cm), PNP - pods number/plant, SNP - seeds number/plant, SWP - seed weight/plant, TSM - 1000 seeds mass (g), PL - pod length, PW - pod width; * significance at $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$; ns – nonsignificant

The quantitative characterization of the main biometric traits showed that the accessions differed in their genetic endowments (Table 4).

BGP was of interest due to the high values of plant height, number of pods and seeds/plant. Fb 08 and FbH 14 were distinguished by the highest seed weight/plant, and the differences between them were statistically insignificant. Fb 04 and FbH 15 were characterized by 1000 seeds mass essentially exceeding that of FbH 98 and BGP. Regarding the traits of pod length and width, the differences between the individual genotypes were insignificant.

Table 4. Biometric characteristic of faba bean accessions

Accessions	FPH	PNP	SNP	SWP	TSM	PL	PW
Fb 02	24.18	7.55	19.68	22.41	121.70	9.073	2.151
Fb 04	25.10	7.87	20.15	22.71	124.92	8.801	2.057
Fb 07	22.52	6.20	19.82	21.30	111.51	10.941	2.034
Fb 08	20.28	9.18	25.02	24.92	102.33	9.108	1.941
Fb 09	24.08	6.28	20.82	20.92	102.20	10.537	2.022
FbH 13	21.68	5.37	18.10	17.35	103.43	10.077	2.002
FbH 14	20.70	6.90	19.32	23.59	120.28	9.228	1.725
FbH 15	22.93	5.87	17.35	20.58	124.74	9.728	1.828
FbH 16	25.88	7.20	19.87	21.47	114.52	10.578	2.134
FbH 98	18.38	5.55	15.69	13.14	84.27	8.474	1.625
BGP	21.37	13.95	39.60	20.97	57.36	6.915	1.435
CV (%)	10.03	32.65	30.22	15.34	19.16	12.23	11.75
$LSD_{0.05}$	0.86	0.90	2.07	2.13	3.72	0.54	0.053

Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions; FPH - 1st pod height (cm), PNP - pods number/plant, SNP - seeds number/plant, SWP - seed weight/plant, TSM - 1000 seeds mass (g), PL - pod length, PW -pod width, CVi, %-coefficient of variation, LSD- Fisher's Least Significant Difference test

Stability assessment

The variation coefficient calculated for seed weight/plant showed a strong variability of the trait for all genotypes – from 36.37% (at FbH 98) to 58.11% (FbH 16) (Table 5).

Table 5. Stability parameters in faba bean accessions

Accessions	CVi	bi	Wi^2	σ^2	YSi
Fb 02	40.76	0.95 ^{ns}	0.08	0.35	10.00+
Fb 04	46.43	1.06 ^{ns}	0.11	0.30	8.00+
Fb 07	44.53	0.83 ^{ns}	0.77	0.92	2.00
Fb 08	43.14	1.20 ^{ns}	1.07	1.46	13.00+
Fb 09	55.24	1.08 ^{ns}	0.16	0.21	4.00+
FbH 13	42.34	0.64*	3.48	5.90	-6.00
FbH 14	50.99	1.05 ns	0.06	0.37	5.00+
FbH 15	39.57	0.63*	3.62	6.14	-4.00
FbH 16	58.11	1.34*	3.15	5.28	5.00+
FbH 98	36.37	0.56**	5.28	9.19	-9.00
BGP	41.50	1.66***	11.81	21.16	6.00+

02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions; CVi - coefficient of variation,]bi - regression coefficient, Wi2 - ecovalence , σ2 - stability variance, YSi - yield and stability index

The linear regression coefficient for some of the genotypes was not significant. Therefore, a strong assessment of stability based on this parameter cannot be done. Accessions that have lower values are evaluated as more stable because they interact less with the environmental conditions. It can be noted that FbH 98 (bi=0.56), FbH 15 (bi=0.63) and FbH 13 (bi=0.64) exhibited stability. The values of "bi" for BGP (1.68) and FbH 08 (1.28) were statistically significant but higher than 1.

Therefore, these genotypes can be considered unstable. Parameters based on the ecovalence Wi2 and stability variance σi2 (OAAC, 2010; Wricke, 1962). Shukla (1972) identified FbH 14, Fb 02, Fb 04, Fb 09 and Fb 07 as the most stable. The remaining genotypes exhibited instability to one degree or another. BGP, FbH 98, FbH 15 and Fb 08 were in a position of greater instability.

The summarizing criterion for stability "YS'" of Kang (1993), considering both stability and productivity, gave a high positive estimation to Fb 08 (13.00+) and Fb 02 (10.00+), followed by Fb 04 (8.00+) and BGP (6.00 +). They combined high values of seed weight/plant and good stability during the different years. The ranks of the stability parameters defined Fb 02 as the best one since it always occupies one of the first three positions (Table 6). Fb 04 was also of interest, followed by Fb 08, which had the first position according to the ranking of "bi" and "YSi" parameters. However, the same accessions did not perform well according to the other parameters, occupying the sixth rank. FbH 14 had a similar manifestation. It was distinguished by high productivity but did not have good positions in some of the parameters.

Table 6. Ranking of stability parameters in faba bean accessions

Accessions	CVi	bi	Wi^2	σ^2	YSi
Fb 02	3.00	1.00	2.00	2.00	2.00
Fb 04	8.00	1.00	3.00	3.00	3.00
Fb 07	7.00	1.00	5.00	5.00	8.00
Fb 08	6.00	1.00	6.00	6.00	1.00
Fb 09	10.00	1.00	4.00	4.00	7.00
FbH 13	5.00	2.00	8.00	8.00	10.00
FbH 14	9.00	1.00	1.00	1.00	5.50
FbH 15	2.00	2.00	9.00	9.00	9.00
FbH 16	11.00	2.00	7.00	7.00	5.50
FbH 98	1.00	3.00	10.00	10.00	11.00
BGP	4.00	4.00	11.00	11.00	4.00

Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions. CVi - coefficient of variation, bi - regression coefficient, Wi2 - ecovalence, σ2 - stability variance, YSi - yield and stability index

GGE biplot analysis

The GGE biplot analysis provides information on the relationship between the productivity level and the degree of its stability (Figure 3). The first two principal components PC1 (94.60%) and PC2 (5.40%) can be considered reliable because they explain 100% of the reasons for variation in the faba bean accessions. For an assessment of each tested genotype, the starting point is the small circle in the biplot center, which represents the so-called "mid" coordinate. BGP, FbH 15, and Fb 07 are located in the small concentric circle, and their productivity was around the group average. However, they have an advantage over others because they exhibit stability over a wider range of environmental conditions. FbH 16 also occupies a good position. It has a relatively high productivity (21.47 g), but its low PC2 value is an indication that it can adapt to more specific environmental conditions.

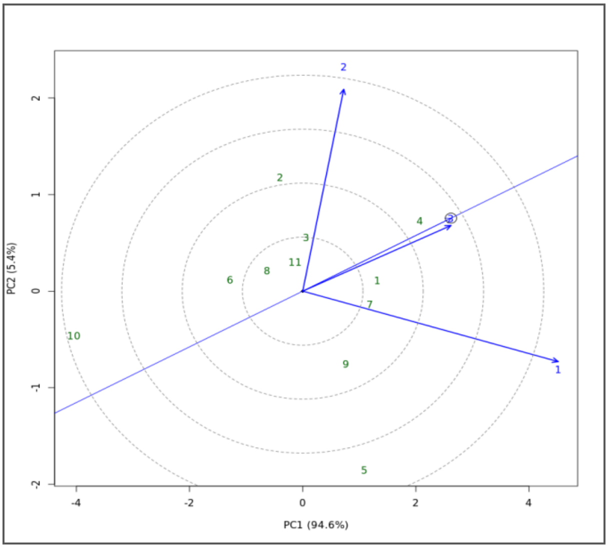

Figure 3. GGE biplot regarding productivity in faba bean accessions - Fb 02, 2 - Fb 04, 3 - Fb 07, 4 Fb 08, 5 - Fb 09, 6 - FbH 13,7 - Fb H 14, 8 - FbH 15, 9 - FbH 16, 10 - Fb 98, 11 - BGP; Vectors 1, 2 and 3 are 2019, 2020 and 2021, respectively

Genotypes characterized by productivity values above the group average and PC2 values close to zero are highly adapted to different environmental conditions. In the present study, Fb 02 (22.41 g) and FbH 14 (23.59 g) are highly productive and located near the smallest concentric circle. The location of Fb 08, which is at the far right of the coordinate system, shows that it is the most productive (24.92 g). However, it falls into a concentric circle at a greater distance from the center, which determines its lower stability. The projection of Fb 04 in the coordinate system suggests that it exhibits a level of stability similar to Fb 08 (its variation under experimental conditions is analogous). The interest in Fb 04 is dictated by the fact that this variety is in the group of high-yielding genotypes (22.71 g). FbH 13 is weakly productive but stable, and FbH 98 occupies the last position in terms of productivity and stability. The analysis shows the importance of contrasting environmental conditions for the differentiation of genotypes according to their productivity and stability.

Reaction to Botrytis fabae

Vicia faba is attacked by many diseases, including fusarium (Fusarium spp.), bacteriosis (*Xanthomonas sp*.), downy mildew (*Peronospora fabae* Jacz.), rust (Uromyces fabae (Persson) de Bary), ascochytosis (Ascohyta fabae *Spegazzini*), chocolate spot (*Botrytis fabae* Sard.), etc. (Stancheva, 2002). During the 3-year experimental period, periodic observations were done to establish the tolerance of the 11 accessions to chocolate spot under a natural background of infestation. Disease symptoms were observed only in 2020, as the optimum conditions for its development being a moderate temperature of 15-18 °C and high relative humidity (over 90%) during several consecutive days (Stancheva, 2002). Characteristic chocolate, rounded, slightly indented spots were formed on both sides of the leaf surface. Similar spots, but elliptical in shape, appeared on stems and pods. The infestation index, calculated according to the formula of McKinney (1923), showed susceptibility to B. fabae in the majority of the studied accessions (Fb 02, Fb 07, Fb 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16) and high susceptibility in one of them (FbH 98) (Figure 4). BGP and Fb 04, with an infestation index of 11.4 and 16.5%, respectively, were determined as moderately resistant to chocolate spot.

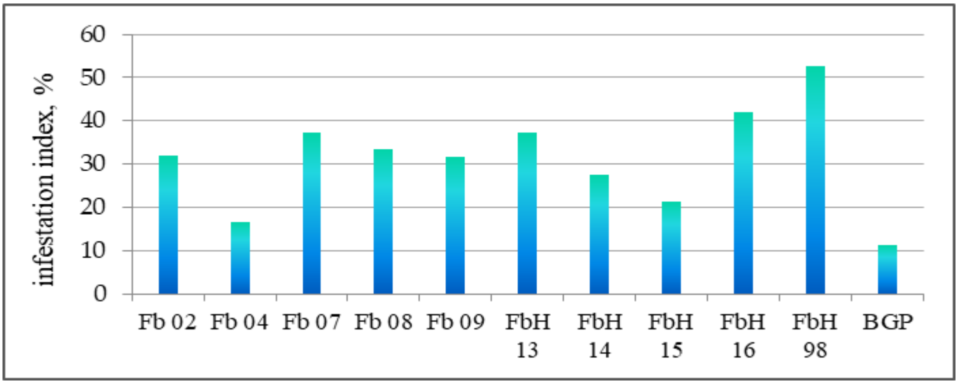

Figure 4. Reaction of faba bean accessions to Botrytis fabae Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions

Biochemical analysis

The seed biochemical analysis concerning CP and CF content showed mean values of 215.96 and 111.74 g/kg^{-1} DM, respectively (Figure 5). Fb 04 and FbH 13 were characterized by a more favorable biochemical composition, in which the CP content was 9.1 and 7.8% higher compared to the average group value. The same accessions were also distinguished by a decreased content of CF, with differences of 5.0 and 7.5%, respectively. Significantly higher protein content, above the group average, was also found in Fb 02, Fb 07 and Fb 16. The complex evaluation regarding main characteristics and parameters (Table 7) determined BGP as the most suitable (rank 1) for organic cultivation conditions in herbaceous forage direction.

It was characterized by a fast growth rate (0.94 cm/day), high values in terms of height and aboveground biomass (88.5 cm and 43.02 g/plant^{-1}, respectively), and the highest tolerance to B. fabae (I = 11.4%).

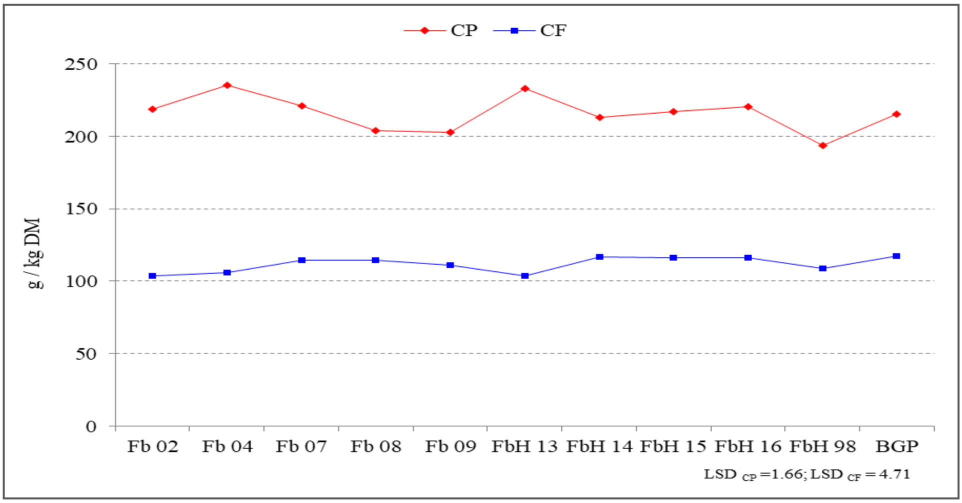

Figure 5. Biochemical composition of seeds in faba bean accessions; Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP - faba bean accessions, g/kg DM - Dry matter content and chemical compositionM CP - crude protein, CF-crude fiber

Table 7. Ranking in terms of main characters and parameters in faba bean accessions

Accessions	**AGB**	**PH**	**TO**	**T**	**Average arithmetic rank**	**SWP**	**CP**	**S**	**T**	**Average arithmetic rank**
Fb 02	4	4	4	6	5	4	5	2.00	6	4
Fb 04	1	2	3	2	2	3	1	3.00	2	2
Fb 07	9	9	8	8	9	6	3	8.00	8	6
Fb 08	3	10	9	7	7	1	9	1.00	7	5
Fb 09	6	8	7	5	7	8	10	7.00	5	8
FbH 13	10	5	5	8	7	10	2	10.00	8	8
FbH 14	5	6	6	4	5	2	8	5.50	4	5
FbH 15	8	7	6	3	6	9	6	9.00	3	7
FbH 16	7	3	2	9	5	5	4	5.50	9	6
FbH 98	11	11	10	10	11	11	11	11.00	10	11
BGP	2	1	1	1	1	7	7	4.00	1	5

Fb 02, Fb 04, Fb 07, F b 08, Fb 09, FbH 13, FbH 14, FbH 15, FbH 16, FbH 98, BGP-faba bean accessions. AGB - aboveground biomass, PH- plant height, ADGR - average daily growth rate, T-tolerance to Botrytis fabae, SWP- seed weight/plant, CP- crude protein, S- stability according to YSi

In grain direction, as exhibiting suitable characteristics for organic production, Fb 04 (rank 2) was identified, which was distinguished by high seed productivity (22.71 g/plant^{-1}) and stability (YSi=8.00+), forage quality (CP=235.60 g/kg DM) and moderate resistance to B. fabae (I=16.5%). Also, accession Fb 04 had good performances in the herbaceous-forage direction.

DISCUSSION

Promising and crucial for faba bean farmers are the Botrytis fabae tolerant accessions that shown good and consistent plant height and grain yield performances in the current investigation. The genotype's performance must be consistent and the winning genotype must be repeatable in the same areas in order to define a mega-environment (Gauch *et al.,* 2013).

A genotype that consistently performs well and has one of the highest yields throughout test settings is considered desirable (Yan and Kang, 2003). Accessions exhibiting strong and stable plant height performance are crucial for the areas where faba beans are mostly farmed for animal feed since plant height and biological yield are highly connected (Maalouf *et al.,* 2015; Kubure *et al.,* 2016).

The duration of the vegetation season is determined by the cultivar and environmental conditions (Animasaun, 2015). The studied accessions were characterized by a low variability of the duration of the germination-ripening period (CV=1.7%), which, expressed in absolute values, was from 88 to 93 days. Similarly, Horaicu *et al.* (2024) found a vegetation of 80-90 days for conditions in Romania. Other researchers reported a more substantial variation in the number of days to maturity for different faba bean genotypes: 112 - 154 days in the Central Region of Saudi Arabia (Ammar et al., 2015), 112 - 132 days in India (Kumar *et al*., 2023), and 112 - 173 days in New Zealand (Martini *et al.,* 2012). In testing 6 genotypes at 4 locations, (Lake *et al.,* 2019) recorded 136-182 days from sowing to maturity for Australian conditions. Varieties with early ripening can avoid severe drought during the summer period. The short growing season is an important characteristic of annual legumes, given the changes in the global climate (Tesfahun, 2018) and the established permanent trend towards warming and drying in Northwest Bulgaria, where the study was carried out (Zhekova *et al*., 2022).

Morphological traits determining better plant competitiveness against weeds in organic farming include greater height, faster daily growth rate and early biomass accumulation (Uhr *et al*., 2014). Paolini and Faustini (2005) also indicated that suitable cultivars for bioproduction are those with medium and greater height as well as with rapid biomass accumulation, which is associated with greater competitive ability. BGP met these requirements to the greatest extent, followed by Fb 04 and Fb 02.

In terms of some of the main quantitative traits related to productivity, the variation in absolute values was as follows: pod numbers – from 5.37 to 13.95 (VC=32.65%), seed numbers – from 15.69 to 39.60 (VC=30.22), seed numbers per plant – from 13.14 to 24.92 g (VC=15.34). A wide range of variation in different agro-morphological characteristics was reported by (Ammar *et al.,* 2015) in a study of 40 faba bean genotypes of different origin (England, Spain, Egypt, Sudan, etc.) in the Riyadh area (Saudi Arabia): from 81.4 to 139.5 cm for plant height, from 8.8 to 44.3 for pods number, from 23.3 to 100.8 for seeds number and from 14.9 to 70.8 g for seed weight/plant. Yüce *et al.* (2023) indicated the following range values for plant height – 25-59 cm, pods number – 1-33, seeds number – 2.40-106, seed weight/plant – 1.80-224.9 g and 1000 seeds mass – 480-2140 g. In a similar experiment, (Abou-Khater *et al*., 2022) also observed a strong variation in the indicated traits. According to the authors, variability is due to the genetic constitution of the genotype and its response to environmental conditions.

In the present experiment, we found that seed productivity (seed weight per plant), as well as all other quantitative traits (except pod length), were highly influenced by environment. In agreement with our findings, previous studies have also found the main factors determining faba bean seed yield to be environmental (Fikere *et al.,* 2008; Temesgen *et al.,* 2015). Opposite results were obtained by Wang *et al.,* (2023) and Neda *et al.* (2021), according to which the genotype is decisive for the manifestation of most quantitative traits. According to Neda et al. (2021), this is indicative of the presence of a high level of genetic diversity in the studied collection and that the breeding methods applied in the future would be effective for trait improvement.

Chocolate spot is one of the most important economic disease that damages the foliage, limits photosynthetic activity and faba bean production (Torres *et al.,* 2006). According to Harrison (1988) and De Notaris *et al.* (2023), plants under organic conditions are particularly affected, as damage caused by the necrotrophic fungus B. fabae can lead to complete defoliation at moderate temperatures and high humidity (Harrison, 1988). Finding cultivars with expressed tolerance to the disease is one of the most effective methods of control, especially in organic farming conditions. However, according to Adhikari (2023), a high tolerance level is difficult to establish because resistance to B. fabae is controlled by multiple genes. According to Gela *et al.* (2022), a cultivar (PBA Amberley) with well-expressed tolerance to the disease was developed in Australia. Considerable differences were found by Beyene *et al.* (2014), who tested 10 cultivars and 50 local populations. The highest level of resistance was observed in ILB-4726, ILB-938 and BPL-710. According to the authors, the resistance is highly heritable, suggesting that phenotypic breeding could be used to improve tolerance to chocolate spot in faba bean. In assessing 11 faba bean genotypes, (El-Abssi *et al.,* 2024) observed high resistance in 6 of them (Nubaria-3, Nubaria-4, Nubaria-5, Sakha-4, Giza-3, Triple White), and medium and low resistance in the remaining genotypes (Giza-843, Misr-3, Sakha-1, Sakha-3, Wadi-1). In the same study, stability was evaluated using different methods and parameters: regression coefficient (bi) and deviation mean square Finlay from linear regression (S2d) were calculated according to Eberhart and Russell (1966), ecovalence (Wricke, 1962) and AMMI's stability value (ASV) (Purchase, 1997). The results showed that Sakha-1, Misr-3, Nubaria-4 and Nubaria-5 demonstrated stable productivity in all tested environments. Finlay *et al.* (1963) used the regression coefficient to assess stability in a collection of 9 faba bean cultivars. The regression coefficient in Gracia (1.12), Pyramid (1.11), and Vertigo (1.19) was considerably higher than 1, indicating larger G×E responses than average. In contrast, Kontu (0.72), Snowdrop (0.62) and SSNS-1 (0.90) demonstrated regression coefficients significantly less than 1, indicating lower sensitivity to the environment. The remaining cultivars (Lynx, Mistral, Taifun) had regression coefficients that did not differ significantly from 1, indicating broad adaptability. Similarly, Yansen *et al.* (2022) estimated the ecological stability of 20 faba bean genotypes and identified several groups with different productive capabilities and specific responses to the environment. They found that genotypes G06, G08, G10, G13, G16, G18, and G20 combined high productivity with stability in different growing environments. G15 had the highest yield but was characterized by high variability and fell into the group of unstable genotypes. According to Abou-Khater *et al*. (2022), an assessment of ecological stability should be done based on several different parameters to obtain a clear idea of the suitability of a given genotype to specific growing conditions. After evaluating 37 faba bean accessions, the authors identified 5 genotypes (IG12983, IG13945, IG13906, IG106453, FB2648) with good productivity and stability. In the present experiment conditions, five stability parameters were calculated, which do not give an unambiguous assessment of the stability of the tested accessions. Our final evaluation was based to a greater extent on Kang's stability index, which considers both stability and productivity. As we have pointed out in previous studies (Mohammed *et al.,* 2023), the differences in stability assessments for individual parameters are due to the fact that different assessment methods are based on different concepts of stability.

Some researchers (Georgieva *et al.,* 2024; Rubiales *et al.,* 2012) recommended the application of GGE biplot analysis for better visualization of results in yield and stability evaluation. According to (Papastylianou *et al.,* 2021) and (Flores *et al.,* 2013; Božović *et al*., 2020; 2022; Rakašćan *et al*., 2021; Popović *et al.,* 2024), the GGE biplot is a good statistical tool for analyzing data from testing genotypes in multiple environments. They viewed the graphical representation of genotype-environment interaction effects using the GGE biplot and believe that this is a way of better exploring the relationships between test environments and the performance and stability of genotypes.

The seed biochemical analysis of the studied accessions in terms of CP and CF content showed a variation from 193.90 to 235.60 g/kg^{-1} DM (CV=5.7%) and from 103.40 to 117.30 g/kg^{-1} DM (CV=4.8%), respectively. Considerably higher CP values have been reported in the conditions of previous investigations: 24.60-30.68% (Papastylianou *et al.,* 2021), 24.7 – 37.2% (Duc *et al*., 1999), 26.6 - 34.9%, 31.8 - 39.7% (Alghamdi, 2009) etc. Obviously, the cultivar and environmental conditions are determinant for this.

CONCLUSIONS

A comparative evaluation of 11 faba bean accessions under organic cultivation conditions was carried out. It was found the accessions differed in vegetation duration, habitus, productivity, stability, biochemical composition and tolerance to Botrytis fabae. For productivity parameters, the current analysis demonstrates a considerable genotype × environment interaction, underscoring the necessity of choosing both broadly suited and genotypes that are well adapted to a particular environment.

The complex evaluation regarding main characteristics and parameters determined BGP as the most suitable (rank 1) for organic production in herbaceous forage direction. The same was characterized by a fast growth rate (0.94 cm/day), high values in terms of height and aboveground biomass (88.5 cm and 43.02 g/plant^{-1}, respectively), and the highest tolerance to B. fabae (I=11.4%).

In grain direction, with suitable characteristics for organic production, Fb 04 (rank 2) was identified. It was distinguished by high seed productivity (22.71 g/plant^{-1}) and stability (YSi=8.00+), forage quality (CP=235.60 g/kg^{-1} DM) and moderate resistance to *B. fabae* (I=16.5%). Also, accession Fb 04 had good performances in the herbaceous-forage direction.

The tolerant germplasm found in this study must be crossed with other cultivars specialized to certain habitats in order to create superior Botrytis fabae tolerant cultivars that are widely adapted to many large environments. Additionally, these lines could be crossed with other cultivars to accumulate economically valuable features in new kinds of faba beans.

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THE EFFECT OF COMPLETE AND PARTIAL REPLACEMENT OF SOYBEAN MEALS WITH HIGH-PROTEIN SUNFLOWERS CONCENTRATE ON THE DIET OF BROILER CHICKENS ON THEIR GROWTH PARAMETERS, MEAT PRODUCTIVITY AND MEAT QUALITY

SUMMARY

Soybean meal (SBM) is the main source of protein in poultry diets, but due to its increasing price, alternative protein ingredients are needed to form a balanced and economical diet. Plant protein sources, such as high-protein sunflower concentrate (HPSC), can be a potential alternative to soybean meal in the diet of broiler chickens. The study aimed to evaluate the effect of full and partial replacement of soybean meal with high-protein sunflower concentrate in broiler chicken diets. For this purpose, 400 one-day-old broiler chickens were randomly divided into four groups (SBM (control group), HPSC, HPSC50cp and HPSC50q) of 100 birds each. The SBM control diet did not contain HPSC, while the HPSC, HPSC50cp and HPSC50q diets were based on complete, 50% crude protein and 50% quantity replacement of soybean meal with high protein sunflower concentrate, respectively. At 42 days of age, 6 broiler chickens were selected from each group of birds for slaughter and meat quality assessment. It was found that the most effective for the body gain of broiler chickens is HPSC50q, which contributes to an increase in body weight by 3.2%, pre-slaughter live weight by 3.2%, bled carcass weight by 4.7%, non-eviscerated carcass weight by 4.1%, semi-eviscerated carcass weight by 4.0% and eviscerated carcass weight by 4.4%, breast muscle weight by 6.6%, thigh by 3.7%, drumstick by 4.5% and wing by 2.0%, as well as a higher yield of bled carcass by 1.3%, non-eviscerated carcass by 0.6%, semi-eviscerated carcass by 0.6% and eviscerated carcass by 1.0% , a higher yield of edible parts of the carcass, namely breast muscles by 0.88%, thigh by 0.22% and drumstick by 0.31%. In addition, HPSC50q contributes to a decrease in the crude fat content in the breast muscles of broiler chickens by 18.8% and in the thigh muscles by 25.0%, while reducing the energy value by 7.5% and 10.6%, respectively, compared to SBM.

Keywords: sunflower concentrate, soybean meal, broiler chickens, live weight, slaughter yield, meat quality, chemical composition of meat

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INTRODUCTION

Optimal growth performance of meat-type poultry depends on the supply of high-quality dietary protein with a well-balanced amino acid profile, accompanied by adequate levels of metabolizable energy, vitamins, and minerals (Ibatullin *et al.,* 2016; Sychov *et al*., 2022; Munawar *et al.,* 2025). Animal protein sources are not preferred for broiler diets due to the risk of contamination and the varying amount of protein available (Hafez and Attia, 2020). Plant-derived protein sources are commonly incorporated into the diets of meat-type poultry (Plyska *et al.,* 2021). Among them, soybean meal is regarded as the most valuable due to its high-quality protein and well-balanced amino acid composition (An and Kong, 2023).

However, despite the fact that Ukraine is an exporter of soybeans, fluctuations in its prices on the international market directly affect both the domestic market and the poultry industry as a whole (Mykhailova *et al.,* 2024). Therefore, plant protein sources such as sunflower (Helianthus annuus) meal may be a potential alternative to soybean meal in broiler chicken diets (Saleh *et al.,* 2021). At the same time, the use of sunflower meal in poultry diets is quite limited due to its high fibre content and low energy and lysine levels (Pilorgé, 2020; Jha and Mishra, 2021; Saleh *et al.,* 2021), which can be partially offset by the use of high-protein sunflower concentrates (Such *et al.,* 2024; Sychov *et al.,* 2025).

Sunflower seeds typically contain 35–40% husk and 60–65% kernel, and sunflower husk contains approximately 50% cellulose and 25% lignin (Munawar *et al.,* 2025). Sunflower meal is produced from sunflower seeds following oil extraction by means of a solvent. It contains, on average, 30–35% protein, 4.6% soluble and 22% insoluble non-starch polysaccharides.

The insoluble non-starch polysaccharides of sunflower meal consist of 41% cellulose, 25% pectic polysaccharides, 24% 4-omethyl-glucuronoxylans, 4.5% galactomannans and 5% fucoxyloglucans (Velázquez-De Lucio *et al.,* 2021). The presence of insoluble non-starch polysaccharides in broiler diets reduces their performance by reducing nutrient absorption due to increased viscosity of digestion thereby reducing feed utilisation efficiency (Jha and Mishra 2021). To reduce the content of insoluble non-starch polysaccharides in sunflower meal, one can use enzymes that break them down (Velázquez-De Lucio *et al*., 2021), or perform additional processing of the meal and use high-protein sunflower concentrates (Such *et al*., 2024).

In recent years, a lot of research has been devoted to studying the possibility of replacing soybean meal in broiler chicken diets with other plant sources of protein. In particular, DMaria de Moraes Oliveira *et al*. (2016) included up to 16% sunflower meal in the diet of broilers from the 1st to the 21st day of their growth and found that adding sunflower meal in a concentration of up to 8% does not affect the growth parameters of the bird, and increasing its content in the diet to 16% already significantly affects productivity. Berwanger *et al.* (2017) found that including up to 10% sunflower meal with an enzymatic complex in the diet can be effectively used in feeding broiler chickens during 21–42 days of growth. Yaqoob *et al.* (2023) also found no adverse effects from including sunflower meal at concentrations ranging from 3 to 9% in broiler chicken diets. Whereas in recent studies, Munawar et al. (2025) demonstrated that soybean meal can be partially replaced by up to 20% sunflower meal in broiler diets without negative effects on growth rate and nutrient digestibility during the growing and finishing periods. However, according to the authors, increasing the level of sunflower meal in the diet significantly increases viscosity of digestion and reduces nutrient digestibility.

However, all studies reported in the scientific literature have concerned the evaluation of the efficacy of complete or partial replacement of soybean meal with sunflower meal in broiler chicken diets. This study aims to evaluate the impact of full and partial replacement of soybean meal in the diet of broiler chickens with high-protein, self-produced sunflower concentrate, as a cost-effective alternative source of protein in poultry feeding, while mitigating the limitations of the use of sunflower meal by increasing the protein content and reducing the fibre content of the concentrate.

MATERIAL AND METHODS

Ethics

Experiments with experimental animals were performed following the rules of the European Convention for the Protection of Vertebrate Animals (Official Journal of the European Union L276/33, 2010) and the Order of the Ministry of Economy of Ukraine «On approval of the requirements for the welfare of farm animals during their housing» of February 18, 2021, were organized. The local Commission approved the experimental protocol on Bioethics of the National University of Life and Environmental Sciences of Ukraine.

Experimental design

The study focused on broiler chickens from the Cobb-500 crossbreed industrial herd. The experiment lasted 42 days, from the placement of day-old chickens for rearing to slaughter. During rearing, broiler chickens were kept in separate floor sections, as recommended by the crossbreed producer Cobb Broiler Management Guide, 2022).

At the age of one day, broiler chickens were divided into four groups of 100 birds each. The differences between the groups were due only to the composition of the diet used. In particular, the difference in feeding of birds of the control and experimental groups was due to the complete or partial replacement of soybean meal in the diet with high-protein sunflower concentrate. Broilers of the control (SBM) group received a complete feed based only on soybean meal, without the addition of high-protein sunflower concentrate. In the diet of broilers from the HPSC group, soybean meal was replaced entirely by sunflower concentrate. In chickens of HPSC50cp and HPSC50q groups, the diet was formulated so that the soybean meal replacement was 50% by crude protein (HPSC50cp) or by quantity (HPSC50q). The compound feeds were used in dry, pellet form.

Poultry feeding

The experimental broiler chickens were fed complete feeds, balanced for all nutrients according to the recommended norms. The composition of the complete feed used for feeding broiler chickens is given in Tables 1–4. The chemical composition of high-protein sunflower concentrate used in broiler diets is given in Table 5.

Table 1. Composition and nutritional value of compound feed for broiler chickens 0-12 days old, %

Indicator	Group of broiler chickens (%)			
	SBM	HPSC	HPSC50cp	HPSC50q
Corn	36.972	44.352	43.058	42.311
Sunflower concentrate SP44	–	35.648	25.000	18.850
Soybean meal SP 41	40.599	0.000	11.942	18.839
Wheat	15.000	15.000	15.000	15.000
Limestone flour Ca 35%	1.598	1.517	1.543	1.557
Cornflour	1.294	0.690	0.869	0.972
Monocalcium phosphate	0.635	0.812	0.756	0.725
Methionine 99.5%	0.372	0.228	0.270	0.295
Lysine hydrochloride	0.309	0.791	0.651	0.570
Salt	0.286	0.017	0.099	0.146
Threonine 99%	0.156	0.221	0.202	0.191
Premix	0.350	0.350	0.350	0.350
Soybean oil	2.429	–	–	–
Sodium sulfate	–	0.374	0.260	0.194
Total	100.000	100.000	100.000	100.000
Nutritional value of compound feed:				
Energy, MJ	12.70	12.88	12.67	12.54
Crude protein, %	22.00	22.00	22.00	22.00
Crude fibre, %	3.81	4.17	4.07	4.02
Digestible lysine, %	1.28	1.28	1.28	1.28
Digestible methionine and cystine, %	0.97	0.97	0.97	0.97

Table 2. Composition and nutritional value of compound feed for broiler chickens 13-28 days old, %

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Corn	43,346	49,803	48,896	48,073
Sunflower concentrate SP44		30,196	22,720	15,950
Soybean meal SP 41	34,430		8,384	15,977
Wheat	15,000	15,000	15,000	15,000
Limestone flour Ca 35%	1,358	1,290	1,309	1,325
Cornflour	2,016	1,501	1,625	1,740
Monocalcium phosphate	0,211	0,360	0,321	0,285
Methionine 99.5%	0,321	0,199	0,228	0,255
Lysine hydrochloride	0,315	0,724	0,625	0,536
Salt	0,289	0,053	0,111	0,163
Threonine 99%	0,142	0,197	0,184	0,172
Premix	0.350	0.350	0.350	0.350
Soybean oil	2.222	–	–	–
Sodium sulfate	–	0,327	0,247	0,174
Total	100.000	100.000	100.000	100.000
Nutritional value of compound feed:				
Energy, MJ	12.98	13.09	12.94	12.80
Crude protein, %	20.00	20.00	20.00	20.00
Crude fibre, %	3.56	3.87	3.80	3.74
Digestible lysine, %	1.16	1.16	1.16	1.16
Digestible methionine and cystine, %	0.88	0.88	0.88	0.88

Data Collection

During the rearing period, the number of broiler chickens removed (due to mortality and culling) was recorded, and their live weight was measured every seven days using "Total Control" scales (Italy). At 42 days of age, 6 broiler chickens were selected from each group of birds for slaughter (3 male and 3 female; selected analogues by average weight in the group) and their pre-slaughter live weight, weight of bled carcass, non-eviscerated carcass, semi-eviscerated carcass and eviscerated carcass, as well as the weight of edible parts - breast, thigh, drumstick, wing, heart, liver and gizzard were determined.

Table 3. Composition and nutritional value of compound feed for broiler chickens 29-35 days old, %

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Corn	46.380	52.276	51.530	50.687
Sunflower concentrate SP44	–	27.724	21.590	14.650
Soybean meal SP 41	31.606	–	6.880	14.663
Wheat	15.000	15.000	15.000	15.000
Limestone flour Ca 35%	1.258	1.196	1.210	1.227
Cornflour	2.386	1.912	2.015	2.131
Monocalcium phosphate	0.089	0.226	0.195	0.159
Methionine 99.5%	0.278	0.166	0.190	0.218
Lysine hydrochloride	0.259	0.635	0.554	0.463
Salt	0.290	0.072	0.120	0.173
Threonine 99%	0.091	0.142	0.131	0.119
Premix	0.348	0.348	0.348	0.348
Soybean oil	2.015	–	–	–
Sodium sulfate	–	0.303	0.237	0.162
Total	100.000	100.000	100.000	100.000
Nutritional value of compound feed:				
Energy, MJ	13.06	13.17	13.05	12.90
Crude protein, %	19.00	19.00	19.00	19.00
Crude fibre, %	3.46	3.74	3.68	3.62
Digestible lysine, %	1.06	1.06	1.06	1.06
Digestible methionine and cystine, %	0.82	0.82	0.82	0.82

As part of the study, the chemical composition of meat obtained from birds selected for slaughter was analyzed using methods described in AOAC (2000) immediately after slaughter. To determine moisture content, meat samples were dried at 110°C for 24 hours by the drying method (AOAC, 950.46) using a Termolab CHOL 58/350 drying oven (Ukraine). The weight was recorded, and the moisture content was calculated. In dry matter, the following was determined: raw ash – by burning the sample in a muffle furnace at a temperature of 550±20 °C for 4 hours (AOAC, 920.153). A SNOL-8.2/1100 muffle furnace (Republic of Lithuania) was used for combustion. The Kjeldahl method (AOAC, 928.08) was used to determine crude protein; a Gerhardt Turbotherm TT–625 (Germany) was used for ashing meat, and a Gerhardt Vapodest–300 (Germany) was used for distillation. Nitrogen was converted to crude protein by multiplying with a factor of 6.25. The analysis was carried out following the steps of cleavage, distillation and titration. Crude fat content was determined by Soxhlet extraction (AOAC, 991.36) by extracting samples with ether by melting the fat in it, using a Velp Scientifica UDK139 (Italy). Benzene was used as an extractant. Nitrogen-free extractives were determined by calculation based on the difference between the percentage of dry matter of crude protein, crude fat and crude ash. To determine the energy value of chicken meat, an IKA C 2000 basic Version 1 calorimeter (Germany) was used.

Table 4. Composition and nutritional value of compound feed for broiler chickens 36-42 days old, %

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Corn	49.247	54.687	54.096	53.236
Sunflower concentrate SP44	–	25.313	20.450	13.370
Soybean meal SP 41	28.868	–	5.454	13.394
Wheat	15.000	15.000	15.000	15.000
Limestone flour Ca 35%	1.235	1.177	1.188	1.208
Cornflour	2.672	2.238	2.320	2.438
Monocalcium phosphate	0.061	0.186	0.161	0.124
Methionine 99.5%	0.215	0.113	0.132	0.160
Lysine hydrochloride	0.202	0.546	0.482	0.388
Salt	0.291	0.091	0.129	0.183
Threonine 99%	0.040	0.087	0.078	0.065
Premix	0.284	0.284	0.284	0.284
Soybean oil	1.885	–	–	–
Sodium sulfate		0.278	0.226	0.150
Total	100.000	100.000	100.000	100.000
Nutritional value of compound feed:				
Energy, MJ	13.15	13.23	13.13	12.99
Crude protein, %	18.00	18.00	18.00	18.00
Crude fibre, %	3.35	3.61	3.56	3.50
Digestible lysine, %	0.96	0.96	0.96	0.96
Digestible methionine and cystine, %	0.74	0.74	0.74	0.74

Table 5. Chemical composition of high-protein sunflower concentrate, g/kg

Indicator	Contents	
	for natural moisture	on dry matter
Moisture	74.1±0.37	–
Crude protein	439.3±0.68	474.5±0.72
Crude fat	9.7±0.08	10.5±0.06
Crude fibre	73.9±0.43	79.8±0.52
Crude ash	68.2±0.27	73.7±0.31
NFE	334.7±1.23	361.5±1.11
NDF	223.7±0.89	241.6±0.97
ADF	78.6±0.31	84.9±0.26
Lignin	10.5±0.11	11.3±0.09

Meat quality assessment was performed 24 hours after slaughter on samples of breast muscle and thigh muscle taken during post-slaughter processing of carcasses and storage in a cold room at a temperature of 4–6 °C. The water holding capacity of breast and thigh meat samples of broiler chickens was determined according to the method described by Barbut S. (2024). A 1 g portion of the meat sample was centrifuged in an Eppendorf 5804R centrifuge (Germany) at 1000 rpm for 15 minutes at 4 °C without NaCl. The extracted water content was then measured and calculated as a percentage of the initial mass of the meat sample. The active acidity of meat was determined using a pH meter "pH-161" (Romania) using two types of electrodes – E536BNC and E531BNC. Cooking losses were determined by cooking meat samples (breasts and thighs) at 90°C for 30 minutes in a hot water bath, followed by cooling to room temperature. The weight of the sample before (W1) and after (W2) cooking was recorded to calculate cooking losses using the following formula 1:

$$Cooking\ loss,\% = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

Statistical analysis

The methods of variational statistics processed the obtained digital results by using software Statistica 10. The significance of differences between groups was assessed using a one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test as a post-hoc testing tool. Checking the distribution of sample data for normality was performed using the Kolmogorov-Smirnov test. If the data distribution was likely to differ from normal, the non-parametric Mann-Whitney U-test was used. Differences between groups were considered significant at P<0.05.

RESULTS AND DISCUSSION

The effect of using high-protein sunflower concentrate in feeding broiler chickens on their viability, body weight and growth rate

The survival of the broiler chicken population throughout the study period corresponded to the regulatory level recommended by the breeder of the cross (Cobb Broiler Management Guide 2022), with no apparent effect of diet composition (Table 6).

Table 6. Broiler chicken survival depending on the level of replacement of soybean meal in the diet with high-protein sunflower concentrate (%, x ± SE, n = 100/group)

Age period, days	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
1–7	100.0±0.00	100.0±0.00	100.0±0.00	99.0±0.10
8-14	99.0±0.09	99.0±0.09	98.0±1.40	98.0±1.71
15-21	98.0±1.40	98.0±1.40	98.0±1.40	98.0±1.40
22-28	98.0±1.40	98.0±1.40	97.0±1.71	98.0±1.40
29–35	97.0±1.71	97.0±1.71	97.0±1.71	97.0±1.71
36–42	96.0±1.96	97.0±1.71	96.0±1.96	96.0±1.96

With regard to broiler body weight, changes were observed under the influence of partial substitution of soybean meal with high-protein sunflower concentrate in the diet (Table 7).

Table 7. Body weight of broiler chickens depending on the level of replacement of soybean meal with high-protein sunflower concentrate in the diet (g, x±SE, n=100/group)

Age, days	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
1	42.3±0.30[a]	42.3±0.38[a]	42.0±0.34[a]	42.2±0.34[a]
7	226.6±1.01[a]	219.6±1.94[b]	226.2±1.54[a]	229.7±1.06[c]
14	572.6±2.63[a]	542.6±4.31[b]	568.3±3.98[a]	581.3±2.15[c]
21	1190.9±18.22[a]	1137.6±20.33[a]	1190.8±21.82[a]	1244.7±19.60[b]
28	1837.3±27.71[a]	1782.5±29.05[a]	1845.4±28.93[a]	1954.8±25.99[b]
35	2601.6±30.11[a]	2513.1±34.69[a]	2579.1±33.77[a]	2707.8±34.47[b]
42	3367.3±31.08[a]	3278.2±35.69[a]	3342.3±36.74[a]	3476.4±34.12[b]

Note: a, b, c - indicate values that differ significantly in one row of the table (P<0.05).

In particular, at the beginning of the studies, namely at one-day age, the weight of broiler chickens was the same in all groups and met the recommendations of the cross producer (Cobb Broiler Management Guide, 2022). However, starting from the second week of growing, broilers of the group HPSC50q were characterised by higher body weight. In particular, at the end of the first week of growing, broilers of the HPSC50q group were characterised by higher body weight by 1.4% ($p<0.05$), in the second week - by 1.5% ($p<0.05$), in the third week - by 4.5% ($p<0.05$), in the fourth week - by 6.4% ($p<0.05$), in the fifth week - by 4.1% ($p<0.05$), compared to the birds of the control group. The final live weight of broiler chickens of the HPSC50q group at 42 days of growing was higher by 3.2% ($p<0.05$), compared to the birds of the SBM group. While the live weight of broiler chickens of the HPSC and HPSC50cp groups at the end of the growing period was at the same level as the control SBM group.

The effect of using high-protein sunflower concentrate on the slaughter quality of broiler chickens

Partial replacement of sunflower meal with high-protein sunflower concentrate in the diet of broiler chickens significantly affected their meat productivity (Table 8). In particular, broiler chickens of the group HPSC50q were characterised by a higher pre-slaughter live weight by 3.2% ($p<0.05$), the weight of the bled carcass by 4.7% ($p<0.05$), the non-eviscerated carcass by 4.1% ($p<0.05$), the semi-eviscerated carcass by 4.0% ($p<0.05$) and the eviscerated carcass by 4.4% ($p<0.05$), compared to the group SBM. As for the mass of edible parts of the carcass of broiler chickens, the effect of partial replacement of sunflower meal with high-protein sunflower concentrate in their diet is also clearly visible. In particular, broiler chickens of the group HPSC50q were characterised by a greater mass of breast muscles by 6.6 % ($p<0.05$), thighs by 3.7 % ($p<0.05$), shins by 4.5 % ($p<0.05$) and wings by 2.0 % ($p<0.05$), compared to the group SBM.

Table 8. Slaughter qualities of broiler chickens depending on the level of replacement of soybean meal with high-protein sunflower concentrate in the diet (g, x±SE, n=6/group)

Indicator, g	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Weight:				
– pre-slaughter live	3364.9±31.60[a]	3272.2±34.83[a]	3340.6±31.33[a]	3471.8±33.89[b]
– bled carcass	3196.7±32.07[a]	3106.2±34.22[a]	3206.0±35.47[a]	3348.2±33.90[b]
– non-eviscerated carcass	3062.1±33.84[a]	2989.3±33.44[a]	3050.5±33.22[a]	3186.8±32.59[b]
– semi-eviscerated carcass	2927.5±34.62[a]	2854.3±34.88[a]	2914.8±32.22[a]	3043.5±35.38[b]
– eviscerated carcass	2572.4±36.39[a]	2483.3±33.08[a]	2563.3±16.38[a]	2686.2±34.77[b]
Weight of edible parts:				
– breast muscles	918.6±18.35[a]	908.4±17.23[a]	919.7±19.91[a]	979.0±19.61[b]
– thigh	469.4±6.26[a]	456.5±5.12[a]	468.4±6.57[a]	486.7±5.34[b]
– drumstick	315.6±4.87[a]	307.9±2.10[a]	314.7±1.89[a]	336.0±4.16[b]
– wing	255.3±1.62[a]	251.6±1.72[a]	253.6±1.67[a]	260.4±1.51[b]
– heart	14.4±0.12[a]	13.4±0.09[a]	14.0±0.14[a]	14.2±0.08[a]
– liver	47.1±0.51[a]	45.8±0.49[a]	47.1±0.34[a]	47.4±0.58[a]
– gizzard	25.9±0.28[a]	25.2±0.27[a]	26.1±0.29[a]	26.2±0.31[a]

Note: a, b - indicate values that significantly differed in one row of the table ($P < 0.05$).

Table 9. Slaughter yield of broiler chickens depending on the level of replacement of soybean meal with high-protein sunflower concentrate in the diet (%, x±SE, n=6/group)

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Carcass output:				
– bled carcass	95.1±0.27[a]	95.0±0.25[a]	95.8±0.29[a]	96.4±0.24[b]
– non-eviscerated carcass	91.2±0.11[a]	91.4±0.08[a]	91.3±0.11[a]	91.8±0.10[b]
– semi-eviscerated carcass	87.1±0.12[a]	87.4±0.11[a]	87.3±0.09[a]	87.7±0.08[b]
– eviscerated carcass	76.4±0.12[a]	76.5±0.16[a]	76.7±0.17[a]	77.4±0.15[b]
Yield of edible parts:				
– breast muscles	27.32±0.176[a]	27.76±0.189[a]	27.53±0.134[a]	28.20±0.122[b]
– thigh	13.80±0.079[a]	13.95±0.078[a]	14.02±0.088[a]	14.02±0.074[b]
– drumstick	9.37±0.036[a]	9.41±0.028[a]	9.42±0.023[a]	9.68±0.038[b]
– wing	7.57±0.061[a]	7.69±0.078[a]	7.59±0.072[a]	7.50±0.051[a]
– heart	0.40±0.008[a]	0.41±0.007[a]	0.42±0.009[a]	0.41±0.004[a]
– liver	1.42±0.052[a]	1.40±0.045[a]	1.41±0.037[a]	1.37±0.041[a]
– gizzard	0.76±0.008[a]	0.77±0.007[a]	0.78±0.009[a]	0.75±0.008[a]

Note: a, b - indicate values that significantly differed in one row of the table ($P < 0.05$).

It was found that the slaughter yield of broiler chickens changed under the influence of partial replacement of sunflower meal in the diet with high-protein sunflower concentrate (Table 9). In particular, broiler chickens of the group HPSC50q were characterised by a higher yield of bled carcass by 1.3% ($p<0.05$), non-eviscerated carcass by 0.6 % ($p<0.05$), semi-eviscerated carcass by 0.6 % ($p<0.05$) and eviscerated carcass by 1.0 % ($p<0.05$), as well as a higher yield of edible parts of the carcass, namely breast muscles by 0.88 % ($p<0.05$), thighs by 0.22 % ($p<0.05$) and drumsticks by 0.31 % ($p<0.05$), compared to the group SBM.

The effect of using high-protein sunflower concentrate on the meat quality of broiler chickens

Studies of the effect of partial replacement of soybean meal in the diet of broiler chickens with high-protein sunflower concentrate on meat quality indicators (Table 10) did not reveal any changes in the technological properties of chicken meat. Some of the identified minor differences in meat quality parameters related only to the complete replacement of soybean meal in the diet of broiler chickens with high-protein sunflower concentrate. In particular, in the HPSC group, a decrease in muscle tissue loss during heat treatment of the breast muscles by 0.9% ($p<0.05$) and thigh muscles by 1.4% ($p<0.05$) was observed.

Table 10. Meat quality of broiler chickens depending on the level of replacement of soybean meal with high-protein sunflower concentrate in the diet (x±SE, n=6/group)

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Breast muscles				
Active acidity, pH	6.1 ± 0.02^a	6.1 ± 0.02^a	6.1 ± 0.03^a	6.1 ± 0.04^a
Water holding capacity, %	39.3 ± 0.38^a	39.6 ± 0.24^a	39.1 ± 0.31^a	39.5 ± 0.28^a
Muscle tissue loss during heat treatment (cooking), %	29.2 ± 0.28^a	28.3 ± 0.33^b	28.8 ± 0.35^a	29.0 ± 0.43^a
Thigh muscles				
Active acidity, pH	6.2 ± 0.02^a	6.2 ± 0.03^a	6.2 ± 0.03^a	6.2 ± 0.04^a
Water holding capacity, %	40.3 ± 0.13^a	40.4 ± 0.29^a	40.7 ± 0.48^a	40.0 ± 0.39^a
Muscle tissue loss during heat treatment (cooking), %	22.8 ± 0.49^a	21.4 ± 0.31^b	22.2 ± 0.42^a	22.5 ± 0.26^a

Note: a, b - indicate values that significantly differed in one row of the table ($P < 0.05$).

The effect of using high-protein sunflower concentrate on the chemical composition of broiler chicken meat

The results of the study of the effect of complete and partial replacement of soybean meal in the diet of broiler chickens with high-protein sunflower concentrate on the chemical composition of meat are given in Table 11. It was found that both with complete and partial replacement of soybean meal with high-protein sunflower concentrate, a decrease in the content of crude fat in the breast muscles and thigh muscles of broiler chickens was observed.

Table 11. Chemical composition of breast and thigh muscles of broiler chickens depending on the level of replacement of soybean meal with high-protein sunflower concentrate in the diet (g/100 g, x±SE, n=6/group)

Indicator	Group of broiler chickens			
	SBM	HPSC	HPSC50cp	HPSC50q
Breast muscles				
Total moisture	75.95±0.298^{a}	77.19±0.161^{a}	77.19±0.189^{a}	76.06±0.227^{a}
Dry matter	24.05±0.112^{a}	22.81±0.109^{a}	22.81±0.098^{a}	23.94±0.123^{a}
Crude fat	6.11±0.143^{a}	5.04±0.128^{b}	5.02±0.171^{bc}	4.96±0.158^{bcd}
Crude protein	82.68±1.014^{a}	83.69±1.765^{a}	83.77±1.561^{a}	83.04±1.547^{a}
Crude ash	8.50±0.145^{a}	9.57±0.151^{a}	9.06±0.303^{a}	9.25±0.176^{a}
NFE	2.71±0.326^{a}	1.70±0.227^{a}	2.15±0.211^{a}	2.74±0.288^{a}
Energy value, kcal	102.3±0.356^{a}	94.8±0.362^{b}	94.9±0.412 ^{bc}	94.6±0.374 ^{bcd}
Thigh muscles				
Total moisture	72.42±0.567^{a}	70.51±0.381^{a}	70.73±0.286^{a}	73.30±0.468^{a}
Dry matter	27.58±0.234^{a}	29.49±0.211^{a}	29.27±0.221^{a}	26.70±0.196^{a}
Crude fat	26.63±0.311^{a}	20.83±0.423^{b}	20.78±0.499 ^{bc}	19.96±0.394^{bcd}
Crude protein	60.09±1.290^{a}	63.72±1.344^{a}	63.30±1.187^{a}	63.43±1.343^{a}
Crude ash	8.09±0.665^{a}	9.29±0.782^{a}	9.34±0.593^{a}	9.80±0.658^{a}
NFE	5.19±0.617^{a}	6.16±0.679^{a}	6.58±0.716^{a}	6.81±0.711^{a}
Energy value, kcal	142.4±0.735^{a}	128.4±0.712^{b}	128.6±0.656 ^{bc}	127.3±0.557^{bcd}

Note: a, b, c, d - indicate values that significant differed in one row of the table ($P < 0.05$)

In particular, chickens in the HPSC group had a lower crude fat content in the breast muscles by 17.5% ($p<0.05$) and thigh muscles by 21.8% ($p<0.05$), compared to the SBM group. Broilers in the HPSC50cp group had a 17.8% ($p<0.05$) lower crude fat content in the breast muscles and 22.0% ($p<0.05$) lower crude fat content in the thigh muscles compared to the control group. Broilers in the HPSC50q group had an 18.8% ($p<0.05$) lower crude fat content in the breast muscles and a 25.0% ($p<0.05$) lower crude fat content in the thigh muscles compared to the SBM group. In addition, the decrease in fat content in the meat of broiler chickens, resulting from the full or partial replacement of sunflower meal in their diet with high-protein sunflower concentrate, led to a decrease in the energy value of the chicken meat. In particular, in the HPSC group, in the diet of which soybean meal was replaced entirely with sunflower concentrate, there was a lower energy value in the breast muscles by 7.3% ($p<0.05$) and in the thigh muscles by 9.8% ($p<0.05$) compared to the SBM group.

In broilers of the HPSC50cp group, a lower energy value of the breast muscles was observed by 7.2% ($p<0.05$) and of the thigh muscles by 9.7% ($p<0.05$) compared to the SBM group. Broilers in the HPSC50q group, however, exhibited lower energy content in the breast muscles by 7.5% ($p<0.05$) and in the thigh muscles by 10.6% ($p<0.05$) compared with the SBM group.

DISCUSSION

Soybean meal is widely used as a protein source in poultry feeds (Barboza Martignone *et al.,* 2024). Despite the fact that Ukraine is an exporter of soybeans, fluctuations in its prices on the international market put pressure on local markets

(Attia *et al*., 2022). Given the need for an alternative source of protein and certain undesirable consequences of using sunflower meal in poultry feeding, we used a new feed product – high-protein sunflower concentrate, and investigated the effectiveness of its complete or partial replacement of soybean meal in the diet of broiler chickens.

This study showed that replacing soybean meal with high-protein sunflower concentrate at a concentration of 50% by volume in the diet of broiler chickens does not have a negative impact on the viability of the birds and contributes to an increase in their body weight at 42 days of age by 3.2% ($p<0.05$). The data obtained are consistent with the results described by Kyrkelanov *et al*. (2020), who describe the successful replacement of soybean meal with high-protein sunflower meal at levels of 5–8–12% and 15–20–22%, respectively, during the starter–grower–finisher periods of growing broiler chickens. In contrast to these results, scientists describe that replacing soybean meal with sunflower meal either has no effect at all or has a negative effect on the growth parameters of broilers. In particular, a recent study by Munawar *et al.* (2025) showed that replacing soybean meal with sunflower meal at a level of up to 20% with enrichment of 100 g/ton of the NSPase enzyme did not have a negative effect on the body weight of broilers. These results are consistent with the data described by Bilal *et al.* (2017), who studied the effectiveness of using sunflower meal in diets with or without the NSPase enzyme and did not find a significant effect on body weight of different concentrations of soybean meal replacement. Similarly, Yaqoob *et al.* (2022), who studied the possibility of replacing soybean meal with sunflower meal at 3%, 6% and 9% in combination with multienzymes, did not observe significant differences in growth parameters of broiler chickens depending on the level of sunflower meal introduction. However, other studies have documented that replacing soybean meal with sunflower meal up to 16% in the diet can negatively affect growth parameters and feed efficiency in 21-day-old broilers, which is explained by the high content of fibre and insoluble non-starch polysaccharides in sunflower meal (DMaria de Moraes Oliveira *et al.,* 2016). Berwanger *et al*. (2017) reported that adding more than 10% sunflower meal to the broiler diet negatively affected the growth performance of broiler chickens during the 21^{st}–42^{nd} day of growth. They explained this adverse effect by an increase in the viscosity of the digested material due to the high content of insoluble non-starch polysaccharides in the diet. These differences can be explained by the fact that, in all the studies described, the sunflower product used to replace soybean meal was sunflower meal, which typically contains 30–34% crude protein, whereas in the present study a high-protein sunflower concentrate with a crude protein content of 44% was used.

The increase in body weight of broiler chickens at slaughter age, caused by replacing 50% of soybean meal in their diet with high-protein sunflower concentrate, contributed to an increase in pre-slaughter live weight by 3.2% ($p<0.05$), the mass of the bled carcass by 4.7% ($p<0.01$), the non-eviscerated carcass by 4.1% ($p<0.01$), the semi-eviscerated carcass by 4.0% ($p<0.05$) and the eviscerated carcass by 4.4% ($p<0.05$), the mass of the breast muscles by 6.6% ($p<0.05$), the thighs by 3.7% ($p<0.05$), the drumsticks by 4.5% ($p<0.05$) and the wings by 5.1 g or 2.0% ($p<0.05$). The difference in pre-slaughter live weight, carcass weight, and individual edible parts of the carcass of broiler chickens may be due to differences in the amino acid profile or biological value of different protein meals, namely soybean and sunflower (Rehman *et al.* 2002). According to Penchev *et al.* (2022), partial replacement of soybean meal with high-protein sunflower meal does not have a significant effect on the carcass characteristics of broiler chickens. At the same time, the authors note that complete replacement of soybean meal with high-protein sunflower meal causes a decrease in carcass weight.

As for the chemical composition, this study found that 50% replacement of soybean meal with high-protein sunflower concentrate contributes to a decrease in the crude fat content in the breast muscles of broiler chickens by 18.8% ($p<0.05$) and thigh muscles by 25.0% ($p<0.05$) while reducing the energy value by 7.5% ($p<0.05$) and 10.6% ($p<0.05$), respectively.

Scientists attribute the reduction in muscle fat content in broilers fed oilseed concentrate to the high availability of long-chain fatty acids in the feed, namely the higher efficiency of conversion of long-chain fatty acids C20:5(n-3) compared to C22:6(n-3) (Kim and Voy, 2021). Diets enriched with various fatty acids have a strong effect on the deposition of polyunsaturated fatty acids in tissue lipids (Kralik et al., 2025). At the same time, the mechanism of modification of adipose tissue deposition by the presence of polyunsaturated fatty acids in the feed is not yet fully understood. The aforementioned reduction in fat content in chicken meat is a significant positive consequence of replacing soybean meal with high-protein sunflower concentrate, as chicken meat is a valuable source of protein, lipids, and minerals for the human body. It also contains bioactive components (vitamins, antioxidants, essential amino acids, trace elements), important in metabolic processes that contribute to the optimal health of consumers (Cartoni Mancinelli *et al*., 2022). There are many scientific studies on the fatty acid content of broiler meat (Long *et al.,* 2020; Ali *et al.,* 2023; Kralik *et al.,* 2023; Qaid *et al.,* 2023), particularly focused on improving the fatty acid profile, usually the polyunsaturated fractions (Dal Bosco *et al.* 2022). Broiler meat with increased content of N-3 polyunsaturated fatty acids (PUFA) is already considered a functional product (Kralik *et al.,* 2025).

At the same time, in the literature, researchers often describe the negative impact of replacing soybean meal with sunflower meal, which is manifested by a decrease in the crude protein content in the meat of broiler chickens. Thus, Penchev *et al.* (2022), who studied the complete and partial replacement of soybean meal with high-protein sunflower meal, noted a decrease in crude protein, moisture, and crude ash in the breast muscles of broilers. A similar negative effect on the content of crude protein and moisture in meat was also described by Aregheore (1998). The authors note that replacing 30% of soybean meal with sunflower meal in the diet of broiler chickens causes a decrease in the moisture and crude protein content of the meat. Similarly, Jankowski *et al.* (2011) found, although insignificant, a negative effect of replacing soybean meal with sunflower meal on the crude protein and crude ash content of turkey meat. In contrast, Laudadio *et al.* (2013) found no difference in the chemical composition of turkey meat when soybean and sunflower meal were used in their feeding, which confirmed the study of Slavica *et al.* (2006).

CONCLUSIONS

It has been established that the most effective way for the body of broiler chickens is to replace 50% of the soybean meal in their diet with high-protein sunflower concentrate. This contributes to an increase in the final body weight by 3.2% ($p<0.05$), pre-slaughter live weight by 3.2% ($p<0.05$), the weight of the bled carcass by 4.7% ($p<0.05$), the non-eviscerated carcass by 4.1% ($p<0.05$), the semi-eviscerated carcass by 4.0% ($p<0.05$) and the eviscerated carcass by 4.4% ($p<0.05$), the weight of the breast muscles by 6.6% ($p<0.05$), the thigh by 3.7% ($p<0.05$), the drumstick by 4.5% ($p<0.05$) and the wing by 2.0% ($p<0.05$), as well as a higher yield of the bled carcass by 1.3% ($p<0.05$), the non-eviscerated carcass by 0.6% ($p<0.05$), semi-eviscerated carcass - by 0.6% ($p<0.05$) and eviscerated carcass - by 1.0% ($p<0.05$), higher yield of edible parts of the carcass, namely breast muscles - by 0.88% ($p<0.05$), thighs - by 0.22% ($p<0.05$) and drumsticks - by 0.31% ($p<0.05$). In addition, 50% replacement of soybean meal with high-protein sunflower concentrate contributes to a decrease in the crude fat content in the breast muscles of broiler chickens by 18.8% ($p<0.05$) and thigh muscles by 25.0% ($p<0.05$) with a decrease in energy value by 7.5% ($p<0.05$) and 10.6% ($p<0.05$), respectively, compared to the control group, whose birds received a standard diet with soybean meal in the composition. Thus, in view of the above results, established that the most effective way for the broiler chickens is to replace 50% of the soybean meal in their diet with high-protein sunflower concentrate.

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THE INFLUENCE OF SEED PRE-TREATMENTS AND SUBSTRATE TYPES ON SEEDLING EMERGENCE OF ACER PALMATUM 'ATROPURPUREUM'

SUMMARY

Acer palmatum Thunb. is an ornamental shrub native to Japan, Korea, and China, widely cultivated in green areas due to its diverse leaf shapes and colors. This study was conducted in the greenhouse at the Didactic and Research Base of "King Mihai I" University of Life Sciences from Timișoara, Romania. The main objective of this paper was to evaluate seedling emergence and growth performance of *Acer palmatum* 'Atropurpureum' under the influence of two pre-germination treatments (90-day cold stratification and hydrothermal treatment by 50 °C water immersion for 24 hours) and three commercial substrates were tested: TS 3 peat, TS 4 medium peat combined with Greenfibre, and Pro Line Tray Substrate peat. Biometrical analyses were performed, there being characterized the influence of the analysed factors solely and combined on seedling height, girth diameter, root number, main root length, number of leaves, and leaf surface area. The stratified seeds grown in Pro Line Tray Substrate peat exhibited the highest values and the most favorable results for all biometrical parameters measured.

Keywords: Japanese maple, seedling, seed pre-treatment, substrate, ornamental shrub

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INTRODUCTION

Acer palmatum Thunb., commonly known as Japanese maple, is a deciduous ornamental species native to East Asia, particularly Japan, Korea, and China. Valued for its aesthetic diversity, the species encompasses a wide range of cultivars and is extensively used in landscape design due to its vibrant foliage, variable form, and adaptability (Dirr, 2009). Among its many cultivars, 'Atropurpureum' is the most appreciated for its deep red foliage and aesthetic appeal, making it a popular choice in temperate climate gardens and urban green areas.

Despite its horticultural value, propagation of *A. palmatum* by seed remains challenging due to pronounced seed dormancy, which limits germination and uniform seedling emergence (Hartmann *et al*., 2011). To overcome this dormancy, pre-sowing treatments such as cold stratification or hydrothermal stimulation are commonly applied (Bewley *et al.,* 2013). Cold stratification, which mimics natural winter conditions, is widely used to break physiological dormancy in temperate woody species, including maples (Bonner and Karrfalt, 2008). Alternatively, hydrothermal treatments have been investigated as a faster method to enhance seed germination by softening the seed coat and activating enzymatic processes (Baskin and Baskin, 2014).

In addition to dormancy-breaking methods, the successful establishment of seedlings highly depends on the proper selection of the growing substrate, which influences water retention, aeration, nutrient availability, and root development (Raviv *et al.,* 2004). Peat-based substrates remain common in containerized seedling production, often supplemented with materials such as wood fibre to enhance structural stability and reduce environmental impact (Gruda, 2012).

The use in the seedlings data analysis of the phenotypic plasticity index (PPI) is relevant for nursery seedlings, because it can predict the potential response of the seedlings to different environmental factors, in practice helping to the optimization of nursery management (dos Santos *et al*., 2023; das Chagas Mendonca *et al*., 2025) by analysing the biometrical parameters of seedlings (Pardos and Calama, 2022) or developed trees (Kurjak *et al*., 2024).

Therefore, this research aims to evaluate the combined effects of two dormancy-breaking pre-treatments (cold stratification and hydrothermal stimulation) and three different commercial substrates, on the early growth of *Acer palmatum* 'Atropurpureum' seedlings. By examining the interaction between pre-sowing treatments and substrate composition, the study seeks to enhance propagation efficiency and promote sustainable practices in the production of this high-value ornamental species, by reducing the use of chemical inputs during the early stages of production of planting material.

MATERIAL AND METHODS

Seed harvest and materials used

Fruits of *Acer palmatum* Thunb. were collected on October 16, 2023, from a private garden in Dumbrăvița, Timiș County (45°48′22″N, 21°14′45″E). Post-harvest conditioning was conducted at the Arboriculture Laboratory, University of Life Sciences "King Mihai I" from Timișoara (Romania). Seeds sampled from healthy, mature trees were air-dried for one week, dewinged, and cleaned of damaged or empty seeds prior to germination (Figure 1).

A polyfactorial experimental design was used to assess the germination stimulation methods and substrate suitability. Two pre-treatments were applied: (1) Cold stratification (**a1**) – 100 seeds were mixed with moist sand and stored at 4 °C and 80% RH for 90 days (Chen *et al*., 2015); and (2) Hydrothermal treatment (**a2**) – 100 seeds were soaked in 50 °C water for 24 hours before sowing (Alipor *et al*., 2019).

From the pre-treated seeds, a total of 180 seeds (90 per treatment) was sown on the 16th of January, 2024, in seed trays, with 10 seeds per cell in three replicates (Šerá, 2023). For seed germination, three commercial substrates were used as presented and described in Table 1: Klasmann TS 3 (**b1**), Klasmann TS 4 medium + clay (**b2**) and Pro Line Bio Tray Substrate (**b3**).

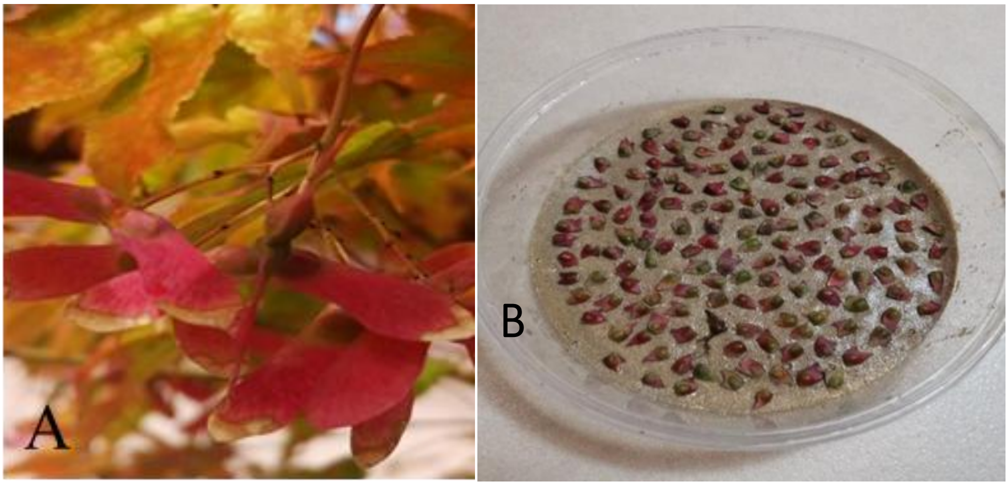


Figure 1. Fruits of *A. palmatum* 'Atropurpureum' before harvest (A) and seeds after samara removal (B) (photo: Poșta D.S.)

Seeds were germinated in seed trays and grown in a climate chamber under controlled conditions (20 °C and 70% humidity level). On the 17th of March, 2024, seedlings were transplanted into 13×13×13 cm pots and transferred into the greenhouse. Manual air ventilation and watering were carried out to maintain air temperature below 27 °C, and soil at field capacity. The greenhouse was covered with a 70% UV protection sunshade.

Table 1. Type of substrates used for seedling emergence

No. of substrates	**1**	**2**	**3**
Substrate type	**Klasmann TS 3**	**Klasmann TS 4 medium + clay**	**Pro Line Bio Tray Substrate**
			
Composition	White peat moderately decomposed (0-25 mm),	White peat 0-25 mm White sod peat 10-25 mm White peat fibre 0-30 mm 20 kg/cbm clay granules	White peat (0-5 mm) Raw materials: TerrAktiv FT Raw materials TerrAktiv Frozen through black peat
Structure	medium 0-25 mm	medium 0-30 mm	extra fine (0-5 mm)
Chemical data	pH value: 6 Fertilizer level (g/l): 1.0 + extra trace element fertilizer	pH value: 6 Fertilizer level (g/l): 1.0 + extra trace element fertilizer	pH value: 6 Salt level (g/l): 0.7-1.3 Horn shaves fertilizer (kg/m^3): 2.0
Nutrients added	Nitrogen (mg N/l): 140 Phosphorus (mg P_2O_5/l): 100 Potassium (mg K_2O/l): 180 Magnesium (mg Mg/l): 100 + all necessary trace elements Iron added as EDTA chelate	Nitrogen (mg N/l): 140 Phosphorus (mg P_2O_5/l): 100 Potassium (mg K_2O/l): 180 Magnesium (mg Mg/l): 100 + all necessary trace elements Iron added as EDTA chelate	Nitrogen (mg N/l): 300-400 Mineralizes N (mg N/l): 50-80 Phosphorus (mg P_2O_5/l): 200-300 Potassium (mg K_2O/l): 300-500 Magnesium (mg Mg/l): 100-200
Physical data	Dry matter: <10 % Water capacity: 75-80% Air capacity: 10-15%	Dry matter: <10 % Water capacity: 70-75% Air capacity: 15-20%	Dry matter: <10 % Water capacity: 78-82% Air capacity: 8-12%

Experimental design and data collection

The experimental factors used were pre-treatment (factor a) and substrate (factor b). The experiment was organized with six variants (2 pre-treatments * 3 substrates), each variant having three replicates. The biometrical data were collected from five plants from each replicate and from all considered variants, the total number of analysed seedlings from biometrical point of view from the experiment was 90.

Seedling parameters analysis

Seedling emergence was monitored daily over 45 days until on 3rd of March, 2024. After 60 days from seedling emergence (figure 2), the seedlings were transplanted and biometric measurements were carried out including seedling height, girth diameter, root number, main root length, number of leaves, and total leaf area. In addition, for all the quantitative variables studied, phenotypic plasticity index (PPI) was calculated according to Valladares *et al.* (2006) applying the following formula:

PPI=(maximum mean value - minimum mean value)/(mean maximum value).

The index varies from zero to one and allows comparisons among traits measured with different units (Valladares *et al*., 2000).

Figure 2. Seedlings before (C) and after (D) transplanting (photo: Poșta D.S.)

Statistical analyses

The primary data were compiled and subjected to statistical analyses to determine the effectiveness of seed pre-treatments, substrate type used and the combined effect of both factors ANOVA analysis was performed first to assess statistically significant differences between the means followed by Tukey's test at $p<0.05$. The software used for ANOVA was JASP 0.19.3 (JASP, 2025).

RESULTS AND DISCUSSION

Seedling growth performance was assessed using several biometric including the root system, where both the number of roots and the length of the primary root were measured to evaluate early development and establishment potential.

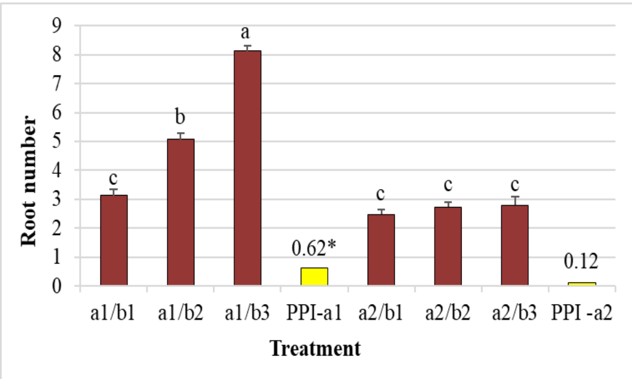
ANOVA regarding the influence of the pre-treatment, substrate and pre-treatment * substrate on the number of roots on the early seedlings of *A. palmatum* is presented in Table 1. The results highlight the highly significant influence of each of the two analysed factors applied and their combined effect on the number of roots per seedling ($p<0.001$).

Table 1. ANOVA – roots number per seedling

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	173.611	1	173.611	245.235	< 0.001
substrate	104.689	2	52.344	73.939	< 0.001
pre-sowing treatment ✻ substrate	86.956	2	43.478	61.415	< 0.001
Residuals	59.467	84	0.708		

Note. Type III Sum of Squares

As illustrated in Figure 3, the number of roots varied according to the applied germination stimulation method and substrate type used. Seeds subjected to hydrothermal treatment displayed relatively uniform root development across all substrate types, with a mean root number of 2.66, showing no statistically significant differences between substrate types.



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS4
a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 3. Root number of *A. palmatum* 'Atropurpureum' seedlings. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at $p<0.05$ according to Tukey's HSD test. Asterisk indicate significant differences within phenotypic plasticity index (PPI) of the two seed pre-treatments;

In contrast, due to cold stratification statistically significant differences were observed in terms of root number across the two experimental combinations: a1/b3 (cold stratification with Pro Line Tray Substrate) and a1/b2 (cold stratification with TS 4 + Greenfibre), which yielded in an average root number of 8.13 and 5.07, respectively.

These values suggest a synergistic effect between cold stratification and specific substrate compositions on root development. Such results are consistent with findings from previous studies emphasizing the role of prolonged stratification and substrate aeration in promoting root system differentiation in temperate woody species (Hartmann *et al.*, 2011; Baskin and Baskin, 2014; Poșta *et al.* 2024).

With regard to the PPI calculated for root number, the results showed that cold stratified seeds resulted in seedlings with high phenotypic plasticity (0.62) in root development, suggesting that these seedlings are more responsive to changes in substrate conditions. On the contrary, hydrothermal treatment produced seedlings with low plasticity (0.12), showing consistent root number regardless of substrate type. The significantly higher PPI in cold-stratified seeds suggests that this treatment may enhance seedling's ability to adjust root traits based on substrate conditions-an important consideration for nursery propagation and substrate selection. These outcomes support the hypothesis that stratification not only enhances germination rates but also promotes more vigorous root architecture, particularly when paired with substrates optimized for moisture retention and air porosity (Criscione, 2021).

Similar results were reported by Fetouh and Hassan (2014) in Magnolia grandiflora seedlings where stratification significantly enhanced root-related traits. Other studies (e.g., on Korean pine and Saskatoon berry) demonstrated that cold stratification improves germination metrics including germination rate, uniformity, and speed and generally enhances early seedling vigour and growth traits (Górnik *et al.,* 2023; Song *et al.,* 2023).

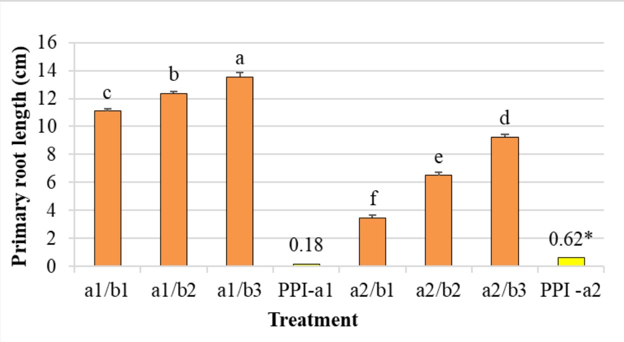
ANOVA analysis of primary root length under the influence of the pre-treatment, substrate and pre-treatment * substrate in the early seedlings of *A. palmatum* is presented in Table 2. As in the case of the number of roots per seedling, the results obtained highlight the highly significant influence of the two analysed factors applied in the experiment and their combined effect on the length of the primary root (p <0.001).

Table 2. ANOVA – *A. palmatum* primary root length (cm)

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	790.914	1	790.914	1113.664	< 0.001
substrate	252.324	2	126.162	177.645	< 0.001
pre-sowing treatment ✻ substrate	41.731	2	20.865	29.380	< 0.001
Residuals	59.656	84	0.710		

Note. Type III Sum of Squares

The length of the primary root also exhibited significant variations across the experimental treatments (figure 4). Seeds subjected to cold stratification demonstrated a considerable improvement in primary root development compared to those treated hydrothermally (50 °C for 24 hours).



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate;
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS4
a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 4. Primary root length of *A. palmatum* 'Atropurpureum' seedlings. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at p<0.05 according to Tukey's HSD test. Asterisk indicate significant differences within phenotypic plasticity index (PPI) of the two seed pre-treatments.

Seed stratification appeared to positively influence root elongation, promoting deeper and more robust root systems as a crucial factor for early seedling vigor and adaptation in nursery conditions. These findings are in accordance with previous results, which reported enhanced root growth in stratified seeds of temperate woody species due to dormancy release and favourable metabolic activation (Bonner and Karrfalt, 2008; Bewley *et al*.,2013; Poșta, 2015).

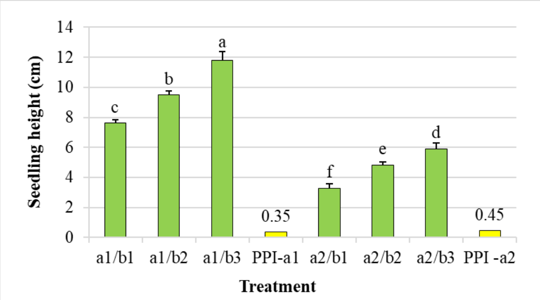
ANOVA analysis of the seedling height of *A. palmatum* under the influence of the pre-treatment, substrate and pre-treatment * substrate is presented in Table 3. The results obtained are evidencing the highly significant influence of the two analysed factors applied in the experiment and their combined effect on the seedling height ($p < 0.001$).

Table 3. ANOVA – *A. palmatum* seedling height (cm)

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	553.040	1	553.040	1625.830	< 0.001
substrate	173.061	2	86.530	254.382	< 0.001
pre-sowing treatment ✻ substrate	9.631	2	4.815	14.156	< 0.001
Residuals	28.573	84	0.340		

Note. Type III Sum of Squares

Seedling height varied significantly across treatments, with average values ranging from 3.27±0.28 cm to 11.78 ± 0.30 cm. The tallest seedlings were recorded in the treatment combining stratified seeds with the Pro Line Tray Substrate, while the shortest seedlings were observed as a result of the hydrothermal treatment (50 °C for 24 hours) on TS 3 peat. This trend is clearly depicted in figure 5, showing statistically significant differences between treatments. PPI were not significantly different between the treatments. PPI-a2 showed slightly higher plasticity than cold stratification, suggesting that hydrothermally treated seedlings show relatively more variability in height in response to substrate, despite their lower seedling height.



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate;
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS4
a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 5. Seedling height of *A. palmatum* 'Atropurpureum'. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at $p<0.05$ according to Tukey's HSD test. PPI - phenotypic plasticity index of the two seed pre-treatments

The enhanced seedling growth in stratified seeds might be attributed to a more balanced germination process, leading to better use of substrate nutrients and moisture. Conversely, reduced seedling height in hydrothermally treated seeds suggests limited physiological processes, possibly due to insufficient dormancy release or thermal stress. These results reinforce the critical role of proper dormancy-breaking methods and optimized substrate selection in maximizing early growth potential in nursery production systems (Ranal and Santana, 2006; Hartmann *et al*., 2011).

In Table 4 is presented ANOVA analysis regarding the influence of the pre-treatment, substrate and pre-treatment * substrate on the girth diameter on the early seedlings of *A. palmatum*. The results obtained show the highly significant influence of the two analysed factors applied ($p < 0.001$). In the case of the combined effect of the factors, the influence on the girth diameter is not significant from statistical point of view.

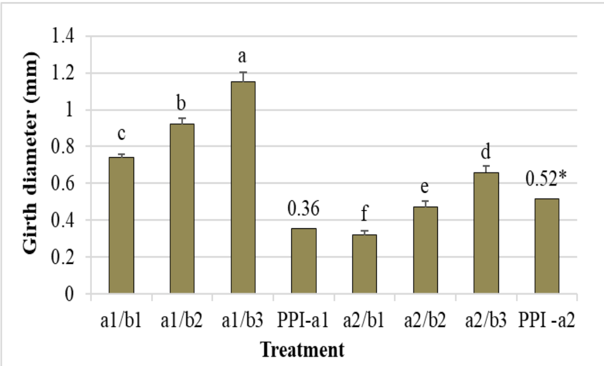
Table 4. ANOVA – *A. palmatum* seedling girth diameter (mm)

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	4.597	1	4.597	1097.846	< 0.001
substrate	2.131	2	1.065	254.424	< 0.001
pre-sowing treatment ✻ substrate	0.015	2	0.007	1.768	0.177
Residuals	0.352	84	0.004		

Note. Type III Sum of Squares

Girth diameter is a key morphological indicator of seedling vigour and structural stability, correlating with both nutrient uptake capacity and transplanting success (Mattsson, 1997). The girth diameter of the seedlings followed a similar variation pattern to that observed in shoot height, with significant differences between substrate types and pre-germination treatments (figure 6).

Seedlings emerged from stratified seeds exhibited larger girth diameters across all substrates tested. Cold stratification led to thicker seedlings, with the best result recorded in Pro Line Bio Tray substrate (a1/b3), confirming its superior performance for overall seedling vigour. While hydrothermal treatment resulted in thinner seedlings overall, it showed higher phenotypic plasticity (PPI=0.52), suggesting that girth in this group is more influenced by substrate quality. The significant PPI difference (0.52 vs 0.36) indicates that although cold-stratified seedlings grow better in absolute terms, hydrothermally treated seedlings are more sensitive to substrate when it comes to girth development.



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate;
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS4
a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 6. Girth diameter of *A. palmatum* 'Atropurpureum' seedlings. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at $p<0.05$ according to Tukey's HSD test. Asterisk indicate significant differences within phenotypic plasticity index (PPI) of the two seed pre-treatments.

In addition to root and stem parameters, the number of leaves was also evaluated as an indicator of photosynthetic potential and seedling vitality.

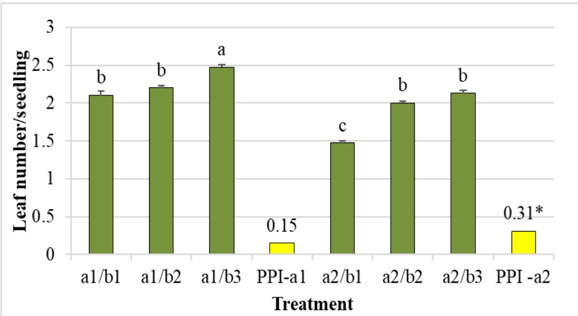
In Table 5 is presented ANOVA analysis regarding the influence of the pre-treatment, substrate and pre-treatment * substrate on the number of leaves per seedling. The results obtained for the leaves number per seedling proved highly significant influence of both analysed factors applied ($p < 0.001$). Also, the combined effect of the factors wasn't statistically significant for the number of leaves per seedling.

Table 5. ANOVA – *A. palmatum* number of leaves per seedling

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	3.600	1	3.600	19.722	< 0.001
substrate	3.800	2	1.900	10.409	< 0.001
pre-sowing treatment ✻ substrate	0.867	2	0.433	2.374	0.099
Residuals	15.333	84	0.183		

Note. Type III Sum of Squares

As shown in figure 7, the leaf number/seedling ranged from 2.00 ± 0.33 to 2.47 ± 0.36, with the highest values observed in stratified seeds, particularly in the a1/b3 combination. Although differences were relatively small, they remain biologically relevant, especially when seedlings are intended for ornamental purposes where early foliage development is desirable. Cold-stratified seedlings produced higher number of leaves, especially in b3 (Pro Line Bio Tray) substrate. However, their PPI was low (0.15), suggesting they maintain stable leaf development regardless of the substrate.



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate;
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 – hydrothermal treatment of seeds, 50 °C - 24 hours/TS4
a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 7. Leaf number/seedling in *A. palmatum* 'Atropurpureum'. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at p<0.05 according to Tukey's HSD test. Asterisk indicate significant differences within phenotypic plasticity index (PPI) of the two seed pre-treatments.

Hydrothermally treated seedlings had greater variability in leaf number across substrates, showing reduced performance in b1. Their PPI was higher (0.31), meaning they are more responsive to substrate differences, even though their average leaf number was lower than cold-stratified seedlings. Previous studies have suggested that substrates rich in organic matter and well-drained structure tend to enhance leaf emergence, particularly in species sensitive to waterlogging and poor aeration (Gruda, 2012).

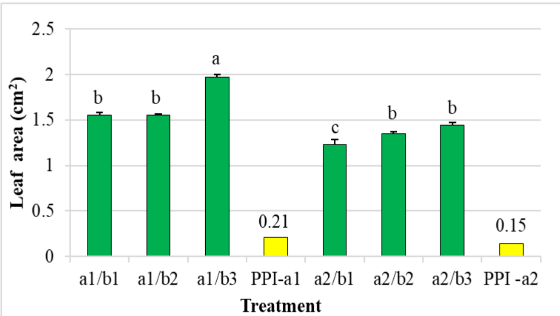
In the case of foliar surface ANOVA analysis (Table 6) the results obtained evidenced similar behaviour with the number of leaves and the girth diameter. Thus, the influence of the pre-treatment, substrate and pre-treatment * substrate on the foliar surface per seedling was highly significant for both analysed factors applied ($p<0.001$); and the combined effect of the factors wasn't significant for this parameter.

Table 6. ANOVA – *A. palmatum* foliar surface per seedling (sq mm)

Cases	Sum of Squares	df	Mean Square	F	p
pre-sowing treatment	2.798	1	2.798	66.407	< 0.001
substrate	1.665	2	0.833	19.756	< 0.001
pre-sowing treatment ✻ substrate	0.431	2	0.216	5.118	0.008
Residuals	3.540	84	0.042		

Note. Type III Sum of Squares

Regarding the leaf area, a strong positive correlation ($r=0.87$) was noted between leaf number and total leaf surface area according to Pearson' correlation. The highest leaf area was recorded in a1/b3 variant (stratified seeds grown in Pro Line Tray Substrate peat), reinforcing the conclusion that this treatment combination supports optimal early growth conditions.



a1/b1 – cold stratification/TS3
a1/b2 – cold stratification/TS4
a1/b3 - cold stratification/Pro Line Bio Tray Substrate;
a2/b1 - hydrothermal treatment of seeds, 50 °C - 24 hours/TS3
a2/b2 – hydrothermal treatment of seeds, 50 °C - 24 hours/TS4 a2/b3 - hydrothermal treatment of seeds, 50 °C - 24 hours/ Pro Line Bio Tray Substrate

Figure 8. Total leaf area/seedling in *A. palmatum* 'Atropurpureum'. The data presented are means±st. error. Different lowercase letters above the bars indicate significant differences between the means at $p<0.05$ according to Tukey's HSD test. PPI - phenotypic plasticity index of the two seed pre-treatments.

In literature, the application of different temperature ranges for the dormancy-release of the seeds combined with different substrates used have an influence the seedling growth, including directly the leaf number and indirect the foliar surface (Costa *et al*., 2021).

The overall results obtained show the statistically significant impact of the pretreatment and substrate considered separately, on all the biometric features analysed on the seedlings of *A. palmatum* 'Atropurpureum'. The combined effect of the applied factors was significant only the root features and seedling height.

The obtained results align with findings from ornamental seedling production, where adequate root and shoot development under stratification and nutrient-balanced substrates enhances establishment success (Raviv *et al*., 2004).

CONCLUSIONS

The findings suggest that both seed pre-treatment and substrate type significantly affected the germination and early growth of *A. palmatum* 'Atropurpureum'. Cold stratification with Pro Line Bio Tray substrate produced the best overall performance, promoting vigorous, stable growth with low PPI values. Hydrothermal treatment yielded lower growth but higher PPI for some traits, reflecting greater sensitivity to substrate composition.

The overall results obtained suggest the development of future research for the obtaining of vigorous planting material of *A. palmatum* in a sustainable manner. The next researches should be focused on the analysis of other developmental stages of the Japanese maple.

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PHENOTYPIC VARIATION OF SEED TRAITS IN NATURAL POPULATIONS OF *ABIES ALBA* MILL. IN BOSNIA AND HERZEGOVINA AND ITS CORRELATION TO GROWTH IN THE FIRST TWO YEARS IN NURSERY

SUMMARY

Abies alba Mill. is one of the most prevalent tree species in Bosnia and Herzegovina. This study aimed to assess the morphological variability of seeds within silver fir natural populations in Bosnia and Herzegovina. Silver fir cones were collected in thirteen natural populations (in situ). Ten trees per population and 30 cones per tree were sampled. Twenty-five well-developed scales and seeds per tree were measured. The seeds were sown in February 2021. The heights and root collar diameters of one-year-old and 2-year-old seedlings were measured. The Kruskal-Wallis non-parametric test showed statistically significant differences among populations for all analyzed traits. A positive correlation was found between some of scale and seed traits, and traits of growth in the first two years. The determined correlation among traits can be used for the selection of the seed for producing fast-growing seedlings. Grouping of populations according to examined traits did not show any geographical pattern, so it is recommended to use the best populations in terms of growth.

Keywords: silver fir, natural populations, seed morphological traits, seedlings growth

INTRODUCTION

The Central Dinarides (Western Balkans) represent one the most important regions for understanding the genetic and morphological diversity of silver fir (*Abies alba* Mill.). Early phylogenetic studies reported unexpected alleles within this area, suggesting a complex evolutionary and postglacial history. Konnert and Bergmann (1995) identified alleles are not typical for the Balkan Peninsula, and similar findings were later documented in the Carpathian region (Longauer, 1996).

Research into mitochondrial DNA revealed that the Dinarides form a major postglacial suture zone where lineages of different biogeographical origins

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.
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converge (Gömöry *et al*., 2004). This work identified two parallel clines: allele 2 dominating along the Adriatic coast and allele 1 prevailing inland. In Bosnia and Herzegovina, both alleles occur; allele 1 primarily in western Bosnian populations, and allele 2 elsewhere, marking the contact zone between the Balkan and Apennine haplotypes (Gömöry *et al.,* 2004; Liepelt *et al*. 2002; Liepelt *et al*. 2009). Later observation of Balkan haplotype alleles west of the contact zone and Apennine alleles east of it suggested hystorical hybridization (Ballian *et al.,* 2012), yet it remains unclear whether such genetic patterns are reflected in morphological traits.

Morphological variation in *Abies alba* cones, seeds, and scales has long been documented across Europe (Gudeski 1966; Hegi, 1981; Aas *et al.,* 1994; Tracz and Barzdajn 2007). In Bosnia and Herzegovina, earlier studies reported pronounced intra- and inter-population variabiliy in cone, seed, and scale traits (Ballian, 2000a; Ballian and Čabaravdić, 2005; Ballian and Halilović, 2016).

Silver fir studies form neighboring Croatia also demonstrated significant provenance-level differences in early seedling growth (Ivanković and Marjanović, 2005). More recent research in Bosnia and Herzegovina confirms that both morphological and growth-related traits vary significantly among populations (Ballian, 2005; Halilović *et al*., 2009b; Halilović *et al*., 2013; Ballian and Halilović, 2016; Višnjić *et al*., 2019; Memišević Hodžić *et al.,* 2021).

In the last few years, new studies from the Balkan region have further highlighted substantial phenotypic variation in silver fir. A recent investigation of needle morphology across the Balkan peninsula demonstrated clear spatial variability linked to climatic factors (Miljković, 2022; Popović *et al*., 2022). Another recent study from Southeastern Europe reported considerable anatomical variability of needles among natural populations, reflecting adaptations to local ecological gradient (Popović *et al*. 2024). Studies in broader Central European and temperate forests have also emphasized variability in growth responses (Bončina *et al.,* 2023) and the ecological importance of early seedling development under different substrate and nutrient conditions (Błońska *et al*., 2023). Work on reproductive performance has additionally shown notable variability in cone production within clonal seed orchards (Teodosiu *et al*., 2023). Provenance-test research from Bosnia and Herzegovina continues to show marked differences among provenances in morphological and growth traits (Memišević Hodžić *et al*., 2021). These findings underscore the continuing importance of understanding regional variability, particularly in genetically complex zones such as the Dinarides.

Ballian (2000b) reported no correlation between seed mass and early growth, but this has not been re-examined using more detailed measurements on larger population samples. Meanwhile, recent studies emphasize the need for identifying early-growth indicators that support forest regeneration, conservation, and climate-resilient management in silver fir populations (e.g. Popović *et al.,* 2022; Popović *et al*. 2024).

This study addresses this gap by exploring whether morphological traits of cones, seeds, and scales correlate with the early growth of seedlings originating from natural populations of silver fir in Bosnia and Herzegovina. Establishing such correlations could help identify seed sources with superior early-growth potential. Therefore, the aims of this research are: (1) to determine the morphological variability of silver fir within and among natural populations in Bosnia and Herzegovina, and (2) to examine the relationship between cone, seed, and scele morphological traits and seedling growth during the first two years.

The results may contribute to improved management, conservation, and regeneration strategies for silver fir in a region of high genetic complexity and ecological importance.

MATERIAL AND METHODS

During the autumn of 2020, silver fir cones were collected from thirteen natural populations in Bosnia and Herzegovina, representing a range of ecological conditions (Figure 1).

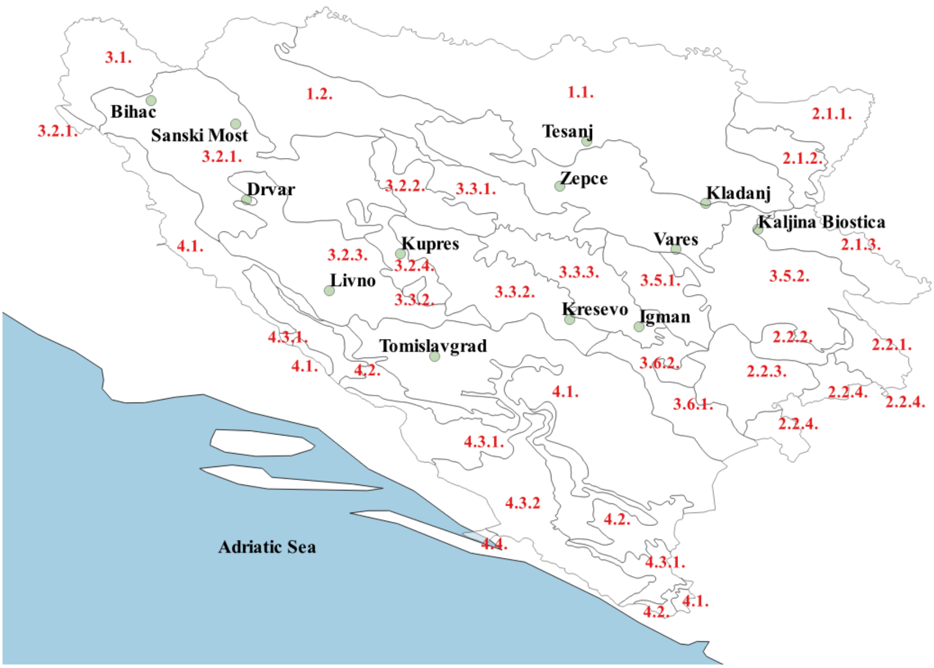

Figure 1. Distribution of researched silver fir populations in Bosnia and Herzegovina

Basic information about each population, including geographical coordinates, altitude, ecological-vegetation regions (Stefanović *et al*., 1983), and approximate tree ages, is presented in Table 1.

Table 1. Investigated populations of silver fir in Bosnia and Herzegovina

No	Population	Locality	Longitude	Latitude	Altitude (m)	Ecological-vegetation area	Area label	Age of trees
1.	Bihać	Risovac	16°03'40"	44°48'19"	898	Inner Dinarides region; West Bosnia limestones-dolomites area; Ključ – Petrovac raion	3.2.1.	100-150
2.	Drvar	Klekovača	16°30'31"	44°24'04"	1169	Inner Dinarides region; West Bosnia limestones-dolomites area; Ključ – Petrovac raion	3.2.1.	51-88
3.	Igman	Crni vrh	18°16'24"	43°47'22"	1245	Inner Dinarides region; Central Bosnia area; Sarajevo – Zenica raion	3.3.3.	69-139
4.	Kaljina Bioštica	Petrovići	18°44'46"	44°07'58"	971	Inner Dinarides region; East Bosnia plateau; Romanija raion	3.5.2.	46-102
5.	Kladanj	Karaula	18°36'11"	44°15'52"	1059	Inner Dinarides region; Zavidovići – Teslić area	3.4.	80-130
6.	Kreševo	Lopata	18°00'09"	43°50'16"	1285	Inner Dinarides region; Central Bosnia area; Vranica raion	3.3.2.	46-148
7.	Kupres	Rastičevo	17°13'20"	44°03'43"	1363	Inner Dinarides region; West Bosnia limestones-dolomites area; Koprivnica raion	3.2.4.	unknown
8.	Livno	Golija	16°52'32"	43°55'32"	1507	Inner Dinarides region; West Bosnia limestones-dolomites area; Glamoč – Kupres raion	3.2.3.	46-146 (208)
9.	Sanski Most	Korčanica	16°26'38"	44°41'23"	889	Inner Dinarides region; West Bosnia limestones-dolomites area; Ključ – Petrovac raion	3.2.1.	129-270
10.	Tešanj	Mekiš	17°59'42"	44°34'37"	360	Pripanonian region; North Bosnia area	1.1.	55-97
11.	Tomislav grad	Vran	17°25'03"	43°39'23"	1471	Mediterranean-Dinarides region; Submediterranean mountain area	4.1.	93-125
12.	Vareš	Dolovi	18°25'29"	44°09'14"	1180	Inner Dinarides region; Zavidovići – Teslić area	3.4.	95-135
13.	Žepče	Mahnjača	17°54'29"	44°27'08"	861	Inner Dinarides region; Zavidovići – Teslić area	3.4.	unknown

Each population was represented by ten mature trees, located 30-50 m appart to minimize spatial autocorrelation. From each tree, thirty medium-sized, well-developed cones were collected.

Morphological measurements

From the central part of medium-sized cone for each tree, twenty-five scales and seeds were selected for measurement. Measured traits of scales and seeds are listed in Table 2 and ilustrated in Figure 2 and 3 (figures modified from Ballian and Halilović, 2016).

Table 2. Measured traits of seeds and scales

No	Scale trait	No	Seed traits
1.	Length (figure 2, trait 1)	1.	Seed length (with wing)
2.	Width (figure 2, trait 2)	2.	Seed width (with wing)
3.	The width of the scale part outside the cone	3.	Seed length
4.	Bract length	4.	Seed width
5.	Bract width at the top of the scale	5.	Seed thickness
6.	Bract tip length		
7.	Scale thickness at the boundary with the outer scale		

All measurements were performed using a digital caliper, with accuracy of 0.01 mm.

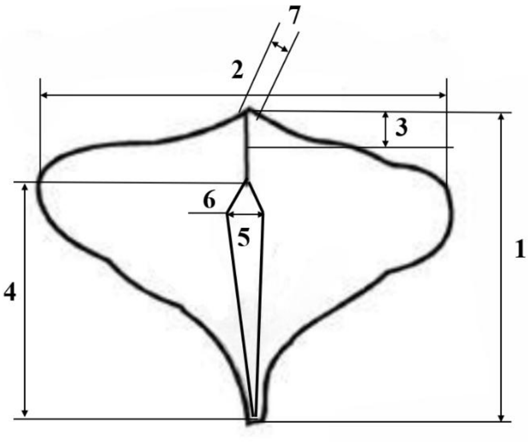

Figure 2. Scale traits

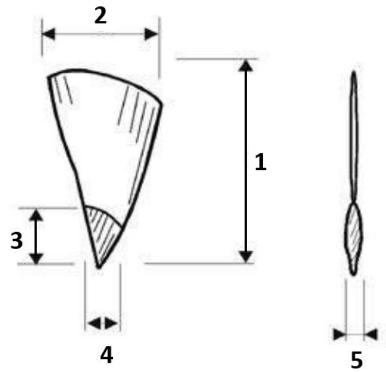

Figure 3. Seed traits.

Seed storage and nursery experiment

After selecting seed for measurement (from the medium-sized cone), the remaining seeds from each population were dried and stored at 0ºC in a cool, dry environment until sowing, with seeds from each population kept separate.

Sowing was conducted on 19 February 2021 at the Busovača Nursery (44°06'11"N, 17°52'44"E; altitude 385 m) in fluvisol soil. Seed were sown 1 cm apart at a depth of 2 cm, and covered with sawdust. Seeds from each population were sown in separate plots in the nursery, ensuring that seeds from different populations were not mixed.

Seedling growth measurement

The height and root collar diameters were measured in all seedlings after the first and second growing seasons using a digital caliper (accuracy 0.01 mm).

Experimental design and replication

The experimental design was completely randomized, with seedlings from each population kept separate to maintain provenance identity. The design ensured ten trees per population x twenty-five seeds/scales per tree for morphological measurements. Seedlings growth measurements were replicated for all individuals grow under the same nursery coniditions, providing a controlled environment for comparison among populations.

Statistival analyses

Normality of data was first tested. Since the data were not normally distributed, non-parametric analyses were performed. Descriptive statistics and the Kruskal-Wallis test were used to assess differences among populations for each trait. Hierarchical cluster analysis was applied to explore grouping patterns among populations based on morphological and growth traits. Pearson's correlation coefficient was calculated to assess relationsips between seed and scale traits and early seedling growth. All statistical analyses were performed using SPSS version 20.0 (IBM Corp., 2011).

RESULTS AND DISCUSSION

Average values of scale traits per population are shown in Table 3. Population Livno had the lowest average values of scale length and scale width (Table 3). Population Bihać had the lowest average value of the width of the scale part outside the cone, and population Kupres had the lowest average of the scale thickness (Table 3). The highest average value of scale length had population Kreševo, scale width population Žepče, the width of the scale part outside the cone population Vareš, and scale thickness population Kladanj (Table 3). The lowest average value of bract length had population Livno, bract width, and bract tip length population Bihać (Table 3). The highest average value of bract length had population Žepče, and bract width and tip length population Livno (Table 3).

Table 3. Average values of scale traits per population (highest average values highlighted in dark grey, lowest average values in light grey)

No	Population	Scale length		Scale width		The width of the scale part outside the cone		Scale thickness		Bract length		Bract width		Bract tip length	
		Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error
1.	Bihać	23.1	0.17	26.2	0.11	5.0	0.04	1.3	0.01	11.1	0.10	5.8	0.03	3.8	0.02
2.	Drvar	22.7	0.19	27.0	0.14	5.3	0.04	1.4	0.01	11.3	0.11	6.2	0.03	4.1	0.03
3.	Igman Sarajevo	27.1	0.18	27.8	0.14	5.4	0.04	1.4	0.01	14.1	0.13	6.1	0.03	4.0	0.02
4.	Kaljina Bioštica	24.7	0.14	27.3	0.15	5.4	0.04	1.3	0.01	12.8	0.09	6.0	0.02	4.0	0.02
5.	Kladanj	26.5	0.19	29.0	0.20	5.8	0.04	1.7	0.01	13.2	0.12	6.1	0.04	4.0	0.02
6.	Kreševo	28.2	0.23	26.7	0.14	5.7	0.05	1.4	0.01	13.9	0.15	6.1	0.04	4.1	0.03
7.	-Kupres	23.8	0.14	27.5	0.17	5.1	0.04	1.3	0.01	12.8	0.11	5.9	0.02	3.9	0.02
8.	Livno	21.7	0.13	26.2	0.10	5.0	0.03	1.4	0.01	10.9	0.09	6.3	0.03	4.2	0.02
9.	Sanski Most	25.1	0.18	27.1	0.18	5.6	0.04	1.6	0.01	12.2	0.13	5.9	0.05	3.9	0.03
10.	Tešanj	25.8	0.24	29.9	0.17	5.4	0.05	1.4	0.01	13.4	0.13	6.1	0.02	4.0	0.01
11.	Tomislavgrad	22.1	0.16	27.5	0.19	5.1	0.03	1.4	0.01	11.0	0.10	6.3	0.03	4.2	0.02
12.	Vareš	26.8	0.16	28.2	0.11	6.2	0.04	1.7	0.01	12.1	0.08	6.1	0.04	4.1	0.03
13.	Žepče	28.0	0.18	30.4	0.18	5.6	0.04	1.3	0.01	14.5	0.13	6.1	0.03	4.1	0.03
-	Total	25.1	0.06	27.8	0.05	5.4	0.01	1.4	0.004	12.6	0.04	6.1	0.01	4.0	0.01

Average values of seed traits per population are shown in Table 4. The lowest average values of seed length (with wing), seed length, seed width, and seed thickness population Livno and population Bihać had the lowest average value of seed width (with wing) (Table 4). The highest average values of seed length (with wing) and seed width (with wing) had population Žepče, seed thickness population Žepče and Tešanj, and seed width population Igman Sarajevo (Table 4).

Table 4. Average values of seed traits per population (highest average values highlighted in dark grey, lowest average values in light grey)

		Seed length (with wing)		Seed width (with wing)		Seed length		Seed width		Seed thickness	
No	Population	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error	Average (mm)	Std. error
1.	Bihać	20.9	0.14	11.5	0.08	10.4	0.05	4.8	0.04	2.9	0.02
2.	Drvar	22.6	0.95	12.0	0.09	10.2	0.05	4.6	0.03	2.9	0.14
3.	Igman Sarajevo	23.8	0.15	12.2	0.09	10.4	0.06	6.4	0.03	2.9	0.02
4.	Kaljina Bioštica	21.9	0.18	12.2	0.12	10.4	0.06	4.8	0.03	3.0	0.02
5.	Kladanj	23.8	0.21	12.7	0.11	11.3	0.08	4.9	0.04	3.0	0.03
6.	Kreševo	24.9	0.18	11.6	0.09	10.9	0.07	4.9	0.03	3.0	0.03
7.	Kupres	22.3	0.14	11.9	0.12	9.9	0.05	4.9	0.04	2.9	0.02
8.	Livno	20.2	0.13	11.8	0.08	9.6	0.06	4.5	0.03	2.6	0.02
9.	Sanski Most	22.3	0.16	12.8	0.12	11.7	0.08	4.8	0.03	2.9	0.02
10.	Tešanj	23.8	0.22	13.0	0.12	11.3	0.08	5.0	0.04	3.3	0.03
11.	Tomislavgrad	20.4	0.18	11.7	0.12	10.1	0.07	4.7	0.03	2.9	0.03
12.	Vareš	23.9	0.15	12.4	0.08	11.6	0.06	5.2	0.03	3.0	0.01
13.	Žepče	25.5	0.17	13.5	0.13	11.4	0.07	5.0	0.04	3.3	0.04
-	Total	22.8	0.09	12.6	0.02	10.7	0.02	5.0	0.01	3.0	0.01

Kruskal-Wallis test of scale and seed traits showed statistically significant differences among populations for all traits (df 12, Sig. <0.005).

Hierarchical cluster analysis based on scale and seed traits (Figure 4) showed that populations Kaljina Bioštica, Kupres, Sanski Most, Bihać, Tomislavgrad, Drvar, and Livno, formed the first cluster, and populations Kladanj, Tešanj, Vareš, Igman, Kreševo and Žepče formed the second cluster.

The clusters did not follow any geographical pattern. Most of populations in the first cluster belong to Inner Dinarides region; West Bosnia limestones-dolomites area (different raions), and area of East Bosnia plateau (population Kaljina Bioštica), but population Tomislavgrad belong to Mediterranean-Dinarides region; Submediterranean mountain area. The second cluster is composed of populations from Inner Dinarides region Central Bosnia area (Igman and Kreševo) and Inner Dinarides region, Zavidovići – Teslić area (Kladanj, Vareš and Žepče). Population Tešanj belongs to Pripanonian region, North Bosnia area (according to Stefanović *et al*., 1983).

In table 5, the mean values of morphological traits of one-year-old and two-year-old seedlings per populations are shown. The lowest average value of height of seedlings had population Kupres, and the highest population Vareš. The lowest average value of root collar diameter had population Bihać and the highest population Igman Sarajevo.

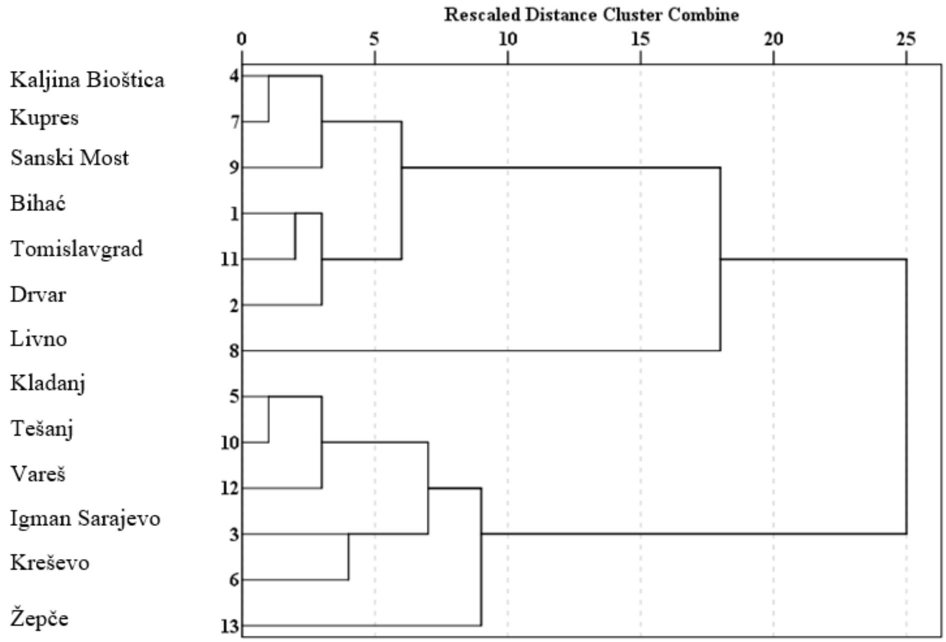

Figure 4. Hierarchical cluster of scale and seed traits

Table 5. Mean values of seedlings' heights and root collar diameters per populations (highest average values highlighted in dark grey, lowest average values in light grey)

No	Population	N 1-y-o	Mean height 1-y-o (cm)	Std. Deviation 1-y-o (cm)	Mean root collar diameter 1-y-o (cm)	Std. Deviation 1-y-o (cm)	N 2-y-o	Mean height 2-y-o (cm)	Std. Deviation 2-y-o (cm)	Mean root collar diameter 2-y-o (cm)	Std. Deviation 2-y-o (cm)
1.	Bihać	296	3.97	1.09	0.87	0.14	276	11.25	2.91	2.90	0.75
2.	Drvar	293	4.26	0.97	1.01	0.13	283	11.90	2.51	3.43	0.69
3.	Igman Sarajevo	236	3.75	0.94	1.23	5.87	170	8.89	2.79	2.71	1.05
4.	Kaljina Bioštica	295	4.14	0.94	0.88	0.14	252	11.15	4.09	2.93	0.75
5.	Kladanj	300	4.08	0.98	0.89	0.17	297	11.23	2.37	3.03	0.73
6.	Kreševo	299	4.12	0.91	0.91	0.14	296	12.88	2.41	3.65	0.78
7.	Kupres	220	3.55	1.66	1.21	5.27	160	10.92	3.22	3.40	1.34
8.	Livno	274	4.30	0.93	0.91	0.16	247	6.73	3.50	6.71	4.18
9.	Sanski Most	300	4.17	1.01	1.01	0.15	297	11.25	2.50	3.41	0.85
10.	Tešanj	300	4.11	2.03	0.93	0.18	281	10.63	2.75	2.95	0.78
11.	Tomislavgrad	192	4.48	0.93	1.01	0.16	172	11.02	2.64	3.14	0.90
12.	Vareš	224	4.25	1.03	0.89	0.17	197	11.25	4.00	3.41	0.93
13.	Žepče	299	5.00	4.54	1.07	0.17	297	16.26	2.92	3.89	0.80
-	Total	3528	4.18	1.76	0.98	2.01	3225	11.35	3.65	3.52	1.72

Results of Kruskal-Wallis test for morphological traits of one-year-old and two-year-old seedlings showed statistically significant differences among populations for both height of plants and root collar diameter (df 12, Sig. <0.005).

The results of hierarchical cluster analysis for morphological traits of one-year-old and two-year-old seedlings are shown in Figure 5. The hierarchical cluster results for morphological traits of seedlings are not similar to those found for seed and scale traits. Populations Kaljina Bioštica, Kladanj, Bihać, Vareš, Tomislavgrad, Tešanj, Kupres, and Drvar are the most alike. The Žepče population was separated from all other populations.

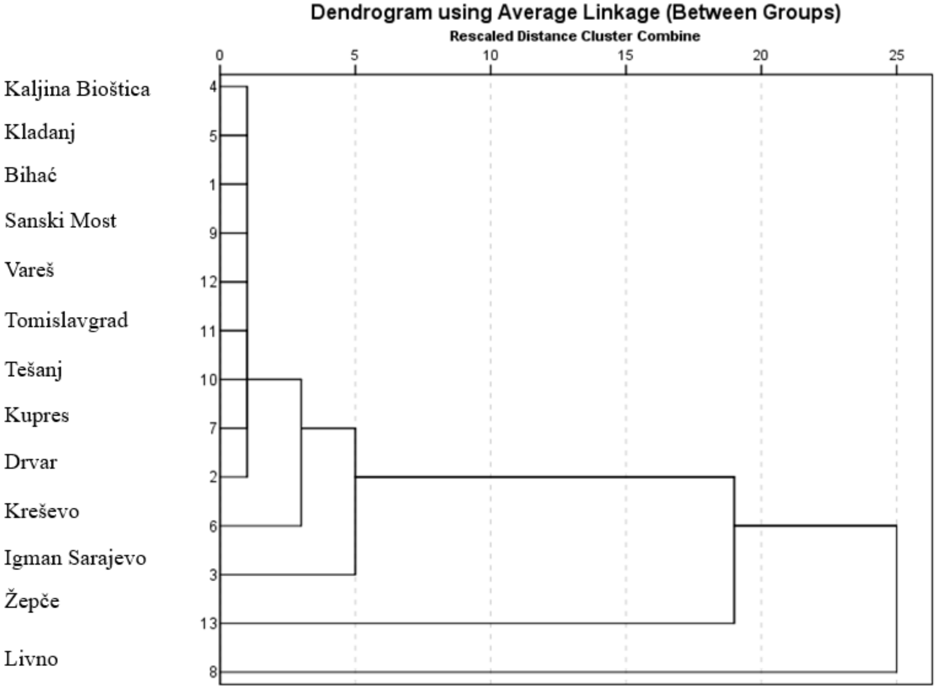

Figure 5. Hierarchical cluster for morphological traits of one-year-old seedlings.

Table 6. Pearson's correlation coefficient among the traits (seeds and scales) and growth in the first two years in the nursery. Significant correlations at the 0.01 level are highlighted in dark grey and significant correlations at the 0.05 level in light grey.

Traits	Traits/Correlation	Scale length	Scale width	Bract length	Seed length with wing	Seed width with wing	Seed length	Seed width	Seed thickness	Height 1-y-o seedlings	Root collar diameter 1-y-o seedlings	Height 2-y-o seedlings	Root collar diameter 2-y-o seedlings
Scale length	Pearson Correlation	1											
	Sig. (2-tailed)												
Scale width	Pearson Correlation	0.446**	1										
	Sig. (2-tailed)	0.000											
Bract length	Pearson Correlation	0.898**	0.492**	1									
	Sig. (2-tailed)	0.000	0.000										
Seed length with wing	Pearson Correlation	0.857**	0.534**	0.822**	1								
	Sig. (2-tailed)	0.000	0.000	0.000									
Seed width with wing	Pearson Correlation	0.013	0.167	0.018	0.041	1							
	Sig. (2-tailed)	0.886	0.057	0.838	0.644								
Seed length	Pearson Correlation	0.687**	0.554**	0.533**	0.676**	0.072	1						
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.414							
Seed width	Pearson Correlation	0.508**	0.471**	0.427**	0.444**	0.059	0.533**	1					
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.506	0.000						
Seed thickness	Pearson Correlation	0.500**	0.572**	0.497**	0.653**	0.081	0.612**	0.480**	1				
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.358	0.000	0.000					
Height 1-y-o seedlings	Pearson Correlation	0.148	0.282**	0.058	0.199*	0.087	0.241**	0.085	0.143	1			
	Sig. (2-tailed)	0.093	0.001	0.515	0.023	0.328	0.006	0.335	0.105				
Root collar diameter 1-y-o seedlings	Pearson Correlation	0.202*	0.118	0.299**	0.220*	0.031	0.114	0.109	0.109	0.040	1		
	Sig. (2-tailed)	0.021	0.180	0.001	0.012	0.730	0.195	0.218	0.216	0.650			
Height 2-y-o seedlings	Pearson Correlation	0.316**	0.266**	0.301**	0.321**	-0.197*	0.326**	0.221*	0.421**	0.223*	0.062	1	
	Sig. (2-tailed)	0.000	0.003	0.001	0.000	0.027	0.000	0.013	0.000	0.012	0.495		
Root collar diameter 2-y-o seedlings	Pearson Correlation	-0.147	-0.156	-0.129	-0.132	0.455**	-0.171	-0.103	-0.188*	0.086	0.034	-0.217*	1
	Sig. (2-tailed)	0.101	0.081	0.149	0.140	0.000	0.056	0.253	0.035	0.336	0.704	0.015	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Results of Pearson's correlation coefficient among the traits (seeds and scales) and growth in the first two years in the nursery are shown in table 6.

DISCUSSION

All investigated seed and scale traits of silver fir showed statistically significant differences among populations, consistent with earlier findings by Ballian and Čabaravdić (2005). The grouping of populations into two main clusters based on scale and seed traits revealed only a weak geographical pattern, with one cluster predominaly from western Bosnia and Herzegovina and the other group from central and eastern regions. This pattern partially corresponds with the molecular differentiation observed by Liepelt *et al.* (2002), who identified distinct haplotypes in Central/Western Europe versus Southeastern Europe and with Gömöry *et al*. (2004), who reported allele 1 in Western Bosnian populations and allele 2 in other Bosnian-Herzegovinian populations. The placement of Kaljina Bioštica population (eastern region) within the western cluster may reflect historical hybridization of different silver fir haplotypes in the Central Dinarides contact zone.

These findings are also consistent with broader biogeographical interpretations. Longauer (1996) suggested multiple refugia along the Dinaric range, while other authors stated that the junction of migration streams from the Balkans and the western refugia was at the eastern limit of Hercynian range (Krippel, 1986; Samek, 1967, in Gömöry *et al*., 2004). Other authors (Horvat-Marolt and Kramer, 1982, in Gömöry *et al.,* 2004) proposed the migration of silver fir from the refugia in Macedonia along the Dinarian range to northern Bosnia and Croatia. Konnert and Bergmann (1995) provided evidence of introgression zones between Balkan and Central European origins. Ballian *et al*. (2012) could not determine the extent of the influence of gene pools from both refugia, but their results supported the established impact of the Apennine pool east of the contact zone through the historical and present hybridization. Research conducted across the species' range supports this complex evolutionary history. For example, Belletti *et al*. (2017) detected substantial intrapopulation and interpopulation variability in Italian silver fir, reinforcing the idea of long-lasting isolation and multiple refugial lineages. Balkans specifically, Miljković (2022) reported significant spatial variation in needle morphology linked to climatic gradients, confirming that morphological traits in the region can reflect both environmental heterogeneity and historical gene flow. Our findings of mixed clustering across regions support this interpretation.

Seedling traits also showed statistically significant differences among populations, similar to earlier studies conducted in Bosnia and Herzegovina (Ballian, 2000b; Ballian, 2005; Halilović *et al*., 2009b, 2013) and neighboring Croatia (Ivanković and Marjanović, 2005). Morar *et al*. (2023) found that silver fir from different provenances vary in tolerance to low-temperature stress, further supporting the importance of provenance-specific responses. Comparisons with recent studies reinforces this: Błońska *et al*. (2023) demonstrated that environmental conditions, such as substrate quality, strongly influence root development and nutritional status of silver fir seedlings, which aligns with out interpretation that nursery conditions and maternal effects likely contributed to the variation we observed. Similarly, Teodosiu *et al*. (2023) found provenance-related variation in cone production, indicating strong reproductive trait differentiation, while Bončina *et al*. (2023) showe that stem growth of silver fir differs significantly among Central European regions. Collectively, these studies emphasize the influence of both genetic background ad local ecological factors, supporting the patterns detected in our seedling growth traits.

The average height of one-year-old seedlings (4.18 cm) was lower than the value reported by Dorofeeva *et al*. (2021), 7.2 cm, who observed that growth depends on temperature sums early in the season and on precipitation later. Given that all populations were grown under identical nursery conditions, the variation observed here most likely reflects genetic differences among populations combined with maternal and micro-environmental effects.

The hierarchical cluster results for morphological traits of one- and two-year-old seedlings differed from those based on seed and scale traits. This is expected, as seedling growth can be influenced not only by genetic factors but also by environmental conditions on the nursery and maternal provisioning effect.

Significant correlations were found between severas seed and scale traits and early seedling growth, suggesting that some reproductive traits may have predictive value for initial seedling vigor. Although most correlations were moderate, they warrant further investigation. Ballian (2000b) found no correlation between seed mass and seedling performance, indicating that seed size does not straightforwardly predict growth, a pattern partly echoed in our results.

No clinal morphological variability was detected, likely reflecting the ecological heterogeneity of the Dinaric region, where silver fir occurs in fragmented subpopulations except in the more continuouts western and eastern Bosnian ranges (Fukarek, 1970; Stefanović *et al*., 1983). The observed diversity and grouping of populations are consistent with the influence multiple glacial refugia and the long-term hybridization documented in previous studies (Konnert and Bergmann, 1995; Gömöry *et al*., 2004; Liepelt *et al*., 2002; Ballian *et al*., 2012). The grouping of both western and easter populations in our dendrograms, along with exceptions, likely reflects historical gene flow, hybridization processes, and the complex demographic history characteristic of silver fir in the Central Dinarides.

CONCLUSIONS

This study provides new evidence of substantial intra- and inter-population morphological variability in silver fir across Bosnia and Herzegovina, confirming the species' high adaptive potential and supporting its long term preservation in naturraly fragmented Dinaric habitats.

A key scientific contribution of this work is the identification of two major morphological clusters of seed and scale traits, broadly reflecting western versus central-eastern population groups. The atypical grouping of the Kaljina Bioštica population with western populations further supports earlier genetic findings that the Central Dinarides represent an active contact zone where Balkan and Appenine haplotypes have historically hybridized.

Another important contribution is the discovery of several statistically significant correlations between seed and scale morphology and early seedlings growth, suggesting that certain cone-related traits may serve as indicators for early selection in nursery conditions.

Despite these contributions, the study has limitations. Seedling growth was assessed in a single nursery environment, which may not fully represent filed performance, and maternal or environmental effects during cone development could not be completely separated from genetic factors. In addition, the study relied exclusively on morphological traits; integrating molecular markers would allow stronger inferences about the genetic structure of populations and the extent of hybridization.

Future research should therefore combine morphological, physiological, and genomic analyses across broader environmental gradients. Long-term provenance trials and reciprocal transplants would help verify whether early growth-morphology correlations persist later in development and under field conditions. Such integrated approaches would substantially improve selection strategies and conservation planning for silver fir in the Dinaric region.

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ASSESSMENT OF THE CARBON FOOTPRINT ASSOCIATED WITH RYE GRAIN INCLUSION IN COMPOUND FEED FOR BROILER CHICKENS

SUMMARY

The carbon footprint of compound feed containing different levels of rye grain during the rearing of broiler chickens was calculated. The study was conducted on five groups of ROSS-308 broiler chickens, each consisting of 120 birds. The control group was fed compound feed based on corn grain and soybean meal without the inclusion of rye grain. Birds in the experimental groups received compound feed containing graded levels of rye. Rye grain was introduced into the compound feed of broiler chickens at inclusion levels of 2–5% between days 11 and 20, 4–10% between days 21 and 30, and 10–25% between days 31 and 42 of age. The carbon footprint of feed ingredients and compound feed was calculated using the FeedPrintNL software (version 2022.00, December 9, 2022) developed by Wageningen University. Greenhouse gas emission values for soybean meal were used according to the Life Cycle Assessment conducted by the Research Institute of Organic Agriculture FiBL and hybrid rye – according to the GFLI database, developed by the independent Blonk consultancy. The calculated greenhouse gas emission values consider only the emissions from raw materials and illustrate the potential for their reduction through the selection of different feed ingredients. The study revealed that the inclusion of rye grain in the compound feed for broiler chicken contributed to a 3.1% to 7.8% decrease in CO_2-eq emissions per tonne of feed.

Keywords: carbon footprint, rye, feeding, poultry

INTRODUCTION

According to the FAO (2013), livestock supply chains emit approximately seven gigatonnes of CO_2-eq/year, with feed production being the primary driver (45%). While poultry accounts for 8% of these emissions (Liu *et al.*, 2015; Attia *et al.,* 2024) and exhibits a lower carbon footprint per unit of mass compared to other species (Williams, Audsley and Sandars, 2006; Peters *et al.,* 2014), the projected demand growth described by Attia *et al.* (2024) necessitates the

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development of sustainable systems (Peter John and Mishra, 2024). Consequently, reducing the "carbon footprint"—defined by Pertsova (2007) as cumulative CO_2, CH_4, and N_2O emissions—through feed optimization remains a priority. Moungsree *et al.* (2024) identify feed production, energy consumption, and manure management as the three primary drivers of GHG emissions in the poultry industry. Feed production, which includes cultivation technology, transport, fertilizer application, and land use change (LUC), exerts a direct influence on the carbon footprint of broiler production. Attia *et al*. (2024) specify that GHG emissions in the broiler industry are distributed as follows: 78% from feed production, 8% from direct on-farm energy consumption, 7% from post-farm processing and meat transport, and 6% from manure storage and processing.

Research by Leinonen *et al*. (2012) demonstrated that feed caused a greater overall environmental impact than any other materials utilized in broiler meat production. Furthermore, Martinelli *et al*. (2020) identified maize and soybean production as the major sources of GHG emissions. Significant interest in the utilization of low-emission feed components through diet modification is noted by Giannenas *et al.* (2017). GHG emissions from various raw materials used in compound feed production can vary substantially, occasionally by factors of tens or even hundreds. For instance, Ershadi *et al.* (2021) estimated GHG emissions from maize grain at 347 kg CO_2-eq/t. In contrast, for maize grown in Ukraine, GHG emissions amount to 481 kg CO_2-eq/t according to the FeedPrintNL software (version 2022.00, December 9, 2022, Wageningen University), which allows for the calculation of GHG emissions for specific cultivation technologies, accounting for actual yield and transport to the processing site.

Protein feeds occupy a significant proportion of poultry diets. An ideal scenario involves meeting the growing demand for protein feeds by increasing the productivity of agricultural lands (Bennetzen *et al*., 2016). However, as emphasized by Riedesel *et al.* (2022), increased productivity may also be accompanied by a rise in GHG emissions per unit area and, consequently, does not always lead to a reduction in GHG emissions per unit of product. A promising avenue lies in the investigation of various feeding strategies, including alternative protein sources that contribute to improved nutrient utilization, thereby reducing nitrogen and carbon emissions. The use of locally produced protein feeds holds significant potential for this purpose (Sychov *et al.,* 2025; Sychov *et al.,* 2022; Plyska *et al.,* 2021).

The utilization of locally sourced ingredients for compound feed manufacturing plays a significant role in mitigating GHG emissions. Thus, when assessing GHG emissions associated with soy-derived products, their provenance is a critical factor, as transport can substantially increase total emissions. GHG emissions also rise significantly when soy is cultivated on land that has undergone a change in land use purpose. Prudêncio da Silva *et al*. (2014) linked the carbon footprint of broilers raised in Brazil to high energy consumption and GHG emissions associated with maize and soybean cultivation, as well as emissions resulting from deforestation (0.39–0.81 kg CO_2-eq/kg maize and 0.39–1.7 kg CO_2-eq/kg soybean). Finnish researchers Usva *et al*. (2023) reported that land use change accounted for 0.55 kg CO_2-eq/kg carcass weight (23%), primarily due to soybean production for feed purposes in Brazil. This view is supported by other researchers (Lopes, Zepka and Deprá, 2024), who assert that increased GHG emissions are associated with higher protein content in broiler feed, which often includes a larger proportion of soy obtained from lands cultivated following deforestation.

Although Arrieta and González (2019) noted that the choice between extruded full-fat soybeans or soybean meal and oil as feed ingredients results in only minor differences in carbon emissions, provenance remains crucial. According to FeedPrintNL software, GHG emissions associated with soybean meal imported into the European Union can amount to 5,728 kg CO_2-eq/t. Conversely, according to Ershadi *et al.* (2021), GHG emissions from soybean meal are 204 kg CO_2-eq/t. For comparison, GHG emissions from locally produced rapeseed meal amount to 607 kg CO_2-eq/t. It is worth noting that, according to the Danube Soya Association, the average carbon footprint of soybean meal produced in Europe from imported soybeans is 1,990 kg CO_2-eq/t, highlighting the significant environmental advantage of using local raw materials and ingredients (Life Cycle Assessment/Carbon Footprint Project - Donau Soja).

The role of amino acids in animal nutrition is considered significant. Calculations by Sturm *et al.* (2022) showed that the use of free amino acids in animal feed has already contributed to avoiding the use of millions of hectares of arable land. In general, the positive effects of including free amino acids in animal feed are realized through two main pathways. First, they improve feed efficiency, thereby reducing the overall demand for feed and, consequently, the negative environmental impact associated with feed production. Second, free amino acids allow for a reduction in the crude protein content of feeds. The implementation of diets with reduced crude protein content is regarded as one strategy to reduce demand for soy in the European Union. This approach also contributes to lowering the overall negative environmental impact of feed production, particularly regarding land use change and deforestation (Sturm *et al.*, 2022).

Since amino acids enhance feed efficiency and reduce nitrogen losses, their usage is expected to increase if regulations aimed at reducing nitrogen emissions are implemented (Vijn *et al.,* 2020; Selle *et al.*, 2022; Sturm *et al.,* 2022). Alves *et al*. (2023) hypothesized that the inclusion of the amino acid lysine HCl in broiler diets would reduce environmental impact; however, their findings did not confirm this. Data modeling in their study did not demonstrate a significant reduction in environmental impact, possibly because lysine production involves the use of crude protein and energy-rich resources. Mosnier *et al.* (2011) emphasized that although amino acid production causes a significant environmental impact, their low inclusion rates result in a negligible contribution to impact categories for broiler and pig diets. Furthermore, diets with reduced crude protein content had higher amino acid inclusion rates. Ershadi *et al.* (2021) reported significantly lower levels of GHG emissions from L-threonine and L-lysine produced by the enzymatic method – 388 kg CO_2-eq/t, whereas emission levels from DL-methionine are estimated at 3010 kg CO_2-eq/t. Overall, Ershadi *et al.* (2021) highlighted that the production of amino acids, including DL-methionine, L-lysine, and L-threonine, requires a large amount of energy compared to other feeds. This may represent an additional source for GHG emission reduction in the production of compound feeds for livestock.

The use of local alternative cereal feeds with minimal GHG emissions is a highly promising direction. One promising cereal crop is rye. Riedesel *et al.* (2022) emphasize that rye has approximately 20% lower GHG emissions per unit area and an 8% smaller carbon footprint compared to wheat. This difference is primarily due to the fact that rye requires approximately 25% less nitrogen than wheat. Expanding rye cultivation in cereal crop rotations offers additional opportunities for feeding and climate change mitigation. Moreover, Macholdt and Honermeier (2017) and Miedaner and Laidig (2019) assert that hybrid breeding increases yield due to the benefits of the heterosis effect. Research by Riedesel *et al.* (2022) showed that the higher yield of hybrid rye varieties (+13%) more than compensates for the resultant higher GHG emissions per unit area (+4%), leading to a lower carbon footprint (-9%) and, accordingly, a reduced climatic impact of hybrids compared to pure lines. The main reason for this is that rye is better adapted for cultivation in extreme conditions, particularly due to its highly developed root system and greater specific root length (Ryser, 2006). Consequently, rye demonstrates higher nitrogen use efficiency and a lower risk of leaching compared to other cereals, including wheat. These findings corroborate previous studies by Wojcik-Gront and Bloch-Michalik (2016), who also identified rye as the crop with the lowest GHG emissions due to the lowest nitrogen requirement among all cereals studied. This can serve as a basis for its use in animal feed to achieve significant reductions in GHG emissions.

The study by Moungsree *et al.* (2024) was based on a "cradle-to-gate" approach, where total GHG emissions from feed production for broilers, laying hens, and pigs were estimated at 650 ± 20, 706 ± 20, and 466 ± 20 kg CO_2-eq/t of feed, respectively. Feed production for laying hens resulted in the highest GHG emissions, being 1.09 and 1.52 times higher than for broilers and pigs, respectively. This is primarily explained by the inclusion of fish meal in diets formulated for laying hens. However, in studies by Usva *et al*. (2023), the climate change impact of the average industrial feed mixture was estimated at 0.96 kg CO_2-eq/kg feed, or 960 kg CO_2-eq/t. A significant portion of the impact (23%) was attributed to land use change, mainly associated with soybean production in Brazil for feed requirements.

For assessing the impact of technological interventions, the study by Leinonen *et al.* (2012) is particularly relevant as it accounted for key parameters such as feed consumption, animal growth, and nutrient assimilation. Simultaneously, Tallentire *et al.* (2017) highlighted that both slaughter age and growth rate significantly influence the feed conversion ratio (FCR). As the production cycle duration increases, energy expenditure for metabolic heat production also increases, thereby reducing feed conversion efficiency. Nacimento *et al.* (2022) reported that reducing the final live weight of broiler chickens from 2.6 to 1.6 kg could reduce the total global warming potential in the Italian poultry sector by approximately 12%.

The application of enzymes in animal feed is a promising strategy for mitigating GHG emissions. For example, a study by Bundgaard *et al.* (2014) demonstrated that the use of an enzyme product based on xylanase, amylase, and protease has the potential to reduce GHG emissions from broiler chicken production by approximately 90 g CO_2-eq per head. Similarly, these findings are supported by Wiedemann *et al*. (2017), who demonstrated that an improvement in the feed conversion ratio by 0.1 led to a reduction in GHG emissions by 3–4.5%. According to Alves *et al*. (2023) and Prudêncio da Silva *et al*. (2014), the assessment of GHG emissions per animal plays a key role in identifying effective emission reduction strategies. The authors suggest that shortening the production cycle and reducing the final live weight of broilers could potentially lower energy consumption and reduce GHG emissions in the Argentine broiler industry. This strategy may contribute to optimizing production efficiency with improvements in feed conversion ratios. Leinonen *et al.* (2012) also emphasized that modifications in feed consumption and composition influence GHG emissions.

In a review by Cesari *et al*. (2017) on the efficiency of broiler production in Italy, emissions ranged from 1.1 to 3.84 tonnes CO_2-eq/kg live weight under identical system parameters and rearing conditions. The average slaughter weight ranged from 1.9 to 2.5 kg, and the fattening cycle lasted from 31 to 42 days. Simultaneously, citing van Horne (2017), Cesari *et al.* (2017) presented results from reports in France indicating a slightly lower weight of 1.9 kg, and from Spain – 2.7 kg. Conversely, the average Argentine chicken weighed 2.88 kg at 49–50 days. On the other hand, although Brazil shares similar production profiles with Argentina, the higher energy usage and carbon footprint of chickens raised in Brazil, estimated by Prudêncio da Silva *et al*. (2014), can be explained by higher energy consumption and GHG emissions throughout the entire production cycle. According to Usva *et al.* (2023), GHG emissions from broiler meat production in Finland were estimated at 2.37 kg CO_2-eq per kg of carcass weight. A study conducted by Duarte da Silva Lima *et al*. (2019) reported a carbon footprint of 2.70 kg CO_2-eq/kg live weight in broiler meat production. The overall results of three review studies by Usva *et al.* (2023) in Finland indicated a carbon footprint in the range of 2.1 to 8.5 kg CO_2-eq/kg carcass weight, with the highest values associated with free-range and alternative production systems.

Pelletier *et al.* (2013) analyzed the carbon footprint of modern intensive production and concluded that further optimization of feed efficiency and the sourcing of feed with the lowest ecological burden are key levers for reducing GHG emission intensity. The use of local crops for animal feeding, which are well-adapted to growing conditions and ensure high yields and low levels of GHG emissions per unit of feed, is highly promising. Such feeds do not have widely investigated optimal usage rates or assessments of their impact on GHG emissions. The utilization of local feeds, such as rye, allows for a reduction in GHG emissions associated with logistics. The search for alternative local diet components with the lowest greenhouse gas emissions is particularly effective.

MATERIAL AND METHODS

A study investigating the potential inclusion of standardized levels of KWS Tayo hybrid rye grain was conducted in 2024 at the Research Laboratory of Animal Nutrition and Feed Quality of the National University of Life and Environmental Sciences of Ukraine, utilizing 600 ROSS-308 broiler chickens. At the age of 10 days, the broilers were randomly allocated into five groups of 120 birds each: one control group and four experimental groups. The broilers in the experimental groups received diets including rye grain at inclusion rates ranging from 2% to 25%.

The study employed a two-step methodological approach integrating biological data with environmental modeling. First, the in vivo experiment determined the actual zootechnical performance of broilers, specifically feed intake and feed conversion ratio (FCR), across different rye inclusion levels. Second, these empirical performance data served as key input variables for the computational modeling using FeedPrintNL software. This integration allowed for a precise assessment of the carbon footprint, translating the physical consumption of specific feed formulations into environmental impact metrics expressed as CO_2-eq/kg per bird and per kilogram of live weight gain.

Within the framework of this study, an assessment of the carbon footprint associated with the inclusion of rye grain in broiler chicken nutrition was performed.

The carbon footprint for corn grain, soybean meal, hybrid rye grain, soybean oil, DL-methionine, L-threonine, L-lysine, L-valine, L-isoleucine, salt, monocalcium phosphate, calcium carbonate, sodium bicarbonate, mineral premix, and vitamin premix was calculated using the FeedPrintNL software (version 2022.00, December 9, 2022), developed by Wageningen University. For corn and rye grain, adjustments regarding cultivation technology were applied during the life cycle assessment (LCA). The greenhouse gas emission potential of the soybean meal used for compound feed preparation is certified in accordance with the LCA conducted by the Research Institute of Organic Agriculture (FiBL), while for hybrid rye, it aligns with the GFLI database developed by the independent consultancy Blonk.

According to FiBL data, greenhouse gas emissions associated with soybean meal produced in the Khmelnytskyi region (Ukraine) from AdamPolSoya soybeans grown under Danube Soya standards amount to 360 kg CO_2-eq/t. Under Ukrainian conditions, soybean meal emissions are estimated by the FeedPrintNL software at 821 kg CO_2-eq/t. In this study, soybean meal obtained from AdamPolSoya soybeans and processed at the enterprise's facilities was utilized; consequently, the emission value of 360 kg CO_2-eq/t provided by FiBL was applied for the calculations.

Greenhouse gas emissions from the hybrid winter rye KWS Tayo used in this study, calculated according to the GFLI database, amounted to 331 kg CO_2-eq/t. For comparison, greenhouse gas emissions from conventional rye varieties constitute 382 kg CO_2-eq/t (GFLI Database – The Global Feed LCA Institute). This emission value is based on a three-year study conducted on farms in Germany. According to the FeedPrintNL software, the range of greenhouse gas emissions from various hybrid rye cultivation technologies in Ukraine lies between 232 and 381 kg CO_2-eq/t of grain. The value of 331 kg CO_2-eq/t was selected based on the identical agronomic practices for hybrid rye in Ukraine and Germany. This figure is consistent with validated emission ranges derived from longitudinal research.

For corn grown in Ukraine using the most prevalent intensive cultivation technology with the application of organic fertilizers, greenhouse gas emissions amounted to 481 kg CO_2-eq/t. Depending on the specific cultivation technology applied, greenhouse gas emissions may range from 250 to 520 kg CO_2-eq/t.

The determination of the carbon footprint per broiler and per kilogram of live weight gain considered only the experimental period (11–42 days of life).

Each group was divided into 6 replicates with 20 birds in each (Table 1).

Table 1. The experimental design of the research and production trial

Group	Number of broilers	Broiler age, days		
		11–20	21–30	31–42
		Content of rye grain in the compound feed, %		
Control: 1	6 × 20	0	0	0
Experimental: 2	6 × 20	2	4	10
3	6 × 20	3	6	15
4	6 × 20	4	8	20
5	6 × 20	5	10	25

The experimental broiler chickens were housed in floor pens with a stocking density of 0.2 m^2 per bird and a feeder space of 3 cm per bird. The birds received a complete compound diet composed of corn grain, soybean meal, soybean oil, essential amino acid supplements (valine, isoleucine, lysine, methionine, and threonine), sodium and calcium additives, as well as a vitamin-mineral premix. The diet provided to the control group did not contain rye grain. Rye grain was incorporated into the diets of the experimental groups in accordance with the experimental design. The proportions of ingredients were adjusted to maintain adequate nutrient and energy levels.

RESULTS AND DISCUSSION

The calculated values are presented in Table 2. According to the FeedPrintNL software, synthetic amino acids and vitamins exhibit the highest levels of greenhouse gas emissions, with values ranging from 3,044 to 7,247 kg CO_2-eq/t. This is primarily attributed to their synthesis methods and the resource intensity of their production.

Greenhouse gas emission levels for table salt (NaCl), calcium carbonate, monocalcium phosphate, and sodium bicarbonate vary depending on their origin and processing methods. In Ukraine, the emission level for carbonates may be as low as 4 kg CO_2-eq/t, whereas emissions for monocalcium phosphate and sodium bicarbonate are significantly higher, at 485 and 486 kg CO_2-eq/t, respectively. A similar trend is observed for the mineral premix utilized in compound feed production, with an emission estimate of 1,176 kg CO_2-eq/t according to the FeedPrintNL software.

The results of greenhouse gas emission calculations for broiler chicken diets containing rye grain, based on the conducted studies, are presented in Table 3.

Table 2. Carbon emissions from input raw materials of feed mill, expressed as CO_2-eq/t

Ingredients	Emissions, kg CO2-eq/t
Corn grain	481
Hybrid rye grain	331
Soybean meal	360
Soybean oil	1010
DL-methionine, 99%	3044
L-threonine, 98%	6487
L-lysine, 96%	6487
L-valine, 96.5%	6397
L-isoleucine, 90%	7247
Salt	174
Monocalcium phosphate	485
Calcium carbonate	4
Sodium bicarbonate	486
Mineral premix	1176
Vitamin premix	6449

Table 3. Total greenhouse gas emissions from the production of compound feed for broiler chickens, kg CO2-eq/t compound feed

Broiler age, days	Group				
	1	2	3	4	5
11–20	468	467	467	466	466
21–30	481	477	475	473	471
31–42	477	467	462	456	451

The carbon footprint of the compound feed for broiler chickens in Group 1 (control, based on soybean meal and corn grain), excluding GHG emissions from feed manufacturing processes, amounted to 468 kg CO_2-eq/t during the first experimental period (11–20 days). This period coincided with the diet containing the highest crude protein content, corresponding to the maximum inclusion of soy products. Increasing the proportion of rye in the feed to 2% and 3% in experimental Groups 2 and 3 resulted in a GHG emission reduction of 1 kg CO_2-eq/t compared to the control group. Similarly, the inclusion of 4% and 5% rye in the diets of experimental Groups 4 and 5 yielded a reduction of 2 kg CO_2-eq/t. Overall, the incorporation of rye into broiler diets during the 11–20 day period provided a reduction in CO_2-eq emissions per tonne of feed of 0.2–0.4%.

During the 21–30 day period, the carbon footprint of the compound feed increased to 481 kg CO_2-eq/t in Group 1 (control) and to 477, 475, 473, and 471 kg CO_2-eq/t in Groups 2–5, respectively. This increase is attributed to a reduction in soybean meal content and a corresponding increase in corn grain levels, which has a higher carbon footprint by 121 kg CO_2-eq/t (compared to the soy source used). Emissions were also influenced by the inclusion of synthetic amino acids, where DL-methionine (99%) contributes 3044 kg CO_2-eq/t, and L-isoleucine (90%) contributes 7247 kg CO_2-eq/t. Nevertheless, the inclusion of 4–10% rye grain in broiler diets allowed for a reduction in CO_2-eq emissions per tonne of feed by approximately 0.8–2.0%.

The final experimental period, covering broiler rearing from 31 to 42 days of age, was the most indicative regarding GHG emissions. During this period, GHG emissions for Group 1 (control) amounted to 477 kg CO_2-eq/t. The inclusion of 10% rye in the compound feed resulted in a 2.1% reduction in GHG emissions, while the inclusion of 25% rye achieved a reduction of up to 5.4%.

Consequently, the inclusion of rye grain in broiler compound feed at levels of 2–5% during days 11–20, 4–10% during days 21–30, and 10–25% during days 31–42 led to a reduction in CO_2-eq emissions per tonne of compound feed in the range of 3.1% to 7.8%.

The quantity of soybean meal within a specific age group remained constant regardless of rye usage in the diet. Given the crude protein content of corn and rye, the diet did not require adjustments to crude protein levels using soybean meal. Thus, soybean meal did not contribute to changes in GHG emissions between groups. The use of rye in diets necessitated the additional use of amino acids, which possess significant GHG emission levels as per Table 1. In the structure of GHG emissions per tonne of feed, amino acids accounted for 1.66–2.01% in Control Group 1, while for the experimental groups, the range was 1.67–2.04%, indicating a negligible impact on overall GHG emission levels. At the same time, the introduction of rye into the diet according to the experimental design reduced emissions associated with corn usage by 4.8% to 17.19%.

Rye grain contains a lower amount of metabolizable energy than corn; consequently, additional soybean oil was used to balance the metabolizable energy levels in the diets. The GHG emission level of soybean oil is 1,010 kg carbon per tonne, which increased the total GHG quantity per tonne of feed. Soybean oil usage in the diet of Control Group 1 ranged from 0.35–0.60%, whereas in the experimental group diets, it increased to 3.1%. Accordingly, within the structure of GHG emissions, soybean oil had a substantial impact on their increase. For Control Group 1, the level of GHG emissions in the overall structure attributed to soybean oil usage was 0.75–1.27%, while in the experimental groups, it ranged from 1.19% to 6.94%. The additional inclusion of soybean oil increased GHG emissions per tonne of compound feed, thereby diminishing the positive effect of utilizing rye in the diet. The search for alternative energy sources and enzyme preparations that enhance energy availability in rye-based diets for broiler feeding, while maintaining low GHG emission levels, is highly promising.

The calculated greenhouse gas emissions for broiler feed preparation account only for emissions associated with raw materials and illustrate the potential for emission reduction through the selection of alternative feed ingredients. These calculations exclude emissions from raw material logistics, grinding, mixing, and pelleting of the finished feed. When considering these additional sources of GHG emissions, it can be assumed that the results align with the conclusions presented by Moungsree *et al*. (2024) during field studies.

Differences in broiler growth rates influence, among other factors, feed consumption per bird, manure output, energy usage, and housing requirements. Consequently, GHG emissions per broiler reflect not only emissions associated with various feed ingredients but also the efficiency of these feeds in terms of live weight at slaughter.

Calculations of greenhouse gas emissions per broiler are presented in Table 4.

Table 4. Total greenhouse gas emissions from broiler feed production, kg CO2-eq per broiler during the experimental period

Broiler age, days	Group				
	1	2	3	4	5
11–20	0.312	0.315	0.314	0.314	0.320
21–30	0.773	0.746	0.763	0.778	0.750
31–42	0.998	1.004	0.980	0.979	0.955
Total	2.083	2.065	2.057	2.071	2.025

Experimental Group 5 exhibited the lowest emission level in terms of kg CO_2-eq per bird. This is attributed to the utilization of compound feed with the lowest GHG emission intensity in this group. Furthermore, a marked reduction in feed consumption per bird was observed in this group.

The calculated emission value for Group 1, which served as the control in the experiment and whose diet was formulated based on corn grain and soybean meal, amounted to 2.083 kg CO_2-eq per broiler. Meanwhile, in Group 2, where the diet contained the lowest inclusion levels of rye throughout the experiment, greenhouse gas emissions decreased by 0.86% per bird. In Group 5, where the diet contained the highest rye inclusion levels, greenhouse gas emissions decreased by 2.78% per bird.

Similar conclusions were reported by Cesari *et al*. (2017), van Horne (2017), Prudêncio da Silva *et al.* (2014), Usva *et al*. (2023), and Duarte da Silva Lima *et al.* (2019).

Total greenhouse gas emissions from feed production per kilogram of live weight of broiler chickens are presented in Table 5.

In Group 1 (control), where broilers received a diet based on corn grain and soybean meal, greenhouse gas emissions averaged 0.836 kg CO_2-eq/kg live weight over the experimental period. In Group 2, the diet of which contained the lowest inclusion levels of rye, the GHG emission value decreased by 2.27%. In Group 5, characterized by the highest rye inclusion levels, the GHG emission value decreased by 1.91%. The most significant reduction was observed in Group 3, where emissions were on average 3.35% lower over the experimental period. In this group, rye inclusion levels were 3% during days 10–20, 6% during days 21–30, and 15% during the 31–42 day rearing period.

It is noteworthy that a progressive increase in greenhouse gas emissions per kg of live weight was observed throughout the entire experimental period. Thus, greenhouse gas emissions per absolute weight gain during the period from day 21 to 30 were 34–37% higher compared to emissions recorded between days 11 and 20 of growth. Furthermore, emissions recorded during the final rearing stage (days 31–42) were 15–29% higher than those observed in the preceding period (days 21–30).

Table 5. Total greenhouse gas emissions from feed production, kg CO_2-eq/kg weight gain during the experimental period

Broiler age, days	Group				
	1	2	3	4	5
11–20	0.596	0.595	0.590	0.589	0.595
21–30	0.810	0.815	0.791	0.816	0.809
31–42	0.986	1.052	0.935	0.942	0.958
Total	0.836	0.817	0.808	0.819	0.820

In addition to the level of feed intake, the Feed Conversion Ratio (FCR) exerts a significant influence on the level of GHG emissions per kg of live weight. Consequently, it is clearly observed that certain experimental groups exhibit higher GHG emission levels per bird—which depend on the kg CO_2-eq/t of feed and the level of feed consumption—yet demonstrate lower levels of kg CO_2-eq/kg live weight.

The obtained results are consistent with data from Leinonen *et al.* (2012), Tongpool *et al.* (2012), Skunca *et al.* (2018), Ershadi *et al.* (2020), Vijn *et al.* (2020), Wiedemann *et al.* (2017), and Ershadi *et al.* (2021), who reported that improving feed efficiency, including the quantity, composition, and nutritional density of consumed feed, holds potential for reducing the environmental impact of broiler meat production.

CONCLUSIONS

The study demonstrated that feed production constitutes the largest contribution to the environmental profile of the entire broiler chicken meat production chain. Inclusion of rye grain into broiler diets at 2–5% during days 11–20, 4–10% during days 21–30, and 10–25% during days 31–42 resulted in a reduction in CO_2-equivalent emissions per tonne of feed by 3.1% to 7.8%. In summary, the results of the calculations highlight the significant potential for reducing greenhouse gas emissions through careful selection of raw materials, taking into account the carbon footprint of each feed ingredient.

Future research should explore in greater detail the options for mitigating the environmental impact of broiler chicken meat production, with particular attention to greenhouse gas emissions per kg of product across different growth periods.

The potential for reducing the overall growing period should also be investigated. In addition, the use of enzyme products warrants further study, as they may enhance feed conversion efficiency and contribute to lower greenhouse gas emissions.

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FIELD EVALUATION OF TOMATO CROP PRODUCTION UNDER SLECI IRRIGATION

SUMMARY

Ghana's diverse agroecological zones support a wide range of crops, with tomato ranking among the most cultivated vegetables due to market demand and quick sell. However, during cultivation, tomato has high water consumption and this sometimes poses a challenge in the face of erratic rainfall patterns and climate change. The daily water needs of a single tomato plant can range up to 6 litres during dry and hot days. Therefore, methods and techniques that can improve water use efficiencies are very important for their production. This study, conducted by the University of Cape Coast under the EU-funded DIVAGRI project, evaluated the performance of self-regulating, low-energy, clay-based irrigation (SLECI) compared to conventional surface drip irrigation (CSDI) for tomato cultivation. The obtained results show that SLECI treatment delivered 78% more tomato yield in comparison with CSDI. SLECI recorded 285.12 m^3/ha water applied during the season and 4.5 kilograms of fresh tomato yield with each m^3 of used water. Furthermore, results showed close values in leaf length, plant height, leaf width, and stem diameter for tomato plants irrigated using both SLECI and CSDI for the duration of 14 to 42 days after transplanting.

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Finally, the results obtained in this study will be useful for all interested parties from the agricultural sector in Ghana and beyond.

Keywords: clay emitters, low-energy irrigation, surface drip irrigation, tomato yield response, water productivity

INTRODUCTION

Water is the critical limiting resource for sustainable food production. Globally, agriculture is the largest consumer of freshwater, and many productive agricultural areas already experience physical water scarcity or high seasonal variability in water availability. The FAO has estimated that billions of people live in agricultural regions with high or very high-water shortages, and projected increases in demand for water (driven by population growth and higher irrigation needs under warming climates) mean that competition for freshwater between agriculture, urban use and ecosystems will intensify in coming decades. Without improvements in water governance, efficiency, and allocation, water scarcity is expected to reduce crop production and undermine rural livelihoods in many vulnerable regions Biswas *et al*., 2025; FAO, 2020).

Water scarcity harms agricultural production through several pathways: reduced soil moisture and plant water stress (lowering yields and quality), changes in planting dates and cropping patterns, increased risk of crop failure during droughts, and degraded soils from unsustainable groundwater extraction and salinization in irrigated areas. Climate change is already amplifying these risks by increasing the frequency and severity of droughts and by altering rainfall seasonality in many regions, making water scarcity worse in the near future (Alotaibi *et al*., 2023). Water scarcity is a prevalent issue, even in nations with sufficient supplies of water (Mishra, 2023). To ensure the sustainability of this vital resource in agricultural production, it is crucial to use water properly. Moreover, it is essential to adopt certain strategies to manage agricultural water use effectively. This includes adopting various water-efficient techniques such: micro-irrigation, irrigation scheduling, drip fertigation (Tanaskovik *et al*., 2016; Tekinel and Kanber, 2002; Howell, 2001; Papadopoulos, 1996), conservation agriculture by use of mulch (Bian *et al*., 2024), use of deficit irrigation practices (Yang *et al*., 2020; Comas *et al*., 2019), innovative water management practices (Biswas *et al*., 2025), etc.

Vegetable production in sub-Saharan Africa is mainly dependent on rainfall and thus highly sensitive to climate variations (Guodaar *et al*., 2016). In recent years, droughts, heavy rains, and increasing temperatures have strongly contributed to a reduction of water availability, soil quality, and increased crop diseases (Asante and Amuakwa-Mensah 2015, Williams *et al*., 2019). In general, Africa has minimal irrigation outside of a few countries, and, in total, only 6 percent of farmland in Africa is irrigated (Zhang and Borja-Vega 2024). Ghana's agriculture is predominantly rainfed, and the country's water resource endowment is uneven in space and time. Literature data indicate substantial untapped irrigable land but a very small proportion under reliable water-managed agriculture. Seasonal rainfall variability, recurrent droughts in some regions, and increasing demand from non-agricultural sectors create localized water stress that constrains crop production, particularly where farmers lack access to irrigation infrastructure or storage to buffer dry spells (Baldwin and Stwalley, 2022; MoFA, 2023; FAO Aquastat, 2010). However, for Ghana condition, supplemental irrigation and irrigation scheduling can significantly enhance productivity, particularly during the growing season when rainfall deficits are most severe (MoFA, 2023; Nalumi *et al*., 2021; Kyei-Baffour and Ofori, 2006).

Tomato is an important horticultural crop in Ghana, produced for both local markets and processing. Yields in Ghana vary widely by region and management, reflecting differences in varieties, pest and disease pressure, access to inputs, and water management. Recent surveys report regional mean yields in the order of several to more than 20 Mt/Ha depending on management intensity and whether production is rainfed or irrigated. Officially, tomato average yield in Ghana is around 8.20 Mt/Ha (MoFA 2023). Tomato's growth stages—especially flowering and fruit set—are highly sensitive to soil moisture deficits; therefore, reliable water supply during these windows is key to securing marketable yield. A mature tomato plant may require up to 6 liters of water per day during hot conditions (Tanaskovikj *et al*., 2014; Đurovka, 2008). Conventional surface drip irrigation (CSDI) has been promoted as a water-saving technology (Tanaskovik *et al*., 2011; Tekinel and Kanber, 2002; Papadopoulos, 1996) but efficiency is often reduced due to surface evaporation and uneven distribution in comparison with sub surface drip irrigation (Ayars *et al*., 1999).

Irrigation development in Ghana has historically been limited by infrastructure, finance, and institutional capacity. Studies that examine the potential to scale up irrigation identify large areas suitable for irrigation but emphasize that realizing this potential requires investment in small-scale, farmer-centred schemes, strengthening water governance, and deploying water-saving technologies that match local socio-economic conditions. Furthermore, studies and extension experience indicate that farmers adopt diverse irrigation practices including small-scale bucket irrigation, motorized pump irrigation, surface flooding, and localized drip systems (Benabderrazik *et al*., 2022; Baldwin and Stwalley 2022; MoFA, 2023; Nalumi *et al*., 2021; Kyei-Baffour & Ofori, 2006).

Effective water management practices are essential for maximizing tomato yield while mitigating the risks associated with climate change. One of the solutions recommended within the DIVAGRI project, which could help growers to achieve respectable yields while using only a fraction of the usual water is SLECI irrigation system. The SLECI technology is a self-regulating subsurface irrigation system that uses the actual suction force of the surrounding soil for regulation of the system's water release. In this technology, water is transferred to the soil via cylindrical clay elements that are hollow inside. The consequence of the self-regulating effect is an efficient, economical, intelligent and innovative use of water (Technical Report on SLECI Pilot Sites Installed within the DIVAGRI Project, 2025; Malchev *et al*., 2022). By delivering water gradually through clay emitters buried below the soil surface, SLECI reduces water loss from evaporation and deep percolation compared to surface irrigation techniques (Agbesi *et al*., 2025; Elmajetni *et al*., 2025). In several studies with different crops, the benefits of SLECI irrigation technologies are presented, including cucumber and mango (Hansmann and Siering, 2018), cherry orchards (Malchev *et al*., 2025; Malchev *et al*., 2022), pepper (Osei *et al*., 2025), lavender, rosemary, maize, gem-squash, cowpea and moringa (Technical Report on SLECI Pilot Sites Installed within the DIVAGRI Project, 2025). This study aims to assess the performance of SLECI compared with a conventional surface drip irrigation for tomato production under Ghana's humid tropical climate. The main objective was to evaluate the effects of SLECI on crop yield. Furthermore, this study aims to advance understanding of SLECI efficacy for smallholder farmers in semi-arid Africa agroecological regions and other similar regions globally.

MATERIAL AND METHODS

The experimental field

The experimental study was conducted with Tomato crop (Solanum lycopersicum) cultivated for one growing season (29 of May to 14 of September, 2023) located at University of Cape Coast's Alexander G. Carson Technology Centre, Central Region, Ghana (5°07' 48.1"N, 1°17' 24.2"W). The demonstration site consisted of two treatments, namely the SLECI sub-surface treatment and conventional surface drip irrigation (CSDI), which serves as the control.

Figure 1. The experimental field showing dug trenches for installing the SLECI emitters (left), and sections of the CSDI treatment (right)

Each treatment covered 19.8 m^2, arranged in ten rows of 22 plants. Tomato seedlings were transplanted at 0.30 m between and within rows, achieving a stand density of 111 000 plants per hectare. The SLECI line with clay emitters was installed under soil, while the control plot received 16 mm tape. Although two flowmeters where available for irrigation measuring, only the AXIOMA Q1 battery-powered device could be used for SLECI treatment. The irrigation water in the CSDI treatment was not measured by flowmeter due to unavailability of a power source. As an alternative solution, CSDI plots were irrigated twice a day (early morning and late evening) by application of about 4.3 liters per day per plant. Furthermore, soil moisture content was measured in both treatment before each irrigation.

Weather and soil conditions

The climate of Ghana is tropical. The eastern coastal belt is warm and comparatively dry. The southwest corner is hot and humid, and the north is hot and dry. Annual average temperatures range from 26.100C in places near the coast to 28.9 0C in the extreme north. It is usually breezy and sunny. Daytime temperatures may rise above 400C in the north. There are two rainy seasons in the south from March to July and from September to October (bimodal rainfall system). The northern part of the country, on the other hand, has only one rainy season (uni-modal rainfall system) from May to October (MoFA, 2023).

Weather steers both plant demand and irrigation supply, while soil properties dictate how long that water remains available. Table 1 summarizes the weather conditions in 2023 recorded from station Nkanfoa, near by the pilot site established at University of Cape Coast Technology Village, Central Region, Ghana. The region where our experimental site was established falls within the tropical savanna climate (Osei *et al*., 2025).

Table 1. Average climate data for Cape Coast for 2023 (Station: Nkanfoa)

Month	Avg Temp (°C)	Humidity (%)	Wind (m/s)	Sun (hours)	Rad (MJ/m²/day)	ETo (mm/day)
January	26.9	75	2.2	5.2	16.1	3.85
February	27.5	76	2.5	5.3	17.1	4.14
March	27.4	80	2.5	5.4	17.8	4.11
April	27.0	84	2.4	5.2	17.4	3.82
May	26.4	87	2.4	4.8	16.2	3.43
June	25.3	88	2.7	4.2	14.9	3.10
July	24.2	88	2.9	4.1	14.9	2.99
August	23.9	87	2.8	4.0	15.2	3.06
September	24.5	87	2.7	4.1	15.6	3.15
October	25.4	86	2.4	4.7	16.2	3.32
November	26.3	83	2.1	4.9	15.8	3.41
December	26.8	78	2.0	4.9	15.3	3.55
Average	26.0	83	2.5	4.7	16.0	3.49

The obtained results showed that the average daily temperatures, keeping the annual mean near 26 °C, while relative humidity rarely drops below 75 %, with an average solar radiation of 16 MJ /m²/day, and an average reference evapotranspiration (ETo) of 3.5 mm/day. The average monthly temperatures are in the frame of the optimum values recommended for tomato crop (Tanaskovikj *et al*., 2014) and vary from 23.9 in August up to 26.4oC in May. Data for relative air humidity during the vegetation season were over the recommended values (Đurovka, 2008) favoring possibilities for fungal diseases appearing. Average seasonal referent evapotranspiration in our research varies from 2.99 in July to 3.43 in May, emphasizing the need for supplemental irrigation even during rainy spells. According to the literature data, the crop demands around 600 mm of seasonal evapotranspiration, with critical moisture periods during flowering and first cluster fruit swell. The soil moisture deficit during flower and fruit stage is reported to have a negative influence on tomato yield (Tanaskovik *et al*., 2016b; Sagheb and Hobbi 2002; Doorenbos *et al*., 1986).

The water physical and chemical properties of the soil at the experimental site are present in Table 2. According to texture classification (USDA-NRCS), the soil at pilot site is loamy sand with 74.6% composition of sand, 11.3% of clay and 14% silt. The results for the soil water capacity (Evett, 2007; ICARDA, 2001) show a wide range between field capacity and wilting point or from 44.2 to 7% volume. The total available water capacity for plants is pretty high or 36.7 % volume. The slightly pH near to neutral reaction is suit for growing tomato crop. The low content of organic matter by 1.28 show need for additional fertilization by organic manure. Therefore, 132 kg of poultry manure was applied per treatment field once before transplanting.

Table 2. Chemical and physical characteristics of the 0-60 cm soil layer

Chemical properties	
Reaction (pH in water)	7.2
Organic matter %	1.28
Physical properties	
Total sand in %	74.6
Silt in %	14.0
Clay in %	11.3
Permanent wilting point (soil moisture retention at 15 bars) in volume %	7.50
Field capacity (soil moisture retention at 0.33 bars) in volume %	44.2
Bulk density in g/cm^3	1.34

RESULTS AND DISCUSSION

The influence of irrigation on tomato yield

Number of fruits per treatment, average yield per harvested plant (g) and total yield (kg/ha) are presented in Table 3. The first date of tomato harvest was 23rd of August while last harvest was at 14th of September 2023. From the obtained results can be concluded that SLECI irrigation treatment produced 79, while conventional surface drip irrigation returned 29 fruits per treatment. From the other hand, SLECI recorded lover average yield per plant or 24.7 vs. 37.7 g. Finally, drip irrigation showed 1.42 t/ha or almost 78% lower tomato fresh yields in comparison with 2.53 t/ha in SLECI irrigation treatment. Our results are below the average range for the country from 8 Mt/Ha, probably due to the absence of chemical fertilizer in both plots and where the irrigation techniques in reality were the dominant factor. The literature data shows that tomato yields were significantly higher when fertilizers were injected through the drip system (Tanaskovik *et al.,* 2011; Cukaliev *et al.,* 2008; Sagheb and Hobbi 2002;

Papadopoulos, 1996). Haynes (1985) reported that if nutrients are applied outside the wetted soil volume, they are generally not available for crop use. The fruit yield of tomato was 28% higher in drip irrigation over furrow irrigation (Shedeed *et al.,* 2009). In other research tomato fruit yield per unit of water supplied was on average one-third better in drip irrigation than in the furrow irrigation. In addition, drip irrigation has resulted in a yield advantage over furrow irrigation (Tanaskovik *et al*., 2016b; Malash *et al.,* 2007). Furthermore, our result implies that buried emitters can sustain respectable yields even in the humid tropics where evaporation losses from conventional drip are assumed to be small. These findings resonate with evidence that subsurface systems buffer crops against extreme evaporative demand (Malchev *et al.,* 2022).

Table 3. Number of fruits per treatment, average yield per harvested plant (g) and total tomato yield (kg/ha)

Treatment	Number of fruits	Average yield per harvested plant (g)	Yield (kg/ha)
SLECI	79	24.7	2534
CSDI	29	37.7	1421

Tomato yields reached 87.6–114.2 t/ha with surface drip, 107.5–128.1 t/ha with subsurface drip at 20 cm, and 105.0–124.8 t/ha with subsurface drip at 40 cm, respectively (Machado et al., 2003). Additionally, in other research, the highest yield in SLECI system has recorded in bell pepper treatment under a burying depth of 5 cm (Osei *et al.,* 2025). Regarding the influence of SLECI irrigation techniques on crop yield performances, our results concur with literature reporting that SLECI exceeded drip yields in maize crop, than almost equaled in gem-squash and moringa crop. From the other hand, in rosemary, lavender and cowpea experiments, results showed that drip better yielding than SLECI (Technical Report on SLECI Pilot Sites Installed within the DIVAGRI Project, 2025). Comparable improvements have been shown for related ceramic emitter systems with melon, wolfberry and apple trees, where subsurface irrigation with ceramic emitter (SICE) was compared with sub-surface or surface drip irrigation, results shown that SICE significantly improved fruit yield (Han *et al*., 2025; Han *et al*., 2023; Cai *et al.,* 2021).

Irrigation water use and soil water content in tomato crop irrigated by SLECI

The results about irrigation water use during the vegetation season in the experimental site are present originally only for SLECI treatment. The total volume of water used in irrigating plants of the SLECI field was 285 m^3/ha. The rainfall recorded by the nearest weather station located at Nkanfoa between the cropping seasons was 406.24 mm. From the other hand, the water consumption for CSDI treatment field has not quantified due to unavailability of a power source.

Anyhow, CSDI treatment was irrigated twice a day (early morning and late evening) by application of about 4.3 liters per day per treatment. Figure 2 present the water consumption volume (in m³) of tomato plants on the SLECI field. The data on water consumption was recorded by using an ultrasonic water meter (Qalcosonic W1, Axioma Metering, USA).

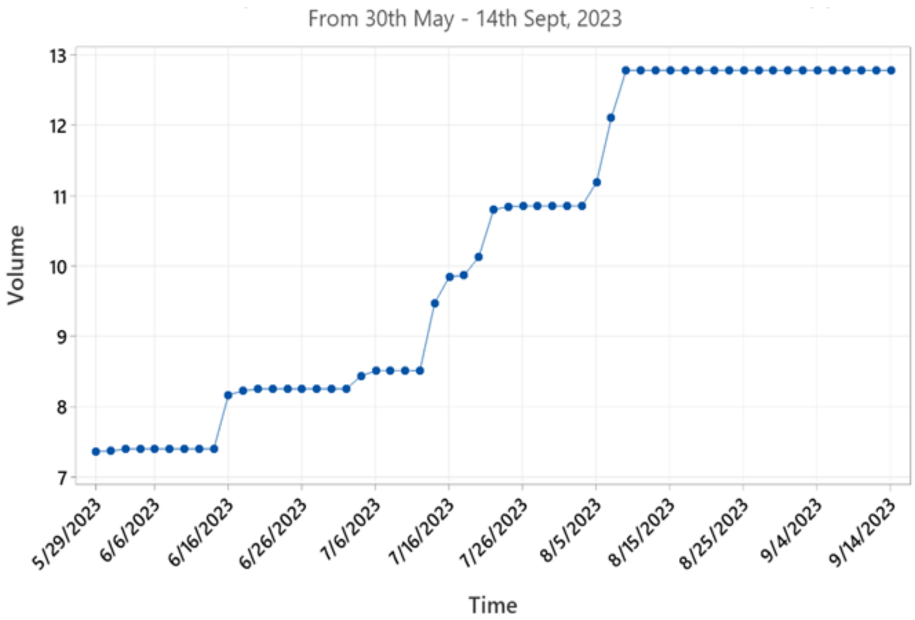

Figure 2. Water consumption for tomato for SLECI field during vegetation (m³)

In Figure 3 is present time series of soil-water content throughout the first sixty days after transplanting of plants. Soil volumetric water content was measured before irrigation in three times per week. The results showed that volumetric content in the drip irrigation shows higher oscillation compared with SLECI treatment. Furthermore, SLECI values hovered a stable oscillation and never approached close to the wilting point threshold. Similar results for stable soil water contents in SLECI in comparison with surface and subsurface drip irrigation are noted in several other research (Technical Report on SLECI pilot sites installed within the DIVAGRI Project, 2025; Malchev *et al.,* 2025; Malchev *et al.,* 2022). In study with apple trees where subsurface irrigation with ceramic emitter's (SICE) was compared with sub-surface drip irrigation (SDI), the variations in soil water contents for SICE were smaller than those for SDI (Cai *et al.,* 2021). Additionally, in other investigations is noted that frequent drip irrigation with small irrigation application rates can obtain best conditions regarding the content of soil moisture, as well as highest yields (Tanaskovik *et al.*, 2016a: Tanaskovik *et al.,* 2016b; Cukaliev *et al.,* 2008; Agele *et al.,* 2006; Tekinel and Kanber, 2002; Papadopoulos, 1996). In other research is evident that irrigation water was utilized more efficiently under the ET controller treatment than under the watermark sensor and control treatment (Al-Ghobari, 2014). The surface drip irrigation controlled by soil moisture sensor treatment resulting in 15–51% less irrigation water applied compared to fixed time irrigation (TIME) treatments (Zotarelli *et al*., 2009).

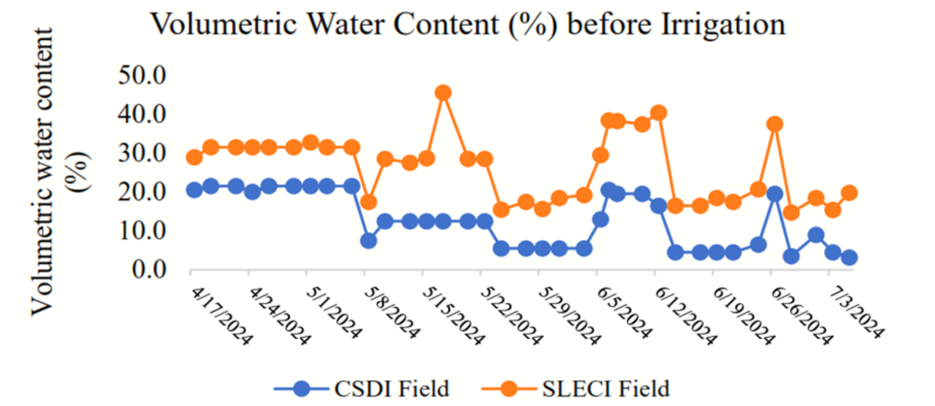

Figure 3. Volumetric water content measured on CSDI and SLECI fields before irrigation

Irrigation water productivity

Irrigation-water productivity (IWP) links the previous two results about obtained yield and applied irrigation water during the season, or simple expressing how many kilograms of crop emerge from each cubic meter poured onto the field. In agriculture, irrigation water productivity is defined as the ratio between the actual crop yield achieved and the water use, expressed in kg/m^3 (Gang *et al*., 2025; Fernandez *et al*., 2020; Cetin and Kara, 2019; Pereira *et al*., 2012). In our analysis, we have used the following equation for estimating the IWP:

$$IWP = \frac{Ya}{Iwa}$$

Where IWP is irrigation water productivity in kg/m^3, Ya is crop yield in kg/ha, while Iwa is irrigation water applied in m^3/ha.

Table 4 lists the values for tomato crop only for SLECI irrigation treatment. The irrigation water productivity (IWP) in tomato crop irrigated by SLECI system show 8.9 kilograms of fresh tomato yield with each m^3 of used water. SLECI is a highly water-efficient system, supplying only small but steady rates of water (Agbesi *et al.,* 2025; Elmajetni *et al.,* 2025; Malchev *et al*., 2022; Hansen and Siering, 2018). The positive influence on SLECI on IWP or water use efficiency is present in several studies with different crops established across Africa (Technical Report on SLECI Pilot Sites Installed within the DIVAGRI Project, 2025; Malchev *et al.,* 2025; Osei *et al*., 2025). Clay-based irrigation reviews emphasize reductions in soil-surface evaporation and deep percolation as the principal drivers of improved IWP (Mahler, 2024). A global meta-analysis of subsurface irrigation reported consistent increases in water productivity across crops and climates (Guo *et al*., 2023). In this context, Rego et al., (1988) indicated that suitable irrigation increasing soil water content as well as nutrient availability, which result in better yield and water use efficiency (Bar-Tal *et al*., 2015; Kafkafi and Tarchitzky 2011; Tanaskovik et al., 2011; Cukaliev *et al*., 2008; Sagheb and Hobbi, 2002; Burt *et al.,* 1998; Papadopoulos, 1996). Compared to conventional drip irrigation (CDI), alternate partial root-zone drip irrigation (ADI) decreased tomato yield slightly, but increased water use efficiency (WUE) by 7.8%, while fixed partial root-zone drip irrigation (FDI) reduced tomato yield and maintained water use efficiency (WUE) (Luo and Li, 2018). Furthermore, results shows that irrigation cut in tomato production did not affect the yield, enhanced fruit quality, and maximized water use efficiency (Lovelli *et al.,* 2017). Greater savings of water for irrigation, the increase in irrigation water use efficiency (IWUE) and the maintenance of yield in cultivars Cedrico, and earlier Amati indicate that regulated deficit irrigation (RDI) could be the more promising strategy for future irrigations of tomato than classical FI approaches (Savic *et al*., 2011).

Table 4. Irrigation water productivity in tomato crop irrigated by SLECI system

Treatment	Tomato yield (kg/ha)	Irrigation water use (m^3/ha)	IWP (kg/m^3)
SLECI	2534	285.12	8.89

The influence of irrigation techniques on leaf length, plant height, leaf width and stem diameter

The average crop performance parameters for plant height, leaf length, leaf width and stem diameter for tomato plants at 14, 28 and 42 days after transplanting (DAT) are presented in Table 5.

Table 5. Crop performance parameters at 14, 28 and 42 DAT for tomato plants under SLECI and CSDI

Crop parameter	14 DAT		28 DAT		42 DAT	
	SLECI	CSDI	SLECI	CSDI	SLECI	CSDI
Plant height (cm)	47.3	57.1	75.9	92.1	93.1	108.7
Leaf length (cm)	11.9	10.0	11.7	11.4	13.2	12.6
Leaf width (cm)	6.7	5.5	5.8	5.3	7.6	6.9
Stem diameter (mm)	9.6	6.3	12.2	11.6	12.9	12.4

Generally, SLECI produced smaller plants, thicker stems, and larger leaves throughout the season. There were noted differences in plant height, leaf length, leaf width, and stem diameter for tomato plants established on both SLECI and CSDI treatments across the 14 DAT, 28 DAT and 42 DAT. However, our results for plant height partially corresponded with those reported in maize, moringa and cowpea present in Technical Report on SLECI pilot sites installed within the DIVAGRI Project (2025), where irrigation technique have not strong influence on plant height.

The best growth parameters for bell pepper plants have achieved with a burying depth of SLECI lines at 10 cm and 80% recommended application fertilizer dosage. This combination resulted in the highest plant height and leaf area (Osei *et al*., 2025). Antony and Singandhupe (2004) indicated that drip irrigation pepper had more plant height and more number of branches compared with surface irrigated crops. As the plant height and branches increased, new nodes for flower and fruit development appeared resulting in an increase in number of fruits and total yield. In other study, drip irrigation enhanced tomato growth more, early in the growing season, than did furrow irrigation, but at later stages, there was little difference between the two irrigation systems (Malash *et al.,* 2005). The tensions of 30 and 60 kPa applied at 10 days after transplanting were the ones that most intensified yield and water productivity without significantly affecting the agronomic development of the crop (Mesquita *et al.,* 2019). Xiao *et al.,* (2023) reported that under aerated drip irrigation, the maximum plant height of tomatoes was 1.1%, 3.4%, and 4.7% higher than that of the control treatments (non-N fertilization, 2/3 of conventional N fertilizer and conventional N fertilization - 210 kg ha^{-1}). Furthermore, drip irrigation recorded significantly higher leaf area index (LAI) (3.15) over furrow irrigation (2.27), reported Shedeed *et al.,* (2009). In other studies, water deficit reduced leaf length and width by 21% and 20% relative to plants irrigated under standard practices (Medyouni *et al.,* 2020). Ullah *et al.,* (2021) obtained better results in spring summer season for plant height and stem diameter of tomato when there was 100% ETc water applied (130.88 cm and 11.29 mm).

Furthermore, a simple linear regression for crop performance parameters at 14, 28 and 42 DAT was fitted in our investigation. The results for linear regression parameters are presented in Table 6.

Table 6. Linear regression parameters for each variable and irrigation technique

Parameter	Slope	Intercept	R^2	p-value
Plant Height – SLECI	1.6357	26.3000	0.9798	0.0909
Plant Height – CSDI	1.8429	34.3667	0.9593	0.1293
Leaf Length – SLECI	0.0464	10.9667	0.6369	0.4117
Leaf Length – CSDI	0.0929	8.7333	0.9980	0.0283
Leaf Width – SLECI	0.0464	10.9667	0.6369	0.4117
Leaf Width – CSDI	0.0929	8.7333	0.9980	0.0283
Stem Diameter – SLECI	0.1179	8.2667	0.9005	0.2043
Stem Diameter – CSDI	0.2179	4.0000	0.8465	0.2563

The linear regression analysis shows strong linear growth trends for tomato plants under both irrigation systems. Plant height increased faster under CSDI (1.84 cm/day) compared to SLECI (1.64 cm/day), with R^2 values above 0.95. Leaf length and width also grew more rapidly and consistently under CSDI (0.093 cm/day, $R^2 \approx 0.998$) than under SLECI, which displayed moderate variability (0.046 cm/day, $R^2 \approx 0.637$). SLECI consistently produced thicker stems, which may be favorable for mechanical stability under field conditions.

Overall, the regression analysis confirms clear treatment-driven differences in growth dynamics, supporting agronomic interpretations relevant for water-saving irrigation systems.

CONCLUSIONS

The results obtained in experimental sites established in Technology Village at UCC showed that SLECI irrigation produced almost 78% higher tomato fresh yields in comparison with 1.42 t/ha in conventional drip irrigation treatment. The results for soil volumetric content showed that the drip irrigation performed higher oscillation compared with SLECI treatment. Furthermore, SLECI values hovered a stable oscillation and never approached close to the wilting point. Generally, SLECI showed significant potential to minimize water loss by evaporation, runoff, and percolation and can be considered a promising new irrigation technology that can increase efficient use of water. Therefore, smallholder farmers are encouraged to use an SLECI irrigation system because it reduces water use and increases yield, allowing water to be available to other economic sectors. Future research should include replicated blocks to nail statistical confidence, test mixed fertilizer programs and monitor multi-year durability of clay emitters under different soil conditions. The SLECI system should also be tested in a greenhouse environment in order to assess its suitability under controlled crop microclimates and weather conditions.

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EFFECT OF SALICYLIC ACID ON THE INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE IN COFFEE PLANTS (*Coffea arabica* L. Var. TIPICA AND GEISHA) TO *Hemileia vastatrix* Berkeley & Broome.

SUMMARY

This study aimed to identify the optimal dose at which Salicylic Acid (SA) induces systemic acquired resistance in coffee plants (*Coffea arabica* L. varieties Tipica and Geisha) against infection caused by Hemileia vastatrix Berkeley & Berkeley and Broome. A completely randomized design was established, with five blocks containing ten plants per variety. Three leaves from each plant were chosen at three different times to assess the severity of the disease. The treatments included various SA concentrations (0.5, 1, and 1.5 mM), water as a negative control (C-), and copper oxychloride as a positive control (C+), which was only applied on day 21 before pathogen inoculation. The plants were sprayed at intervals of 21, 14, and 7 days, and on day 0, the fungus was inoculated at a concentration of 0.5 mgL^{-1}. Subsequently, SA applications were made on 7, 14, and 21 days. For the statistical analysis of the data, an ordinal logistic regression with a cumulative logit link was used to quantify the association between variables using odds ratios and to estimate the probability of disease occurrence according to the levels of rust severity shown. The results showed that in both varieties, the treatment with 1.5 mM SA preserved 27 healthy leaves out of the initial 60, suggesting that SA at this concentration activated the coffee plant's defense mechanism, protecting it from disease attack.

Keywords: Salicylic acid; *Coffea arabica*; Hemileia vastatrix, severity

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INTRODUCTION

Coffee rust, the most devastating disease affecting coffee crops worldwide, causes annual production losses exceeding US$1 billion (Kahn 2019; Secretaría de Agricultura y Desarrollo Rural 2018). In Mexico, after its first report in 1981, the disease became a widespread epidemic in 2013 and 2014, resulting in losses of up to 50% of production (Food and Agriculture Organization of the United Nations, FAO 2015).

In response to this threat, various disease control programs have been implemented, including the renewal of coffee plantations with resistant varieties (Ejea 2009). However, it has been noted that some of the introduced cultivars have been losing this trait over time, because of the pathogen's adaptation breaking down the resistance barrier.

To monitor coffee rust and provide early warning systems and damage severity assessments, fixed and mobile plots have also been established (Organización de las Naciones Unidas para la Alimentación y la Agricultura, FAO 2015; Secretaría de Agricultura, Ganadería Desarrollo Rural Pesca y Alimentación 2013). The use of agrochemicals has been another tactic. Notwithstanding the dangers this poses (Couttolenc-Brenis *et al.,* 2021), it is crucial to stress that coffee plantations offer significant environmental services, including the production of oxygen, mitigation of climate change, reduction of erosion, and conservation of biodiversity, supporting a diverse range of species (Nava and Hernández 2017). Despite their efficacy, excessive use of fungicides can damage soil and vegetation (Loland and Singh 2004) and even cause pathogen resistance (Organización de las Naciones Unidas para la Alimentación y la Agricultura, FAO 2015), jeopardizing the sustainability of production and ecosystem health.

Salicylic acid (SA) is a potent phytohormone and signaling molecule that plays a key role in plant immune response to biotic stress, inducing systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Loland and Singh 2004; Mishra *et al.,* 2024). As a result, it is becoming a viable substitute and has proven effective in protecting various crops from biotrophic and hemibiotrophic pathogens (Ullah *et al*., 2023; Sillero *et al.,* 2012). Its exogenous application has triggered defense mechanisms in plants like wheat against septoria (Zymoseptoria tritici) (Ramos Cabrera *et al.,* 2020); in bananas against Colletotrichum sp. in banana fruits after harvest (Berumen-Varela *et al*., 2015).

Applying SA exogenously can increase a plant's resistance to bacterial, viral, and fungal diseases (Tannuri *et al.,* 2021). By modifying immune-related genes, this substance also enhances plant growth and development (Singh 2023). Given the magnitude of economic losses and the risks associated with conventional control methods for coffee rust, it is essential to explore alternatives such as the use of SA. The objective of this research is to determine the optimal concentration of SA as a bioregulator to strengthen the systemic resistance of coffee plants to coffee rust. By investigating its effectiveness in key commercial varieties such as Tipica and Geisha, we seek to develop more environmentally friendly disease management strategies, contributing to the long-term sustainability of coffee production.

MATERIAL AND METHODS

Location

The study was conducted from March to April 2023 at the Faculty of Agronomy of the Universidad Veracruzana, located in Xalapa, Veracruz, at coordinates 19° 30' 96"N and 96° 55' 06" W. The average temperature at the 1,365-meter-high research site is 18°C.

Fifty plants of the "Tipica" variety that were five months old after germination were chosen for this study. We also selected an equal number of four-month-old "Geisha" variety plants. The "Alarcón" nursery, located at 19° 25' 29.70" N and 96° 6' 58.90" W, in the "Ejido San Marcos de León," Veracruz, Mexico, at an elevation of 1,115 meters above sea level, provided the plant materials. The selection criteria ensured that the plants were robust and healthy, free of rust disease symptoms, and untreated with fungicides.

Determination of the AS effect on systemic acquired resistance of coffee plants during rust infection

The fully randomized block design consisted of ten plants of the rust-prone "Tipica" variety and an equal number of plants of the rust-resistant "Geisha" variety (Tannuri *et al.*, 2021). To track them and assess the severity of the disease on three different dates, each plant had three leaves marked (Table 1). Before the pathogen inoculation, the initial assessment was completed at the start of the experiment. Twenty-six days after infection, the second assessment was conducted, and fifty days after inoculation, the third. Weeds were controlled by hand during the study, and irrigation was used as needed.

Three SA treatments (0.5, 1, and 1.5 mM) were used. On the day of spraying, the SA solutions were made in distilled water, beginning with a concentration of 2.0 mM SA. On days 21, 14, and 7, SA was sprayed prior to the pathogen inoculation. A hand sprayer was used to inoculate the pathogen on day 0 (Ramos Cabrera *et al.*, 2020; Sillero *et al.*, 2012). SA sprays were created at 7-, 14-, and 21-days following pathogen infection to disrupt the pathogen cycle (Cerna-Chávez *et al.*, 2019). Water was used as a negative control to prevent drift contamination, and the recommended dose of 3.7 gL^{-1} copper oxychloride (García *et al.,* 2021) was applied on day 21 before pathogen inoculation (Organización de las Naciones Unidas para la Alimentación y la Agricultura, FAO 2001).

When the plants were sprayed, they were covered with plastic. For inoculum preparation, leaves with abundant rust pustules were collected in the "Ejido San Marcos de León". A total of 70 to 100 rust-infected leaves were collected for inoculum preparation. Using a camel brush, the rust pustules were collected in sterile tubes. The inoculum was then diluted to 0.5 $\mu g\ mL^{-1}$ at the time of inoculation (Pereira *et al.*, 2012) and kept in microtubes at -20°C for a maximum of 48 hours (Pale-Ezquivel *et al.*, 2024). This was then applied to each of the treatment blocks. The spraying was carried out so that the drops reached the underside of the leaf.

Table 1. Modified severity scale to establish rust damage based on the technique of the "Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria (2016)

Category	**Severity (%)**	**Reference image**
0	0%	
1	1% Chlorotic spot visualization	
2	From 2 to 6%.	
3	From 7 to 19%	
4	From 20 to 44%.	
5	From 45% to 69	
6	From 70 to 84%	
7	From 85% to defoliation	

Evaluation of rust severity

A modified seven-grade scale was used based on the methodology proposed by the "Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria" (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2016). A seventh category corresponding to the defoliation effect was included (Table 1).

This scale measures the progress of the disease by considering the area of the leaf's underside and assessing the percentage of disease present. The first category is 0, which indicates a healthy leaf with no visible signs of disease. The subsequent categories (1, 2, 3, and 4) correspond to the development of typical disease symptoms.

Category 1 is characterized by the development of chlorotic spots on the leaves, which turn yellow. In category 2, there is evidence of disease progress ranging from 2 to 6%; small pustules are visible. In category 3, disease progress is observed in the range of 7 to 19%; a yellowish powder corresponding to the disease spores can be seen. In the later categories (5, 6, and 7), the leaves exhibit different stages of disease progression. In category 7, leaf senescence occurs due to nutrient deficiency and weakening of the leaves.

Statistical analysis

Descriptive analyses of the data were performed using tables and stacked bar charts. The probability of rust severity reaching or surpassing a particular level was then estimated by fitting an ordinal logistic regression model with cumulative logit link (Angarita-Fonseca 2015; Bombelli *et al.*, 2013), taking into account the effects of treatments and coffee varieties. Strong evidence of the relationship between the explanatory variables (varieties and treatments) and the likelihood of observing greater severity was provided by the odds ratios. The analysis was performed in R statistical software (version 2023.03.0 Build 386) with the support of the MASS, VGAM, and Brant libraries.

RESULTS AND DISCUSSION

The results of the degree of progress and the variability in the frequency of rust severity are shown in Table 2. It was observed that, from the second evaluation, a reduction in the number of healthy leaves was observed in all treatments due to the progress of the disease, placing them in the different severity categories, referring to the damage caused to the leaf area of the coffee leaves (Table 2; Fig. 1 and 2).

It draws attention to the significant increase in the number of leaves affected for the same category and treatment across the different evaluation dates. The negative control (water) and 1.0 mM SA treatments had the most affected leaves in the most recent evaluation, with 10 and 16, respectively. However, 30 and 27 leaves remained healthy in the 1.5 mM SA treatment and the positive control (fungicide), respectively.

Table 2. Progress in the frequency distribution of severity observed in coffee leaves

No. Assessment	Category: % severity	Treatments				
		Water (C-)	0.5 mM SA	1.0 mM SA	1.5 mM SA	Fungicide (C+)
1 2 3	0: 0% no damage	60 19 10	60 32 22	60 26 16	60 43 28	60 45 30
2 3	1: 1 chlorotic spot	11 10	7 9	11 8	5 11	3 1
2 3	2: from 2 and up to 6%	15 10	6 5	8 7	6 6	3 7
2 3	3: From 7 and up to 19%	10 12	9 7	9 9	3 8	6 6
2 3	4: From 20 to 44%.	2 11	5 8	5 13	3 2	3 11
2 3	5: From 45% to 83%.	3 0	1 5	1 3	0 4	0 3
2 3	6: From 70 to 84%	0 2	0 3	0 0	0 0	0 2
2 3	7: From 85% to defoliation	0 5	0 1	0 4	0 2	0 0

It's interesting to note that the 1.5 mM SA treatment preserved 27 of the 60 leaves evaluated in the prior reading. Furthermore, 11 leaves in category 1 were impacted, suggesting that the rust damage in the leaves treated with this method was not particularly severe. The leaves from this treatment were categorized using a higher severity progression (Table 2). The control treatment (C-) water presented ten affected leaves in the last reading in category 1; this was the highest value observed; likewise, in categories 2 and 3 there were 10 and 12 leaves, respectively. In Category 2, the 0.5 mM SA treatment showed 5 affected leaves, while the C+ (fungicide) treatment showed 7 leaves (Table 2).

Similarly, in category 3, (C-) had twelve damaged leaves, (C+) had six, and the 1.0 mM SA treatment had nine leaves. The 1.0 mM SA treatment had 13 damaged leaves in category 4, which was comparable to the (C+) treatment's 11 damaged leaves. With two affected leaves, the 1.5 mM SA treatment produced the lowest value (Table 2). Five leaves in category 5 were found to be affected by the 0.5 mM SA treatment in the most recent reading, whereas the control group (C-) showed no signs of leaf damage. On the last observation date in category 6, it was observed that the 0.5 mM SA treatment had three diseased leaves, while the 1.0 mM and 1.5 mM SA treatments showed no diseased leaves (Table 2). (C-) with five leaves and 1.0 mM of SA with four leaves were the treatments that had the greatest effect in category 7, which corresponds to leaf loss; the (C+) treatment did not exhibit any damage. At the conclusion of the study, it was found that (C+) had the greatest number of healthy leaves (30), while 1.5 mM SA had the closest record (27); in both situations, a variable efficacy is indicated, being effective in reducing some severities but not in others (Table 2).

Table 3. Rust symptoms following inoculation in the varieties "Típica" and "Geisha," corresponding to treatment 1.5 mM SA.

Evaluation dates	**Varieties**	
	TIPICA	**GEISHA**
1: day 0 start of the experimentation		
2: day 26 after inoculation		
3: day 50 post-inoculation		

It should be mentioned that each of the two varieties, “Tipica” and “Geisha,” had 150 healthy leaves (experimental units) at the start of the study, which corresponded to the total of all the treatments; these were distributed in accordance with the severity scale used in this study after infection and as the rust progressed. The damage inflicted by the 1.5 mM SA treatment on the two study varieties during the three evaluation dates is displayed in Table 2.

At the end of the last evaluation, the “Geisha” variety retained 76 healthy leaves in total, in contrast to the “Tipica” variety, which retained only 29 leaves. In both cases, the highest number of diseased plants were located between categories 1 and 4 (Fig. 2, Table 3).

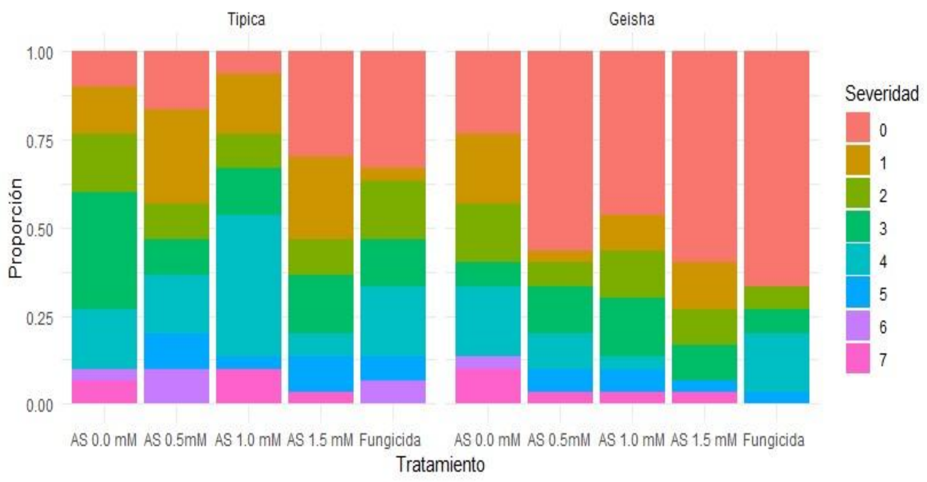

Fig. 2. Distribution of rust severity by variety and SA treatments

The number of diseased leaves observed in the last evaluation for the “Tipica” variety was 121 while for the “Geisha” variety, there were around 74 affected leaves. It should be noted that more than 50% of the affected leaves were distributed among categories 1, 2, 3, and 4 (Fig. 2, Table 4).

Table 4. Frequency distribution by variety, rust severity and date of assessment

No. Assesment	**Category: % severity**	**Varieties**	
		Típica	**Geisha**
1	0: 0% no damage	**150**	**150**
2		56	109
3		29	76
2	1: 1 chlorotic spot	31	6
3		25	14
2	2: from 2 and up to 6%	17	21
3		19	16
2	3: from 7 and up to 19%	32	5
3		26	16
2	4: from 20 to 44%.	12	6
3		30	15
2	5: from 45% to 83%.	2	3
3		9	6
2	6: from 70 to 84%	0	0
3		6	1
2	7: from 85% to defoliation	0	0
3		6	6

In the first categories, from 1 to 4, the highest number of diseased plants was distributed for both coffee varieties.

The plants treated with 1.5 mM SA were characterized by the distribution of affected leaves. More than 50% of the experimental units fell into the initial categories of the severity scale, indicating a significant reduction in rust progression. As a result, the severity of symptoms observed in this treatment was lower than in the other treatments. Additionally, many of the affected leaves exhibited rust at the infected sites, which further limited the spread of the disease (Fig. 3). This effect can be attributed to the activation of reactive oxygen species (ROS) due to the accumulation of SA, which triggered a restriction in the area affected by the infection.

Fig. 3. Oxidation effect observed in the rust-affected sites, which may have contributed to slowing the progression of the disease

The reference categories for the ordinal logistic regression (OLR) model were the fungicide-corresponding positive control treatment (C+) and the "Tipica" variety. Plants treated with 0.0 mM SA significantly increased the probability of finding affected leaves in high-severity categories compared to plants treated with SA and fungicide. With an odds ratio (exponent) of 2.375 and an ordinal regression coefficient (ORC) of 0.8650 ($p=0.00908$) as a positive and significant result, it was clear that this treatment increased the probability of severity by 1.37 times when compared to the applied fungicide (Table 4). Despite being 0.16 times more susceptible than the fungicide, the 1.5 mM SA treatment showed a (ORC) of -0.1748, a non-significant result ($p=0.60530$), indicating that this treatment significantly protected the plant from rust, which is why it does not present a high severity index (Table 5).

The “Geisha” variety's ORC was -1.0835 ($p=4.40e-07$), which is highly significant and negative. This indicates that the “Geisha” variety is less prone to rust, meaning that it has a much lower chance of falling into a higher severity category than the "Tipica" variety (Table 5; Figs. 1 and 2). In comparison to the "Tipica" variety, the “Geisha” variety decreased severity by 66.2%, according to the odds ratio of 0.338 (Table 5; Figs. 1 and 2).

The main conclusions of this study indicate that spraying a 1.5 mM solution of salicylic acid (SA) on the "Tipica" and "Geisha" coffee varieties every seven days resulted in effects that were statistically comparable to those obtained with copper oxychloride. A common contact, preventive, and protective fungicide used to lessen rust damage to coffee crops is copper oxychloride.

Table 5. Rust severity in SA treatments compared to that observed with the fungicide (C+)

SA Treatments (mM)	OCR*	P (≤0.05)	Odds ratio (exponent)	Severity compared to (C+)
0.0	0.8650	0.00908	2.375	1.37 times more susceptible
0.5	0.3305	0.31998	1.392	0.39 times more susceptible
1.0	0.7119	0.03165	2.038	1.03 times more susceptible
1.5	-0.1748	0.60530	0.840	0.16 times more susceptible

*ORC (Ordinal Regression Coefficient)

Plants treated with 1.5 mM SA were 0.16 times more susceptible to the disease than those treated with the fungicide, indicating that this treatment offers protection that is very similar to that of copper oxychloride, with the added benefit of avoiding environmental contamination.

The ability of salicylic acid (SA) to trigger different defensive responses against pathogens in a variety of crops has attracted a lot of attention in recent years. It is acknowledged as a crucial signaling molecule in plant defense responses to these pathogens. According to research, SA inhibits the catalase enzyme and, via a variety of mechanisms, increases systemic and local disease resistance. Because of this, SA and its derivatives have been used extensively to create systemic acquired resistance to various pathogens. Similar to our findings, Dieryckx *et al.*, (2015) discovered that there was inhibition of Botrytis cinerea mycelial growth; however, methyl salicylic acid was the most effective in preventing fungal growth, followed by acetylsalicylic acid and salicylic acid. They conclude that there is circumstantial evidence for the role of SA as a signaling molecule to induce resistance.

Tannuri *et al.*, (2021) analyzed the response of seven-year-old coffee plants (in the production stage) of the "Topazio" variety treated with SA at a concentration of 0.15gL^{-1}, with the foliar application once a month for five months, and found that the incidence and severity of rust were reduced from 36.3 to 54.7% when compared with untreated plants, results similar to those obtained in our study.

In the control of septoria, or wheat leaf spot, Ramos-Cabrera *et al.,* (2020) observed that plants treated with 1 mM SA for two years reduced the necrotic area, even with lower values than the control in the first four leaves; however, in terms of pycnidia cover, the results were not significant compared to the control. Similarly, SA at 0.5 mM sprayed on detached rice leaves inhibited Rhizoctonia solani fungal biomass growth and lesion formation (Sillero *et al.,* 2012), according to Kouzai *et al.,* (2018) found that broad bean plants treated preventively with 5 mM SA had less Uromyces viciae-fabae rust infection than the control.

In order to confirm the effect of SA in inducing systemic resistance in *C. arabica* against the coffee cherry disease Colletotrichum kahawae (Alemu *et al.*, 2019) found that applying 10 mM SA reduced the disease by 50–65% in high and moderately susceptible coffee hypocotyls. Our results, which demonstrated that plants treated with 1.5 mM SA had less severe rust, are corroborated by these studies. There are two phases to systemic acquired resistance (SAR). The first step is the identification of an invasive pathogen, which sets off signaling cascades that cause local defense reactions. Salicylic acid (SA) builds up inside cells because of this process. Reactive oxygen species (ROS) levels rise as SA builds up, and genes encoding pathogenesis-related proteins (PR) and the development of the hypersensitive response (HR) are expressed. A type of programmed cell death known as the hypersensitive response takes place to limit the pathogen's area of infection. Chen *et al.*, (1993) proposed a mode of action of SA on the basis that it binds to and inhibits the enzyme catalase Loake and Grant (2007). Catalase inhibition activates other reactive oxygen species that seem to be involved in the signaling cascade for the expression of defense genes and may increase the concentration of hydrogen peroxide ($H2O_2$) molecules that may have antibiotic activity against pathogens, according to multiple studies (Rangel-Sánchez *et al.,* 2010).

Breeding programs involving coffee varieties have been developed because H. vastratrix rust poses significant challenges. However, it can take years of research and be very expensive to obtain a cultivar resistant to this disease. It is now recognized that the coexistence of the rust fungus and the coffee plant results in mutual co-evolution, as evidenced by the fungus's virulence towards the plant and the wide genetic diversity of plant resistance to the fungus (Avelino and Rivas 2014). More than 55 rust races have been identified in recent years (Ingelbrecht *et al.*, 2023), demonstrating the difficulty of obtaining genetic materials with the resistance genes required for total immunity to this disease.

In coffee production, rust-resistance breeding has been an important tactic. Renovating coffee plantations with rust-resistant cultivars of two species-*Coffea arabica* (60%) and Coffea canephora (40%)—has been one strategy. *C. arabica* is especially prone to rust, and some of its popular varieties, like "Typica," "Bourbon," and "Geisha," are highly valued for their outstanding cup quality. Studying the various cultivars of this species is therefore of great interest. In the early stages of rust infection (germination, appressoria, and haustoria formation), Couttolenc *et al., (*2021) assessed the local response of two cultivars, "Oro Azteca" and "Garnica." They discovered that resistance in "Oro Azteca" was significantly higher than in "Garnica," with 8.2% and 53.3%, respectively, because of increased expression of the pathogen recognition gene CaNDR1 and those linked to SA, such as CaNPR1, CaPR1, and CaPR5.

The "Tipica" variety was more vulnerable to rust, as evidenced by the increased signs and progression of the disease compared to the "Geisha" variety, which is in line with our study's findings. Further research on the expression of these genes in the "Tipica" and "Geisha" varieties would be intriguing.

CONCLUSIONS

The results obtained help confirm the effectiveness of salicylic acid (SA), which, according to previous research, activates the defense mechanisms of coffee plants affected by coffee rust. The study observed a significant reduction in the pathogen's infection rate. More than 50% of the affected leaves were classified in the lowest severity categories, indicating a significant decrease in the spread of coffee rust. Furthermore, the research found that using 1.5 mM of SA can help reduce reliance on agrochemicals for rust control. These chemicals are expensive and can irreparably damage crop health.

Conflict of Interest

The authors declare that they have no financial interests or personal relationships that could have influenced the work presented in this document.

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COMPARATIVE ANALYSIS OF RESULTS OBTAINED FROM DIFFERENT SHAPES OF SAMPLE PLOTS IN PARTIAL INVENTORY OF HIGH BEECH FORESTS IN MONTENEGRO

SUMMARY

The paper presents the results of a comparative analysis of the number of trees, stand volume and the position of the height curve within the height overdiameter curves, obtained through a partial survey of high beech forests on different shapes of sample plots: concentric circles used in Montenegro (CC CG), concentric circles used in Serbia (CC RS) and angle count sampling (WZP RS) applied in the Serbian forest inventory. Data processing was conducted in Osnova software and within a GIS environment. Height curves were modelled using the Näslund function, while spatial interpolation of results was performed using the Spline with Barriers method. Regarding the average number of trees and average volume, although the CC CG method produced the lowest values, it is difficult to determine which plot shape is superior. The reason lies in the differing elements of partial survey designs among the tested plot types (number, size, sampling intensity, number of detailed subplots, etc.). However, since the studied stand belongs to selection forests, which represent extremely heterogeneous structural forms, we consider that priority should be given to angle count sampling (WZP RS). This method is based on unequal probabilities of tree selection for measurement, specifically the likelihood of a tree being selected is proportional to its diameter which undoubtedly contributes to a more reliable determination of stand volume under the given conditions. Furthermore, we consider that the method of selecting trees for height measurement in the forest inventory of Montenegro does not ensure a representative sample for accurate modelling of the height curve. As a result, poorer height classes are determined, leading to lower stand volume values. Spatial visualization through raster analysis facilitates planning and decision-making in forest management planning, as it provides a more realistic insight into internal stand variations that are often overlooked in conventional tabular presentations. In this context, GIS software and raster analyses represent

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efficient tools for the assessment, comparison and visualization of field data results, enabling better decision-making and improvement of forest resource management.
Keywords: partial inventory, concentric circles, angle count sampling, comparative analysis of results, raster analysis, spatial interpolation

INTRODUCTION

The numerous and complex objectives of contemporary forest management planning necessitate the development of a planning system that at all levels, from operational to strategic, has an integrated character (Medarević, 2006; Golubović, 2024). Over time, the goals of forest management have evolved from the aim of ensuring continuous production, yield and revenue in wood (a monofunctional approach) toward the objective of multifunctional utilization of the overall forest potential within forest areas, as encompassed in the globally accepted definition of sustainable forest management. Therefore, every decision-maker in forestry must take into account a variety of criteria that influence the selection of management strategies and methods (Medarević *et al*., 2006; Pantić *et al*., 2015; Golubović, 2024). The fundamental assumption for developing realistic forest management plans is the availability of a wide range of highly reliable information about the forest resources. Such information is provided by forest inventory – a discipline that establishes the informational foundation of forestry (Banković *et al*., 2002). Forest inventory involves the collection, processing, evaluation and presentation of information about forest resources, their spatial distribution, structural composition, temporal (dynamic) development, degree of utilization and other relevant characteristics (Banković and Pantić, 2006; Pantić *et al.,* 2013a). In parallel with the overall development of forestry, particularly with the advancement of forest management planning, forest inventory has also necessarily evolved. This development has progressed from terrestrial methods of data collection using simple measuring instruments and manual data processing, through the introduction of modern measuring equipment and specially designed software packages for data processing, to the application of aerial photogrammetry and remote sensing (Borota, 2018). Furthermore, Geographic Information Systems (GIS) have become an indispensable component of the forest management planning process. Since most activities in forest management planning involve working with spatial data, the need for their efficient storage, processing and analysis has become increasingly pronounced. In this context, GIS represents a set of functions that enable advanced management of spatially referenced data, as well as their visualization and interpretation (Pantić *et al.,* 2013b). Raster analysis constitutes an essential component of modern data analysis in forestry, as it allows for the visualization and assessment of the spatial distribution of various data types, both attribute and numerical. Understanding the spatial heterogeneity of these values is of great importance for assessing the condition and potential of forest resources, as well as for making timely and sound decisions in forest management planning. Given the importance of forest inventory from the perspective of forestry as a whole and the sophisticated techniques it employs for data collection, processing and database development at various levels of forest spatial division, the objective of this research was defined accordingly. It lies in determining the most suitable shape of sample plots used for partial inventory in high beech forests of Montenegro, based on a comparative analysis of the number of trees, stand volume and the position of the height curve within the height overdiameter curves (height class determination). The study also takes into account the fundamental elements of measurement on different plot types, such as sample size, measurement intensity and the number of measured tree heights. Pure and mixed beech forests account for 21.4% of the total forest area of Montenegro, 33.9% of the total growing stock and 28.5% of the volume increment, with relatively high values of these elements expressed per hectare (Dees *et al.,* 2013). In studies conducted in Biogradska Gora, Čurović *et al.* (2011) reported high productivity within different subassociations of beech forests, ranging from 365 to 775 m³/ha. Regarding biodiversity, even 58 vascular plant species were recorded on a relatively small area within these forests (Čurović *et al.,* 2020). These findings point to the exceptional multifunctional importance of Montenegrin beech forests, not only at the local but also at the regional level. Therefore, the reliability of inventory data is a necessary prerequisite for preserving the ecological and structural stability, biological diversity and high productivity of these forests, which was the main motivation for the present research.

MATERIAL AND METHODS

Montenegro ranks among the most forested countries in Europe. In order to ensure the rational use and preservation of this natural resource for future generations, it is necessary to establish a planning and decision-making system that meets the numerous demands of modern society toward forests, while maintaining the functional permanence and stability of forest ecosystems. One of the prerequisites for rational and realistic planning is the existence of a wide range of highly reliable information about forests, provided through forest inventories.

Data collection

Data for this research were collected in stand 12/b of the management unit "Kaludarsko–Dapsićke šume," located in the municipality of Berane, in the northeastern part of Montenegro. Forest management in this area is carried out by the regional unit Berane within Administration for Forest and Hunting Management. According to its characteristics, the stand belongs to high beech forests. Data were collected through a partial inventory (Table 1), in accordance with the methodology applied in:

A. In Montenegro, the concentric circles method is applied for stands larger than 10 ha, arranged in a 100x100 m grid. On every second plot, for each tree species and diameter class of 26–65 cm, the heights of two trees per diameter class are measured.

B. In Serbia, until mid-2024, experts considered the previously applied methodology far superior to the one prescribed by the new Regulation (2024). It allowed for the selection of sample plot shapes, sample size and number of detailed subplots in accordance with the variability of the population (the stand), expressed through a certain degree of homogeneity.

Table 1. Basic elements of partial inventory according to methodologies A and B

Shape of sample plots	Stand area [ha]	Number of sample plots - n [no.]	Number of detailed sample plots - n_d [no.]	Plot size [are/counting factor]	Sampling intensity - Pp [%]	Distance - S [m]
CC CG	12,45	10	4	1, 2, 5, 10	8,0	100,0
CC RS	12,45	28	6	1, 2, 5, 10	22,5	66,7
WZP RS	12,45	28	6	2	37,8	66,7

Note: CC CG – concentric circles Montenegro; CC RS – concentric circles Serbia; WZP RS – angle count sampling plots Serbia.

It is important to note that the centers of the concentric circles and the circular angle count sampling plots used in Serbia were identical, which enabled a more accurate comparison of the results obtained from these two types of sample plots. Using GIS tools, the sample plots were systematically distributed across the stand area (a simple systematic sampling design). For the center of each sample plot, precise coordinates were determined and subsequently transferred to the field using handheld GPS devices during fieldwork activities.

Data processing

Data processing was carried out using classical dendrometric and statistical methods integrated into the "Osnova" software. To ensure a more consistent comparison of the obtained results and the derivation of relevant conclusions, the same version of the software was used for processing data collected from CC CG, as well as from CC RS and WZP RS. In addition to the measured tree diameters (d, cm) and heights (h, m), the input elements in the data processing procedure also included the stand area and the data describing site and stand characteristics. Numerous output parameters were generated through the data processing procedure, with this research focusing primarily on the number of trees, stand volume, their distributions and the height curve.

Depending on the shape of the sample plot, correction factors were applied to convert each tree within a given diameter class to a per-hectare basis and by summing these values N/ha was obtained for each sample plot within the stand. The arithmetic mean of N/ha across all sample plots represented the final N/ha value for the sample. This value, together with the standard error of the estimate, defines the range within which the number of trees in the population (the stand) varies.

The best statistical parameters in modeling the relationship h= f(d) (height curve) were obtained using the Näslund function:

$$h = 1{,}30 + \frac{d^2}{(a + b \cdot d)^2}$$

The application of the Näslund function for modeling the relationship between tree height and diameter is widely used in European forestry practice and has been confirmed in contemporary studies (Mehtätalo *et al.,* 2015; Sharma and Breidenbach, 2015; Liziniewicz *et al.,* 2016; Holmström *et al.,* 2018; Lidman *et* *al.,* 2021; Mensah *et al.,* 2022; Persson *et al.*, 2022; Ogana *et al.,* 2023; Siipilehto *et al.,* 2023). The resulting height curves were used to determine the height class, since the stand volume was calculated using the tariff method:

$$V = \sum_{i=1}^{n} n_i \cdot v_i$$

v_i – volume of the mean tree in the diameter class,
n_i – number of trees in the diameter class expressed per hectare.

The procedure for calculating V/ha at the level of each sample plot, the calculation of the average volume of the sample and the estimation of the total stand volume based on the sample volume and the standard error of the estimate is identical to the procedure used for calculating the number of trees per hectare.

For the spatial analysis of the data obtained from field measurements, the Spline with Barriers method was applied within the ArcMap 10.8 software. The main objective of the interpolation was to display the spatial distribution of the number of trees and volume per hectare, based on the sample plots established within the stand. The input values used in the interpolation consisted of the number of trees and volume values positioned at the centers of all sample plots. Each sample plot represented a location where the number of trees was measured and the volume calculated, both converted to a per-hectare basis (Table 2). The Spline with Barriers method enables the generation of a continuous surface without abrupt transitions between sample plots, while treating stand boundaries as barriers that prevent the calculation of values beyond the actual limits of the analyzed area. This method is widely used in spatial analyses, as it provides a realistic representation of variability and prevents interpolation across physical barriers, as demonstrated by its applications in ecological and geomorphological studies (Hogg *et al.*, 2016; Hogg *et al.,* 2021; Brophy *et al.*, 2019; Wardell and Huvenne, 2022; Brenko *et al.,* 2024; Wang *et al.,* 2024; Bosso *et al.*, 2025). This approach enabled the visualization of spatial variability of the analyzed parameters (number of trees and volume) within the specific forest complex. After creating individual rasters for each of the applied sample plot types used in the partial inventory (CC CG, CC RS and WZP RS), a comparison of results was performed to assess the level of agreement and deviation among the different approaches. For this purpose, the Raster Calculator function was used, allowing arithmetic operations on rasters within the GIS environment. By combining the corresponding rasters of tree numbers and volumes, formulas were applied to calculate the percentage differences per pixel. The calculation of these differences resulted in new rasters that visually and numerically represent the deviations per spatial unit, or per pixel.

The following formulas were used to calculate the differences in the number of trees and volume per hectare between the sample plot rasters:

$$R_1 = \frac{CC\ CG - CC\ RS}{CC\ RS} \cdot 100\%$$

$$R_2 = \frac{CC\ CG - WZP\ RS}{WZP\ RS} \cdot 100\%$$

$$R_3 = \frac{CC\ RS - WZP\ RS}{WZP\ RS} \cdot 100\%$$

RESULTS AND DISCUSSION

Number of trees, volume, distributions

The lowest number of trees and the lowest sample volume were obtained through the partial inventory using CC CG, amounting to 529 trees/ha and 338.2 m³/ha (Table 2). For CC RS, these values were 614 trees/ha and 502.9 m³/ha, while for WZP plots the number of trees was 679 trees/ha and the volume 461.4 m³/ha. The standard error of the estimate for the number of trees ranged from 19.3% for CC RS to as high as 38.6% for CC CG. In terms of volume, this error ranged between 10.3% for WZP RS and 18.8% for CC CG. Considering the structural composition of the stand, if the values obtained from WZP sample plots are assumed to be the most relevant, the deviations (R_2) amount to -22.1% for the number of trees and -26.7% for the volume, while deviations (R_3) are -9.6% and 9.0%. The differences (R_1) are -13.8% for the number of trees and as much as -32.8% for stand volume (Table 3). Similar relationships between numerical elements obtained from concentric circles and WZP plots were reported in previous studies (Matérn, 1972; Bitterlich, 1984; Schreuder *et al.*, 1987; Scott and Alegria, 1989; Packard and Radtke, 2007; Piqué *et al.*, 2011; Leiter and Hasenauer, 2023).

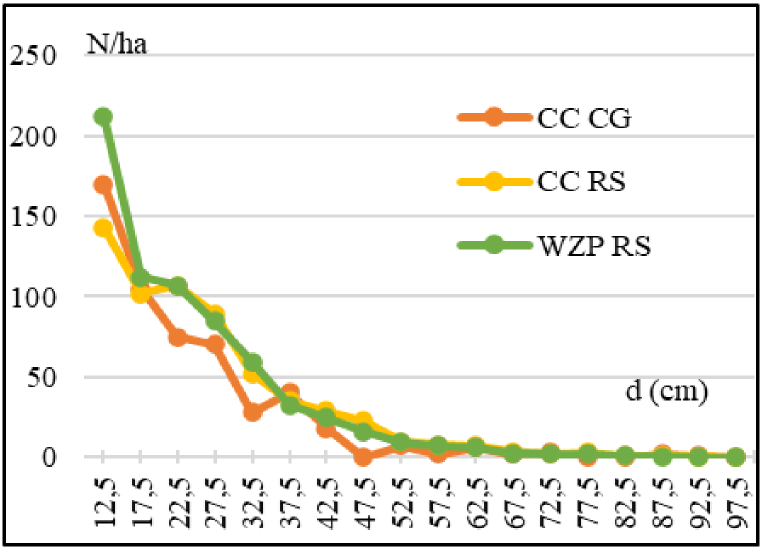
From the distribution of the number of trees by diameter classes (Figure 1), it can be seen that the stand structure is very close to a typical selection structure, even though in forest management plans it is defined as an uneven-aged stand with a long regeneration period, which implies age diversity.

The distributions of the number of trees and volume obtained through partial inventory on the tested shapes of sample plots show significant differences (Graphs 1 and 2, Table 3), confirming the findings of other authors who compared fixed (CC) and variable sample plots (WZP) (Lowell, 1997; Fallah *et al.,* 2000; Rios *et al*., 2000; Motz *et al*., 2010; Piqué *et al.,* 2011; Alijanpour *et al.,* 2018). The variation ranges of the distributions are pronounced (12.5–92.5/97.5 cm), as a consequence of the presence of trees of different dimensions, confirming that the stand is indeed of a selection type.

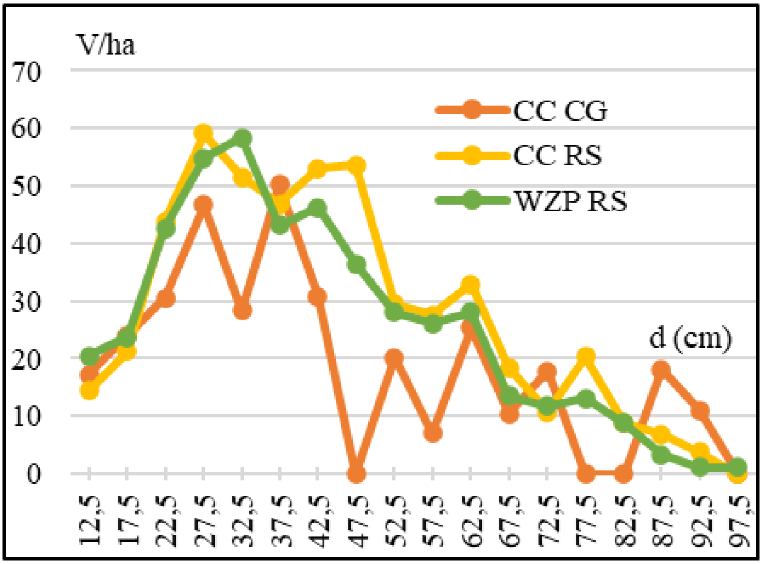
Table 2. Number of trees and volume per hectare obtained from the tested shapes of sample plots

Number of sample plot	CC CG		CC RS		WZP RS	
	N	V	N	V	N	V
	no./ha	m^3/ha	no./ha	m^3/ha	no./ha	m^3/ha
1	100	371,8	450	687,2	378	556,0
2	490	269,1	220	677,5	211	441,1
3	560	368,1	330	902,1	268	667,6
4	110	204,5	30	82,2	58	106,4
5	610	237,9	500	632,8	404	516,0
6	680	545,4	490	371,0	468	337,7
7	960	255,2	770	513,1	779	454,3
8	960	353,5	950	596,8	1174	598,2
9	140	427,7	410	448,0	505	537,3
10	680	362,7	930	420,1	1562	495,9
11	-	-	210	470,2	223	417,9
12	-	-	650	465,8	763	456,1
13	-	-	660	363,0	763	315,0
14	-	-	700	364,1	917	402,5
15	-	-	350	395,3	363	351,7
16	-	-	700	406,5	800	382,0
17	-	-	480	443,8	485	450,7
18	-	-	890	766,0	712	641,8
19	-	-	490	633,6	502	599,8
20	-	-	530	538,3	594	442,9
21	-	-	1070	675,2	1219	663,1
22	-	-	360	381,3	434	411,4
23	-	-	420	343,1	564	257,6
24	-	-	1330	618,4	1209	578,2
25	-	-	970	447,9	1288	382,5
26	-	-	500	528,9	445	457,5
27	-	-	560	473,5	580	507,1
28	-	-	1250	436,0	1354	495,0
$\bar{N}, \bar{V}$ *(ha)*	529	338,2	614	502,9	679	461,4
sN, sV (%)	38,6	18,8	19,3	12,1	21,6	10,3

Note: $\bar{N}, \bar{V}$ (ha) - average number of trees and volume per hectare in the sample; sN% – standard error of the estimate for the number of trees; sV% – standard error of the estimate for volume.



Graph 1. Distribution of the number of trees by diameter classes



Graph 2. Distribution of volume by diameter classes

Differences in the number of trees by diameter classes are significant and fall within the intervals: R_1=(–73.3)–185.7%, R_2=(–72.2)–900.0% and R_3=(–32.7)–300.0%. Differences in volume by diameter classes are also large, amounting to R_1=(–74.3)–189.5%, R_2=(–72.9)–900.0% and R_3=(–29.3)–245.5% (Table 3). It should be noted that in Table 3, a value of –100% represents the absence of trees or volume in the respective diameter class.

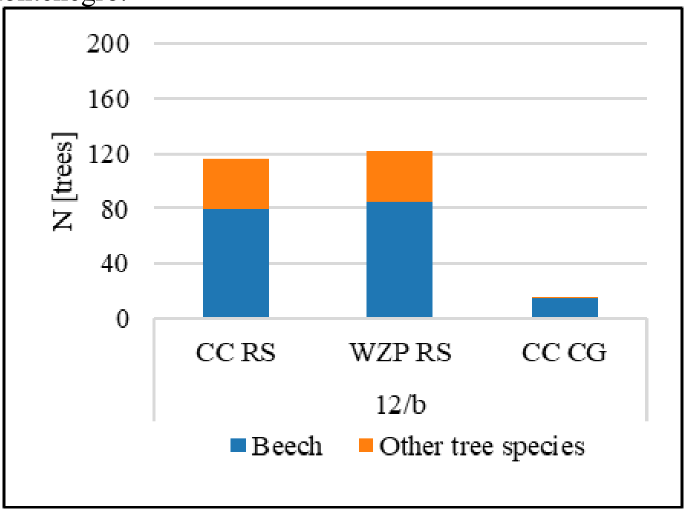
Table 3. Differences in the distributions of the number of trees and volume obtained from the tested shapes of sample plots

d (cm)	R_1=(CC CG–CC RS)/CC RS*100		R_2=(CC CG–WZP RS)/ WZP RS*100		R_3=(CC RS–WZP RS)/WZP RS*100	
	N/ha R_1 (%)	V/ha R_1 (%)	N/ha R_2 (%)	V/ha R_2 (%)	N/ha R_3 (%)	V/ha R_3 (%)
12,5	19,0	18,6	-20,0	-16,1	-32,7	-29,3
17,5	3,1	9,0	-6,3	1,3	-9,2	-7,1
22,5	-30,0	-30,5	-29,8	-28,6	0,2	2,8
27,5	-21,6	-21,0	-17,2	-14,8	5,7	7,8
32,5	-46,3	-45,0	-53,0	-51,3	-12,6	-11,5
37,5	14,3	7,5	23,1	16,4	7,7	8,3
42,5	-37,1	-41,7	-27,7	-33,3	14,9	14,5
47,5	-100,0	-100,0	-100,0	-100,0	44,0	47,1
52,5	-27,1	-31,8	-23,9	-28,1	4,3	5,3
57,5	-73,3	-74,3	-72,2	-72,9	4,2	5,3
62,5	-15,5	-22,8	-3,2	-9,6	14,5	17,1
67,5	-44,4	-43,8	-23,1	-23,5	38,5	36,0
72,5	66,7	67,3	57,9	51,7	-5,3	-9,3
77,5	-100,0	-100,0	-100,0	-100,0	61,1	55,0
82,5	-100,0	-100,0	-100,0	-100,0	-15,4	4,8
87,5	185,7	162,3	400,0	432,4	75,0	102,9
92,5	150,0	189,5	900,0	900,0	300,0	245,5
97,5	-	-	-100,0	-100,0	-100,0	-100,0
Difference at stand level	-13,8	-32,8	-22,1	-26,7	-9,6	9,0

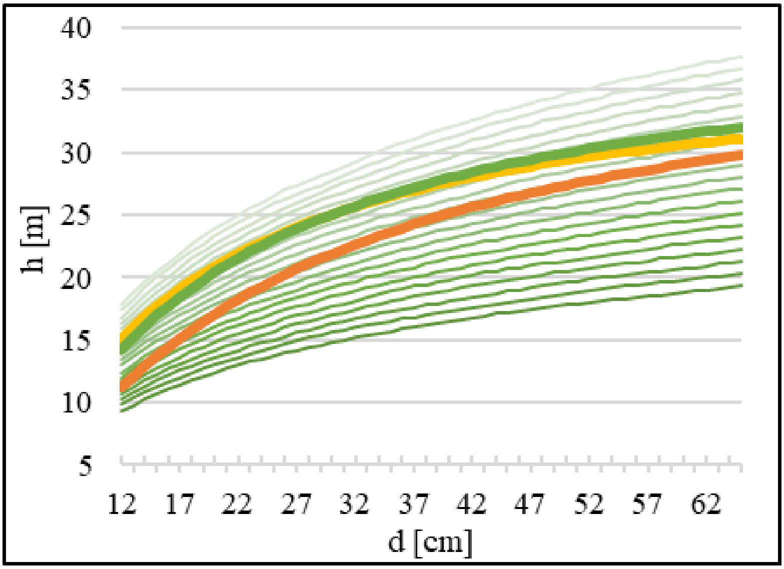
The analysis of differences in the average number of trees and volume per hectare in the sample, as well as differences in their distributions by diameter classes, does not allow for a definitive determination of the sample plot shape that provides the most accurate results under the given conditions. The reasons, among others, lie in the significantly lower sampling intensity of CC CG, which is 8%, compared to 22.5% for CC RS and 37.8% for WZP, as well as in the fact that the CC CG plots, due to their 100 m spacing, were located in different parts of the stand compared to CC RS and WZP RS plots, whose centers were positioned at the same locations, 67 m apart. The non-coincidence of the centers of the tested sample plots certainly affects the quality of the analyses and the conclusions drawn. Since the stand in question is of the selection type, representing an extremely heterogeneous structure, a complete inventory would yield the most accurate results. However, the Montenegrin Regulation (2016) does not recognize this measurement method, while the Serbian Regulation (2024) allows its optional application. Therefore, under the given conditions, preference should be given to angle count sampling. The reason lies in the fact that this type of partial inventory is based on unequal selection probability that is, the probability of a tree being selected for measurement is proportional to its importance for the final result. The selection structure is characterized by a large proportion of thin trees, hence, during measurement, priority must be given to trees that represent the main volume carriers large-diameter trees which is precisely the principle of unequal selection probability on which WZP is based.

Height curve, height classes

Based on the measured heights from the detailed sample plots, the relationship h=f(d) was modeled, resulting in height curves that were used to determine the stand's belonging to a specific height class (tariff series). The number of detailed sample plots for CC RS and WZP RS was 6 each (Table 1), with tree heights measured for every tree above the inventory threshold, resulting in 79 and 85 measured beech heights, respectively, as the dominant tree species on these plot types. For CC CG, 4 detailed plots were established; however, due to the measurement method applied (two heights per tree species within diameter classes of 26–65 cm), only 14 beech heights were obtained, which is considerably lower than the realistically possible and required number (Graph 3). Since the quality of the height curve directly depends on the number and accuracy of the measured tree heights (Sharma, Parton, 2007; Nowak *et al.,* 2008; Pretzsch, 2009; Alijanpour *et al*., 2018; Leiter, Hasenauer, 2023; Ogana *et al*., 2023; Siipilehto *et al.,* 2023), it is evident that the quality of the curve for CC CG is lower compared to the curves obtained from the other tested sample plot types. The position of the CC CG curve within the height bundle is significantly lower than that of the other two curves (Graph 4), which directly affected the determination of the tariff series and, consequently, the stand volume. These findings indicate the need to re-examine the validity of the height measurement methodology applied in the partial inventory of forests in Montenegro.



Graph 3. Number of measured tree heights



Graph 4. Position of height curves within the height overdiameter curves

Raster analysis

Raster analysis provided additional insight into the spatial distribution of the number of trees and volume within the studied stand, obtained through partial inventory using the tested shapes of sample plots. It also enabled a visual examination of the influence of plot shape, measurement intensity (sample size) and the scope of input data on interpolation results and the spatial structure of the forest resources. Essentially, this analysis served as a logical continuation of the previous comparisons, aimed at a more detailed examination of the differences in results that arose from the application of different sample plot shapes in the partial inventory.

The spatial distribution of the number of trees obtained from CC CG (Figure 1 – left) shows a relatively uniform pattern, with fewer transition zones between distribution groups. This pattern is a consequence of the smaller number of sample plots, which limits interpolation possibilities and reduces spatial resolution. One large zone of high density (over 1,200 trees/ha) stands out, though without pronounced internal heterogeneity. The inventories based on CC RS (Figure 1 – center) and WZP RS (Figure 1 – right) display a much more complex spatial structure. A larger sample and more even stand coverage resulted in a higher degree of data variability and the occurrence of local extremes. In the case of CC RS, areas of higher density are noticeable in the western and eastern parts of the stand, while WZP RS, although following the same layout, shows slightly higher values in the central and southeastern zones. These shapes more accurately reflect the actual heterogeneity of the stand.

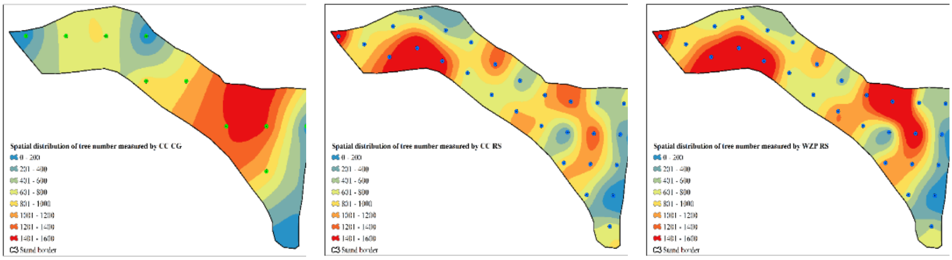


Figure 1. Spatial distribution of the number of trees (left – CC CG; center – CC RS; right – WZP RS)

The percentage differences in the number of trees obtained from the tested shapes of sample plots are shown in Figure 2.

•CC CG:CC RS (Figure 2 – left): The differences reach extreme values, exceeding 4,000% in zones where CC CG data are absent or minimal. Such large deviations indicate insufficient representativeness of the CC CG sample for a stand structure similar to a typical selection structure. Most areas are displayed in blue-green tones (differences up to 500%), but in the outer zones, CC CG plots can be observed where the number of trees is underestimated compared to the reference CC RS.

•CC CG:WZP RS (Figure 2 – center): Spatially, the deviations occur in zones that are poorly covered by CC CG plots, which once again indicates the limited reliability of this plot type in terms of interpolation accuracy.

•CC RS:WZP RS (Figure 2 – right): When comparing the number of trees obtained from these two sample plot types, the smallest mutual deviations were recorded, with most differences around ±30%. This outcome is expected, as both plot types use the same grid layout (distribution across the stand area), meaning that the differences arise solely from the distinct sampling methods, specifically, the way trees are selected for measurement. The sample size and the number of measured trees directly influence the results, making them more reliable compared to those obtained from CC CG.

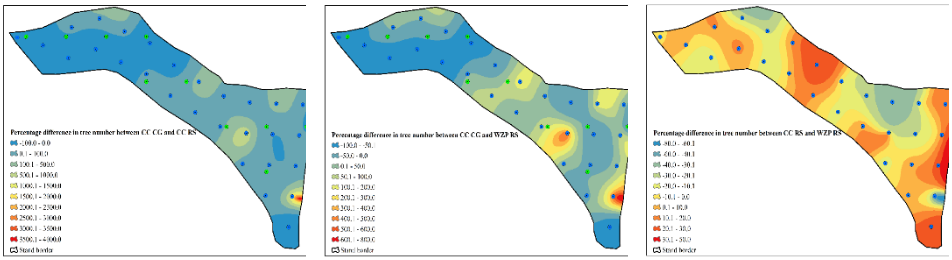


Figure 2. Percentage differences between N/ha obtained from the tested shapes of sample plots (left – CC CG:CC RS; center – CC CG:WZP RS; right – CC RS:WZP RS)

These results clearly demonstrate that the methodological approach and the scope of collected data have a decisive impact on the spatial interpretation of results. Due to the small number of sample plots and the limited amount of input data, CC CG produces results that are not sufficiently stable, particularly in the boundary zones of the stand. In contrast, CC RS and WZP RS, owing to a considerably larger sample and greater volume of input data in the processing stage, provide not only statistically more reliable values but also a spatial representation that more accurately reflects the actual field conditions. Furthermore, such spatial analysis enables the identification of local zones and differences that would otherwise remain hidden in aggregated tabular presentations. It is important to note that in certain zones, the WZP RS raster detects a higher number of trees than CC RS, which suggests that angle count sampling more effectively captures larger trees, even at greater distances. This reflects the fundamental principle of unequal selection probability and represents a necessary condition for obtaining reliable results in structurally heterogeneous situations such as uneven-aged and selection forests.

As in the case of the number of trees, methodological differences among the tested sample plot types led to distinct spatial distributions of volume, with this variability being more pronounced than in the case of tree numbers (Figure 3).

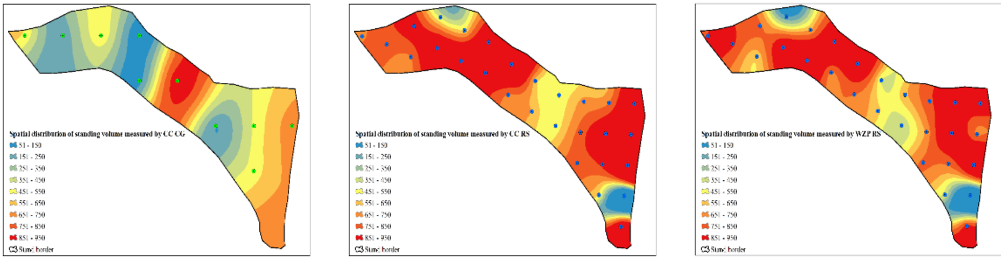


Figure 3. Spatial distribution of volume (left – CC CG; center – CC RS; right – WZP RS)

The CC CG plots (Figure 3 – left) show a very uniform and simplified spatial distribution of volume. The values of this parameter are mainly concentrated in the central and eastern parts of the stand, but without clearly defined zones of high concentration. This uniformity results from the smaller number of input data and insufficient spatial coverage to capture all internal variations within the stand. The absence of higher volume values in certain zones indicates that this plot type did not include larger trees (volume carriers), which is consistent with the tabular data (Table 2) showing the lowest average sample volume of 338.2 m^3/ha. On the other hand, CC RS (Figure 3 – center) and WZP RS (Figure 3 – right) display a more complex and differentiated volume structure. Both plot types show several zones with high volume values (over 750 and even 950 m^3/ha), mostly distributed in the western and southeastern parts of the stand. The fact that these zones do not always coincide with the areas of the highest number of trees indicates that the increased volume results from a greater number of measured large-diameter trees, which represents the main advantage of angle count sampling plots in structurally heterogeneous stands. Thus, these spatial patterns align with the characteristics of a stand with a structure close to the selection type, where volume is not distributed proportionally to the number of trees but largely depends on the inclusion of individual large trees.

The percentage differences in volume obtained from the tested shapes of sample plots are shown in Figure 4.

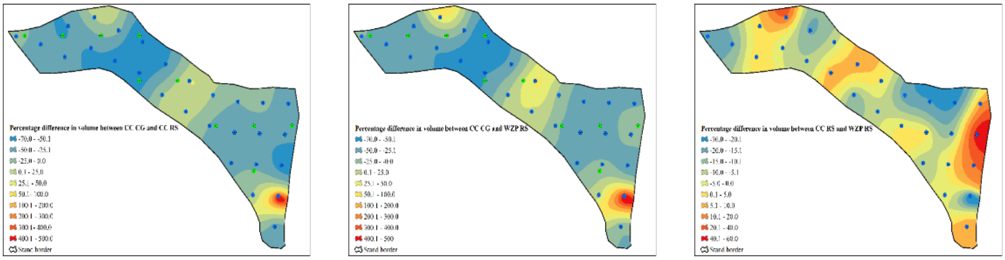


Figure 4. Percentage differences between V/ha obtained from the tested shapes of sample plots (left – CC CG:CC RS; center – CC CG:WZP RS; right – CC RS:WZP RS)

•CC CG:CC RS (Figure 4 – left): The differences are significant across a large portion of the stand, most commonly ranging from 50% to over 300%, while in a smaller part of the stand they reached values exceeding 500%. These differences are a direct consequence of the CC CG inventory insufficiently capturing large-diameter trees, which resulted in a systematic underestimation of the stand volume.

•CC CG:WZP RS (Figure 4 – center): The distribution pattern is similar to the previous comparison (CC CG:CC RS). In most parts of the stand, the differences range between 100% and 300%, which is a consequence of the fact that angle count sampling plots more effectively capture large-diameter trees that contribute significantly to the total volume. One zone with an extremely high difference is also noticeable here, further indicating the limitations of using CC CG with a relatively low sampling intensity under structurally heterogeneous stand conditions.

•CC RS:WZP RS (Figure 4 – right): The differences are much smaller, mostly around ±10%, with local exceptions reaching up to ±20%. These values confirm the previous conclusions regarding the reliability of inventories conducted on these two sample plot types. Since CC RS and WZP RS plots have the same number and spatial arrangement, the observed differences result from the distinct methods of tree selection for measurement. In certain zones, WZP RS shows higher volume values than CC RS, which is likely due to the inclusion and measurement of larger trees captured within the selected counting angle but not encompassed by the CC radius.

Volume has proven to be a parameter more sensitive than the number of trees to the shape and spatial arrangement of sample plots, the range of tree diameters and the number of measured trees in higher diameter classes. This indicates that a partial inventory with a denser and more systematic grid of sample plots, which also includes a greater number of trees, particularly those of larger dimensions, provides a more accurate representation of the spatial distribution of numerical stand attributes. The CC CG method shows clear limitations in interpolation, producing a simplified spatial representation that may lead to misinterpretation in forest management planning, silvicultural decisions and related applications.

CONCLUSIONS

By synthesizing the research results, the following conclusions can be drawn regarding the selection of the most suitable plot shape for partial inventory under the given conditions:

1. Partial inventory conducted on different plot shapes, even when applied within the same stand, may produce significantly different values of numerical elements and their distributions. Consequently, these differences arise from variations in sample size and sample type, the intensity of the inventory, the number of detailed sample plots and the number of trees whose heights are measured.

2. In the present case, it is difficult to determine which of the tested plot shapes is superior, due to the differing elements used in partial inventory. Considering the structural characteristics of the stand and in the absence of a complete (total) inventory, priority should nevertheless be given to angle count sampling, because this method is based on selection with unequal probability, the probability of selecting a tree is proportional to the importance of that element for the final result.

3. The method of height selection applied in the partial inventory of Montenegro generally does not provide a sufficient amount of data for constructing a reliable height curve. As a result, the curve tends to be positioned lower within the height overdiameter curve (producing a tariff series inferior to the actual one), which consequently leads to underestimated volume, volume increment and the feasible allowable cut in production forests. In light of these findings, it is recommended that the height-measurement methodology used in the forest inventory of Montenegro be reassessed and revised where necessary.

4. By applying raster analysis in a GIS environment, a more detailed insight was obtained into the spatial distribution of the number of trees and volume per hectare for the tested plot shapes, as well as into the percentage differences of these elements. The spatial distribution of tree numbers and volume enables more precise planning, improves the organization of field activities, and contributes to the optimization of silvicultural measures implemented within the stand. Spatial visualization facilitates decision-making regarding regeneration, tending operations, stand utilization, and similar activities, as it provides a realistic understanding of internal stand variability, which is often overlooked in traditional tabular presentations of stand conditions.

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ANALYSIS OF THE INFLUENCE OF CLIMATE CONDITIONS ON HAZELNUT FLOWERING AND POLLINATION

SUMMARY

In the hazelnut orchards in Bosnia and Herzegovina, continental cultivars are predominantly cultivated, though Mediterranean cultivars have been increasingly grown in recent years. Hazelnut yield in orchards is quite variable between years and depends on the influence of weather conditions on reproductive biology during the entire organogenesis cycle, and especially during the flowering and pollination periods. As a result of adverse weather conditions, uneven flowering times of male and female inflorescences of dominant cultivars and pollinator cultivars are registered, accelerated and shortened pollen shedding time, freezing of catkins that are in the elongation and pollen shedding phase and lack of pollination of late-flowering hazelnut cultivars.

An analysis of the influence of climatic factors on the hazelnut flowering process and the compatibility of cultivars for cross-pollination was conducted out in the area of northwestern Bosnia and Herzegovina in the period from 2017 to 2020. The following cultivars were analyzed: Romai, Istarski Dugi, Hall's Giant, Tonda Gentile Romana and Tonda Gentile delle Langhe.The results of the study show that during certain years there is a significant influence of weather conditions on the flowering and pollination of hazelnut, where early and accelerated pollination and uneven flowering time of the observed cultivars is registered.

Keywords: pollen shedding, stigmatic styles, climate change

INTRODUCTION

The hazelnut (*Corylus avellana* L.) is an anemophilous (wind-pollinated), monoecious fruit species that blooms in the winter period (Germain, 1994) when other fruit species are in the dormancy phase. In the Northern Hemisphere, under moderately continental and Mediterranean climates, hazelnut flowering occurs from early December to late March, depending on cultivar characteristics,

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weather conditions, altitude, and terrain exposure (Solar and Štampar, 2011; Ilić *et al.,* 2018). The flowering type can vary depending on weather conditions, with cultivated hazelnut cultivars most commonly exhibiting protandry (Piskornik *et al.,* 2001). The duration of male flowering ranges from 3 to 75 days, while female flowers take from 5 to 105 days (Modic, 1969; Santos and Silva, 1994; Ilić *et al*., 2018). Over the past two decades, the Balkan Peninsula has experienced rising average temperatures and a significant reduction in snow days in lower-altitude regions (Trbić *et al.,* 2017). Winters are increasingly characterized by warm spells and frequent temperature fluctuations, from –10 to +20 ºC, which directly affect hazelnut flowering and pollination (Ilić *et al.,* 2021). Indicative changes in flowering dynamics are manifested in the following: a reduction in the flowering period of catkins and pollen shedding by up to 25.6 percent on a multi-year level (Črepinšek *et al.,* 2011; Mercuri *et al.,* 2016); uneven flowering times between catkins of pollinator cultivars and female flowers of dominant cultivars in orchards (Ilić *et al*., 2018; Benewitz *et al.,* 2019) and the absence of pollination in late-flowering cultivars (Baldwin, 2009). The occurrence of low temperatures after a warm period can affect the functionality of the male gametophyte, which is found in different micro-phenophases of development (Mićić *et al*., 2022). Hazelnut cultivars are characterized by varying degrees of resistance to low temperatures. At the time of pollen shedding, catkins freeze at temperatures of –5 to –7 ºC (Miljković, 2018, cited by Romisondo, 1978), while at temperatures of –2 to –5 ºC, degeneration of male gamets occurs reducing the possibility of fertilization (Šoškić, 2005 citing Milošević, 1997). On the other hand, female inflorescences exhibit greater resistance to low temperatures during the flowering time. Krpina *et al.* (1994) and Vujević *et al.* (2014) note that the continental cultivars Romai, Istarski Dugi and Hall's Giant did not suffer significant damage in terms of yield in those years when temperatures during flowering ranged from –5 to –19.5 ºC.

The aim of this research is to analyze the influence of climatic conditions on flowering phenology and the possibility of cross-pollination of cultivated hazelnut cultivars in the territory of Bosnia and Herzegovina. The results of the research should create the possibility for the application of biological fertility control and solving the problem of low and irregular fruiting of hazelnuts.

MATERIAL AND METHODS

Monitoring the influence of climatic factors on the flowering phenology and pollination synchrony of cultivated hazelnut cultivars was conducted in a hazelnut orchard located in the Laktaši municipality (Šušnjari), Bosnia and Herzegovina, at an altitude of 200-250 m, coordinates N 44°50'43.3" 17°17'12.7"E. The orchard was established in 2012. year, covering an area of 6 ha with planting distance 5×3 m. The hazelnuts were grown on their own roots with a bush training system. The research was conducted during the flowering season 2017/2018, 2018/2019, and 2019/2020 and included the following hazelnut cultivars: Romai, Istarski Dugi, Hall's Giant, Tonda Gentile Romana (TGR) and Tonda Gentile delle Langhe (TGDL). Hazelnut flowering phenology was studied from November to March, focusing on different micro-phenophases of development depending on the type of inflorescence:

a) male inflorescences (start of flowering - elongation of the catkins and pollen shedding in <10 percent of catkins; full flowering - pollen shedding in >10 percent of catkins; end of flowering - drying of >90 percent of catkins).

b) female inflorescences (start of flowering - appearance of red stigmatic tips in >10 percent of female inflorescences; full flowering – red stigmatic tips present in >10 percent of inflorescences; end of flowering - drying of >90 percent of the stigmatic tips which turn dark).

Field activities and monitoring of the flowering phenophase were conducted visually on a weekly basis. In the event of high air temperatures, the observation were conducted two to three times per week, depending on the weather conditions. For the analysis, the northwestern side of the orchard was selected, covering an area of 0.3 ha, where five uniformly vigorous bushes of each cultivar were monitored. Due to the pronounced influence of weather conditions on the flowering dynamics of male and female inflorescences, as well as the simultaneous occurrence of different micro-phenophases of flowering depending on the position of the inflorescences on the plant (Figure 1), all micro-phenophases were represented in the phenogram as full flowering phases. During the research, the occurrence of dichogamy, the influence of air temperature on the phase of pollen shedding and the flowering synchrony of cultivars for mutual cross-pollination were recorded, which was analyzed subsequently. The meteorological station of the Hydrometeorological Institute of the Republic of Srpska, which provided the air temperature data, is located 8 km away from the hazel orchard in a straight line and is located at an altitude of 163 meters above sea level. The chi-square test was used to test differences in flowering length of male and female inflorescences between years and between male and female inflorescences within a year.

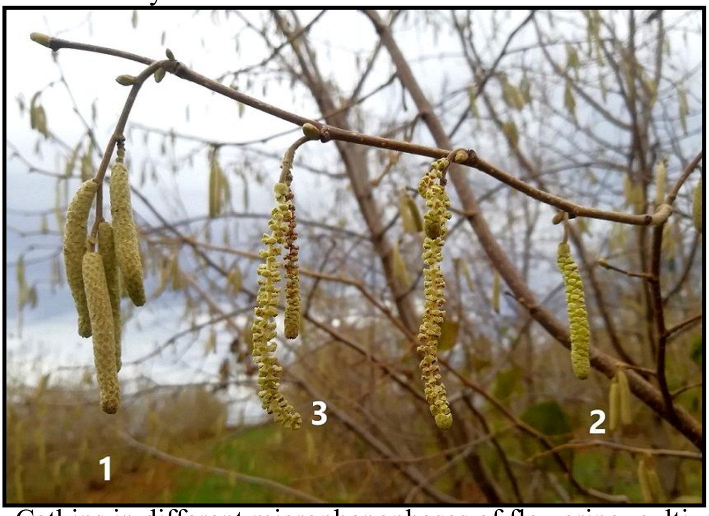


Figure 1. Catkins in different microphenophases of flowering, cultivar TGDL (phase 1 - catkins are completely dormant; phase 2 - elongation of catkins before the start of pollination; phase 3 –dried catkins that have finished pollination)

RESULTS AND DISCUSSION

The results of the research on flowering phenology of male and female hazelnut inflorescences during the 2017/2018 season are presented in Figure 2, while meteorological data with air temperatures are shown in Graph 1.

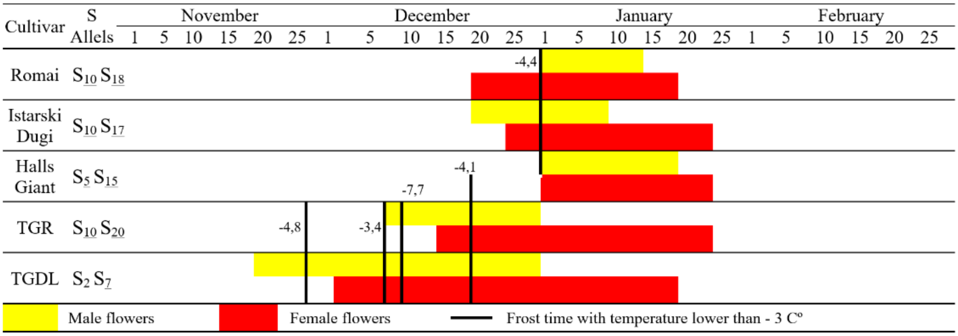
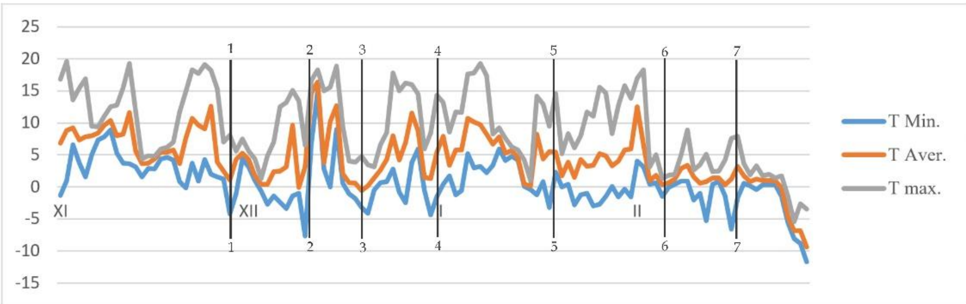


Figure 2. Total flowering phenophases of hazelnut during the 2017/2018 season

Flowering of male inflorescences lasted from November 20 to January 20 (totaling 60 days), while female bloomed from December 5 to January 25 (totaling 50 days). The earliest start of flowering was observed in the TGDL cultivar, with male inflorescences on November 20 and female inflorescences on December 5. The latest flowering start was recorded in the Hall's Giant cultivar, at the beginning of January for both male and female inflorescences. The warm weather conditions during November, December and January (Graph 1) with maximum daily temperatures reaching up to 20 ºC, accelerated the hazelnut flowering process, particularly pollen shedding, which concluded by mid-January for most cultivars.



Graph 1. Minimal, average and maximal daily air temperatures, November 2017 - March 2018 (Republic Hydrometeorological Institute of the Republic of Srpska, 2024).

In early-flowering cultivars TGDL and TGR, pollen shedding ended on December 25, at a time when female flowering in other cultivars had not yet begun. These two cultivars were capable of cross-pollinating each other and could also pollinate the Romai cultivar at the onset of its flowering. The Romai and Istarski Dugi cultivars flowered uniformly and were able to pollinate the late-flowering cultivar Hall's Giant, which in turn could pollinate unpollinated female flowers across all cultivars in the orchard. Due to the presence of the S10 allele in pollen of TGR, Romai and Istarski Dugi, their mutual cross-compatibility is questionable. During the pollen shedding period, several temperature fluctuations (from –8 to +20 ºC) were recorded on 6 occasions, which affecting initiation of flowering and the freezing of pollen in the catkins.

During the second year of measurement, significant differences in flowering timing were observed among the studied cultivars (Figure 3).

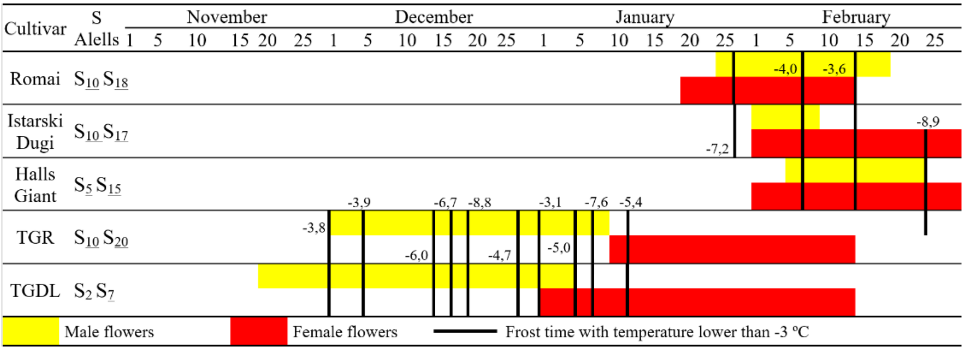
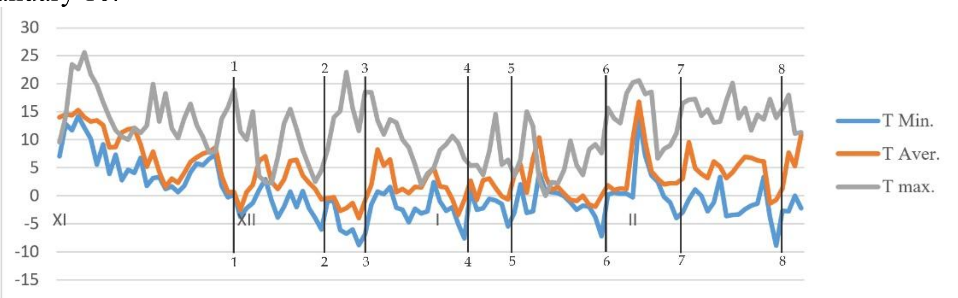


Figure 3. Total flowering phenophase of hazelnut during the 2018/2019 season.

Flowering of male inflorescences lasted from November 17 to February 25 (totaling 97 days), while flowering of female inflorecences lasted from December 1 to March 1 (totaling 90 days). The earliest start of pollen shedding was observed in the TGDL cultivar (November 17) and the Hall's Giant cultivar had the latest (February 5). Similarly, the earliest female flowering initiation was recorded in TGDL, with the latest flowering observed in Istarski Dugi and Hall's Giant (February 1). The warm November weather, featuring a maximum temperature of 26.2 ºC (Graph 2), promoted earlier male flowering and pollen shedding in TGDL and TGR cultivars, which completed their flowering cycle by January 10.



Graph 2. Minimal, average and maximal daily air temperatures, November 2018 - March 2019 (Republic Hydrometeorological Institute of the Republic of Srpska, 2024).

The TGR and TGDL cultivars could not pollinate other cultivars in the orchard because the female flowers of the other cultivars bloomed much later.

However, in the final phase of male flowering, the TGR cultivar was able to pollinate the female flowers of TGDL that had just begun flowering. The flowering of Romai, Istarski Dugi and Hall's Giant cultivars was synchronized, allowing for mutual cross-pollination among them. These cultivars could also pollinate the female flowers of TGR and TGDL, assuming compatibility issues are disregarded. During the entire flowering period, temperature fluctuations were recorded on 8 occasions with morning frosts as low as –8.9 ºC and daytime temperatures up to 22.2 ºC.
During the third year of observation, flowering began the earliest (Figure 4).

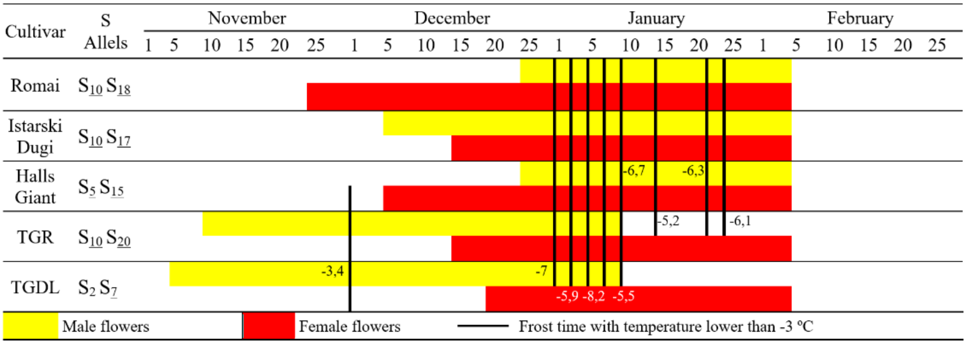
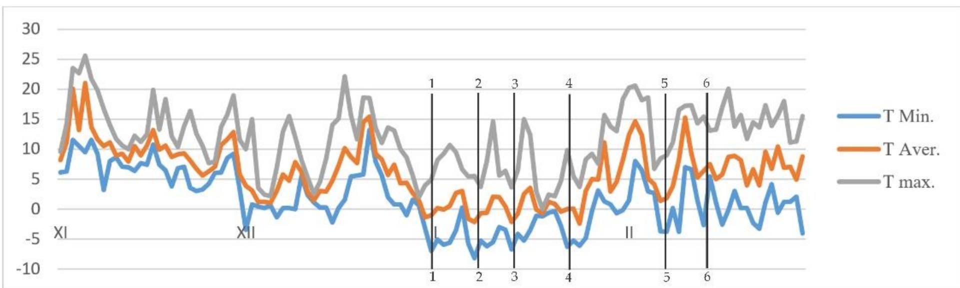


Figure 4. Total flowering phenophase of hazelnut during the 2019/2020 season

The flowering of catkins lasted from November 6 to February 5 (totaling 92 days), while the flowering of female flowers lasted from November 25 to February 5 (totaling 72 days). In the early-flowering cultivar TGDL, flowering started on November 6, whereas it lasted in the Romai and Istarski Dugi cultivars, beginning in early January. The earliest start of female catkin flowering was in the mid-flowering cultivar Romai on November 25, and the latest was in the early-flowering cultivar TGDL on December 20, which was completely opposite of what was expected.



Graph 3. Minimal, average and maximal daily temperatures, November 2019. - March 2020., Republic Hydrometeorological Institute of the Republic of Srpska, 2024).

Higher air temperatures in November and December, along with milder temperatures in January, led to earlier and more uniform flowering across all cultivars without significant differences, as observed in previous years. Extremely early pollen shedding was recorded at the beginning of November, when approximately 50 percent of the catkins from the TGDL and TGR cultivars released pollen due to unusually high night and daytime temperatures reaching up to 26 ºC. Colder weather in December and January showed pollen release from the remaining catkins in the orchard, extending the process over the next 50 to 60 days. However, a sudden rise in temperatures in early February accelerated and shortened the flowering period, causing it to end within just a several days. The flowering synchronization was quite uniform, increasing the chances of successful cross-pollination of the cultivar, although temperature fluctuations were recorded on several occasions.

The flowering of male inflorescences was the shortest in the TGR cultivar, 19 days during the 2017/2018 year, and the longest in the TGDL cultivar, 66 days during the 2019/2020 year (Table 1).

Table 1. Testing the difference in the flowering time of male and female inflorescences with the Chi-square test and the occurrence of dichogamy.

Cultivar	Year	Σ number of days of flowering male and female inflorescences						Type of dichogamy
		♂	$\chi^2_{0,05}$	♀	$\chi^2_{0,05}$	♂ $\chi^2_{0,05}$	♀ $\chi^2_{0,05}$	
Romai	2017/18	21	15.30**	31	38.20**	0.05^{ns}	0.45^{ns}	Protogyny
	2018/19	24		30		0.80^{ns}	1.51^{ns}	Protogyny
	2019/20	37		71		14.45**	37.02**	Protogyn.
Istarski Dugi	2017/18	22	19.65**	32	14.11**	0.20^{ns}	0.25^{ns}	Protandry
	2018/19	37		49		14.45**	5.60*	Protandry
	2019/20	30		52		5.00*	8.25**	Protandry
Hall's Giant	2017/18	20	14.65**	27	23.67**	0.00^{ns}	1.82^{ns}	Homogamy
	2018/19	22		29		0.20^{ns}	1.02^{ns}	Protogyny
	2019/20	37		62		14.45**	20.82**	Protogyny
TGR	2017/18	19	1699.1**	41	2.31^{ns}	0.05^{ns}	1.02^{ns}	Protandry
	2018/19	39		38		18.05**	0.25^{ns}	Protandry
	2019/20	61		41		84.05**	1.02^{ns}	Protandry
TGDL	2017/18	35	153.50**	42	10.45**	11.25**	1.20^{ns}	Protandry
	2018/19	47		46		36.45**	3.45^{ns}	Protandry
	2019/20	66		49		102.05**	5.60*	Protandry

The flowering of female inflorescences was the shortest with the cultivar Hall's Giant 27 days during 2017/2018, and the longest with the cultivar Romai 71 days during the year 2019/2020. Testing the difference in the flowering of male and female inflorescences between years shows highly significant differences in all cultivars, except for the female inflorescences of the cultivar TGR, which had no significant differences. Testing the difference between male and female inflorescences within years, shows highly significant differences during the 2019/2020 year in male inflorescences in all observed cultivars, as well as in the TGDL cultivar during all observed years. In the flowering of female inflorescences, highly significant differences were manifested in certain years only in the cultivars Romai and Hall's Giant. All cultivars exhibited different types of dichogamy depending on the year and weather conditions. Throughout all years of observation, the TGDL, TGR and Istarski Dugi cultivars showed protandry, while Romai showed protogyny. Hall's Giant cultivar alternated between protogyny and homogamy depending on the year.

According to the available research results in the field of hazel flowering phenology indicate different research results depending on the region and the influence of climate conditions. The flowering of male and female hazel inflorescences in our study was much earlier and shorter compared to other studies conducted in BiH (Skender *et al.,* 2019; Ilić *et al.,* 2021). Similar results were recorded in relation to the area of Croatia (Vujović *et al.,* 2014), Slovenia (Solar and Štampar, 1997; Solar and Štampar, 2011) and Poland (Piskornik, 2001) where the observed cultivars bloomed for an average of two to three months, from January to the end of March. Cristofori *et al*. (2018) states that in the area of central Italy, the cultivar TGDL showed early flowering of male inflorescences during November, in accordance with our research, and much later pollination of the cultivar TGR, which is contrary to our measurements. The non-coordinated synchronization of flowering of cultivars, which was recorded in our research during 2018/2019, is stated by Bennewitz *et al.* (2019) in the territory of Chile in different locations with dominant and pollinator cultivars as well as Baldwin (2009) in the territory of Australia for late flowering cultivars. Mićić *et al.* (2024) claim that the essence of a successful cultivar composition implies that the microphenophases of anther shedding of pollen and the ability of the pistil to accept pollen of compatible cultivars coincide in at least 50 percent of the flowering time during two of the five observed years.

The dynamics and synchronization of the flowering in our research indicates unevenness between the shedding of pollen of Mediterranean cultivars and flowering of female inflorescences of the other observed cultivars, whereby they do not coincide in the flowering period which is greater than 50 percent. The flowering time of male hazelnut flowers in Italy is shortened by up to 25 percent due to the influence of climatic factors (Mercuri *et al.,* 2016) as well as in Slovenia (Črepinšek *et al.,* 2011), which is in accordance with our research that was recorded during 2017/2018 and 2018/2019 in cultivars Romai, Istarski Dugi and Hall's Giant. Regarding the occurrence of dichogamy in hazelnut, Piskornik *et al.* (2001) and Turcu *et al.* (2001) state that the type of manifestation of dichogamy depends on the weather conditions during the flowering period, whereby cultivars that are considered exclusively protandric cultivars can also exhibit a protogynous type of flowering. In research in the region, Skender *et al.* (2019) state the occurrence of protandry in cultivars Romai, Istarski Dugi and Hall's Giant in BiH, which is in agreement with our research, except for Romai cultivar. While Krpina et al. (1994) and Vujević *et al.* (2014) in Croatia report variability and protogyny in Romai, Istarski Dugi and Hall's Giant cultivars, which is in agreement with our research, with the exception of the Istarski Dugi cultivar which was protandric in our research.

CONCLUSIONS

The analysis of the influence of climatic conditions on the phenology of hazelnut flowering in the Banja Luka region shows a strong influence of air temperatures on the dynamics and duration of flowering of male and female inflorescences in all observed cultivars. During some years, extremely early pollen shedding of Mediterranean cultivars was recorded, uneven flowering of male and female inflorescences and shortened and accelerated flowering of temperate continental cultivars. Temperature fluctuations started and stopped the flowering process on several occasions, with cold snaps of –9 C° interrupting the flowering of male inflorescences at different stages of development. In order to avoid the problems noted in this study it is necessary to take into account the choice of dominant cultivars and pollinator cultivars when establihing new hazelnut orchard. For each dominant cultivar, it is necessary to use two or three compatible pollinators with synchronized shedding of catkins that cover the entire complete stage of flowering of female inflorescences of dominant cultivar in orchard. Also, it is necessary to conduct regular biological control of flowering and the functionality of the male gametophyte, and based on this implement adequate measures of early pollen collection and supplementary artificial pollination.

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FROM WASTE TO GROWTH: MUSHROOM RESIDUES IN HORTICULTURE

SUMMARY

The rapid global expansion of mushroom cultivation has established it as an important agribusiness sector and a promising strategy for sustainable management of agricultural residues. Nevertheless, this growth has resulted in the annual accumulation of millions of tons of spent mushroom substrate (SMS), creating significant environmental and logistical challenges. On average, approximately five kilograms of SMS are generated for every kilogram of fresh mushrooms harvested. In North Macedonia, the mushroom industry similarly produces considerable quantities of SMS, which presents an opportunity for its reutilization as a growing substrate for horticultural crops. In this context, the present study investigated the physical and chemical properties of SMS derived from the fast-growing oyster mushroom (*Pleurotus ostreatus*). The results demonstrated that the substrate exhibits favorable physical characteristics, including low bulk density, homogeneous texture, and low electrical conductivity, making it suitable for incorporation into horticultural substrate mixes. Chemically, the substrate was found to be low in nitrogen and phosphorus but contained moderate to sufficient levels of potassium, while its exceptionally high organic matter content enhances its potential as a soil conditioner. Literature review indicates that mushroom residues are primarily employed as components of growing media in vegetable and ornamental crop production. Additionally, SMS can serve as a fertilizer, soil amendment, or mulch, providing multifunctional benefits in sustainable horticultural practices. Overall, oyster mushroom SMS represents a valuable resource for horticulture, combining desirable physical properties with nutrient supplementation potential and contributing to circular waste management strategies in the mushroom industry.

Keywords: spent mushroom substrate, growing medium, composition, Pleurotus ostreatus, horticulture

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INTRODUCTION

Peat remains the predominant substrate in horticultural crop production; however, its unsustainable extraction and escalating cost have driven research and industry efforts toward identifying alternative substrates or partial substitutes (Zeljković *et al.,* 2015). Mushroom cultivation, a rapidly growing agricultural sector, utilizes various organic wastes but also generates around 100 million tons of spent mushroom compost annually, posing environmental and industry challenges (Mwangi *et al.,* 2024). For every 1 kilogram of fresh mushrooms produced, approximately 5 kilograms of fresh spent mushroom substrate (SMS) are generated (Diego *et al*., 2021). Although numerous mushroom species are cultivated globally, around 70% of total production comes from just three: the white button mushroom (*Agaricus bisporus*, 32%), the shiitake mushroom (*Lentinus edodes*, 25%), and the oyster mushroom (*Pleurotus* spp., 14%) (Uzun, 2004). Mushroom residues are commonly referred to as Spent Mushroom Substrate (SMS) or Spent Mushroom Compost (SMC). Spent mushroom substrate (SMS) refers to the fresh residual material left immediately after mushroom production, consisting of organic and mineral substances as well as microbial biomass and enzymes (Catal and Peksen, 2020; Prasad *et al*., 2021), whereas spent mushroom compost (SMC), also called weathered-SMS or composted SMS, is the same substrate after further decomposition or composting for several months (typically 3–24 months), a process recommended by many authors before its use in horticulture (Prasad *et al*., 2021; Alves *et al.,* 2024). The use of SMS offers notable advantages, particularly because its cost is approximately 20% of that of cattle manure compost, owing to reduced transportation requirements and the absence of a composting phase (Danai *et al*., 2011). In North Macedonia, mushroom cultivation is a growing industry and data on the availability of production residues are lacking. At the same time, the main cultivated species are *Pleurotus ostreatus* (oyster mushroom) and *Agaricus bisporus* (button mushroom). The most commonly used substrate for industrial oyster mushroom (*Pleurotus ostreatus*) cultivation in North Macedonia typically consists of 15 kg of wood pellets (derived from deciduous trees) and 10 liters of water, supplemented with calcium sulfate ($CaSO_4$) and calcium carbonate ($CaCO_3$). These additives stabilize pH, enhance substrate structure, and suppress the growth of undesirable microorganisms. The exact proportions of calcium sulfate and calcium carbonate can vary depending on the cultivated mushroom species and local agrochemical conditions, but generally constitute 2–5% of the total substrate mass. This substrate is also suitable for the cultivation of several other mushroom species, including Lion's Mane (*Hericium erinaceus*), Maitake (*Grifola frondosa*), Shiitake (*Lentinula edodes*), and Reishi (*Ganoderma lucidum*). In Israel, the substrate for mushroom cultivation typically consists of compost made primarily from poultry manure, wheat straw, and gypsum, along with a casing layer composed of black peat amended with lime (Danai *et al.,* 2011). The composition of spent mushroom substrate (SMS) in Turkey varies depending on the mushroom species cultivated (Çatal and Pekşen, 2020). For Pleurotus ostreatus, three different substrate mixtures have been reported: (1) 95% paddy straw and 5% poplar sawdust, (2) 60% wheat straw, 20% paddy straw, and 20% corn cobs, and (3) 70% oak sawdust, 20% wheat bran, 5% cottonseed pulp, 3% marble dust, and 2% gypsum. According to Rinker (2017), Pleurotus species are grown on wood sawdust and plant fibers supplemented with locally available proteins and carbohydrates. The main application of mushroom residues in vegetable production is as a component of growing media (Charles Heuser *et al.,* 2007; Younis *et al*., 2023) for seedling production of crops such as tomato (Medina *et al*, 2009; Eudoxie and Alexander, 2011; Zhang *et al*., 2012; Ünal, 2015; Priadi *et al.,* 2016; Collela *et al.,* 2019; Alves *et al.,* 2024), pepper (Medina *et al.,* 2009; Demir, 2017), lettuce (Kwack *et al*., 2012; Marques *et al.,* 2014; Liu *et al*., 2018), eggplant (Sönmez *et al*., 2016), cucumber (Zhang *et al.,* 2012), melon (Van Tam and Wang, 2015) and zucchini (Medina *et al*., 2009). In sustainable fruit production, mushroom residue is used as fertilizer, mulch, or an alternative pesticide and should be applied every few years (Uzun, 2004). A few studies have examined the use of spent mushroom substrate in fruit production (Danai *et al.,* 2011; Orluchukwu and Adedokun, 2015; Prasad *et al*., 2021). Although ornamental production is rapidly expanding, limited research has been conducted on the use of spent mushroom substrate, primarily focusing on woody ornamentals (Chong and Rinker, 1994), geraniums and surfinia (Zeljković *et al.,* 2015), and chrysanthemums (Atikmen *et al.,* 2014). Furthermore, spent mushroom substrate can enhance microbial diversity (Huang et al., 2023), aid in disease control (Mwangi *et al.,* 2024), support biogas production (Kumar *et al.,* 2022), contribute to bioremediation efforts (Rinker, 2017), and serve as a material for biodegradable containers in horticulture (Postemsky *et al.,* 2016).

Considering the potential value of mushroom residues and the need to minimize waste, this study addresses two objectives. The first objective is to characterize the physical and chemical properties of spent mushroom substrate from *Pleurotus ostreatus*, one of the fastest-growing cultivated mushrooms, which can be harvested within approximately one month after inoculation. The second objective is, based on available literature, to highlight the various applications of mushroom residues in horticultural practices.

MATERIAL AND METHODS

In order to achieve the first objective a detailed analysis of the physical and chemical properties of a spent mushroom substrate of oyster mushroom (*Pleurotus ostreatus*) was conducted at the accredited Laboratory for Soil and Fertilizer Analysis (L-04), Faculty of Agricultural Sciences and Food – Skopje. The laboratory is accredited according to ISO/IEC 17025, and the analytical procedures follow validated national (MKC), international (ISO, ASTM, EN) and internal AL methods.

1. Physical analyses

The following physical parameters were analyzed:

Moisture (%) – determined according to MKC ISO 8190:1992 by oven-drying at 105 °C to constant weight using a drying oven, with precision ±0.1 g.

Texture, color, and scent – determined by organoleptic evaluation, commonly used for characterizing compost-like organic substrates. Observations were performed under standard daylight conditions, following internal AL protocols.

Density (g/L) – measured according to MKC ISO 1237:2009 by using graded 1 L volumetric cylinders, with the material compacted under standardized conditions to ensure reproducibility.

Electrical conductivity (EC, mS/cm) – measured according to ISO 11265:2004 with a conductivity meter, equipped with a 325 conductivity cell. Measurements were performed at 25 °C and corrected automatically for temperature.

2. Chemical analyses

The chemical analysis of the substrate included the following parameters:

pH (H_2O) – determined according to MKC ISO 13037:2011 using a meter with a electrode.

Organic matter (%) – determined by loss-on-ignition method following ASTM D2974-14, using a muffle furnace at 550 °C.

Ash (%) – obtained as a complementary parameter from the same ASTM procedure.

Total nitrogen (mg/L) – measured according to EN 15750:2009 using the Kjeldahl digestion–distillation method on digestion unit and distillation system, with titration performed using 0.01 N HCl.

Phosphorus (P_2O_5, mg/L) – extracted with ammonium-lactate solution following the validated AL method used at FASF-Skopje. Determination performed by UV-Vis spectrophotometry.

Potassium (K_2O, mg/L) – extracted identically (AL method) and measured using a flame photometer, calibrated with K standard solutions (0.5–10 mg/L K).

3. Sample preparation

For sample preparation it was used fresh spent mushroom substrate from oyster mushroom (Pleurotus ostreatus) from producer in Negotino just after oyster production. Substrate samples were air-dried at room temperature (22–25 °C), homogenized manually, and sieved through 2 mm mesh (for chemical assays) and 10 mm mesh (for physical structure characterization).

Moisture and density measurements were performed on fresh, non-dried samples to preserve the natural physical status of the spent mushroom substrate.

4. Quality Assurance / Quality Control (QA/QC)

All analyses were performed in duplicate, and results are reported as the mean. Standard reference materials (SRMs) for organic substrates and composts were used where available. Instruments were calibrated daily using certified calibration standards for pH, EC, N, P, and K.

Blank samples and control solutions were included in each analytical batch. The relative standard deviation (RSD) for repeated measurements was kept below 5%, fulfilling the laboratory's internal QA requirements.

In order to achieve the second objective utilization of mushroom residues in horticulture, information was gathered and presented from peer-reviewed articles published between 1994 and 2024. These articles were sourced from reputable journals indexed in databases such as Scopus, Web of Science, ResearchGate, and Google Scholar.

RESULTS AND DISCUSSION

Physical and chemical properties of the Spent mushroom substrate (SMS) of the oyster (Pleurotus ostreatus)

The physical analysis of the oyster mushroom substrate indicated that it possessed moderate moisture, a uniform texture, a mixed dark-to-light coloration, a characteristic odor, suitable density, and an electrical conductivity within an acceptable range. The chemical analysis further showed that the substrate exhibited slightly acidic pH, very high organic matter content, low ash levels, and a nutrient profile characterized by low nitrogen and phosphorus availability, along with moderate to sufficient potassium supply. Overall, the substrate demonstrates high organic quality, appropriate physical properties, and a generally favorable chemical composition for use in cultivation (Table 1).

Table 1. Physical and chemical properties of the Spent mushroom substrate (SMS) of the oyster (*Pleurotus ostreatus*)

	Parameter	Method	Values	Unite Measure
Physical parameters				
1.	Moisture	MKC ISO8190:1992	55,02	[%]
2.	Texture	organoleptic	homogeneous	/
3.	Color	organoleptic	brown-black-white	/
4.	Scent	organoleptic	characteristic	/
5.	Density	MKCISO 1237:2009	215	[g/L]
6.	EC	ISO 11265:2004	0,9	[mS/cm]
Chemical parameters				
1.	pH	MKCISO 13037:2011	5,60	/
2.	Organic matter	ASTM D2974 – 14	98,32	[%]
3.	Ash	ASTM D2974 – 14	3,02	[%]
4.	Total nitrogen (N)	EN 15750:2009	212	[mg/l]
5.	Phosphorus (P_2O_5)	AL Method validated at FASF - Skopje	178	[mg/l]
6.	Potassium (K_2O)	AL Method validated at FASF - Skopje	301	[mg/l]

Table 2. Physical and chemical characteristics of spent mushroom substrate (SMS) and spent mushroom compost (SMC)

Parameter	SMS-PO[2] (Medina et al., 2009)	White button mushroom SMC (Kwack et al., 2012)	*Flam Mulina velutipes* SMS (Zhang et al., 2012)	100% SMW[3] (Sendi et al., 2013)	SMS (Van Tam and Wang, 2015)	Aged SMC (Sönmez et al., 2016)	Spent oyster mushroom compost (Priadi et al., 2016)	FSMC[1] (Demir, 2017)	Agaricus bisporus SMS (Collela et al., 2019)	*Agaricus bisporus* SMS (Catal, and Peksen,2020)	Fresh SMS of white button mushroom (Alves et al., 2024)
M	-	-	-	-	-	-	73.36 %	-	-	-	-
BD	-	0.12 g cm^{-3}	0.413 g cm^{-3}	-	0.308g cm^{-3}	-	-	-	325.0 kg/m^3	-	0.308kgL^{-1}
TPS	-	-	79.8%	-	89.73%	-	-	-	67%	-	-
AP	61(% (v/v)	-	24.5%	-	36.47%	-	-	-	-	-	-
WHC	324 mL L^{-1})	-	55.3%	-	53.26 %	-	-	-	62.3%	277.51%	-
pH	6.9	7.0	5.28	6.10	7.65	6.66	8.4	7.14	6.9	6.23	7.42
EC	1.03dS m^{-1}	4.97dS m^{-1}	2.45$mScm^{-1}$	1.33dS m^{-1}	3.50 dS m^{-1}	6.85dS m^{-1}	-	10.91dS m^{-1}	2.54	6.60dS m^{-1}	5.81dS m^{-1}
N	1.12 %	-	10.78	0.34%	0.218g kg^{-1}	1.663%	0.32 %	1.786%	8.7g kg^{-1}	2.32%	15.1g kg^{-1}
NO_3	2 mg L^{-1}	79 mg L^{-1}	-	-	-	-	-	-	-	-	-
P	252mg L^{-1}	34 mg L^{-1}	2.69gkg^{-1}	0.16%	11.49g kg^{-1}	25280$mgkg^{-1}$	0.29 %	28280$mgkg^{-1}$	542.96 mg/dm3	0.68%	5.0g kg^{-1}
K	1875mg L^{-1}	1879 mg L^{-1}	1.34gkg^{-1}	0.53%	13.85g kg^{-1}	13170$mgkg^{-1}$	0.11 %	20860 $mgkg^{-1}$	600.0 mg/dm3	2.43%	18.6g kg^{-1}
Ca	20.1gkg^{-1}	1317 mg L^{-1}	30.1gkg^{-1}	0.51%	33.45g kg^{-1}	43210$mgkg^{-1}$	-	41910 $mgkg^{-1}$	52.7 cmol/dm3	4.73%	66.2g kg^{-1}
Mg	3.9gkg^{-1}	231 mg L^{-1}	4.52gkg^{-1}	0.15%	7.79g kg^{-1}	6648.0$mgkg^{-1}$	-	5148.0 $mgkg^{-1}$	3.0 cmol/dm3	0.55%	6.6g kg^{-1}
CEC	-	-		-	-	-	-	-	58.73 cmol/dm3	-	-
BS	-	-	81.2%	-	-	-	-	-	-	-	-
OM	-	-		-	-	-	-	-	15.04 dag/kg	66.98%	-
C:N	-	-		-	-	-	32	-	-	16.74	-
C	-	-		-	-	-	10.35%	-	-	38.85%	-

M (Moisture), BD (Bulk density), TPS (Total pore space), AP (Air porosity), WHC (Water holding capacity), CEC (cation exchange capacity) OM (organic matter) [1]FSMC (fresh spent mushroom compost) [2]SMS-PO (Spent mushroom substrate of *Pleurotus ostreatus*) [3]SMW (Spent mushroom waste)

Table 2 presents the physical and chemical characteristics of mushrooms residues as reported in the reviewed literature. According to Medina *et al*. (2009) spent mushroom substrate from *Pleurotus ostreatus* has very high air porosity, pH near neutral, moderate EC, high N and P, but very high K content which make it not directly usable as growing media. On the other hand, composted oyster spent mushroom substrate showed higher moisture content (73.36%), high pH levels (8.4), low nitrogen levels, and moderate P and K content (Priadi *et al*., 2016).

The physical and chemical properties of spent mushroom substrate from white button mushroom (*Agaricus bisporus*) vary considerably, but it is generally characterized by elevated pH and electrical conductivity, moderate to high nitrogen concentrations, medium to high phosphorus levels, and consistently high potassium content (Kwack *et al*., 2012; Collela *et al*., 2019; Catal and Peksen, 2020; Alves *et al.,* 2024). *Flammulina velutipes* SMS exhibits a pH comparable to that of the oyster mushroom substrate, but it has a notably higher electrical conductivity and substantially greater concentrations of nitrogen, phosphorus, and potassium, indicating a richer nutrient profile that could influence its suitability for use as a growing medium or soil amendment (Zhang *et al.,* 2012).

Utilization of Mushroom residues in horticulture

The use of low-cost, renewable materials such as spent mushroom substrate and spent mushroom compost offers an environmentally sustainable alternative to chemical inputs and peat, supporting the sustainable production of potted plants and seedlings (Mwangi *et al.,* 2024; Younis *et al*., 2023). Overall, media containing spent mushroom compost enhanced tomato seedling growth, with the best results observed in media containing spent paddy straw mushroom compost (Priadi *et al.,* 2016). The addition of 100% spent mushroom compost to the growing media significantly improved tomato seedling quality, yielding the best results in hypocotyl length, seedling length, root length, and both shoot and root fresh weights (Ünal, 2015). Seedlings grown in spent mushroom substrate (SMS) from *Agaricus bisporus* exhibited faster germination rates and speeds, and outperformed other substrates in terms of tomato seedling quality (Collela *et al.,* 2019). Tomato seedlings grown in SMS were 32% taller and had 12% more leaves at 5 weeks after seeding compared to those grown in peat at 6 weeks, demonstrating that SMS is a more effective medium for seedling production (Eudoxie and Alexander, 2011). Using 100% composted SMS as a substrate did not impair cherry tomato seedling growth, while fresh SMS negatively affected germination and growth, likely due to excess salts, though it significantly increased chlorophyll content (SPAD), leading to the recommendation that fresh SMS can be used at 20% as a peat substitute without harming seedling development (Alves *et al*., 2024). The optimal substrate for tomato and cucumber seedlings is a spent mushroom substrate (SMS) mixed with perlite at a 2:1 ratio or with vermiculite at a 4:1 ratio, depending on the amendment used (Zhang *et al.,* 2012). Spent mushroom media (SMM) from *Flammulina velutipes* mixed with 0–30% vermiculite showed a high germination rate in pepper, while SMM from *Pleurotus eryngii* with 20% vermiculite achieved 100% germination. Pepper growth improved with increasing vermiculite content, indicating that Flammulina velutipes SMM with up to 30% vermiculite can be effectively used as a horticultural growing medium for pepper cultivation (Chan-Jung *et al.,* 2009). Similar results were obtained by Demir, 2017 where a mixture of 70% aged spent mushroom compost with 30% perlite and aged spent mushroom compost alone performed comparably to a 70% peat and 30% perlite mixture as a seedling medium for pepper, based on germination rate, stem diameter, height, leaf number, and seedling macroelement content. Fresh spent mushroom substrate (SMS) should not be used alone for pepper cultivation; instead, it is recommended to mix it with commercial peat at a 25% ratio for successful horticultural use (Medina *et al.,* 2009; Zhu *et al*., 2019). Among six tested spent mushroom composts (SMCs), white button mushroom SMC was the most suitable growing medium for lettuce transplant production when properly mixed, while sawdust-based SMCs require careful treatment to improve their physical and chemical properties for use as growing media (Kwack *et al*., 2012). According to Liu *et al.* (2018), a substrate mixed with 50% spent mushroom substrate (SMS) effectively improves the growth of lettuce seedlings. Using spent mushroom compost at a 45% ratio provides the best conditions for lettuce seedling germination and growth, leading to greater biomass and vigor in 27-day-old plants (Marque *et al.,* 2014). Growing media significantly affected seedling quality, with peat and perlite mixtures generally yielding the best results, while eggplant seedlings grown in a mixture of new and old spent mushroom compost (70% SMC + 30% perlite) had the highest nutrient content, leading to the conclusion that spent mushroom compost can be effectively used in seedling growing media (Sönmez *et al*., 2016). Spent mushroom substrate (SMS) can be used up to 80% in mixtures with manure compost for honeydew melon seedling production, but 40% SMS provides the most suitable conditions for seedling growth (Van Tam and Wang, 2015). In addition to seedling production, spent mushroom substrate or compost can be used as manure (Ahlawat *et al*., 2009), to improve microbial diversity (Huang *et al.,* 2023), and even for biogas production (Kumar *et al*., 2022).

In sustainable fruit production, spent mushroom compost (SMC) serves as a fertilizer, soil amendment, mulch, or alternative pesticide, and is recommended for application at intervals of several years to maintain soil health and productivity (Uzun, 2004). Using spent mushroom substrate as a soil enrichment material for pineapple resulted in better growth and yield, with no significant difference in fruit number and fresh weight compared to inorganic fertilizer (Orluchukwu and Adedokun, 2015). SMS offers significant advantages, minimal transport and no composting, making it beneficial for both avocado and mushroom growers; however, its commercial use in avocado orchards requires careful monitoring of soil electrical conductivity (salinity) to prevent potential damage (Danai *et al*., 2011). Growing media greatly affects overall strawberry performance; replacing peat with 15% and 25% (v/v) fresh *Agaricus bisporus* spent mushroom substrate (A-SMS) and *Lentinula edodes* spent mushroom substrate (L-SMS) in soilless strawberry production resulted in better growth and compatibility compared to commercial peat and Pleurotus spent mushroom substrate (P-SMS) (Prasad *et al*., 2021).

Research on woody ornamentals has shown that incorporating spent mushroom substrate (SMS) at rates of 25% or 50%, which are preferred in commercial container nursery practices, significantly promotes excellent growth of nursery crops (Chong and Rinker, 1994). The use of spent mushroom compost from button mushroom (*Agaricus bisporus*) cultivation, combined with a proportion of commercial substrates, is recommended for producing *Pelargonium peltatum* L. and *Petunia hybrida* Juss. seedlings due to its positive effects on root and shoot development, economic viability, and environmental suitability (Zeljković *et al.,* 2015). Fresh and exhausted mushroom composts were used without pretreatment as growth substrates for chrysanthemum cultivation. The results indicate that fresh mushroom compost is most effective at a 12.5% ratio, with 25% as a secondary option, while exhausted mushroom compost is best used at 25%, with 50% as an alternative in later stages (Atikmen *et al.,* 2014).

Compared to other organic materials, spent mushroom substrate offers three key benefits for intensive horticulture: no weed seeds, slow nutrient release, and a beneficial microbial community that helps protect plants from pathogens (Diego *et al*., 2021).

CONCLUSIONS

Based on the results of this study, it can be concluded that the spent mushroom substrate (SMS) of oyster mushroom possesses favorable physical properties, including low bulk density, favorable structure, and low electrical conductivity. Regarding chemical properties, the substrate is relatively low in nutrients, except for potassium, and exhibits a slightly acidic pH, making it suitable for a wide range of horticultural crops. Its extremely high organic matter content further enhances its value as a soil conditioner. Oyster mushroom SMS can be incorporated into substrate mixes for vegetable production. According to the reviewed literature on the utilization of mushroom residues in horticulture, its primary application in vegetable production is as a substrate substitute for seedling cultivation. In fruit production, these residues can be used as a 25% component of growing substrates, as a fertilizer, soil amendment, mulch, or alternative pest control agent. For ornamental horticulture, oyster mushroom SMS can be applied at rates of 25–50% as a substitute for conventional growing media.

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POLYPHENOLS AND ANTHOCYANIN PROFILE OF BILBERRIES (*VACCINIUM MYRTILLUS* L.) FROM NORTH MACEDONIA

SUMMARY

In this study, physico-chemical properties, phenolic compounds, and antioxidant capacity of bilberries (*Vaccinium* spp.) fruits grown in three main localities in Sharr Mountain were investigated. The bilberries localities are: B1 (Kobilica, 1936m altitude), B2 (Kodra e Diellit, 1698 m), B3 (Tetovo, 467m). B3 locality is characterized by the significant ($P<0.05$) higher dry matter values (18 g/100g) as well as higher total acidity (1.32%) than the other localities. Based on the environmental conditions that prevailed during the vegetation, the B1 bilberries location recorded significantly higher values ($P<0.05$) of polyphenol content 651.36±0.50mg GAE/100g. Also, bilberries B1 and B2 localities showed higher amounts of antioxidant values (717.72±17.17 and 634.99±15.61 mg Trolox equivalents (TE)/100 g) compared to B3 locality (447.68±18.73 mg TE/100 g). These findings indicate that growing altitude is a major environmental factor determining the concentration of phenolic compounds and antioxidant activity in wild *Vaccinium myrtillus* L. populations in North Macedonia.

Keywords: natural; HPLC; DPPH; tannins; phenolic acids; flavonoids.

INTRODUCTION

The bilberry fruit belongs to the botanical family of Ericaceae that grows in the form of bushes in temperate climates on soils with pH 3.5-4.0 (Michalska and Łysiak 2015; Krzewinska, 2004). Bilberry (*Vaccinium myrtillus* L.), also known as European blueberry, is a low-growing deciduous shrub of the Ericaceae family native to northern Europe and widespread in mountainous regions of the western Balkans (Nestby *et al*. 2011; Zhang *et al*. 2016; Zorenc *et al*. 2016; Skupień, 2006). Numerous studies have compared the phenolic content and antioxidant capacity of wild bilberries (*V. myrtillus*) with those of cultivated highbush blueberries (*V. corymbosum*) (Taruscio *et al.,* 2004). Studies have shown that bilberry fruits contain numerous components that benefit human health especially vitamin C, carotenoids and polyphenols showing strong antioxidant capacity (Shivembe and Ojinnaka, 2017).

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Bilberries are available on the market in the form of berries and are consumed as such (fresh, frozen, or dried), and bilberries are also available in the form of semi-finished products (juices, concentrates, canned). Phenolic compounds in bilberries vary greatly, depending on the cultivar, season, and growing location. It is reported that the plant's growth location and interaction with the ecosystem influence the phytochemical compounds in blueberry fruits (Zoratti *et al*., 2015; Correia *et al.,* 2016). Bilberries are recognised for their exceptionally high anthocyanin and phenolic content, which confer strong antioxidant, anti-inflammatory, and cardioprotective properties (Zhang *et al.* 2016, Može *et al*. 2011, Lätti *et al*. 2009; Brambilla *et al.* 2008; Cho *et al*. 2004). Information regarding the high antioxidant efficiency of wild bilberries has aroused considerable popular interest in these fruits from consumers who want to know how bilberries can contribute to the nutritional quality of their diet. Antioxidants are necessary to prevent and protect the body against the action of variable free radicals, which cause damage to various biomolecules in the body and lead to many health problems (Bayazid *et al.* 2021). Natural antioxidants are preferred by consumers because of the quality and safety compared to unnatural colorants, antioxidants and nutraceuticals (Wang *et al.* 2017).

North Macedonia, spanning 2,571,000 ha, values its forests as a key part of its national heritage. Forests cover 835,056 ha and host about 16% of all Balkan endemic or subendemic species. Favorable natural conditions-its climate, terrain, geology, and hydrology-support rich biodiversity, including over 3,000 higher plant species, about 1,000 with medicinal uses, and roughly 500 fully medicinal plants; (https://mk.macedonism.org/Makedonska-Enciklopedija/shumarstvo/). The Sharr Mountain range in North Macedonia, with its broad altitudinal gradient and diverse microclimates, hosts extensive natural populations of *V. myrtillus* and offers an ideal setting to study environmental effects on fruit quality. (Abdi *et al.,* 2016). Limited published data are available on the phenolic composition and antioxidant properties of wild bilberry (*Vaccinium myrtillus* L.) fruits from the Balkan region. Existing studies have focused mainly on populations from Slovenia (Može *et al*., 2011; Mikulic-Petkovsek *et al*., 2012), Croatia (Jakobek *et al.,* 2007), Romania (Bunea *et al*., 2011), Serbia (Jovancevic *et al.,* 2011), Montenegro (Brašanac-Vukanović *et al.,* 2018), and Bosnia and Herzegovina (Akagić *et al.,* 2014). These wild types, distinguished by their higher acidity and unique flavor yet still underutilized compared to cultivated varieties, present significant scientific and economic interest for value-added products, especially amid growing regional and global demand for organic and functional foods (Veliu *et al.,* 2025).

No published data exist on the phenolic composition or antioxidant properties of bilberry fruits from North Macedonia. Therefore, the purpose of this research is to investigate differences and similarities among localities at various latitudes and elevations, with special emphasis on the antioxidant values of bilberries, including their anthocyanin and phenolic compound content.

MATERIAL AND METHODS

Material

Bilberry fruits from three different locations were collected: two natural populations growing at altitudes of 1829 m and 1936 m, and one population located at 467 m. The fruits were harvested during the first and second weeks of August 2022 (Figure 1). Following collection, five samples from each location were vacuum-sealed and refrigerated until analyses were conducted. The samples were evaluated for various parameters, including pH, dry matter, total acidity, color, total phenolic content, individual phenolic profile, and antioxidant capacity, using established analytical methods. Depending on the requirements of each method, the samples were either homogenized, diluted, or analyzed in their solid form.

Figure 1. Sampling localities in the Sharr Mountain, North Macedonia: B1 – Kobilica (1936 m), B2 – Kodra e Diellit (1829 m), B3 – Tetovo (467 m).

Methods

Chemical composition, Total phenolic and Antioxidant capacity assays

Dry matter content, pH, and total acidity were measured using standard AOAC procedures (AOAC, 1990). Samples (5 g) were extracted twice with 50 mL of 80 % aqueous methanol by homogenisation (Ultra-Turrax, 10 000 rpm, 2 min) followed by shaking (2 h, 25 °C). Extracts were combined, centrifuged (2800×g, 10 min), and filtered (0.45 µm) before analysis (Goztepe *et al.,* 2022). The Total Phenolic Content (TPC) of the bilberry samples was analyzed following a modified version of the method described by Singleton *et al*. (1999). In summary, 2.5 mL of Folin–Ciocalteu reagent (diluted 1:10 v/v) and 2 mL of Na_2CO_3 solution (7.5 g/100 g) were added to the methanol-diluted cranberry extracts. The mixture was placed in a test tube, sealed, and incubated in the dark for 30 minutes. After incubation, the absorbance was measured at 760 nm using a

UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). Total phenolic content was expressed as gallic acid equivalents (GAE) in mg per gram of dry matter (Figure 2).

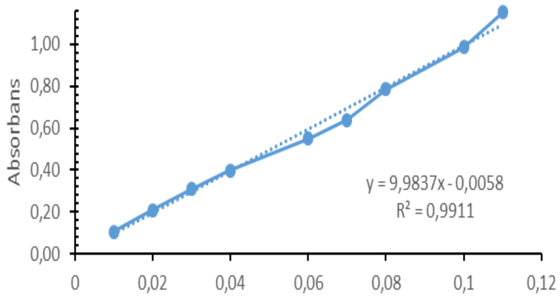

Figure 2. Gallic acid standard calibration curves

The antioxidant capacity of the extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. A 0.1 mL aliquot of blueberry extract was combined with 4.9 mL of DPPH• solution (0.1 mmol/L in methanol). After incubating the mixture in the dark for 30 minutes, the absorbance was recorded at 517 nm (Singh *et al.*, 2002). Results were expressed as milligrams of Trolox equivalents per gram of dry matter (mg TE/g DM). For the ABTS assay, the procedure was based on Arnao *et al.* (2001) with minor modifications. A fresh $ABTS^+$ radical solution was prepared for each measurement. A 100 µL portion of blueberry extract was mixed with 2 mL of the $ABTS^+$ solution and left to react in the dark for 2 hours. Absorbance was then measured at 734 nm using a spectrophotometer, and antioxidant activity was expressed as milligrams of Trolox equivalents (TE) per gram of dry matter. The Trolox standard curve showed linearity within the ranges of 100–1998 µM for DPPH and 10–799 µM for ABTS (Figure 3a, 3b).

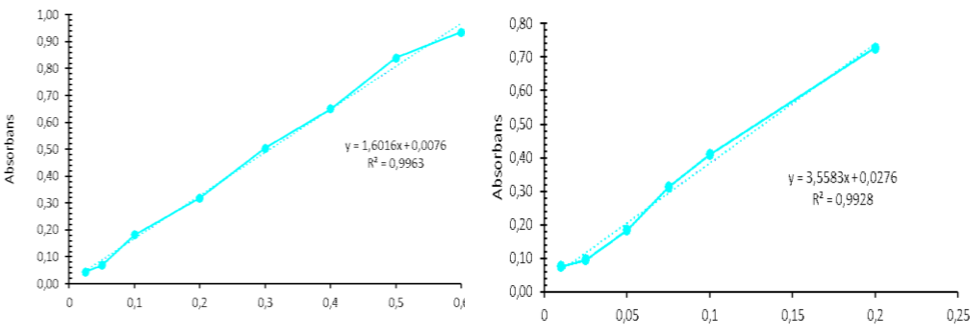

Figure 3. DPPH and ABTS standard troloxa calibration curves (a, b)

HPLC analysis of phenolics and anthocyanin profiles

Individual phenolic compounds were quantified by high-performance liquid chromatography with diode-array detection (HPLC-DAD) following the method of Özcan *et al*. (2023) with minor modifications, using a Shimadzu LC-20AD system (Shimadzu Corp., Kyoto, Japan). Extracts were prepared in methanol/water (50:50, v/v) at a liquid-to-solid ratio of 10:1 (v/w), centrifuged, and filtered through a 0.45 µm PTFE membrane prior to injection. Separation of phenolic acids was performed on a Waters Atlantis C18 column (250 mm×4.6 mm, 5 µm) using a linear gradient elution program with solvent A (formic acid/water, 1:99, v/v) and solvent B (formic acid/acetonitrile, 1:99, v/v) at a flow rate of 1 mL/min. The gradient conditions were as follows: 10–60% B from 0–15 min; 60% B (isocratic) from 15–20 min; 60–10% B from 20–25 min; and 10% B (isocratic) from 25–30 min. Chromatograms were monitored at 278, 320, and 360 nm, with spectral data collected from 190–400 nm. Phenolic acids were identified by comparing retention times and UV–Vis spectra of sample peaks with those of authentic standards. Quantification of phenolic compounds—including gallic acid, protocatechuic acid, catechin, 4-hydroxybenzoic acid, syringic acid, ellagic acid, m-coumaric acid, o-coumaric acid, chrysin, caffeic acid, p-coumaric acid, ferulic acid, myricetin, quercetin, kaempferol, rutin, sinapic acid, and chlorogenic acid—was performed using calibration curves generated for each standard. Results are expressed as mg per 100 g dry matter; compounds below the limit of quantification were reported as not detected (nd).

Determination of color in bilberries

The color of the three bilberry types was measured using a chromameter (Konica Minolta CR-400, NJ, USA). Color parameters included L* (lightness), where positive values indicate greater lightness and negative values indicate darkness; a* (green–red axis), where positive values reflect stronger green tones and negative values stronger red tones; and b* (yellow–blue axis), where positive values denote more intense yellow hues and negative values more intense blue hues. Additional color characteristics, Hue (overall color tone) and Chroma (color intensity), were also calculated to quantify color properties.

The color shades (Chroma) were calculated according to the formula:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

a* and b* are the components of chroma of the color in the CIE color space.
a* represents the green-red axis.
b* represents the blue-yellow axis.

Statistical analyses

Three localities (samples B1, B2, and B3) were included in a randomized design, with five samples per locality and two independent replicates (trials), to analyze response variables related to composition, color, and phenolic compounds. Analysis of variance (ANOVA) was performed using the general linear model procedure (SAS, 1995), with locality treated as a fixed effect and replicates as a random effect, and significance ($P<0.05$) determined using Duncan's test. Correlation analyses were conducted using the Kruskal-Wallis test and Pearson correlation as a non-parametric approach. To investigate relationships among phenolics within localities, principal component analysis (PCA) and heat mapping were carried out using SPSS software (IBM Corporation, 21.0, Armonk, NY, USA).

RESULTS AND DISCUSSION

Physicochemical parameters, total antioxidant and phenolics

The physicochemical properties of bilberry samples from different locations are presented in Table 1. Dry matter content was 18.0±0.26% for the B1 location, 17.6±0.14% for B2, and 16.8±0.29% for B3. These values are higher than those reported by Yörük (2019), who found dry matter levels ranging from 11.70 to 13.11 g/100 g. The pH of the bilberries varied between 2.40 and 2.77 across locations, which is slightly lower than values reported by Türkben *et al.* (2008) (2.77–2.95) and Yörük (2019) (3.03–3.16). Titratable acidity ranged from 1.56 to 1.77 g/100 g, with B3 showing the highest acidity and B1 the lowest (0.76±0.04). Yıldız (2012) reported acidity values of 0.27–1.65 g tartaric acid/100 g in 13 bilberry varieties collected from various regions of Turkey. Similarly, Türkben *et al.* (2008) found total acidity levels of 0.90–1.23 g citric acid/100 g in *Vaccinium myrtillus* L. samples from different areas of Uludağ. Overall, these results support previous findings by Zeng *et al.* (2020) and Correia *et al.* (2016), confirming that growing location significantly influences dry matter content, pH, and total acidity.

Table 1. Physical-chemical parameters of bilberries

Paremeter	*Unit*	*B1*	*B2*	*B3*
Dry matter	g/100 g	18.0±0.26^{c}	17.6±0.14^{b}	16.8±0.29^{a}
pH	-	2.77±0.02^{b}	2.87±0.02^{c}	2.49±0.01^{a}
Total acidity	% citric acid	1.56±0.021^{a}	1.59±0.043^{b}	1.77±0.021^{c}
L	-	32.47±1.15^{c}	25.18±2.94^{b}	22.33±1.58^{a}
a	-	3.60±0.39^{a}	6.74±0.17^{c}	4.83±1.47^{b}
b	-	-2.89±0.92^{c}	-4.75±0.17^{a}	-3.36±0.80^{b}
C	-	4.61±0.008^{a}	8.23±0.43^{c}	5.88±0.057^{b}

Means ± SD, n = 3. Values in the same row with different superscript letters differ significantly (Duncan test, $P < 0.05$).

The measured *L, a,* and *b* values of the bilberry fruits revealed notable differences in their color characteristics. The *L* values ranged from 22.33 to 32.47, while the *a* values varied between 3.60 and 6.74, and the b values ranged from –2.89 to –4.75 (Table 1). Among the samples, B1 exhibited the highest lightness (32.33±1.15), followed by B2 (25.18±2.94) and B3 (22.33±1.58). Sample B2 showed the greatest a value (6.74±0.17) and the highest chroma (C=8.23±0.43). Overall, these results demonstrate that location has a clear impact on the color attributes of the different bilberry samples.

Table 2. Total phenolic compounds, antioxidant capacity of bilberries

Localities	*Total polyphenol content (mgGAE/100g)*	*DPPH antioxidant capacity (mg TE/100 g)*	*ABTS antioxidant capacity (mg TE/100g)*
B1	2034.12±25.29[b]	717.72±17.17[b]	651,36±0.50[c]
B2	2008.82±22.48[b]	634.99±15.61[b]	409.97±2.50[a]
B3	1317.48±84.31[a]	447.68±18.73[a]	498.11±1.50[b]

aAbbreviation: DM, dry matter. Values are means ± SD; means within a column with different superscript letters differ significantly (P<0.05).

Samples from the highest altitude (B1, 1936 m) exhibited significantly higher total phenolic content than those from B2 and B3 (P<0.05). The antioxidant capacity of the bilberries from the three localities was calculated using the DPPH free radical and the ABTS free radical. The results showed that the B1 bilberries sample has higher antioxidant capacity compared to the B2 and B3 samples, whichh showed slightly lower antioxidant properties compared to the B1 sample. The marked increase in total phenolic content and antioxidant capacity with altitude agrees with previous reports and is attributed to greater UV-B exposure and cooler temperatures that enhance phenylpropanoid pathway activity (Zeng *et al*., 2020; Rossi *et al*., 2022).

Similarly, in samples harvested from localities exposed to the sun were detected higher amounts of total phenolics in comparison with those grown in shadow with total phenol from 3.92-5.24 mg GA/g dm (Jovancevic *et al.* 2011). Değirmencioğlu *et al.* (2017) evaluated the antioxidant capacity of bilberries (*Vaccinium myrtillus*) grown in Balıkesir using the DPPH and ABTS assays. They reported DPPH values ranging from 8.56 to 19.23 µmol TE/g and ABTS values between 4.26 and 9.56 µmol TE/g. In a similar study, Jakobek *et al*. (2007) measured the antioxidant capacity of Slovenian bilberries and found much higher values: 125.52 µmol TE/g using the DPPH method and 53.28 µmol TE/g using ABTS. Bunea *et al*. (2011) analyzed wild bilberries from Romania using the ABTS assay and reported antioxidant capacities ranging from 43.08 to 56.65 µmol TE/g. HPLC-DAD analysis revealed slight differences in the anthocyanin profiles of the first two locations (Figure 4; B1, B2). Peak identification was based on spectral characteristics and retention times. Although the qualitative anthocyanin composition was similar across sites, the peak areas varied. The B1 location exhibited 17 distinct anthocyanin peaks, while the third location (B3) showed fewer and weaker peaks, with a total of 14 identified anthocyanins.

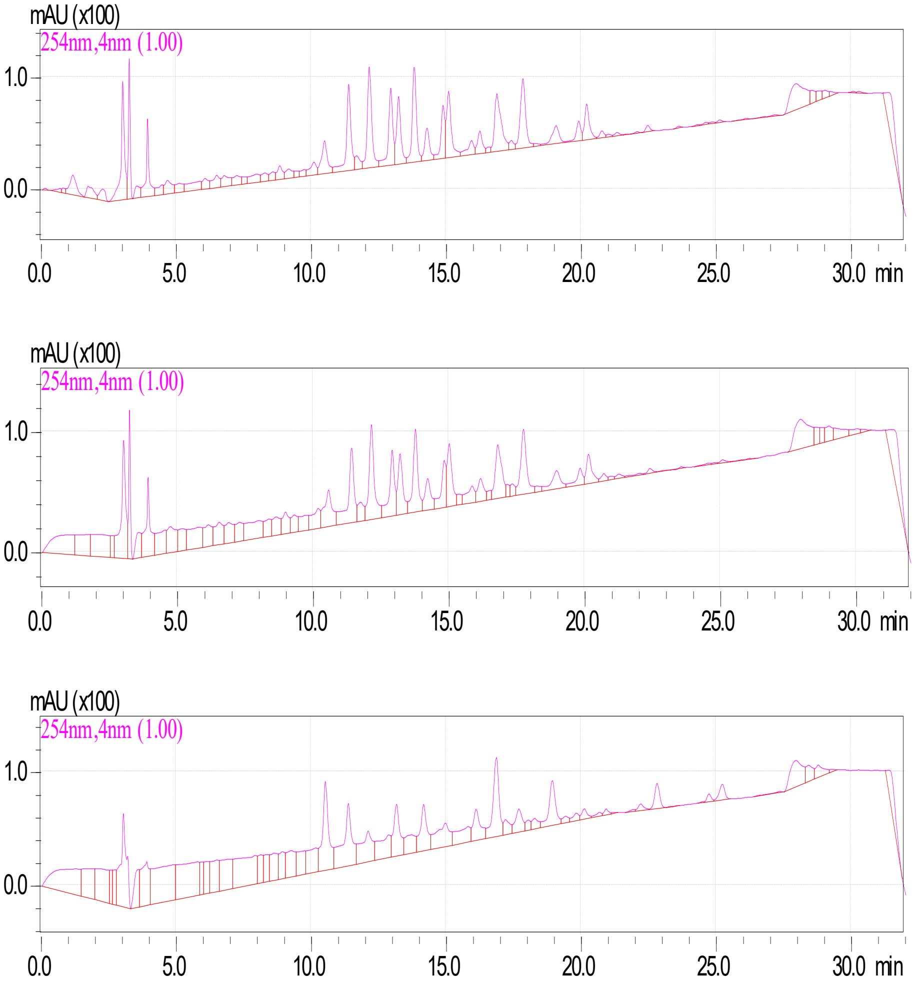

Figure 4. Representative HPLC-DAD anthocyanin chromatograms (520 nm) of bilberry extracts from localities B1, B2, and B3.

Phenolic compounds

A total of 17 phenolic compounds were identified in the bilberry fruits (Table 2). Flavonoids represented the largest group, with catechin, myricetin, and rutin occurring at notably higher concentrations in samples from the B3 location. Phenolic acids formed the second major group, with chlorogenic acid being most abundant in B3, while protocatechuic acid was present at higher levels in B1. The third group of polyphenols consisted of tannins, represented primarily by ellagic acid. Previous studies have consistently demonstrated that wild fruits are rich sources of health-promoting compounds such as gallic acid, chlorogenic acid, hydroxybenzoic acids, and rutin. Other important contributors to their nutritional value include quercetin-3-galactoside, kaempferol-3-O-glucoside, ellagic acid, and various ellagic acid derivatives. These findings emphasize the nutritional benefits and dietary importance of wild fruits (Deng *et al*., 2014; Natić *et al*., 2019; Drkenda *et al.,* 2014). The phenolic compound profiles varied among the bilberry samples from different locations. Catechin levels were significantly higher ($P<0.01$) in the B3 sample, while protocatechuic acid and ellagic acid were most abundant in the B1 sample. Only small amounts of quercetin and caffeic acid were detected, which aligns with previous reports indicating that these compounds typically occur in minor quantities in bilberries (Okan *et al*., 2018; Zimmer *et al*., 2014). Jakobek *et al.* (2007) analyzed methanolic extracts of bilberries (*Vaccinium myrtillus*) from Slovenia using HPLC and reported concentrations of 54.90 mg/kg p-coumaric acid, 9.57 mg/kg ferulic acid, and 136.97 mg/kg quercetin. In another study, Değirmencioğlu *et al*. (2017) examined bilberries grown in Balıkesir and identified a wide range of phenolic compounds, including 9.25–532.97 mg/kg vanillic acid, 28.79–995.15 mg/kg syringic acid, 1.81–20.05 mg/kg p-coumaric acid, 4.13–24.87 mg/kg ferulic acid, 1.62–6.93 mg/kg quercetin, and 4.42–79.37 mg/kg naringin.

Table 3. Phenolic compounds (mg/100g) of bilberries

Poliphenolic compounds	***B1***	***B2***	***B3***
PHENOLIC ACIDS			
Protocatechuic acid	52.35	49.61	n.d
4-Hydroxybenzoic acid	4.83	1.88	n.d
Syringic acid	0.91	0.32	9.73
m-Coumaric acid	0.01	n.d	n.d
o-Coumaric acid	n.d	0.17	n.d
Caffeic acid	1.60	1.51	2.20
p-Coumaric acid	1.29	1.06	0.80
Ferulic acid	1.50	0.48	0.38
Chlorogenic Acid	38.10	7.58	111.00
Sinapic Acid	1.23	0.14	0.03
FLAVONOIDS			
Catechin	66.67	45.81	164.56*
Myricetin	8.79	8.76	8.71
Quercetin	4.31	0.00	0.00
Kaempferol	1.73	1.69	0.00
Rutin	7.01	5.71	4.15
TANINS			
Ellagic acid	29.54	21.18	7.04

**significance at p<0.01*

3.5. Principal component analysis (PCA) and heatmap

Principal component analysis showed that the first two components accounted for 99.3% of the total variance (PC1: 82.9%, PC2: 16.4%). PC1 was strongly and positively associated with protocatechuic acid, ellagic acid, total phenolics, and antioxidant capacity, effectively separating the high-altitude samples (B1 and B2) from B3. PC2 was mainly influenced by catechin and chlorogenic acid, providing additional distinction of the B3 samples (Figure 5).

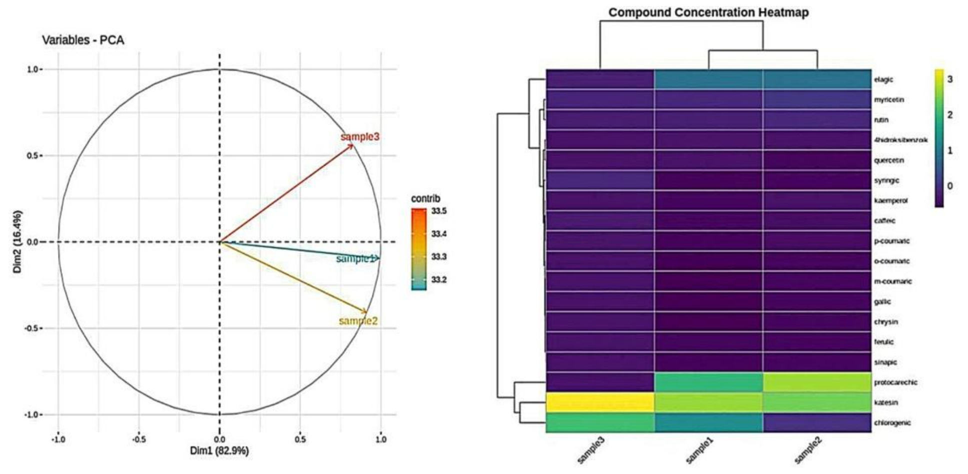

Figure 5. Principal component analysis (PCA) biplot (left) and heatmap (right) of phenolic compounds in bilberry fruits from the three localities

CONCLUSIONS

Altitude was the main factor influencing the phenolic content and antioxidant capacity of wild bilberry (*Vaccinium myrtillus* L.) fruits in the Sharr Mountain, North Macedonia. Fruits collected at 1829–1936 m exhibited significantly higher total phenolic content (589–651 mg GAE/100 g DM) and antioxidant capacity (612–718 mg TE/100 g DM by DPPH; 612–689 mg TE/100 g DM by ABTS) than fruits from 467 m. Individual phenolic compounds and anthocyanin profiles also varied with altitude, with higher concentrations of protocatechuic acid, ellagic acid, and total anthocyanins at the two higher sites. Protecting natural bilberry stands across the full altitudinal gradient is therefore relevant for preserving biodiversity and maintaining a source of high-quality raw material for the regional food and nutraceutical sectors. This study provides the first characterisation of phenolic and antioxidant properties of *V. myrtillus* fruits from North Macedonia.

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Gordan S. KARAMAN[1]

TWO SPECIES OF GENUS *NIPHARGUS* Schiödte, 1849 (FAM. NIPHARGIDAE) NEW FOR FAUNA OF ALBANIA (CONTRIBUTION TO THE KNOWLEDGE OF THE AMPHIPODA 346)

SUMMARY

From the subterranean waters of Vjosa and Shushica Rivers in southern Albania, with its tributaries, two species of genus *Niphargus* (*Crustacea, Amphipoda: Niphargidae*) known in some adjacent regions, are discovered at the first time in Albania, *Niphargus* (*Orniphargus*) *lourensis lourensis* Fišer *et al*., 2006, and *Niphargus* (*Jovoniphargus*) *multipennatus* Sket, 1956. As specimens of both species were rather damaged and not adult, both species are here only partially redescribed and figured, to recognize and confirm existence of these species in Albania.

Keywords: Taxonomy, subterranean waters, *Amphipoda*, *Niphargus*, southern Albania.

INTRODUCTION

The subterranean fauna of Crustacea on Balkan is moderately studied, despite the presence of numerous various rivers, torrents, springs, caves, lakes, etc. The similar situation is also in Albania, where diversity of the subterranean fauna of *Crustacea Amphipoda* is only moderately known. The present knowledge of this fauna suggests the presence of various endemic taxa as well as presence of some taxa known in the adjacent countries (Greece, Northern Macedonia, Serbia, Crna Gora) or entire Balkan. The results of investigation of subterranean *Niphargus* species (Fam. *Niphargidae*) from southern Albania, presented in this work, support these suggestions, where two known Niphargus species (from Greece and Balkan) are discovered at the first time in Albania.

Thanks to Dr Griebler from the University of Wien (Austria), I had the possibility to study Amphipoda material collected in Albania in the years 2021 and 2023 during the realization of the APPEAR project VjoSusDev of the Austrian Development Cooperation implemented by the OeAD. A part of these studies is presented in this work.

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Notes: The author declares that has no conflicts of interest. Authorship Form signed online.
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MATERIAL AND METHODS

The studied material was preserved in 70 percent ethanol. The specimens were dissected using a WILD M20 microscope. Dissected body-parts were submersed in a mixture of glycerin and water for study and drawing using a camera lucida. After study, body-parts were submerged in Liquid of Faure and covered by a thin cover glass creating definitive microscope slides. All illustrations were inked manually. The studied material was collected using various methods (Boxshall *et al.,* 2016).

Some morphological terminology and setal formulae follow G. Karaman's terminology (Karaman G., 1969, 2012) for mandibular palpus article 3 (A=A-setae on outer face of article; B=B-setae on inner face of article; D=short D-setae along lateral margin of article; E=long E-setae at distal part of article), as well as for distal corner of propodus of gnathopods 1 and 2 (S=corner palmar S-spine on outer face; L= slender serrate L-spines sitting near S-spine on outer face; M=row of long facial corner submarginal M-setae on outer face; R=subcorner R-spine on inner face).

Terms "setae" and "spines" are used based on its shape, not origin. The present study is based on morphological, ecological and zoogeographical data. In References are cited also number of figures as very important data in description of various taxa.

TAXONOMICAL PART

Order AMPHIPODA Latreille, 1816
Suborder SENTICAUDATA Lowry & Myers, 2013
Family NIPHARGIDAE G. Karaman, 1962 (sensu Bousfield, 1977: 303; 1982: 267).

Genus *NIPHARGUS* Schiödte, 1849

NIPHARGUS (ORNIPHARGUS) LOURENSIS LOURENSIS Fišer, Trontelj & Sket, 2006

Figures 1-5H, 7

Niphargus lourensis Fišer, Trontelj & Sket, 2006: 2288, figs. 7, 16–19; Sarbu, Brad, Băncilă, & Ştefan, 2024: 6;

Niphargus lourensis lourensis G. Karaman, 2018: 20; G. Karaman, 2020: 20, figs. 1-7; Ntakis, A., Karaouzas, I., Fišer, C. & Stoch, F., 2020: 531.

LOCUS TYPICUS: Spring of Louros River, Vouliasta, Ionannina, Greece.

MATERIAL EXAMINED:
ALBANIA:

VJ 09 at the bank of the Vjosa River (not a tributary) = the site is near the village of Gjonc; coordinates: 40.55689993797279, 19.636117233056243 , 1 juv. (4.9.2023), 1 juv. (24.4.2023) (leg. Ch. Griebler).

VJ 14 at the Vjosa River near the village of Leshnje; coordinates: 40.39202490519476, 19.822210337422828, 1 exp (8.9.2023) (leg. Ch. Griebler).

DESCRIPTION:

FEMALE not quite adult, 6.5 mm (VJ 14): Body moderately slender, metasomal segments with 1-2 dorsoposterior marginal setae (Fig. 1D); urosomal segment 1 with 2 spines at each dorsolateral side, urosomal segment 2 with 3 spines at each dorsolateral side, urosomal segment 3 naked (Fig. 3E). Urosomal segment 1 on each ventroposterior corner with one spine near basis of uropod 1 peduncle (Fig. 3E).

Epimeral plates with acute ventroposterior corner progressively increasing towards epimeral plate 3, epimeral plate 2 with 1 ventral spine, epimeral plate 3 with 2 ventral spines and rather concave posterior margin bearing several setae (Fig. 1D).

Head with short rostrum and subrounded lateral cephalic lobes (Fig. 1A). Antenna 1 reaching nearly half of body; peduncular articles progressively shorter (ratio: 58:46:31) main flagellum consisting of 18 articles (some articles with poorly visible single short aesthetasc); accessory flagellum short, 2-articulated, reaching nearly half of peduncular article 3 (Fig. 1B).

Antenna 2: article 3 poorly longer than broad, with distoventral group of setae (the longest seta exceeding diameter of article itself). Article 4 at dorsal margin with several short setae, along ventral margin with several setae of different length (the longest seta exceeding diameter of article itself); article 5 nearly as long as article 4 but more slender, along dorsal margin with several single or paired setae, along ventral margin with 3 bunches of setae; flagellum nearly as long as last peduncular article but more slender than article 5, consisting of 7 articles (Fig. 1C), antennal gland cone short.

Mouthparts well developed. Labrum broader than long, convex distally (Fig. 5A). Labium broader than long, inner lobes well developed, narrowed, outer lobes convex distally (Fig. 5B).

Left mandible: incisor with 5 teeth, lacinia mobilis with 4 teeth and 4 rakers. Right mandible: incisor with 4 teeth, lacinia serrate, accompanied by 5 rakers. Mandibular palpus 3-articulated: first article naked, second article with 10 setae; third article subfalciform, poorly longer than second one, provided with nearly 18 lateral D-setae and 5-6 distal long E-setae, on outer face appear one bunch of 3 A-setae (Fig. 1E), on inner face are attached 4 B-setae (Fig. 1F).

Maxilla 1: inner plate with 2 setae, outer plate with 7 spines bearing 1-4 lateral strong teeth each (Fig. 5D). Palpus 2-articulated: first article naked, second article not reaching distal tip of outer plate spines and bearing 3 distal setae (Fig. 5C).

Maxilla 2 longer than broad, both plates with numerous distal setae, inner plate with 3 distomesial setae (Fig. 1G).

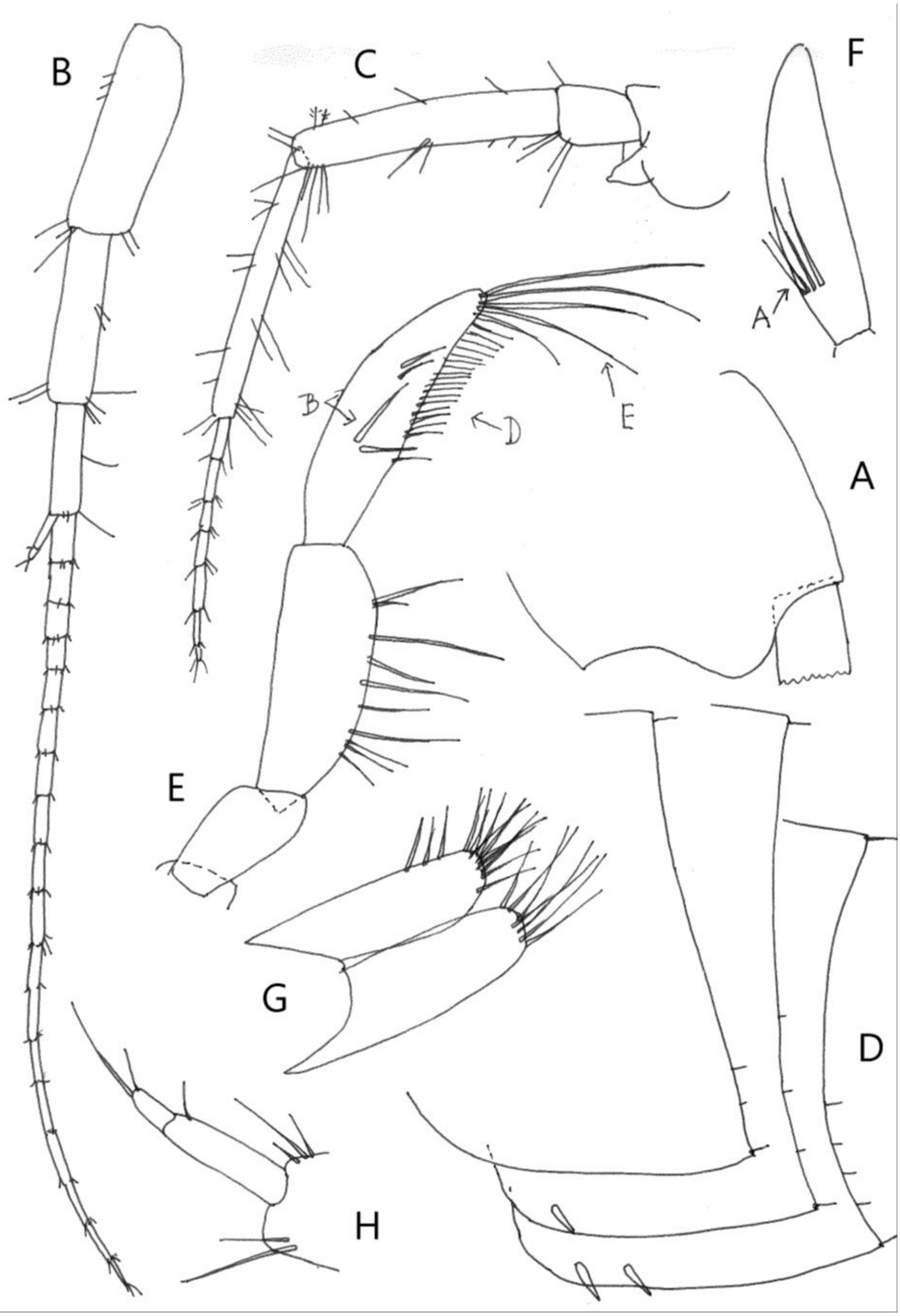

Fig. 1. *Niphargus lourensis lourensis* Fišer *et al.,* 2006, Albania, LK 7, VJ 552 [VJ 14 GWB], female juv. 6.5 mm.: A=head; B=Antenna 1; C=antenna 2; D=epimeral plates 1-3; E=palpus mandible inner face [D=marginal D-setae; E=distal E-setae; B=facial B-setae]; F=mandible palpus distal article, outer face [A=facial A-setae]; G=maxilla 2; H=maxilliped palpus distal article.

Maxilliped well developed: inner plate short, with 4 distal spines mixed with single setae; outer plate reaching nearly to the half of palpus article 2, along mesial margin with row of 7-8 single spines and with several distal setae; palpus article 3 at outer margin with distal group of setae, along mesial margin with group of setae; article 4 at outer margin with one median seta, at mesial margin with one seta near basis of the nail, nail exceeding half of article 4 (Figs. 1H, 5E).

Coxae moderately short. Coxa 1 nearly as long as broad, with ventroanterior part subrounded, bearing 3 setae (Fig. 2A). Coxa 2 longer than broad (ratio: 70:45), with subrounded ventral margin bearing 5-6 setae (Fig. 2D).

Coxa 3 longer than broad (ratio: 65:42), with 5-6 setae along convex ventral margin (Fig. 3A). **Coxa 4 rather longer** than broad (ratio: 62:48), with 6 setae at ventral convex margin, posterior excavation shallow (Fig. 3C).

Coxae 5-7 much shorter; coxa 5 remarkably broader than long (ratio: 55:37), anterior lobe subrounded (Fig. 4A). Coxa 6 bilobed, broader than long (ratio: 48:33) (Fig. 4C). Coxa 7 entire, much broader than long (ratio: 50:24) (Fig. 4F).

Gnathopods 1-2 moderately large. Gnathopod 1: article 2 with long setae in proximal part and rather shorter setae in distal part of both margins; article 3 with one distoposterior bunch of setae. Article 5 shorter than propodus (ratio: 35:55), along anterior margin with distal group of setae (Fig. 2A). Propodus rather smaller than corresponding coxa, trapezoid, nearly as long as broad, along posterior margin with 5 transverse groups of setae (Fig. 2B); palm poorly convex, inclined rather over half of propodus-length, defined on outer face by corner S-spine accompanied laterally by 2 L-spines (Fig. 2C) and 2 unequal corner facial M-setae (Fig. 2B), on inner face by one R-spine. Dactylus reaching posterior margin of propodus, along outer margin with 2-3 median setae, along mesial margin with 4-5 short setae (Fig. 2B).

Gnathopod 2 distinctly larger than gnathopod 1; article 2 with longer proximal marginal setae and short setae in distal part; article 3 with distoposterior group of 3 short setae; article 5 rather shorter than propodus, with group of distoanterior marginal setae (Fig. 2D). Propodus trapezoid, nearly as long as broad, along posterior margin with 6 transverse groups of setae. Palm slightly convex, inclined remarkably over half of propodus-length (Fig. 2E), defined on outer face by corner S-spine accompanied by one L-spine sitting partially behind S-spine, accompanied by 2 unequal corner facial M-setae, on inner face by one R-spine (Fig. 2F). Dactylus with 4 short single median setae along outer margin, along mesial margin with several short setae (Fig. 2E).

Pereopods 3-4 moderately strong. Pereopod 3: article 2 with several long setae in proximal part. Articles 4-7 of different length (ratio: 50:30:35:14), scarcely setose (Fig. 3A), articles 4-5 at both margins with single short setae or spine-like setae; article 6 at anterior margin with single short setae, at posterior margin with 4 single or groups of setae or spine-like setae. Dactylus short and rather strong, with one slender spine-like seta or seta at inner margin near basis of the nail, at outer margin with one median short plumose seta, nail rather shorter than pedestal (Fig. 3B).

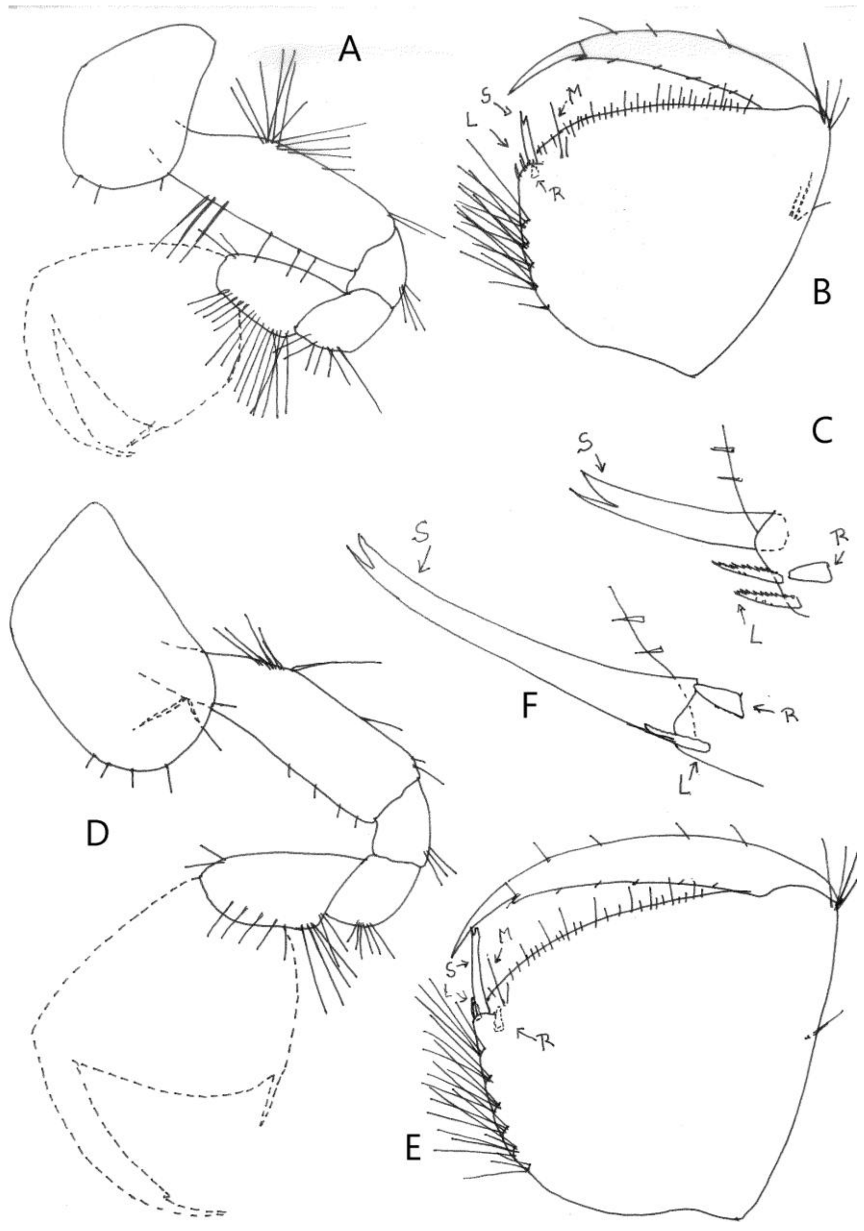

Fig. 2. *Niphargus lourensis lourensis* Fišer et al., 2006, Albania, LK 7, VJ 552 [VJ 14 GWB], female juv. 6.5 mm.: A-B=gnathopod 1, outer face [S=corner S-spine; L-lateral L-spines; M=corner facial M-setae; R=subcorner R-spine, inner face); C=gnathopod 1 propodus, distal corner, inner face [S=corner S-spine; L- lateral L-spines; R=subcorner R-spine, inner face); D-E=gnathopod 2, outer face [S=corner S-spine; L- lateral L-spines; M=corner facial M-setae; R=subcorner R-spine, inner face] F=gnathopod 2 propodus, distal corner, inner face [S-spine; L- lateral L-spines; R=subcorner R-spine, inner face].

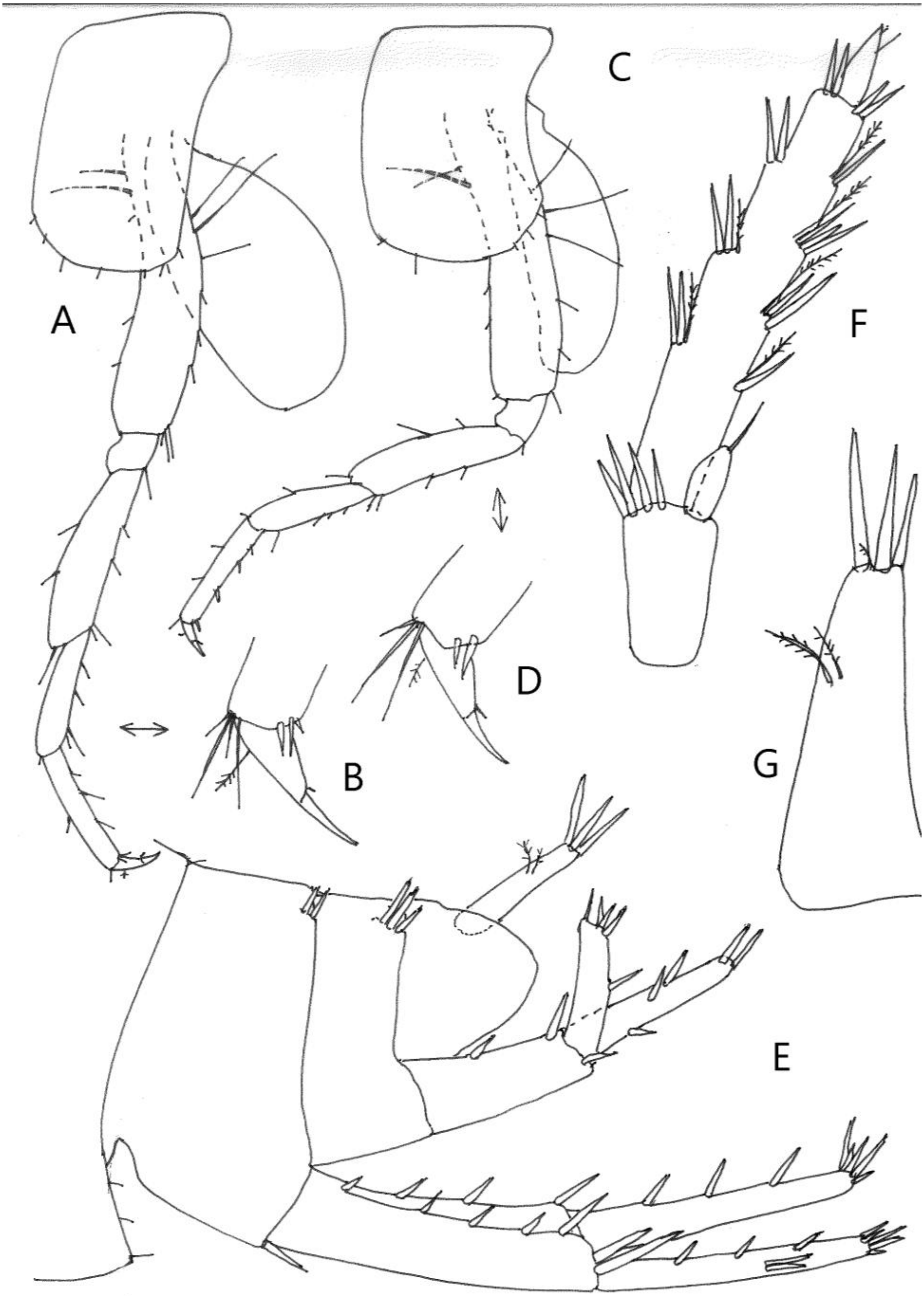

Fig. 3. *Niphargus lourensis lourensis* Fišer *et al.,* 2006, Albania, LK 7, VJ 552 [VJ 14 GWB], female juv. 6.5 mm.: A-B=pereopod 3; C-D=pereopod 4; E=urosome with uropods 1-2; F=uropod 3; G=telson.

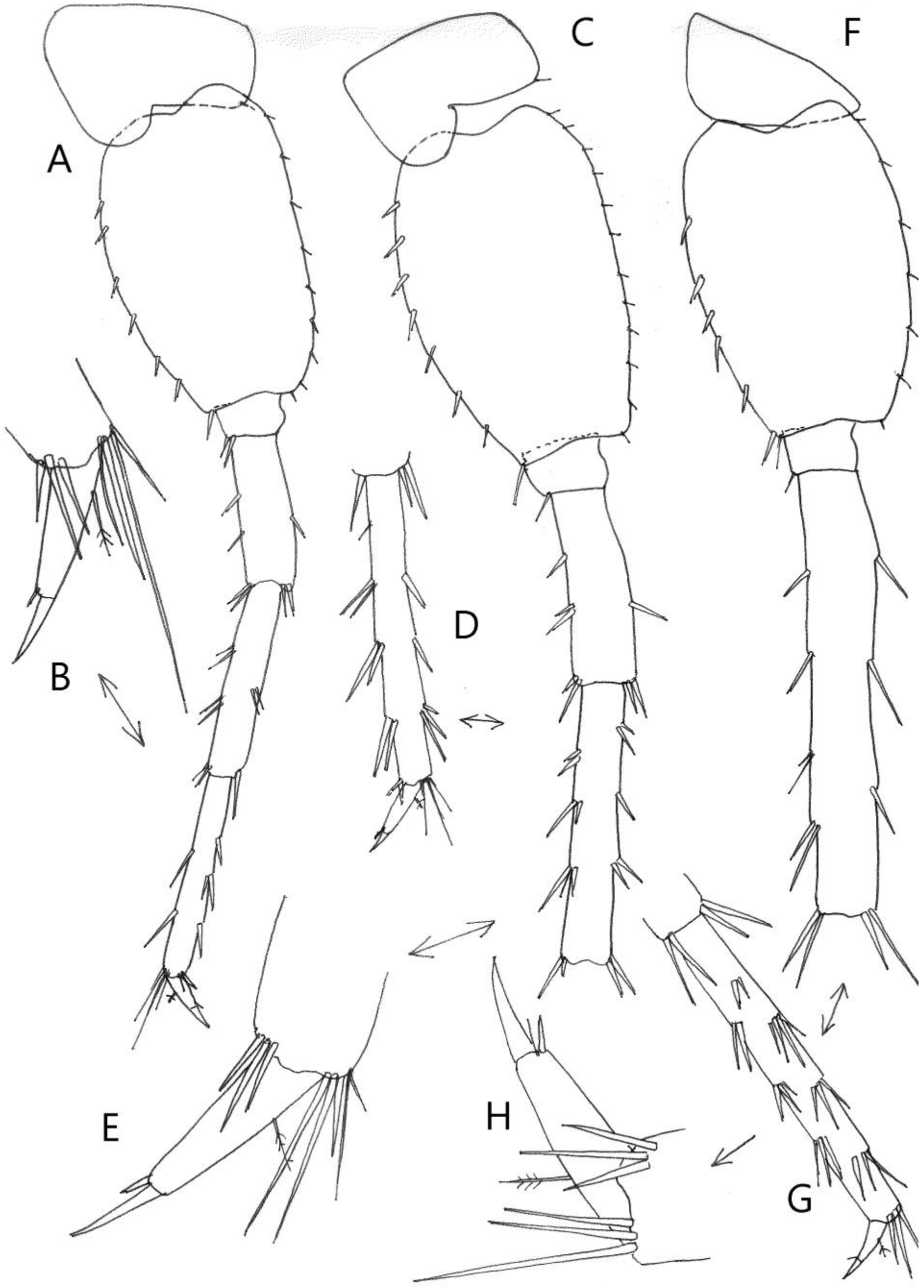

Fig. 4. *Niphargus lourensis lourensis* Fišer *et al*., 2006, Albania, LK 7, VJ 552 [VJ 14 GWB], female juv. 6.5 mm.: A-B=pereopod 5; C-D-E=pereopod 6; F-G-H=pereopod 7.

Pereopod 4 very similar to pereopod 3, including its pilosity. Articles 4-7 of different length (ratio: 41:31:30:12), article 6 along posterior margin with 4 single slender spines (Fig. 3C). Dactylus short and strong, at inner margin with one spine-like seta, at outer margin with one median plumose seta, nail rather shorter than pedestal (Fig. 3D).

Pereopods 5-7 moderately strong. Pereopod 5 rather shorter than pereopods 6-7; article 2 dilated, longer than broad (ratio: 84:52), anterior margin remarkably convex, provided with 7-8 single short spines, along posterior less convex margin with 9 short setae, ventroposterior lobe not distinctly developed (Fig. 4A). Articles 4-7 of different length (ratio: 39:53:55:18), anterior margin of articles 4-5 with several spine-like setae or slender spines, along posterior margin with 2 groups of spines on each article; article 6 along both margins with several longer slender spines, at top with group of longer setae. Article 6 distinctly shorter than article 2. Dactylus with slender spine at inner margin near basis of the nail, and one media plumose seta at outer margin, nail shorter than pedestal (Fig. 4B).

Pereopod 6: article 2 longer than broad (ratio: 90:57), along anterior convex margin with 8 single spines, along posterior less convex margin with 12 short setae, ventroposterior dilatation not forming lobe (Fig. 4C, D). Articles 4-7 of different length (ratio: 51:75:83:24), articles 4-6 along both margins with single and groups of spines (the longest spines exceeding diameter of article itself). Article 2 only slightly longer than article 6. Dactylus at inner margin with one slender spine near basis of the nail, at outer margin with one median plumose seta, nail shorter than pedestal (Fig. 4E).

Pereopod 7: article 2 dilated, longer than broad (ratio: 85:56), anterior convex margin with 6-7 single spines, along convex posterior margin with 7 short setae, ventroposterior lobe absent (Fig. 4F, G). Articles 4-7 of different length (ratio: 50:68:95:22), articles 4-6 along both margins with single or groups of spines, the number of spines increasing towards article 6. Article 2 is shorter than article 6. Dactylus at inner margin with spine mixed with one lateral short seta, at outer margin with one median plumose seta; nail shorter than pedestal (Fig. 4H).

Pleopods with 2 retinacula. Peduncle of pleopod 1 with 3 single setae along anterior margin (Fig. 5F); peduncle of pleopod 2 with one median seta at posterior margin (Fig. 5G); peduncle of pleopod 3 with 2 single setae at posterior margin (Fig. 5H).

Uropod 1: peduncle with dorsoexternal and dorsointernal row of strong spines (Fig. 3E); inner and outer ramus of the same length, both provided with several lateral and 5 unequal distal spines.

Uropod 2: inner ramus only poorly longer than outer one, both rami with single lateral and group of distal spines (Fig. 3E).

Uropod 3 relatively short, strong, not produced; peduncle slightly longer than broad, with row of distal strong spines (Fig. 3F); inner ramus short, scale-like, with distal strong seta; outer ramus strong, at inner margin with 5 groups of strong spines mixed usually with one plumose seta; second article short, not exceeding diameter of first article, narrow, bearing one lateral and 1 subdistal short seta.

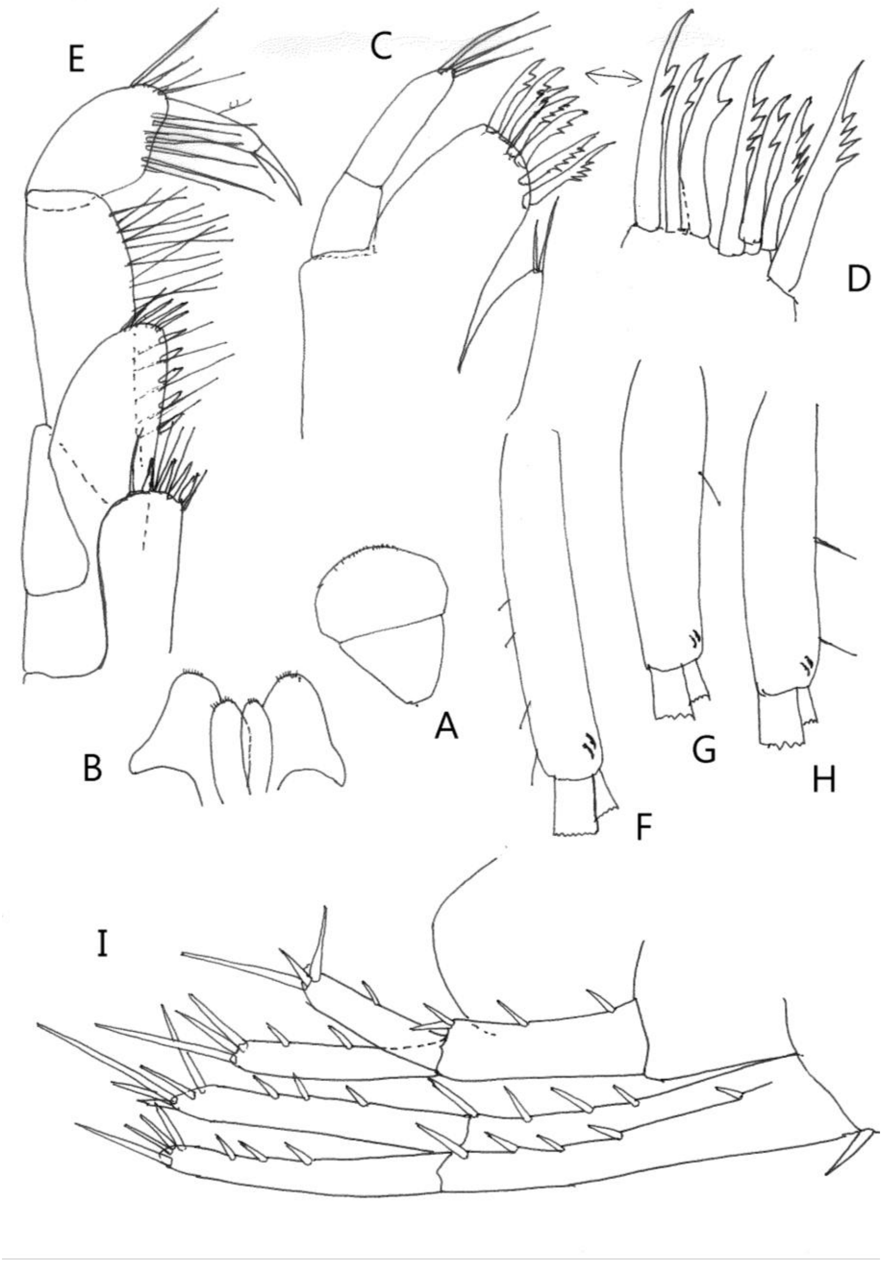

Fig. 5. *Niphargus lourensis lourensis* Fišer *et al*., 2006, Albania, LK 7, VJ 552 [VJ 14 GWB], female juv. 6.5 mm.: A=labrum; B=labium; C-D=maxilla 1; E=maxilliped; F=peduncle of pleopod 1; G=peduncle of pleopod 2; H=peduncle of pleopod 3. *Niphargus multipennatus* Sket, 1956, Albania, AL 5 (Vjo 003-Shus 17): left bank of the Shushica River, juv. 3.3 mm: I=urosome with uropods 1-2.

Telson longer than broad, deeply incised, each lobe with 3 unequal distal spines, lateral and facial spines absent; a pair of short plumose setae sitting rather over half of outer margin of each lobe (Fig. 3G).

Coxal gills ovoid, not exceeding ventral tip of corresponding article 2 (Fig. 3A, C). Oostegites not fully developed, without lateral setae.

REMARKS AND AFFINITY

The single not fully developed female corresponds by its morphological characters to *Niphargus lourensis lourensis* Fišer *et al*., 2006. To support this conclusion, we described specimen from Albania more in detail.

At the moment, *N. lourensis lourensis* was known as endemic from Greece only, in various localities mentioned by Fišer *et al*. (2006) and by G. Karaman (2020). G. Karaman (2018) recognized the various populations of *Niphargus* from Skiros Island (Greece) by several morphological differences as a distinct subspecies, *Niphargus lourensis skirosi* G. Karaman, 2018.

By this way, *Niphargus lourensis lourensis* is the new known species for fauna of Albania.

Sarbu *et al*. (2024) cited this species for Sulfur cave as hypogenic cave located in the Vromoner Canyon, on the border between Greece and Albania (40.0961 N, 20.6789 E). It has a small entrance that opens into the Sarandaporo River, a tributary of the Aaos/ Vjosa River.

Niphargus lourensis belongs to the well limited group of species, subgenus *Orniphargus* S. Karaman (1950) by all its characters (short uropod 3, large propodus of gnathopods 1-2 with several setae along outer margin, mouthparts, epimeral plates, uropod 1, telson). Some authors use a different name for it, as morphotypes (Fišer *et al*., 2009), species-aggregate (Fišer *et al.,* 2006), complex of species (Ntakis *et al.,* 2015), molecular phylogenies (Borko *et al*. 2019), etc.

***NIPHARGUS* (*JOVONIPHARGUS*) *MULTIPENNATUS* Sket, 1956**
Figures 5 I, 6, 7

Niphargus jovanovici multipennatus Sket, 1956: 71, fig. 3; Sket, 1959: 105; Sket, 1972: 106, figs. 25-42; G. Karaman, 1972: 5; G. Karaman, 1974: 19; Barnard, J. & Barnard C., 1983: 692;

Niphargus (*Jovaniphargus*) *jovanovici multipennatus* S. Karaman, 1960: 77;

Niphargus multipennatus G. Karaman, 1980: 19; G. Karaman & Ruffo, 1986: 528; G. Karaman, 1988: 25; G. Karaman, 1994: 32; G. Karaman, 1997: 45; Kralj, 2001: 148. G. Karaman, 2021: 13-19, figs. 6-9.

MATERIAL EXAMINED:

ALBANIA:

AL 5 (Vjo 003-Shus 17): left bank of the Shushica River between the new and old bridge of Gjorm (Ura e Gjormit), 2.5 km north of the city of Gjorm, June 2021 (Latitude 40.3384000249207, Longitude 19.6387849655002}, one damaged juv. female mixed with *Fingerhadzia zorae* G. Karaman, 2024 (leg.: C. Karwautz, G. Rasch & C. Griebler).

LOCUS TYPICUS: Stožice near Ljubljana, Slovenia.

DISTRIBUTION: Slovenia, Croatia, Bosnia & Herzegovina, Serbia (Kosovo & Metohia), Albania (new) (Fig. 9).

REMARKS.

Despite the single specimen from Albania was very juvenile of 3.3 mm and damaged, based on its existing morphological characters we recognize that this specimen belongs to the species *Niphargus multipennatus* Sket, 1956.

Head with short rostrum and subrounded lateral cephalic lobes (Fig. 6A), metasomal segment 3 with 1 dorsoposterior marginal seta, epimeral plate 3 strongly angular (Fig. 6E). Urosomal segment 1 on both ventroposterior corners with one strong spine near basis of the uropod 1-peduncle (Fig. 5 I).

Articles of main flagellum in antenna 1 moderately long (Fig. 6B), some of them with one longer aesthetasc rather shorter than article itself) (Fig. 6B). Mandibular palpus article 1 naked, article 2 with 2 setae, article 3 subfalciform, with 4 distal E–setae 2 D-setae and 1 A-seta (Fig. 6C) [reduced number of setae because very juv.].

Maxilliped: inner plate with 3-4 distal spines, mesial margin of outer plate with row of short spines, palpus article 4 with one seta at ventral margin near basis of the nail and one median seta at outer margin (Fig. 6D).

Gnathopods 1-2 with propodus large, almost ovoid, of nearly subequal size, with one median seta on outer margin of its dactylus, and strongly inclined palm.

Gnathopod 2-propodus strongly inclined, defined on outer face by corner S-spine accompanied by one spine-like L-spine and by 2 corner facial M-setae (Fig. 6F), on inner face by 1 subcorner R-spine; dactylus rather exceeding diameter of propodus, along outer margin with one short median seta, along inner margin with several short setae.

Pleopods 1-3 with 2 retinacula, peduncles scarcely setose or naked.

Uropod 1: peduncle longer than rami, provided with dorsoexternal and dorsointernal row of strong spines (Fig. 5 I); rami of the same length, bearing 2-3 lateral strong spines and 5 unequal strong distal spine each (the longest distal spine on inner ramus slightly exceeding half of ramus-length.

Uropod 2: peduncle with 1-2 lateral and distal spines; inner ramus with 2 lateral and 4-5 distal unequal strong spines (the longest spine on inner ramus reaching 2/3 of ramus length) (Fig. 5 I); outer ramus rather shorter than outer one, with one lateral and 3-4 unequal distal spines (the longest spine poorly shorter than ramus itself).

Uropod 3 missing.

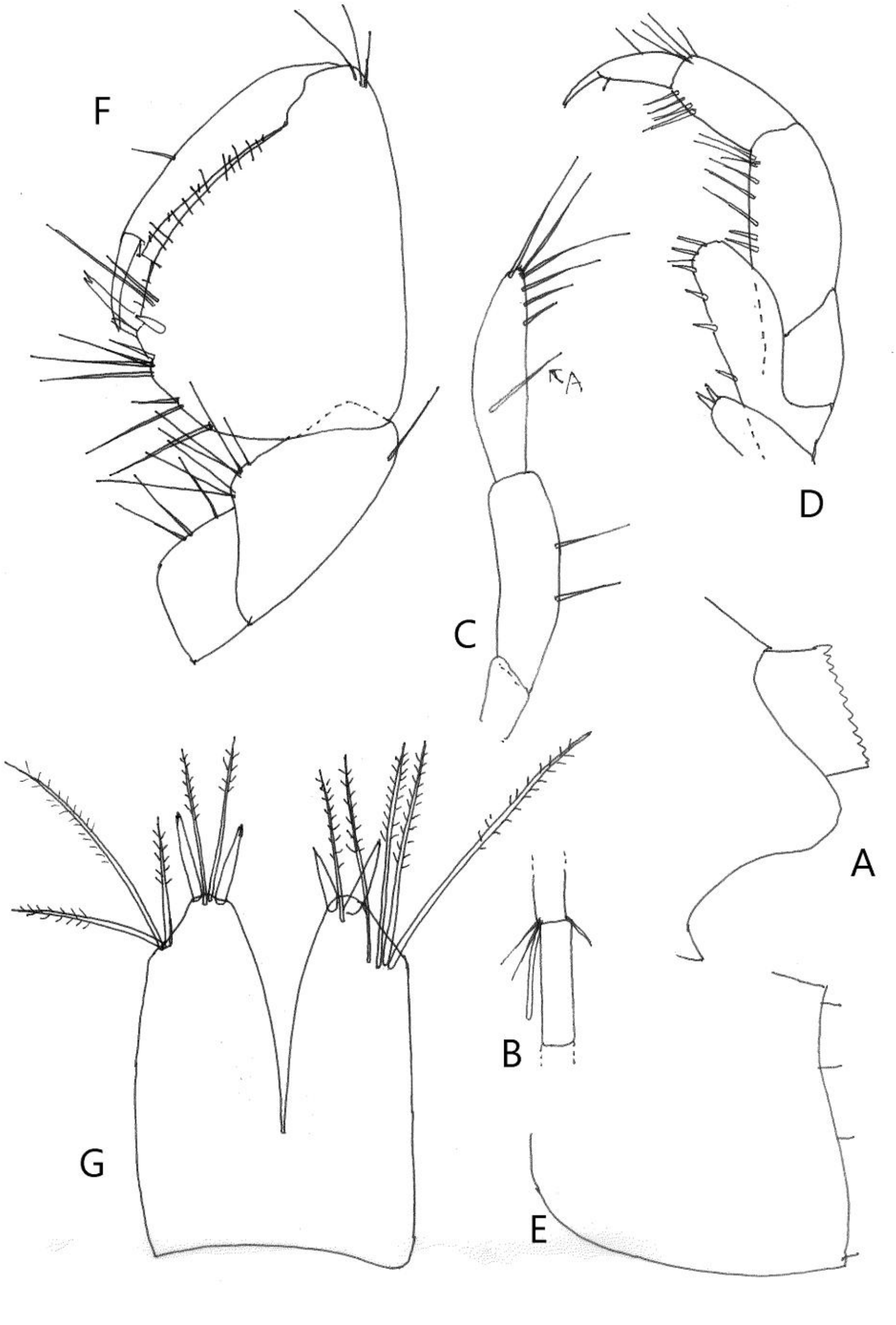

Fig. 6. *Niphargus multipennatus* Sket ,1956, Albania, AL 5 (Vjo 003-Shus 17): left bank of the Shushica River, juv. 3.3 mm: A=head; B=aesthetasc on antenna 1 main flagellum; C=mandibular palpus, outer face; D=maxilliped; E=epimeral plate 3; F=gnathopod 2 propodus, inner face; G=telson.

Telson is the most characteristic of this species: longer than broad (ratio: 95:75), incised almost 2/3, each lobe tapering distally, bearing 2 distal spines and 5 very long plumose setae (Fig. 6G).

Discovery of additional specimens from this locality will probably support our determination.

We used the term Subgenus *Jovaniphargus* S. Karaman, 1960 for that group of morphologically similar taxa named often by various authors as *N. jovanovici* Group of species, species aggregate, etc.

The recent authors usually ignore subgeneric level of some morphologically well-defined subgenera ("‘the obsolete subgenus *Phaenogammarus* Dudich, 1941, etc. (Copilaș-Ciocianu, 2017), etc. (G. Karaman, 2025b).

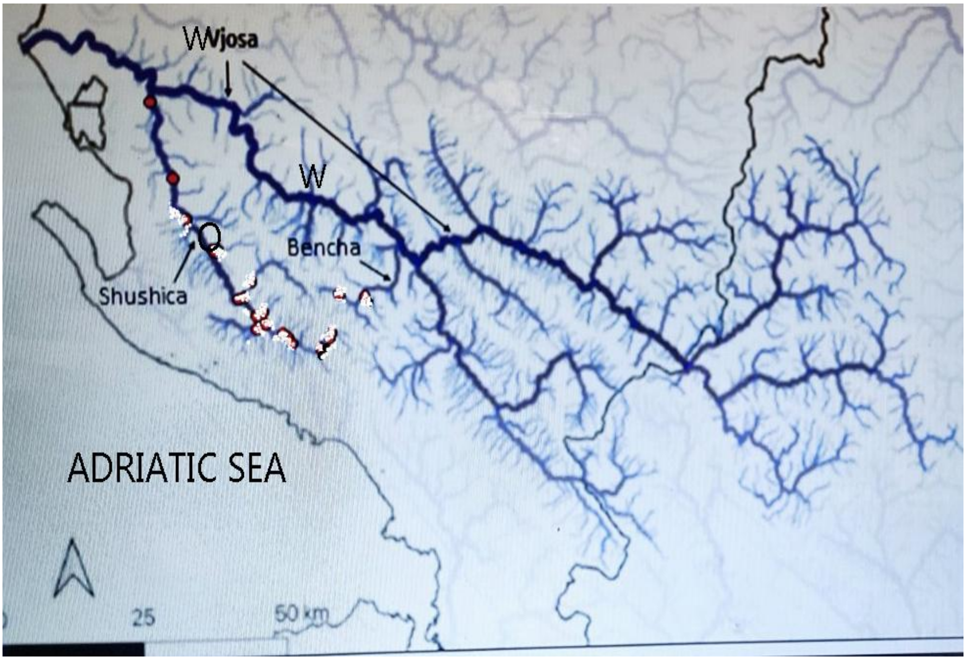

Fig. 7. Localities of two *Niphargus* species in Albania:
O=*Niphargus multipennatus* Sket 1956
W=*Niphargus lourensis lourensis* Fišer *et al*. 2006

KEY TO THE SPECIES OF *NIPHARGUS* IN ALBANIA:

1. Dactylus of gnathopods 1-2 with one median external marginal seta……………………………2
-- Dactylus of gnathopods 1-2 with 3 or more median external marginal setae …………………… 3

2. Telson with 5-6 distal plumose long setae on each lobus…………. ***MULTIPENNATUS*** Sket 1956
---Telson with 2 short lateral plumose setae on each lobus……………. ***GRIEBLERI*** G. Karaman 2023

3. Telson with distal spines only……………………………………………………………………4
-- Telson with distal, lateral and facial spines……………………………………..……………… 5

4. Spines on maxilla 1 outer plate with several teeth each. Uropod 1 peduncle with dorsointernal row of spines. Urosomal segment 1 with 2 spines on each dorsolateral side
....……***LOURENSIS LOURENSIS*** Fišer et al. 2006
-- Spines on maxilla 1 outer plate with 1 lateral tooth; Uropod 1 peduncle with dorsointernal row of setae (except distal spine); urosomal segment 1 with 1 seta on each dorsolateral side
…………………………………………………………………***LEPUSHENSIS*** G. Karaman 2025a

5.-Uropod 1 in male with inner ramus distinctly longer than outer ramus…***TOMORI*** G. Karaman 2011
---Uropod 1 both rami in male nearly of equal length………………………………………………6

6. Epimeral plate 3 strongly produced, plates 2-3 with subventral setae. Pereopods 5-7 stouter
……………………………………………………………..………O***HRIDANUS*** S. Karaman 1929
----Epmeral plate 3 obtusely angular, plates 2-3 with subventral spines, pereopods 5-7 more slender
…………………………………………………………………***BENCAENSIS*** G. Karaman 2025b

ACKNOWLEDGEMENTS.

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