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MEAT PERFORMANCE AND PHYSICO-CHEMICAL EVALUATION OF WOOD PIGEON (*Columba palumbus*) MEAT

SUMMARY

The present study evaluated the meat performance and physico-chemical properties of the wood pigeon (*Columba palumbus*), one of the most widespread game species in Europe, with the aim of assessing its nutritional potential and suitability as an alternative source of high-quality protein. A total of 20 pigeons (10 males, 10 females) were analysed for carcass characteristics, overall chemical composition, amino acid and fatty acid profiles, cholesterol content and colour parameters. The average live body weight was 476.7 g, with significantly higher values in males (514.8 g) than in females (457.7 g; $P=0.031$). The carcass yield averaged 67.2%, which is comparable with domesticated meat-type pigeons. Breast muscles contained 24.1% protein, 0.72% intramuscular fat, and 46 mg/100 g cholesterol, confirming the lean and protein-rich nature of this species. Amino acid analysis revealed lysine, leucine and arginine as the predominant essential amino acids, highlighting its high-quality protein profile. The fatty acid composition was dominated by monounsaturated fatty acids (47.2%), followed by saturated fatty acids (31.9%) and polyunsaturated fatty acids (17.0%), with oleic acid being the most abundant fatty acid. Wood pigeon meat was darker ($L^* \approx 35$) than conventional poultry meat, reflecting its greater myoglobin content. Compared with domesticated pigeons and various poultry species, *C. palumbus* meat was found to be leaner, richer in protein, and nutritionally favourable in terms of amino acid and lipid composition. These findings confirm its value as a meat with potential to diversify sustainable protein sources in the human diet.

Keywords: wood pigeon, meat, chemical composition, amino acids, fatty acids, meat colour

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INTRODUCTION

The increasing global demand for high-quality animal protein highlights the importance of identifying sustainable alternatives to conventional poultry species such as chicken and turkey. Among various poultry species, pigeon meat stands out for its premium qualities due to its rich nutritional profile and unique taste. Among wild avian species, the wood pigeon (*Columba palumbus*), one of the most widespread game birds in Europe, has attracted growing attention as a potential source of nutritious and lean meat.

Pigeon meat represents a traditional yet currently marginal component of global animal production. Its consumption is markedly lower compared with major meat types, primarily due to the absence of industrial-scale production and the fact that pigeon rearing for meat (“squab production”) is carried out predominantly on small, regionally oriented farms (Hunter *et al.*, 2010; Calder, 2015). Market reports show that global pigeon meat production constitutes only a minor segment of the poultry sector and is geographically unevenly distributed, with greater significance in certain regions of Asia, the Middle East, and the Mediterranean, where its consumption is historically rooted (Simopoulos, 2016). Within the European Union, pigeon meat is not recorded as a distinct category in livestock production statistics but is classified under “other meats,” a group that over the long-term accounts for less than 1% of total meat consumption, highlighting its limited market prevalence (Mensink *et al.*, 2003; Eur-Lex, 2013). Its wider expansion is also constrained by specific legislative and sanitary requirements governing the production and marketing of non-standard animal-derived foods, including EU regulations related to animal products and consumer health protection, which emphasize strict conditions for processing and food-safety control (Hunter *et al.*, 2010). In addition to regulatory constraints, cultural and consumer preferences play an important role, as pigeon meat is perceived in many European and non-European countries primarily as a culinary specialty than a regular dietary component. Overall, the prevalence of pigeon meat consumption remains low, and its production and utilization are significant mainly within specific traditional and regional contexts.

Pigeon meat is recognized for its high nutritional value, characterized by low fat content and high digestibility, making it a desirable source of protein for human consumption (Chang *et al.*, 2023a; Chang *et al.*, 2023b). Due to its favourable chemical composition, pigeon meat is often recommended for health-conscious individuals and those seeking lean protein options. Understanding the nutritional makeup of pigeon muscle, particularly regarding conventional components such as water, crude protein, and crude fat, is essential for evaluating its value as a food source (Winiarska-Mieczan *et al.*, 2016).

In addition to proximate and nutritional parameters, physico-chemical and technological traits play a crucial role in determining the overall performance of meat (Sule *et al.*, 2024). Comparative evaluations have revealed that pigeons, both domestic and wild, typically produce leaner and more protein-rich muscle tissues than conventional poultry, although their meat can be perceived as darker and less tender due to the predominance of oxidative muscle fibres adapted for sustained flight (Listrat *et al.*, 2016).

Despite its nutritional advantages and cultural significance in game meat traditions, scientific data on the meat quality of the wood pigeon remain limited. Most previous investigations have concentrated on domestic pigeons (*C. livia domestica*) (Pomianowski *et al.*, 2009; Sule *et al.*, 2024), leaving a gap in comprehensive characterization of *C. palumbus*. Understanding its proximate composition, as well as its physico-chemical properties, is therefore essential for assessing its potential role as a sustainable protein source in modern diets.

The aim of this study was to evaluate the meat performance and physicochemical properties of wood pigeon meat by assessing its basic chemical composition, amino acid and fatty acid profile, and colour. By comparing available data with data from other pigeon species and conventional poultry, this work provides new insights into the nutritional value, processing suitability, and quality attributes of *C. palumbus* meat.

MATERIAL AND METHODS

The biological material used in the study was 20 wood pigeons (10 females, 10 males) from the hunting area in Piešťany (Slovak Republic). The pigeons were not fed; they consumed only natural forage. The age of the pigeons (approximately 3 months) was estimated based on their development of plumage, ossification of the skull (pneumatization of the skull) and the stage of wing feather interweaving in accordance with EU and hunting biology standards. The pigeons were shot legally (Hunting Act of the Slovak Republic 274/2009 Coll.). After killing they were weighed using a WTC 2000 laboratory scale with an accuracy of 0.01 g (RADWAG, Poland) and transported in cooling boxes at 4 °C to the Institute of Food Sciences of the Slovak University of Agriculture in Nitra for further analyses.

Slaughter and measurements

After defeathering, the carcasses were weighed using a laboratory scale WTC 2000 with an accuracy of 0.01 g (RADWAG, Poland) and subsequently meat and carcass yields were determined by dissection according to Haščík *et al.* (2024). Samples were taken from the most valuable parts of the carcass (breast muscle - musculus pectoralis major and thigh muscle - musculus biceps femoris) during dissection and stored at 4 °C for meat-quality assessment, including colour, and analysed 24 hours after slaughter. At the same time, additional organs were extracted and weighed, including shanks, head, digestive system, giblets, heart, liver, gizzard, neck, CY (%), breast part, thigh part, wings. Carcass yield was calculated as the weight of the carcass together with offal as a percentage of the live weight of the pigeons. The yield of the breast muscle and the yield of the thigh muscle were calculated as a percentage of the eviscerated body weight.

Chemical composition

The basic chemical composition of the wood pigeon meat samples (water, crude protein, crude fat, and cholesterol content) was examined using the

INFRATEC 1265 instrument (Germany), which operates in transmittance mode within a range of 2 nm from 850 to 1050 nm. After being homogenized, the 50 g samples were put into a 90×90×15 mm glass cup and scanned twice. Each sample's spectrum was calculated as $\log 1/T$ (T =transmittance) and represented the average of five scan locations. The results are given in g/100 g. Every determination was made three times.

Amino acid composition

The determination of amino acids was performed using the method of Straková *et al.* (2015) on an automatic amino acid analyser AAA 400 (Ingos a.s., Prague, Czech Republic). The pigeon meat (5 g) was placed in a 50 ml polypropylene centrifuge tube and then 15 ml of hydrochloric acid solution with a concentration of 0.02 mol/l was added and mixed in a vortex mixer until the sample was fully dispersed. The mixture was made up to 25 ml and then centrifuged for 10 minutes at 8800 rpm and 4 °C. Then 2 ml of supernatant was collected, and 2 ml of 8% salicylic acid solution was added. After 30 minutes of maintenance at 4 °C and 10 minutes of centrifugation at 10,000 rpm, the supernatant was removed and washed with 2 ml of n-hexane. The mixture was centrifuged for 10 min at 10,000 rpm and then filtered through a 0.22 µm membrane before analysis using an automatic amino acid analyser L-8900 (Hitachi High-tech Co., Ltd., Shanghai, China). For the determination of individual amino acids, acid hydrolysis in 6 N HCl at 110 °C for 24 h was performed. Quantification was based on the colour reaction of amino acids with the oxidising agent ninhydrin. After conversion to 100% dry matter, the resulting amino acid values were expressed in grams of amino acid content per 100 g of muscle. Two sets of determinations were performed.

Fatty acid composition

Fat was extracted from the lyophilized samples using Soxhlet extraction with petroleum ether. The methodology of Soxhlet extraction was adapted from Luque de Castro and Priego-Capote (2010) and modified by ISO 12966-2:2017: preparation of methyl esters of fatty acids, animal and vegetable fats, and oils. The fat content was expressed as g/kg of dry matter and fatty acids methyl ester profile in %.

The chemical reagents used for fat and fatty acid determination were: petroleum ether 40–65 °C (Sigma–Aldrich, Steinheim, Germany), methanol for chromatography (Sigma–Aldrich, Steinheim, Germany), NaHSO₄ H₂O (Centralchem, Bratislava, Slovakia), Na₂SO₄ (Centralchem, Bratislava, Slovakia), KOH (Centralchem, Bratislava, Slovakia), HCl 33–36% (Centralchem, Bratislava, Slovakia), filter medium Hyflo Super Cel (diatomaceous earth) (Sigma–Aldrich, Steinheim, Germany), anhydrous N-hexane for chromatography (Sigma–Aldrich, Steinheim, Germany), 42 component standard FAME mix 10 mg/mL in methylene chloride containing C4–C24 FAME (2–4% relative concentration), manufacturer Supelco, catalog number 47885-U chromatography (Sigma–Aldrich, Steinheim, Germany). Apparatus: vacuum oven (Fisher

Scientific, Waltham, MA, USA) temperature adjustable (100 ± 3 °C). Tecator Soxtec System HT 1043 Extraction unit (Gemini, Apeldoorn, the Netherlands) apparatus at a flow rate of 10 cycles/h or 10 mL/min.

Meat colour measurement

Instrumental colour measurements of meat samples were performed using a spectrophotometer (Konica Minolta CM-2600d, Osaka, Japan) with the setting Specular Component Included (SCI). D65 light source and a 10° observer, with a port 8 mm in diameter were used. The white plate calibration was performed at 23 °C, as suggested by the manual. The results were coordinates in the colour interface of the Commission International de l'Eclairage (CIE) $L^* a^* b^*$ system ($L^*=0$, black; $L^*=100$, white; $100+a^*=$ redness; $-a^*=$ greenness; $+b^*=$ yellowness; $-b^*=$ blueness). Using the optically passive glass aperture cover that came with the colorimeter to ensure a consistently level sample surface, colour measurements were made at three random positions on each sample (Bianchi *et al.*, 2005). A white tile was used to calibrate the colorimeter (Minolta calibration plate: C:Y=93.66, $=0.3150$, $y=0.3217$).

Statistical analysis

All analyses were performed in triplicate, unless the description indicates otherwise. Results were reported as means $\pm$ standard deviations of measurements. Data were analysed using Student t-test at a significance level of $P\leq 0.05$ ($\alpha=0.05$) to test for differences between mean values. Data were analysed using XLSTAT® software (version 2018.5.52280, Addinsoft, New York).

RESULTS AND DISCUSSION

Meat performance

The average meat performance values of Wood pigeon (*C. palumbus*) are given in Table 1.

Table 1. Meat performance of Wood pigeon (*C. palumbus*) (g)

Parameter	♀	♂	♀+♂	P-value (♀:♂)
Live BW	457.70 $\pm$ 42.07	514.80 $\pm$ 21.37	476.73 $\pm$ 45.18	0.031
CW	275.08 $\pm$ 28.78	304.83 $\pm$ 10.33	284.99 $\pm$ 27.76	0.078
Shanks	7.26 $\pm$ 0.60	7.45 $\pm$ 0.72	7.33 $\pm$ 0.62	0.643
Head	16.41 $\pm$ 1.91	16.15 $\pm$ 0.69	16.33 $\pm$ 1.57	0.799
Digestive system	23.55 $\pm$ 4.65	23.80 $\pm$ 4.78	23.63 $\pm$ 4.47	0.932
Giblets	33.80 $\pm$ 4.47	37.85 $\pm$ 1.85	35.15 $\pm$ 4.20	0.119
Heart	5.78 $\pm$ 0.67	7.03 $\pm$ 1.12	6.19 $\pm$ 1.00	0.033
Liver	10.48 $\pm$ 2.26	10.95 $\pm$ 1.11	10.63 $\pm$ 1.91	0.705
Gizzard	10.14 $\pm$ 1.61	11.43 $\pm$ 1.33	10.57 $\pm$ 1.59	0.200
Neck	7.41 $\pm$ 1.26	8.45 $\pm$ 0.77	7.75 $\pm$ 1.19	0.165
CY (%)	67.50 $\pm$ 3.48	66.63 $\pm$ 2.67	67.21 $\pm$ 3.13	0.671
Breast part	138.88 $\pm$ 17.08	158.83 $\pm$ 8.23	145.53 $\pm$ 17.34	0.045
Thigh part	27.93 $\pm$ 3.88	32.85 $\pm$ 2.25	29.57 $\pm$ 4.10	0.043
Wings	56.85 $\pm$ 5.54	61.75 $\pm$ 1.90	58.48 $\pm$ 5.13	0.123

Notes: BW – live body weight; CW – carcass weight; CY – carcass yield; mean $\pm$ SD (standard deviation); means significant differences between rows ($P\leq 0.05$) determined with Student t-test.

The average live body weight of wood pigeons in this study was 476.7 g, with males (514.8 g) being significantly heavier than females (457.7 g; $P=0.031$). Correspondingly, carcass weights were higher in males (304.8 g) than in females (275.1 g), although this difference did not reach statistical significance ($P=0.078$). Carcass yield averaged 67.2%, which is consistent with previously reported values for domestic meat-type pigeons, typically ranging from 65 to 70% (Pomianowski *et al.*, 2009; Sule *et al.*, 2024). The breast portion accounted for the largest share of the carcass (145.5 g on average), with males showing a significantly greater breast-muscle weight (158.8 g vs. 138.9 g; $P=0.045$). Similarly, thigh weights were higher in males (32.9 g) compared to females (27.9 g; $P=0.043$).

No significant differences were observed between sexes in liver and digestive tract weights, as well as stomach and neck weights. These patterns correspond with the findings of Haščík *et al.* (2024) in California pigeons. Male feral pigeons showed significantly larger hearts compared to females (7.0 g vs. 5.8 g; $P=0.033$). The relative weight of offal (35.15 g) suggests that wood pigeons provide an additional source of edible by-products, although in lower quantities than domestic pigeons (Haščík *et al.*, 2024), but their commercial use in game species remains limited.

Chemical Composition

The proximate composition of breast and thigh muscles of wild wood pigeon (*C. palumbus*) confirmed the species' lean and protein-rich nature (Table 2). Breast muscle contained on average 73.51% moisture, 24.09% protein, 0.72% intramuscular fat, and 0.046 g/100 g cholesterol, whereas thigh muscle contained 72.74% moisture, 23.31% protein, 1.47% fat, and 0.049 g/100 g cholesterol. A significant sex-related difference was found only in intramuscular fat of breast muscle, with males exhibiting higher fat levels (1.02%) than females (0.57%; $P=0.005$). Protein and cholesterol contents did not differ between sexes.

Table 2. Chemical composition of the most valuable parts of Wood pigeon (*C. palumbus*) (g/100 g)

Parameter	♀	♂	P-value (♀:♂)	♀ + ♂
Breast muscle				
Water	73.53±0.71	73.48±0.36	0.893	73.51±0.62
Total Protein	24.25±0.34	23.79±0.27	0.056	24.09±0.38
Crude Fat	0.57±0.22	1.02±0.10	0.005	0.72±0.28
Cholesterol	0.044±0.00	0.050±0.01	0.053	0.046±0.01
Thigh muscle				
Water	72.91±0.48	72.41±0.61	0.185	72.74±0.58
Total Protein	23.18±0.45	23.58±0.32	0.178	23.31±0.45
Crude Fat	1.52±0.34	1.37±0.37	0.534	1.47±0.36
Cholesterol	0.050±0.01	0.046±0.01	0.312	0.049±0.01

Notes: mean±SD (standard deviation); means significant differences between rows ($P\leq 0.05$) determined with Student *t*-test.

These results highlight that wild wood pigeon meat is a nutritionally valuable lean meat, with higher protein levels and much lower fat compared with those of domesticated pigeons. Pomianowski *et al.* (2009) reported that meat-type pigeons such as Wrocław, King, and Europigeon had breast protein levels ranging from 21.7 to 23.6%, while intramuscular fat was substantially higher (4.3–7.1%). Kokoszyński *et al.* (2020) also documented fat levels exceeding 4% in Carrier and King pigeons, together with slightly lower protein values (~22%). In contrast, *C. palumbus* displayed protein levels above 24% and fat below 1%, making it considerably leaner than all investigated domesticated breeds.

Cholesterol concentrations in *C. palumbus* breast muscle (~46 mg/100 g) were like or slightly higher than those reported in domesticated pigeons (23.6–44.4 mg/100 g; Pomianowski *et al.*, 2009). Despite minor variability, both domestic and wild pigeons present cholesterol contents comparable to chicken breast (~47 mg/100 g; Ponte *et al.*, 2004), and markedly lower than in turkey (34–85 mg/100 g; Komprda *et al.*, 2002) or ostrich (63–68 mg/100 g; Horbańczuk *et al.*, 1998).

Amino acid composition

The amino acid profile of *Columba palumbus* meat (Table 3) demonstrated high nutritional quality, with breast muscles containing higher concentrations of most essential amino acids than thigh muscles.

Table 3. Amino acid composition of Wood pigeon (*C. palumbus*) meat (g/100 g)

AA/Group	Muscle	♀	♂	P-value (♀:♂)	♀ + ♂
Threonine	Thigh	1.02±0.09	0.96±0.10	0.421	1.00±0.10
	Breast	1.13±0.10	0.99±0.08	0.047	1.08±0.11
Valine	Thigh	0.94±0.06	0.90±0.06	0.333	0.93±0.06
	Breast	1.02±0.08	0.96±0.07	0.283	1.00±0.08
Methionine	Thigh	0.73±0.07	0.68±0.07	0.359	0.71±0.07
	Breast	0.80±0.07	0.79±0.06	0.906	0.80±0.07
Isoleucine	Thigh	0.91±0.10	0.85±0.11	0.462	0.89±0.11
	Breast	1.07±0.11	0.99±0.09	0.266	1.05±0.11
Leucine	Thigh	1.82±0.18	1.71±0.21	0.423	1.79±0.20
	Breast	2.09±0.21	1.91±0.16	0.175	2.03±0.21
Phenylalanine	Thigh	0.92±0.09	0.87±0.10	0.428	0.91±0.09
	Breast	1.07±0.11	0.98±0.08	0.222	1.04±0.11
Lysine	Thigh	1.94±0.20	1.83±0.23	0.459	1.90±0.21
	Breast	2.30±0.24	2.13±0.20	0.296	2.25±0.24
Cysteine	Thigh	0.31±0.03	0.30±0.03	0.478	0.31±0.03
	Breast	0.33±0.02	0.31±0.02	0.156	0.32±0.03
Histidine	Thigh	1.01±0.10	0.93±0.11	0.250	0.98±0.11
	Breast	1.02±0.10	0.98±0.08	0.480	1.01±0.10
Arginine	Thigh	1.45±0.15	1.38±0.17	0.473	1.43±0.16
	Breast	1.73±0.18	1.59±0.14	0.248	1.69±0.18

Notes: mean±SD (standard deviation); means significant differences between rows ($P \leq 0.05$) determined with Student *t*-test.

Lysine (2.25 g/100 g), leucine (2.03 g/100 g), and arginine (1.69 g/100 g) were the predominant essential amino acids, confirming the superior protein quality of pigeon breast meat. A significant sex effect was observed only for threonine in breast muscle, with higher concentrations in females (1.13 g/100 g) than in males (0.99 g/100 g; $P=0.047$).

These results are consistent with earlier findings in domesticated pigeons, where sex-related differences in amino acid composition were negligible (Pomianowski *et al.*, 2009). When compared with domestic meat-type breeds such as King, Carrier, and Europigeon, *C. palumbus* showed comparable or higher values for lysine and leucine (Kokoszyński *et al.*, 2020). The high lysine and leucine content reflects the metabolic specialization of pectoral muscles and underlines the suitability of wild pigeon meat as a high-quality protein source.

Dietary proteins of animal origin provide a complete profile of essential amino acids, making meat a nutritionally dense component of the human diet. Essential amino acids such as lysine, leucine, threonine and histidine - whose intake must be ensured through food (WHO/FAO/UNU Expert Consultation, 2007) - perform diverse physiological functions. Lysine plays a critical role in collagen synthesis and tissue repair, and its biochemical relevance extends to epigenetic regulation through lysine residues in histone modification (Shi and Whetstone, 2007), which underlines the importance of adequate lysine intake during growth and regeneration. Arginine, although conditionally essential, is indispensable during metabolic stress due to its role as the precursor of nitric oxide and its involvement in vascular regulation and wound healing (Wu and Morris, 1998). Leucine, a branched-chain amino acid, functions as a potent anabolic signal via activation of the mTORC1 pathway and thus directly regulates skeletal-muscle protein synthesis and post-exercise recovery (Kimball and Jefferson, 2006; Wolfe, 2017). Threonine is required for the synthesis of mucins that maintain intestinal barrier integrity, and deficiencies impair mucosal protection and gastrointestinal immune function (Bertolo and Burrin, 2008). Histidine serves as a precursor for biologically active compounds such as histamine, carnosine, and anserine, contributing to antioxidant defense, tissue growth, and immune modulation (Brosnan and Brosnan, 2020). Given that deficits of essential or conditionally essential amino acids compromise immune competence and increase susceptibility to meat, by providing these amino acids in an optimal ratio, constitutes a key dietary source supporting growth, tissue repair, metabolic homeostasis, and immune resilience, particularly in periods of elevated physiological demand.

Fatty acid composition

The fatty acid profile of *C. palumbus* revealed a balanced composition, dominated by monounsaturated fatty acids (MUFA), with significant contributions from saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) (Table 4).

Table 4. Fatty acid composition of Wood pigeon (*C. palumbus*) meat (g/100 g)

FA/Group	Muscle	♀	♂	P-value (♀:♂)	♀ + ♂
Lauric	Thigh	0.10±0.01	0.11±0.01	0.684	0.10±0.01
	Breast	0.09±0.01	0.09±0.00	0.180	0.09±0.01
Myristic	Thigh	1.34±0.02	1.37±0.04	0.168	1.35±0.03
	Breast	1.41±0.01	1.38±0.03	0.028	1.40±0.02
Palmitic	Thigh	24.36±0.20	24.46±0.24	0.506	24.40±0.22
	Breast	24.21±0.18	24.13±0.24	0.578	24.18±0.20
Heptadecanoic	Thigh	0.29±0.03	0.30±0.01	0.935	0.30±0.03
	Breast	0.22±0.03	0.28±0.02	0.004	0.24±0.04
Stearic	Thigh	10.78±0.17	10.86±0.11	0.480	10.81±0.16
	Breast	10.47±0.24	10.52±0.11	0.691	10.49±0.21
Oleic	Thigh	35.75±1.61	34.63±3.57	0.513	35.38±2.50
	Breast	14.55±7.09	19.36±4.16	0.007	19.48±9.53
Vaccenic	Thigh	4.77±0.06	4.80±0.08	0.578	4.78±0.07
	Breast	4.97±0.10	4.91±0.03	0.231	4.95±0.09
Linoleic	Thigh	6.68±0.82	5.85±1.06	0.199	6.41±0.99
	Breast	6.64±0.41	9.21±1.92	0.008	7.50±1.67
Conjugated linoleic	Thigh	0.13±0.02	0.13±0.01	0.983	0.13±0.02
	Breast	0.10±0.02	0.12±0.01	0.138	0.11±0.02
α-Linolenic	Thigh	0.27±0.03	0.26±0.03	0.426	0.27±0.03
	Breast	0.24±0.03	0.28±0.02	0.035	0.25±0.03
Eicosenoic	Thigh	0.52±0.07	0.57±0.08	0.250	0.54±0.07
	Breast	0.50±0.13	0.61±0.11	0.216	0.54±0.14
Arachidonic	Thigh	1.67±0.28	1.79±0.28	0.518	1.71±0.29
	Breast	1.65±0.23	1.79±0.31	0.451	1.70±0.27
Eicosapentaenoic	Thigh	0.09±0.02	0.09±0.02	0.812	0.09±0.02
	Breast	0.06±0.02	0.09±0.01	0.040	0.07±0.02
Docosapentaenoic	Thigh	0.13±0.01	0.14±0.01	0.367	0.13±0.01
	Breast	0.12±0.01	0.12±0.01	0.906	0.12±0.01
Docosahexaenoic	Thigh	0.03±0.00	0.04±0.00	0.496	0.03±0.00
	Breast	0.03±0.01	0.03±0.00	0.748	0.03±0.01
Omega 3	Thigh	0.57±0.06	0.54±0.08	0.436	0.56±0.007
	Breast	0.62±0.04	0.61±0.04	0.602	0.62±0.04
Omega 6	Thigh	12.46±0.56	11.35±1.02	0.049	12.09±0.91
	Breast	11.05±0.77	12.45±0.69	0.019	11.52±0.99
Σ SAFA	Thigh	34.79±1.30	34.26±0.80	0.507	34.61±1.18
	Breast	31.70±0.84	32.23±0.31	0.299	31.88±0.75
Σ MUFA	Thigh	49.41±0.55	49.69±0.71	0.500	49.50±0.62
	Breast	46.68±1.33	48.15±1.38	0.136	47.17±1.51
Σ PUFA	Thigh	14.05±0.83	13.56±1.35	0.496	13.88±1.06
	Breast	16.92±1.47	17.31±1.46	0.701	17.05±1.48

Notes: mean±SD (standard deviation); means significant differences between rows ($P \leq 0.05$) determined with Student t-test

In breast muscles, SFA accounted for 31.9%, MUFA for 47.2%, and PUFA for 17% of total fatty acids, while in thigh muscles the respective values were 34.6%, 49.5%, and 13.9%. Palmitic acid (24.2%) and stearic acid (10.5%) were the principal SFAs. Oleic acid represented the dominant MUFA, with significantly higher concentrations in males (19.36 g/100 g) than in females (14.55 g/100 g; $P=0.007$). The PUFA fraction was mainly represented by linoleic (7.5%), arachidonic (~1.7%), and α -linolenic acid (0.25%). Minor but nutritionally relevant amounts of long-chain n-3 PUFA, including eicosapentaenoic and docosapentaenoic acids, were also detected.

The predominance of MUFA, particularly oleic acid, is consistent with previous studies in domesticated pigeons (Pomianowski *et al.*, 2009; Kokoszyński *et al.*, 2020). However, the higher PUFA proportion observed in *C. palumbus* is in line with findings in wild pigeons by Sakhawat *et al.* (2023), who demonstrated a more favourable PUFA/SFA ratio and higher n-3 fatty acid content in wild compared with farmed pigeons. This difference reflects the ecological impact of diet, with wild pigeons consuming seeds, acorns, and green vegetation, while domesticated breeds are reared on cereal-based diets.

When compared with conventional poultry, the fatty acid composition of *C. palumbus* reveals specific differences. The PUFA content is lower than that of chicken breast (20–25%; Ponte *et al.*, 2004), but like ostrich meat (10–12%; Horbańczuk *et al.*, 1998), whereas the MUFA fraction is considerably higher than in both chicken and turkey (Komprda *et al.*, 2002). This places wood pigeon meat in an intermediate position between poultry and ratites, combining the benefits of MUFA predominance with adequate levels of essential PUFA.

Dietary fatty acids present in meat, particularly palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1n-9), play distinct physiological roles that influence lipid metabolism, energy homeostasis, and cardiometabolic health. Palmitic and stearic acids represent major saturated fatty acids (SAFA); however, their metabolic effects differ—palmitic acid is associated with increased LDL-cholesterol and pro-inflammatory signaling, whereas stearic acid is considered neutral due to its rapid desaturation to oleic acid via stearyl-CoA desaturase (SCD1) (Mensink *et al.*, 2003; Hunter *et al.*, 2010). Oleic acid, the predominant monounsaturated fatty acid (MUFA) in meat, is linked to improvements in plasma lipid profile, insulin sensitivity, and modulation of inflammatory pathways, contributing to its recognized cardioprotective potential (Schwingshackl and Hoffmann, 2012). From a broader perspective, the balance between saturated (SAFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) in the diet is crucial: while excessive SAFA intake may elevate cardiovascular risk, MUFA and PUFA - especially n-3 and n-6 PUFA - participate in membrane fluidity, eicosanoid synthesis, and regulation of gene expression related to lipid metabolism (Calder, 2015; Simopoulos, 2016). Meat contributes substantially to total fatty acid intake and, depending on species and feeding practices, may serve as an important source of oleic acid and essential PUFA, thereby influencing overall dietary fat quality.

Colour attributes of wood pigeon meat

Instrumental colour analysis confirmed the dark, myoglobin-rich character of wood pigeon meat. Breast muscle exhibited $L^*=35.0$, $a^*=13.4$, $b^*=10.8$, while thigh muscle showed $L^*=35.6$, $a^*=13.6$, $b^*=11.6$. No significant sex differences were observed (Table 5).

Table 5. Evaluation of Wood pigeon (*C. palumbus*) meat colour

Muscle	L^*	a^*	b^*
Breast ♂	35.63±6.58	13.75±1.73	11.50±3.19
Breast ♀	34.72±4.80	13.16±4.07	10.49±2.66
P-value	0.788	0.793	0.573
Thigh ♂	33.70±6.51	12.65±2.16	10.02±3.92
Thigh ♀	36.59±4.76	14.00±1.80	12.35±2.16
P-value	0.397	0.274	0.203
Breast ♂ + ♀	35.02±5.16	13.36±3.38	10.83±2.75
Thigh ♂ + ♀	35.63±5.29	13.55±1.95	11.57±2.91
P-value (♂ + ♀)	0.779	0.866	0.526

Notes: mean±SD (standard deviation); means significant differences between column ($P \leq 0.05$) determined with Student t-test

Compared with chicken breast (L^* 50–55; Paleari *et al.*, 1998), wood pigeon breast is markedly darker, reflecting its greater myoglobin concentration. Elevated a^* values indicate intense redness, a characteristic linked to oxidative metabolism of flight muscles (Listrat *et al.*, 2016). Similar values have been described in other game birds, including partridge and pheasant, which share the classification as dark game meats.

The results clearly demonstrate that wild wood pigeon meat combines high protein (~24%), very low intramuscular fat (<1% in breast), low cholesterol (~46 mg/100 g), and dark, myoglobin-rich muscle colour. Compared with domesticated pigeons (King, Carrier, Wrocław, Europigeon, Mondain), wild *C. palumbus* is consistently leaner, richer in protein, and nutritionally superior in lipid quality, though with slightly higher cholesterol. When contrasted with poultry species such as chicken, turkey, and ostrich, wood pigeon meat offers comparable or lower cholesterol levels and higher nutritional density, while its darker colour sets it apart (Haščík *et al.*, 2024).

These characteristics underline the nutritional and functional value of *C. palumbus* as a wild game species, with potential as a premium meat product for consumers seeking lean, high-protein, and micronutrient-rich alternatives to conventional poultry and domesticated pigeons.

CONCLUSIONS

The results show that wood pigeon meat is a nutritionally valuable food, combining high protein content, very low intramuscular fat content and a balanced fatty acid profile dominated by monounsaturated fatty acids. Its amino acid composition underlines its role as a source of high-quality protein, while cholesterol levels remain comparable to those of conventional poultry. Its dark, myoglobin-rich colour reflects its oxidative muscle metabolism, which distinguishes it from domesticated pigeons and chickens, but also places it among other game species. Compared with domestic breeds, wood pigeon meat is leaner and has better protein composition, confirming its potential as a health-promoting game meat. Its nutritional properties support the role of wild pigeons in contributing to sustainable and diverse protein sources in the human diet.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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THE IMPACT OF SALINE IRRIGATION ON SOIL SALINIZATION IN THE ARID REGION OF ALGERIA

SUMMARY

Water and soil salinity are among the most important challenges facing the agricultural sector in arid and semi-arid regions. In water-scarce areas, the use of saline water for irrigation is a widespread practice. This study aimed to assess the quality of irrigation water in the arid region of Messaâd and evaluate its impact on soil salinity. The analysis revealed that the electrical conductivity (EC) of irrigation water ranged from 0.595 to 24.8 mS/cm, while the sodium adsorption ratio (SAR) values ranged from 1 to 12.37 mEq/L. In soil, EC values ranged from 0.117 to 13.69 mS/cm and SAR values ranged from 1.46 to 12.26 $\mu\text{mol/litre}$. According to the Piper plot analysis, the irrigation water in Al Messaâd is mainly of the calcium chloride and chloride type. The soil texture classification indicates that the soils in the vicinity of irrigated Al Messaâd are mostly sand, sandy loam, sandy loam, and sandy loam. The results indicate that there is a clear influence of irrigation water salinity on soil salinisation, especially in the eastern parts of the region where salinity levels are significantly higher.

Keywords: Soil salinity, Irrigated Perimeter, PCA, Messaâd, Piper plot, Algeria

INTRODUCTION

Food self-sufficiency is challenged in semi-arid and arid regions due to limited fertile land, irrigation water, and the salinity or brackishness of available water sources, hindering agricultural production. The use of saltwater for crop irrigation in these regions has led to soil salinisation. This has reduced agricultural productivity by as much as 27 billion dollars per year (Singh, 2022). In some regions, agricultural production has decreased by up to 97%. Also, according to Wang *et al.* (2021), global food production has already decreased by

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50% in the twenty-first century due to soil salinization. Although the relationship between soil salinity and irrigation water has been the subject of several studies on the five continents (Gholami *et al.*, 2025; Demo *et al.*, 2025; Nicolas *et al.*, 2023; Tedeschi and Xue, 2025).

In Algeria, the studies of this phenomenon touched just the Algerian Sahara area, where the annual rainfall is almost null, such as: Zaiz and Boutoutaou (2022) in Oued Souf, Abdenmour *et al.* (2020, 2021) in Biskra, Selmane *et al.* (2024) in Ourgla, and Benslama *et al.* (2020) and Kraïmat *et al.* (2025) in Ghardaia. These areas are located in the saharian part of Algeria and are characterized by a very harsh arid climate, high evapotranspiration, and an increasing salinization of soils. Due to drought and the lack of surface water, farmers rely on groundwater as the sole source of irrigation.

However, there aren't still number of studies in the high plateaus region of Algeria (northern part of the Algerian desert) such as the province of Djelfa, that treat the soil salinization problem. Recognize as a transition zone from a semi-arid to an arid climate where the local geology, specifically the presence of marls, gypsum, and evaporite formations in the soil profile affects the soil salinity. It's also influenced by surface water dynamics, particularly in the Messaad region. Therefore, it would be very interesting to focus research on assessing water quality for irrigation using indicators such as Sodium Absorption Ratio (SAR) and Electrical Conductivity (EC).

The aim of this work is to identify the quality of irrigation water in the Messaad area in Djelfa province, to detect if this water has an impact at soil and it is to propose crops that are adapted to this irrigation water.

MATERIAL AND METHODS

Study area

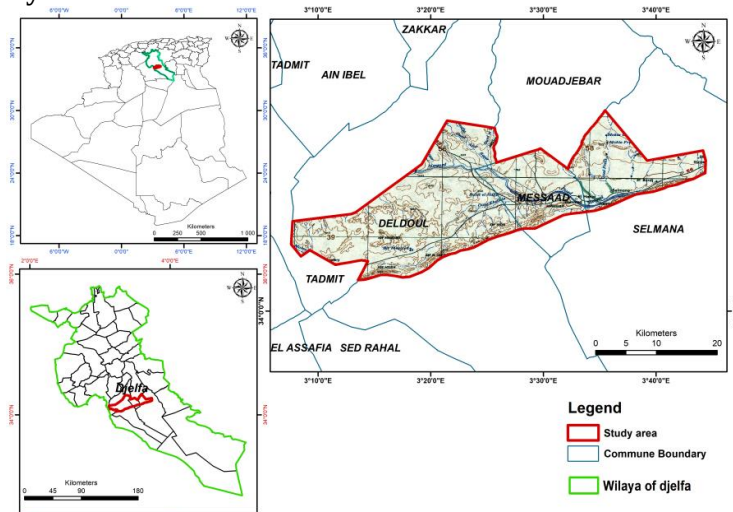


Figure 1. Location of the study area of the Messaad region in Djelfa Province, Algeria

This work was performed in the arid area of Messaâd, which located 400 km south of Algiers (Algeria). At an altitude ranging from 600 to 859 meters above sea level, the area spans 657.37 square kilometers. It's located between 34° 17' 48'' and 34° 2' 54'' North latitude, and 3° 7' 42'' and 3° 44' 30'' East longitude (figure 1). This region has an arid climate, with an average maximum temperature of 36° C and an average minimum temperature of 7° C. The mean annual rainfall is 180 mm (DPSB, 2018; DSA, 2021).

Field work

Messaâd has 76 farms (DSA, 2021). An inquiry regarding soil salinity was conducted with 67 of the 76 farmers in the study area. After the inquiry, soil and water samples are taken once in the irrigation season in September 2023 from the farms and were collected at a 20 cm depth. Using a GPS Venture HC receptor, coordinates (latitude and longitude) were recorded for each of the 67 sampling locations (figure 2). Whereas, water samples were taken from the irrigation borehole on the farms. One-litre bottles were used to collect water samples.

Laboratory work

Soil samples were prepared for laboratory analysis by air drying, crushing and sieving using a 2 mm sieve. The EC, calcium, magnesium, sodium, and particle size were determined in the soil, while in the irrigation water, calcium, magnesium, sodium, chloride, bicarbonate, potassium, sulfate, and electrical conductivity were analyzed.

The EC was measured with a conductivity meter AZ86021 (equipped with a temperature correction device, allowing a direct reading at the reference temperature of 25°C, and using the soil extract with a ratio of 1/5 soil/water. The SAR parameter was also determined by preparing soil extracts (for soil) and using collected water samples, measuring ion concentrations (Na⁺, Ca²⁺, Mg²⁺). The calculation of SAR from these values using the formula:

$$SAR = \frac{[Na^+]}{\sqrt{\frac{Ca^{++} + Mg^{++}}{2}}} \dots\dots\dots eq(1)$$

Statistical analysis

The Principal Component Analysis (PCA) was used to identify homogeneous irrigated perimeters in the study region in terms of salinity. After laboratory analysis, the collected water and soil data was processed to classify homogeneous boreholes and soils in the irrigated perimeters using the following parameters:

- For borehole water: ECw, SARw, pH, Na, K, Ca, Cl, SO₄ and HCO₃,
- For soil: ECs, SARs, pH, limestone, Organic Matter (OM), Moisture, Na and Ca+Mg.

It should be noted that the statistical analyses were made with Statistica 12.

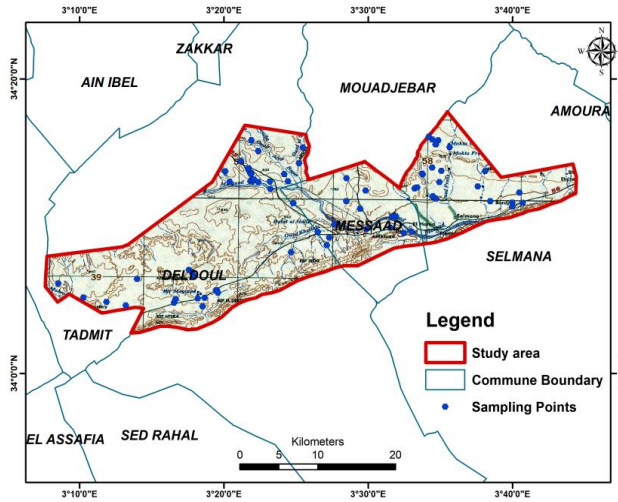


Figure 2. Map of sampling sites

RESULTS AND DISCUSSION

Survey results

The results of the survey of farmers in the Messaâd region are presented in Table 1. We divided the study region into three zones: East, Centre and West.

Table 1. Surveying zones characteristics of the study area

Zone	Number of farmers	Surface area (ha)	Irrigation technique	Crop type	Salinity problem
Center	15	1 - 30	By spraying	1-2	2
West	35	70 - 80	By spraying	1-2-3-4	3
East	17	1 - 140	By spraying	1-2-3-4	6
total	67				11

(1) Fruit growing; (2) Vegetable growing; (3) Cereal growing; (4) Olive growing

The number of farms surveyed in the study area was 67. The area cultivated by each farmer ranged from one hectare to 140 hectares. Agriculture in Messaâd area is based on family farming, which explains the small and medium-sized cultivated areas. On the one hand, the irrigation system used on the farms is sprinkler irrigation, and on the other hand, the predominant crop is fruit trees (apricots and pomegranates). According to the survey results, 11 farms reported salinity issues in these farms, mostly in the east.

We observed that, according to the respondents, the choice of this type of crops is related to the adaptation of the cultivated varieties to the climatic conditions of the region on the one hand, and on the other hand to the age of planting trees in the region, a tradition that is passed down from generation to generation.

Chemical analysis of irrigation water

The results of the chemical analysis of the water used for irrigation allow us to classify the boreholes, used in irrigation, according to their EC and SAR values (figure 3).

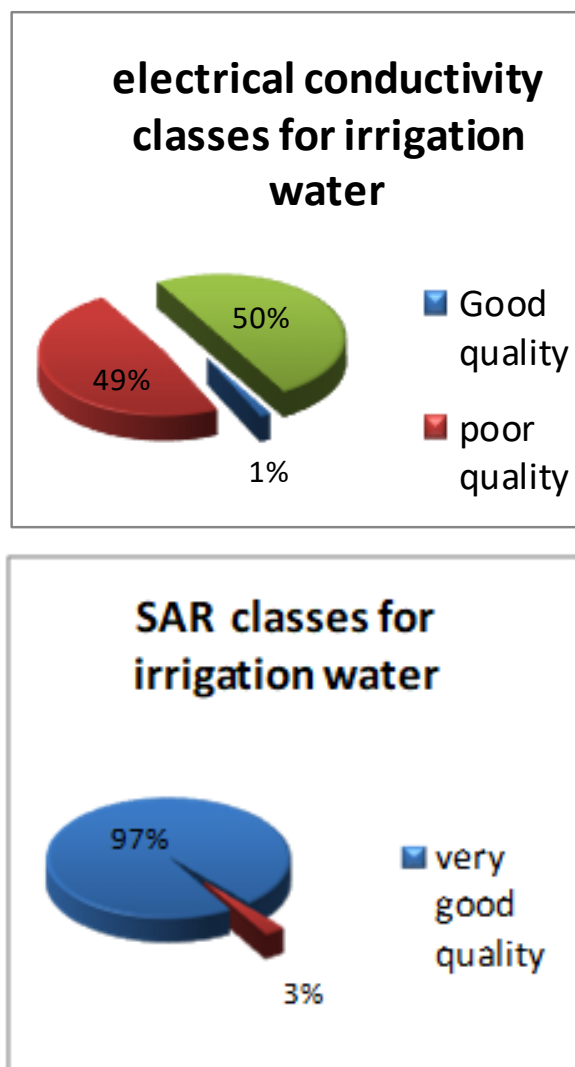


Figure 3. Classification of irrigation water of according to the EC index and the SAR index

The Messaâd irrigated area is represented by 67 irrigated perimeters, where 33 boreholes are highly saline ($0.75 < EC < 2.25$ m/cm) and 34 very highly saline with average EC values above 2.25 m/cm (Daoud and Halitem. 1994; Benaissa and Bessati 2017).

Whereas, according to the SAR index, 33 boreholes have very good water quality ($SAR < 10$) and only two boreholes have good water quality (Richards, 1954).

Irrigation water in the eastern part of the study area has a higher salinity level compared to the western part and the center of Messaâd.

Classification of irrigation water using Piper's method

The Messaâd center

The analysis of irrigation water from boreholes in the Messaâd center has shown the dominance of magnesium over other cations, while the triangle of anions is dominated by chlorine. Irrigation water in the central irrigated perimeters is therefore of the hyper-chloride-calcium type (Figure 4).

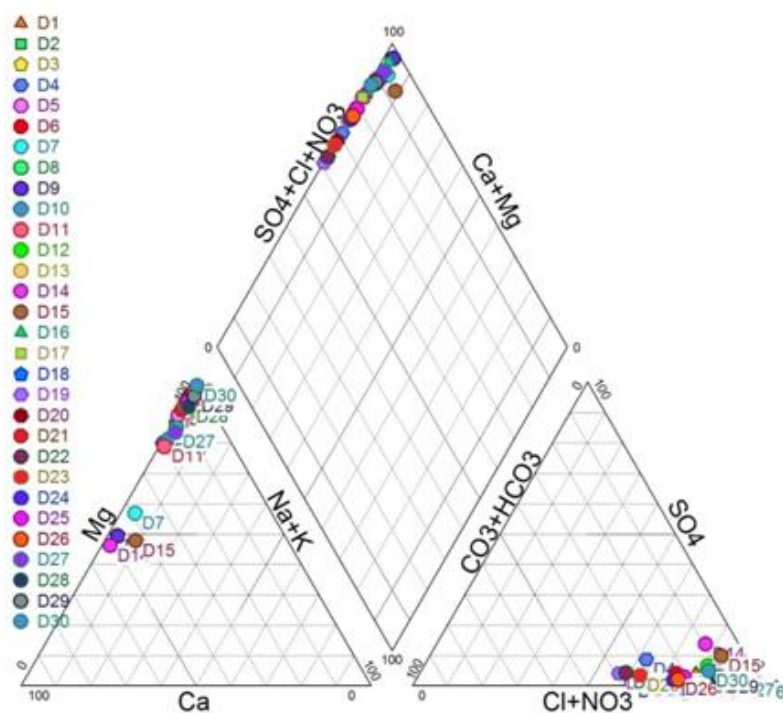


Figure 4. Classification of Piper water from irrigation boreholes in the Messaâd irrigated perimeters

The western Messaâd perimeter

We found from the Piper classification of water from the western Messaâd boreholes that chlorine is the most dominant anion on the right-hand side of the diagram, while magnesium is the most dominant cation on the left-hand side. All the western boreholes are of the calcic hyperchloride type (Figure 5).

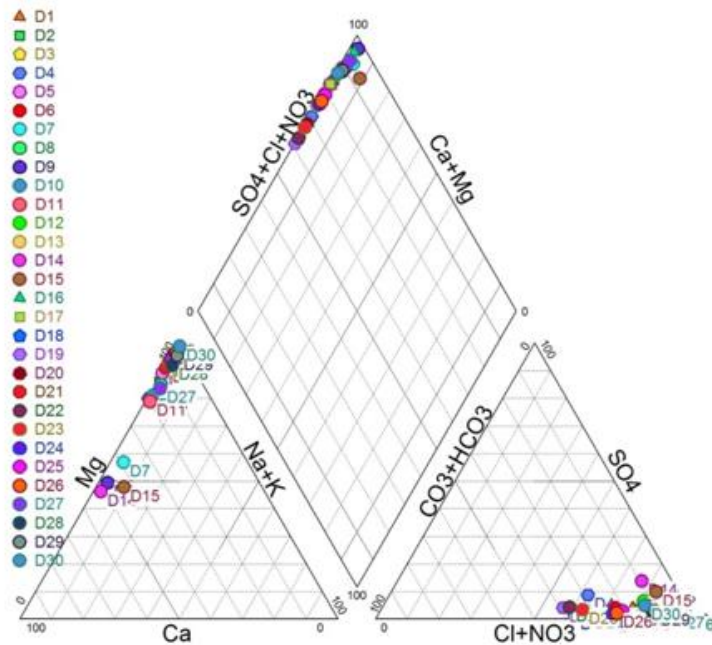


Figure 5. Piper's water classification of irrigation boreholes in irrigated perimeters - west of Messaâd

Eastern Messaâd perimeter

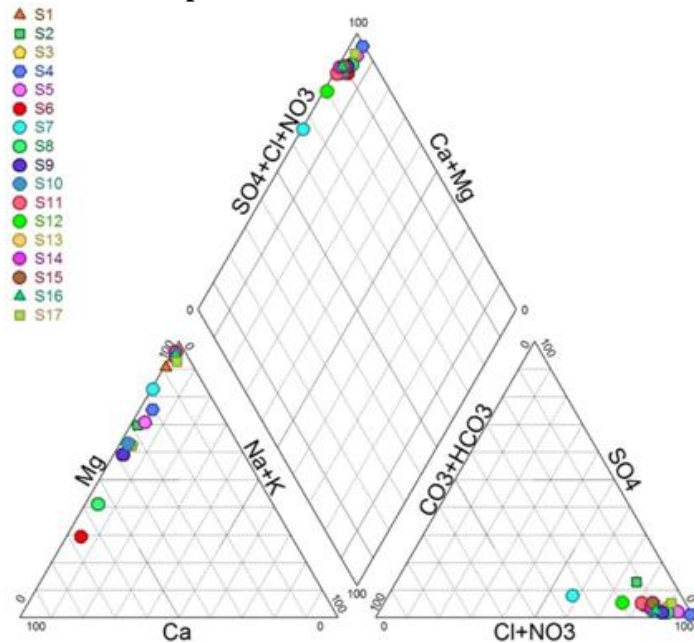


Figure 6. Piper's water classification of irrigation boreholes in irrigated perimeters - east of Messaâd

The agricultural perimeters to the east of Messaâd are irrigated with water of the hyper-chloride-calcium type. On the one hand, in 15 boreholes, the dominant cation is magnesium and in the other boreholes (two) no dominant cations; on the other hand, the dominant anion is chloride in 17 boreholes (Figure 6).

Soil salinity classification in the Messaâd irrigated perimeters

The results of the EC and the SAR index for irrigated soils are shown in the figure 7.

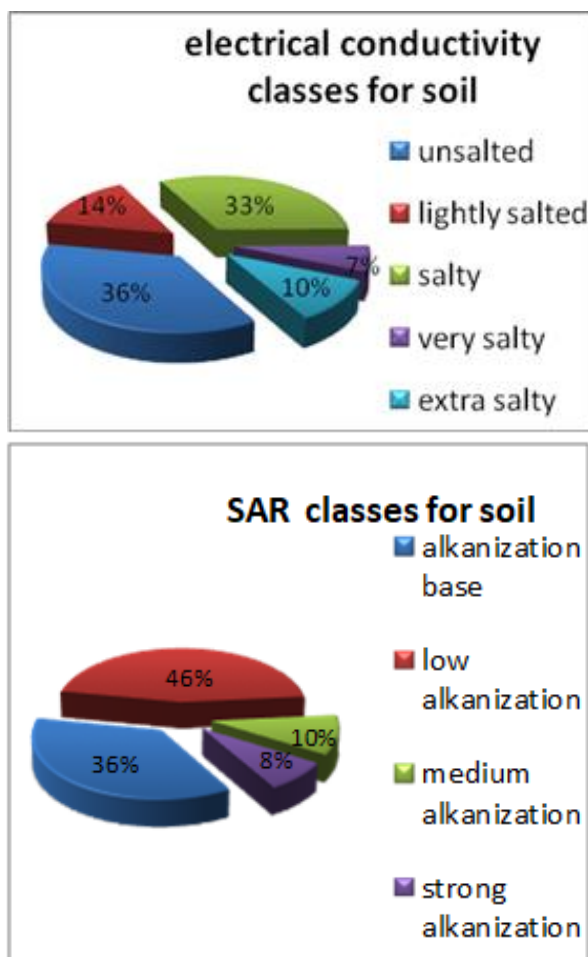


Figure 7. Classification of Messaâd perimeters according to EC values and SAR index

At the Messaâd center perimeters, we noticed that: the soil of two sites is not salty ($CE < 0.6$ ms/cm), four sites with a slightly salty soil ($0.6 < CE < 1.2$ ms/cm), six sites are characterized by salty soil ($1.2 < CE < 2.4$ ms/cm), two sites recorded a CE between: 2.4 and 4 ms/cm, which represents very salty soil and only one site extremely salty ($CE = 5.32$ ms/cm).

The majority of soils on farms in the western part of the study region are classified as non-salty (32 sites), where EC values are below 0.6ms/cm, the soil on one farm is slightly salty ($EC < 1$ ms/cm), the soil on five farms is salty ($1 < EC < 2$ ms/cm) and the soil on just one farm is classified as very salty ($2 < EC < 4$ ms/cm). Whereas, and according to standards, the majority of irrigated perimeters in the East (14 perimeters out of 22), are classified as very salty and extremely salty where the EC value ($4.55 < EC < 13.69$ ms/cm), the soil at three sites is no salty ($CE < 0.6$ ms/cm), the soil in two perimeters is slightly salty ($0.6 < CE < 1$ ms/cm) and the soil in three perimeters is salty ($1 < CE < 2$ ms/cm) (Mathieu and Pieltain., 2009; Aubert., 1960).

We classified the soils in our study area according to the criteria mentioned by Servant and Servat (1966), who classified saline soils according to the SAR index. On farms located on the perimeter of Messaâd, we found that over 85% of farm soils were weakly alkaline ($4 < SAR < 8$ meq/L).

Soil texture classes in the Messaâd region

The particle size analysis of the studied soils revealed textural classes ranging from sandy to sandy-loam and loamy-sand, indicating a dominance of coarse particles over fine ones. These textural types are typical of arid environments and significantly influence the physical behavior of the soil, particularly in terms of permeability and water retention (Figure 8).

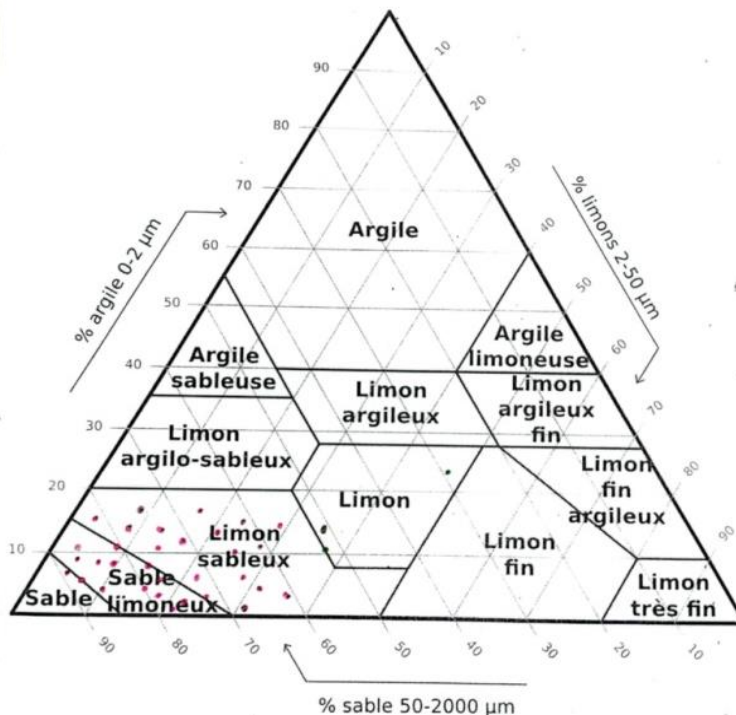


Figure 8. Soil texture triangle

The arid zone soils are generally saline, alkaline, gypsiferous and calcareous, especially in the north. Exchange capacity is limited and water holding capacity is very low. Desert soils are low in organic matter, generally less than one per cent, and lack nitrogen (Pouget, 1977; Halitim, 1988).

After analyzing the obtained results (EC and SAR), we observed that salinity is higher in irrigated soils in the eastern part of the study area, where over 63% of agricultural soils were classified as highly saline and very saline ($EC > 4$ m/cm). These results showed a highly significant difference between the soils of the eastern perimeter and those of the other perimeters. The salinity phenomenon is associated with highly saline irrigation water and climatic conditions (arid bioclimatic phase) (Daliacopoulos *et al.* 2016; Wu *et al.* 2019; Jiang *et al.* 2019; Bensalama *et al.* 2020).

It should be noted, that the findings of EC values recorded in the Messaâd region were in good agreement with those obtained by Azlaoui *et al.* (2021), Ben Amara *et al.* (2024) and Rabouh (2025) in ain Oussara Plain located in the same province. Also, they are lower than those found in the soil of El Ghrous, an arid region in Biskra, which ranged from 36 mS/m to 854 mS/m, and the EC values noted by (Bradai *et al.*, 2022), in the arid plain of Mina (Algeria), where the average (EC) of soil samples ranged from 135 to 2842 mS/m (Berkane *et al.*, 2021).

Concerning SAR index values, soils from irrigated perimeters in the Messaâd region recorded similar values to those found in the arid region of Zelfana (southern Algeria), in an irrigated farm where SAR values ranged from 1.33 to 16.98 meq/l (Benslama *et al.*, 2020).

These results show that soil salinity levels increase with irrigation, which is confirmed by numerous studies in Algeria (Berkane *et al.*, 2021; Abdenmour *et al.*, 2020; Abdenmour *et al.*, 2021; Benslama *et al.*, 2020; Semar *et al.*, 2019; Mostefa *et al.*, 2022) and in different countries (Lhissoui *et al.*, 2014; Gorji *et al.*, 2015; Asfaw *et al.*, 2018).

Homogeneous irrigated perimeters according to the PCA analysis

Principal Component Analysis (PCA), a more in-depth statistical analysis, was used to examine the effect of irrigation water on soil salinity and their relationship. It should be noted that we have used abbreviations to make the results easier to read: B: borehole; F: farm.

Homogeneous borehole irrigation water in terms of salinity in the study area

The results of the PCA of the borehole irrigation water in the irrigated perimeters of Messaâd are represented in figure 9.

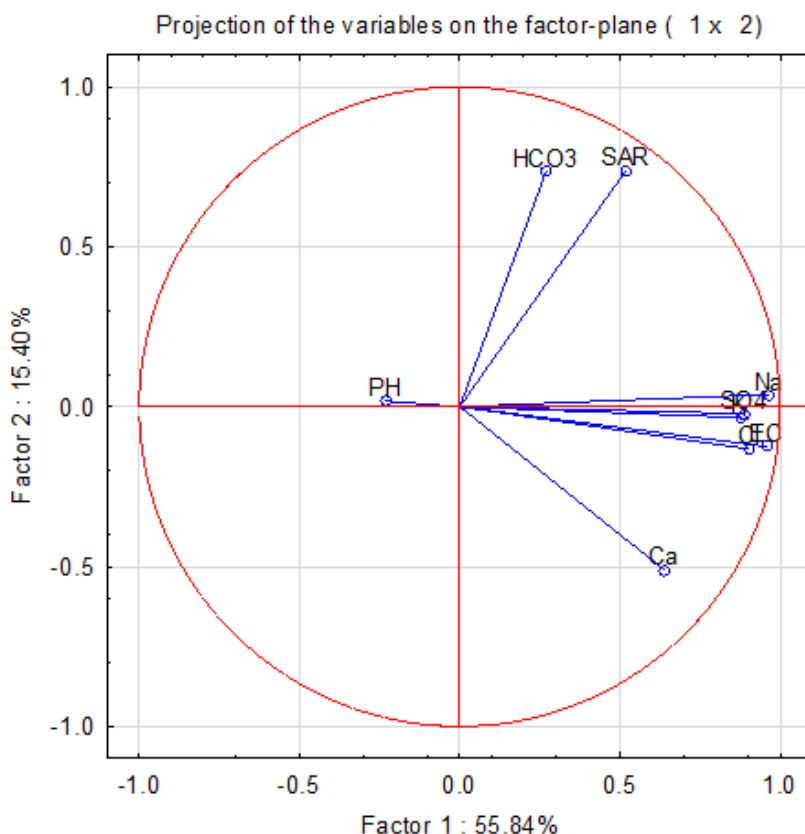


Figure 9. The PCA projection of the variables (ECw, SARw, pH, Na, K, Ca, Cl, SO₄ and HCO₃) on the plane (axis 1 - axis 2).

The projection of the variables on the (1x2) plane (figure 10) shows that the nine variables chosen are well correlated with excellent quality of representation (factor 1: 55.84%; factor 2: 15.40%).

Analysis of the projections of individuals linked to axes 01 and 02 revealed that the farms located on the positive side of the axe 1 are within the following perimeters: B2, B3, B4, B5, B6, B8, B9, B10, B12, B13, B15, B17, B18, B23, B28, B30, B31, B41, B42, B43, B45, B48, B49 and B61.

On the other hand, they situated the following irrigated perimeters on the negative side of the axe 1: B68, B14, B65, B66, B29, B64, B14, B59, B67, B33, B62, B63, B34, B11, B47, B51, B37, B39, B38, B1, B22, B50, B44, B20, B24, B32, B46, B36, B25, B35, B21, B7, B26, B40, B55, B57, B54, B52, B53, B27, B58, B60, B19, B56.

Compared with axis 2, we note on the positive flank of this axis the presence of the following farms: B3, B5, B6, B11, B12, B13, B14, B15, B17, B28, B29, B30, B31, B33, B34, B59, B61, B62, B63, B64, B65, B66, B67 and B68.

Whereas the farms positioned on the negative side of the axe tow are: B1, B7, B19, B20, B21, B22, B24, B25, B26, B27, B32, B35, B36, B37, B38, B39, B40, B44, B46, B47, B50, B51, B52, B53, B54, B55, B56, B57, B58 and B60.

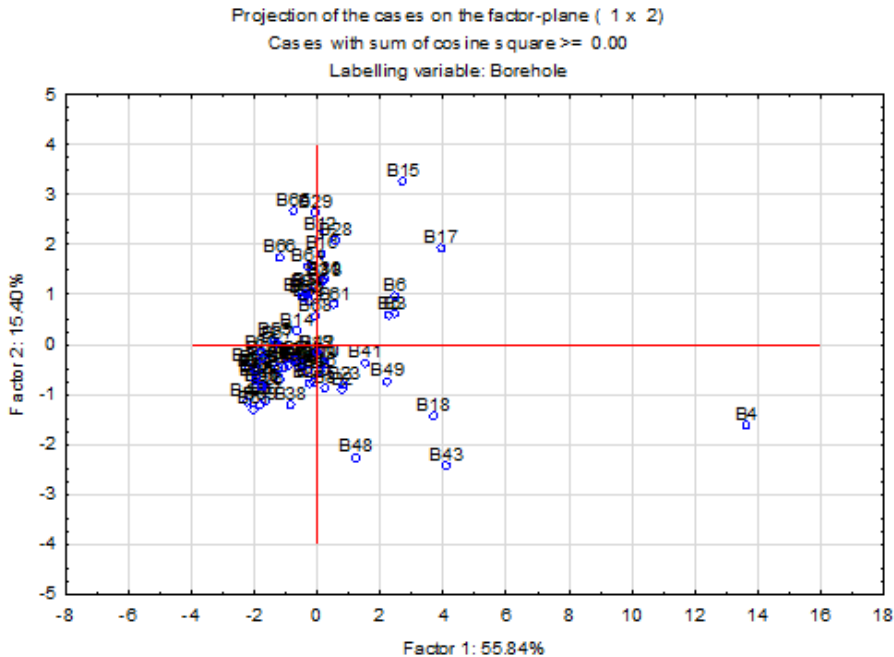


Figure 10. The projection of individuals (borehole).

Interpretation of projections

The irrigated perimeters (B3, B5, B6, B12, B13, B15, B17, B28, B30, B31 and B61) located on the positive side of the two axes are characterised by a high EC and SAR values ($2.32 < EC_w < 6.19 \text{ ms/cm}$); ($4.45 < SAR_w < 12.37 \text{ meq/l}$). On the positive side of Axis 1 and the negative side of Axis 2, we observed 13 irrigated perimeters (B2, B4, B8, B9, B10, B18, B23, B41, B42, B43, B45, B48 and B49) have very high values of ECw varied from: 2.64 to 24.8 ms/cm and an SARw equivalent averages between: 2.14 and 8.75 meq/l.

On the other hand, the farms were grouped together on the positive side of axis 2 and the negative side of axis 1 (B11, B14, B29, B33, B34, B59, B62, B63, B64, B65, B66, B67 and B68), with equivalent electrical conductivity of water (ECw) rates between: 1.45 and 2.51 ms/cm. The SARw index inscription rates range from: 3.47 to 7.27 meq/l.

Furthermore, the largest group is present at the level of the two negative flanks of axes 1 and 2, with 30 irrigated perimeters (B1, B7, B19, B20, B21, B22, B24, B25, B26, B27, B32, B35, B36, B37, B38, B39, B40, B44, B46, B47, B50, B51, B52, B53, B54, B55, B56, B57, B58 and B60) recording ECw values between: 0.59 and 3.23 ms/cm. Additionally, SAR index values varied between: $1.041 < SAR_w < 4.078 \text{ meq/l}$.

Homogeneous soil farms in terms of salinity in the study area

The figure 11 shows the results of the Principal Component Analysis (PCA) of the homogeneous soil farms

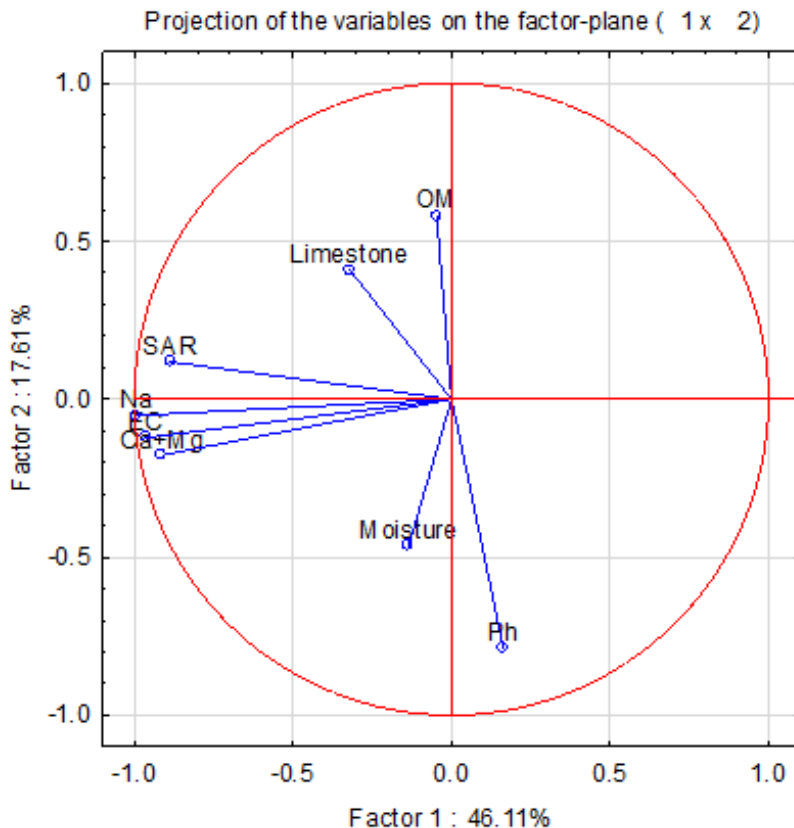


Figure 11. The PCA projection of the variables (ECs, SARs, pH, limestone, Organic Matter (OM), Moisture, Na and Ca+Mg)

The nine variables that have been selected are well correlated with excellent representation quality (factor 1: 46.11%; factor 2: 17.61%), as shown by the projection of the variables on the (1 x 2) plane (figure 11).

Interpretation of projections

We have revealed a cloud of points in the projection of individuals (figure 12), which has enabled us to distinguish 4 clearly individualised groups, as follow as:

-First group: F1, F3, F8, F24, F26, F27, F29, F35, F47, F48, F52, F60, F65, F66, F67, F69, F73 and F74,

-Second group: F4, F7, F9, F10, F11, F12, F15, F16, F17, F20, F25, F28, F30, F33, F37 and F68,

-Third group: F5, F13, F14, F18, F19, F21, F22, F32, F34, F36, F71 and F76,

-Fourth group: F2, F6, F23, F38, F39, F40, F41, F42, F43, F44, F45, F46, F49, F50, F51, F53, F54, F55, F56, F57, F58, F59, F61, F62, F63, F64, F70, F72, F76 and F77.

The first and fourth groups are on the positive side of axis 1, while the second and the third group are on the negative side of axis 1. In relation to axis 2, the first and second groups are on the positive side, and the third and fourth groups on the negative side.

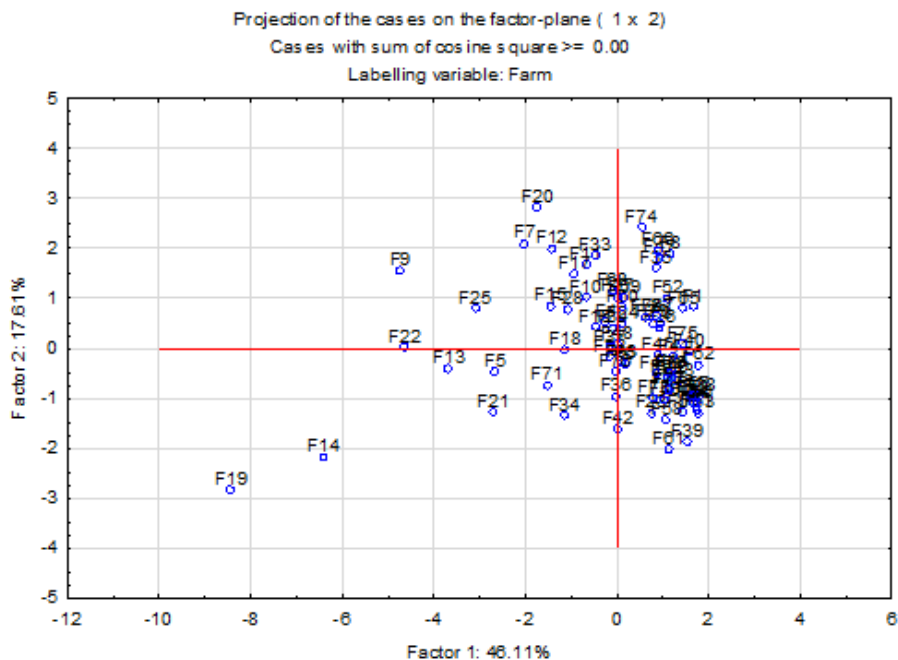


Figure 12. The projection of individuals (farms).

The ECs values in the first group vary between: 0.17 and 1.62 ms/cm. On the other hand, the SARs index has the following values: 1.64 to 6.50 meq/L. The second group comprises farms with EC averages ranging from 0.827 to 8.48 ms/cm, and SARs values ranging from: 5.74 and 12.26 meq/l. In the farms of third group, the SARs index has transcribed values of: 6.55 and 12.26 meq/l. In contrast, the average values of the ECs were recorded as 1.19 and 13.69 ms/cm. Finally, in the farms of the fourth group, the ECs recorded values between: 0.132 and 1.499 ms/cm and the SARs index transcribed values ranging from 1.46 to 6.92 meq/l.

The PCA analysis confirms the classification of the boreholes according to their EC and SAR values. The water with the highest salinity level was found in eight of the 12 boreholes (the PCA boreholes projection of individuals: B3, B5, B6, B12, B13, B15, B17) on the eastern perimeter of Messaâd, which have high EC and SAR values. The majority of boreholes with low EC and SAR values are located in the center and west of Messaâd.

Concerning soil classification, the PCA farms projection of individuals show that the farms have EC values between 0.827 and 8.48 ms/cm and SAR up to: 5.74 meq/l, are sited in the eastern of Messaâd (F4, F5, F7, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F20, F21 and F22). These results prove that water salinity impacts soil salinity. In other words, using saline water for irrigation affects the soil which reduces agricultural productivity. Furthermore, high salt concentration significantly reduces the infiltration rate by clogging soil pores (particularly with sodicity), destroying soil structure, and changing the chemistry of the root zone resulting in water stress and crop failure, creating a cycle of degradation and erosion.

CONCLUSIONS

The aim of this study was to assess the quality of the irrigation water in the Messaâd arid region, determine its impact on the soil salinity. Where the identification of the water and soil salinity was based on two main parameters: the EC and the SAR index.

The found results shown that the EC values of water irrigation were varied from: 0.595 to 24.8ms/cm, and the SAR averages were ranged between one and 12.37mea/l. in contrast, the soil EC rates were various from: 0.117 and 13.69ms/cm, and the SAR index transcribed values between: 1.46 and 12.26 meq/l. the texture soil classification demonstrate that the Messaâd soil perimeters are sandy to sandy-loam and loamy-sand.

According to Piper's water classification, the borehole irrigation water in the arid Messaâd region is hyper-chloride-calcium type. Based on the EC and SAR values, the salinity level of the irrigation water in the eastern perimeters of the study area is higher than in the western perimeters and centre. Similar results were obtained for the soil in the irrigated areas of Messaâd, where the soils in the eastern farms were most affected by salinity.

On the other hand, PCA analysis of the water and soil data classified all the boreholes and farms with high EC and SAR values into the same homogeneous groups in terms of salinity, confirming the previous results.

Irrigation water in the Messaâd region impacts soil salinity, especially in the eastern areas. For this reason, irrigation techniques should be modified to avoid salt buildup for sustainable soil management and salt accumulation would be reduced by effective irrigation advised, such as drip systems. Moreover, time monitoring salinity requiring EC and SAR measurements of the soil, reserving surface cover, adding organic matter, and growing salt-tolerant crops can improve soil health and productivity.

Future studies should broaden their scope to include diverse new technologies such as remote sensing data to map and spatially estimate soil salinity. Research should also explore the effects of the Unmanned Aerial Vehicles and their role in detecting salt-affected areas with high spatial accuracy, and integrated with field data (EC, SAR) for precise, cost-effective assessment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TRADITIONAL FOOD AND MEDICINAL USES OF LEAFY VEGETABLES IN NORTH MACEDONIA

SUMMARY

This ethnobotanical study explores the traditional knowledge and use of leafy vegetables in North Macedonia, focusing on both culinary and medicinal aspects. In 2024, structured interviews were conducted with 67 respondents from five regions of the country, gathering information on the use of twenty-one species from ten botanical families. The majority of the species belong to the families Apiaceae and Asteraceae. All respondents reported consuming leafy vegetables, with most of them also using wild species. The most frequently cited plant was spinach (*Spinacia oleracea*), with a citation frequency index of 0.58. These plants are most frequently prepared in the form of salads, soups, stews, teas, juices, smoothies, and pies, or used as spices. Apart from their dietary roles, these plants are traditionally used to support various health functions, including those of the cardiovascular, gastrointestinal, immune, urinary, and respiratory systems. Certain species are also associated with benefits such as detoxification, the treatment of anemia and diabetes, weight management, improved bone health, and anticancer effects. Despite their significance, ethnobotanical research on leafy vegetables in North Macedonia remains scarce, highlighting the need for more in-depth studies. Continued research is crucial to safeguard this traditional knowledge and to better understand the broader cultural, ecological, and health-related values of these plants.

Keywords: ethnobotany; leafy vegetables; North Macedonia; traditional knowledge; medicinal plants

INTRODUCTION

Vegetables are the consumable portions of plants, including leaves, stems, roots, flowers, seeds, fruits, bulbs and tubers and fungi, which can be eaten

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entirely or in sections, either fresh or prepared as a component of a main course (Nnamani *et al.*, 2010). However, when the edible portion of a vegetable primarily consists of leaves, it is classified as a leafy vegetable. They are also known as potherbs, greens, vegetable greens, leafy greens, or salad greens (Vainio-Mattila, 2000; Filipović *et al.*, 2025). Leafy vegetables are consumed primarily for their dietary benefits, but they are also used in treating various health conditions. Leafy greens are highly versatile, hardy, and capable of withstanding harsh climates. They grow naturally in the wild, and most do not require formal cultivation, as they are easily found in open fields (Raghuvanshi *et al.*, 2001).

Leafy vegetables offer strong nutritional and medicinal benefits and can help meet dietary needs. According to Randhawa *et al.* (2015), the minimal calorie content of leafy greens makes them perfect for weight control. Additionally, leafy greens contain essential nutrients, abundant in dietary fiber, low in fats, and rich in folate, vitamin C, vitamin K, magnesium, and potassium. They are also a valuable source of phytochemicals such as β -carotene and flavonoids. According to Oluwole *et al.* (2021), the bioactive substances found in leafy greens possess antioxidant, anti-inflammatory, and various other physiological properties.

Ethnobotanical research serves as a crucial connection between flora and human societies within a particular ecosystem. It explores how people utilize plants for their well-being, whether as food or folk remedies. The Balkan region heavily depends on wild plant species, demonstrating a strong reliance on natural resources. Ethnobotanical investigations in the Balkans are highly significant due to the area's vast biodiversity, cultural richness, and longstanding tradition of plant-based knowledge, as well as their role in fostering rural development and eco-tourism (Pieroni *et al.*, 2013).

Wild leafy vegetables have been documented as part of the diet in coastal, rural, and remote mountainous communities in Bosnia (Redžić, 2006; Redžić, 2007; Redžić, 2010; Lakić *et al.*, 2022), Bulgaria (Nedelcheva, 2013), Greece (Kostakis and Lazaridis, 2019), Albania (Pieroni, 2008; Pieroni, 2010; Pieroni *et al.*, 2014a; Pieroni *et al.*, 2014b), Croatia (Łuczaj *et al.*, 2013a; Łuczaj *et al.*, 2013b; Dolina and Łuczaj, 2014), and Turkey (Dogan *et al.*, 2004; Kargioglu *et al.*, 2008; Sarper *et al.*, 2009; Simsek *et al.*, 2012; Dogan, 2012; Dogan *et al.*, 2013; Dogan *et al.*, 2015).

The number of ethnobotanical studies in North Macedonia is very limited (Pieroni *et al.*, 2013; Rexhepi *et al.*, 2013; Miskoska-Milevska *et al.*, 2020; Josimovska and Miskoska-Milevska, 2025; Miskoska-Milevska *et al.*, 2025; 2026). Research on leafy plants in North Macedonia highlights their historical and cultural importance. Traditionally, rural communities utilized a diverse array of leafy plants for culinary, medicinal, and spiritual purposes. Various ethnic groups in North Macedonia, including Macedonians, Albanians, Serbs and others have adapted plant usage to suit their cultural traditions and ecological surroundings (Rexhepi, 2018; Rexhepi and Bajrami, 2021).

The objective of this study was to compile and analyse the key culinary and medicinal aspects of certain leafy vegetables used in North Macedonia.

MATERIAL AND METHODS

This ethnobotanical survey was conducted across the territory of North Macedonia, located in the central region of the Balkan Peninsula. The country experiences a transitional climate between Mediterranean and continental influences, characterized by hot, dry summers and cold, wet winters.

Traditional knowledge about leafy vegetables was obtained through an ethnobotanical field survey conducted in 2024, involving direct structured interviews with randomly selected 67 respondents from five regions of North Macedonia: Skopje, Northeastern, Vardar, Pelagonia, and Southwestern. Face-to-face interviews were used to gather information on the practical use and knowledge of leafy vegetables among respondents of different age groups. Respondents shared their knowledge and experiences regarding the use of wild and cultivated leafy vegetables as food and folk remedies. This study presents the scientific and folk names of leafy vegetables, along with their culinary and medicinal applications.

Taxonomic identification of plants is based on standard references for the Macedonian flora (Micevski, 1985-2005). Species taxonomy and nomenclature follow the World Flora Online (<https://www.worldfloraonline.org>).

A total of twenty-one plant species were documented in this study: *Allium ursinum* L., *Angelica sylvestris* L., *Anethum graveolens* L., *Apium graveolens* L., *Petroselinum crispum* (Mill.) Fuss., *Calendula arvensis* L., *Cichorium intybus* L., *Lactuca sativa* L., *Taraxacum officinale* F.H. Wigg., *Brassica oleracea* var. *viridis* L., *Brassica oleracea* var. *sabauda* L., *Eruca vesicaria* subsp. *sativa* (Mill.) Hegi., *Beta vulgaris* subsp. *cicla* (L.) Schübl. and G. Martens), *Chenopodium album* L., *Spinacia oleracea* L., *Ocimum basilicum* L., *Malva sylvestris* L., *Rumex patientia* L., *Rumex acetosa* L., *Portulaca oleracea* L., and *Urtica dioica* L. The Relative Frequency of Citation (RFC) index was calculated for each mentioned plant species. The RFC was determined using the formula: $RFC = FC/N$, where FC is number of respondents who mentioned the use of the species and N is total number of respondents in the survey (Sansanelli *et al.* 2017). The RFC value ranges from zero (when nobody referred to a plant as useful) to one (when all respondents referred to a plant as useful).

Descriptive statistics, including frequencies and percentages, were used to summarize the data obtained through a Microsoft Excel spreadsheet, which was also used for drawing graph. Further analysis was carried out to compare the field data with existing ethnobotanical literature (Pulliah, 2002; Guarrera, 2003; Leporatti and Ivancheva 2003; Brussell, 2004; Ishtiaq *et al.*, 2007; Jana and Shekhawat, 2010; Malik *et al.* 2011; Rexhepi *et al.*, 2013, Dogan *et al.*, 2013; Street *et al.*, 2013; Nedelcheva, 2013; Łuczaj *et al.* 2013b, Ouhaddou *et al.*, 2014; Dogan *et al.*, 2015; Vasas *et al.*, 2015; Saidi *et al.*, 2015; Fortini *et al.*, 2016; Mueen Ahmed and Parasuraman, 2016; Rexhepi, 2018; Bhat *et al.*, 2020;

Miskoska-Milevska *et al.*, 2020; Oladimeji and Kumar, 2023; Mehraj and Alam, 2024; Miskoska-Milevska *et al.*, 2025).

RESULTS AND DISCUSSION

Socio-demographic characteristics of participants

In the research, a total of 67 respondents were interviewed. Of these, 40.3% were above 50 years of age, 31.3% were in the 36-50 age group, and 28.4% were in the 18-35 age group. The largest percentage of the surveyed individuals (68.7%) were female (Table 1). The majority of respondents were Macedonian (83.6%), followed by Albanian (7.4%), Serbian (3%), Turkish (3%), and Roma (3%). Regarding geographical distribution, 70% of the respondents were from the Skopje region, 15% from the Northeastern region, 7.5% from the Vardar region, 6% from the Pelagonia region, and 1.5% from the Southwestern region. In terms of education, most respondents had secondary-school education (59.7%), 23.9% had a university degree, 13.4% had primary-school education, and 3% had other forms of education.

Table 1. Age distribution of the respondents interviewed

Age	Male	Female	Total number	Percentage
18-35	4	15	19	28.4%
36-50	7	14	21	31.3%
Above 50	10	17	27	40.3%
Total	21	46	67	100%

In this survey, all respondents answered that they practice consuming leafy vegetables. A smaller percentage of the interviewees (41.8%) practice growing leafy vegetables, while a larger percentage (58.2%) do not. Half of the respondents collect wild leafy vegetables, while the other half do not practice this. The majority of the interviewees (89.6%) consume wild leafy vegetables. The main sources of information about the use of leafy vegetables were the Internet (40.7%), literature (37.4%) and older people (21.9%).

Diversity of leafy vegetables

According to the results presented in Figure 1, twenty-one species from ten botanical families were identified as leafy vegetables. The majority of the species belong to the families Apiaceae (4), Asteraceae (4), Brassicaceae (3), and Chenopodiaceae (3).

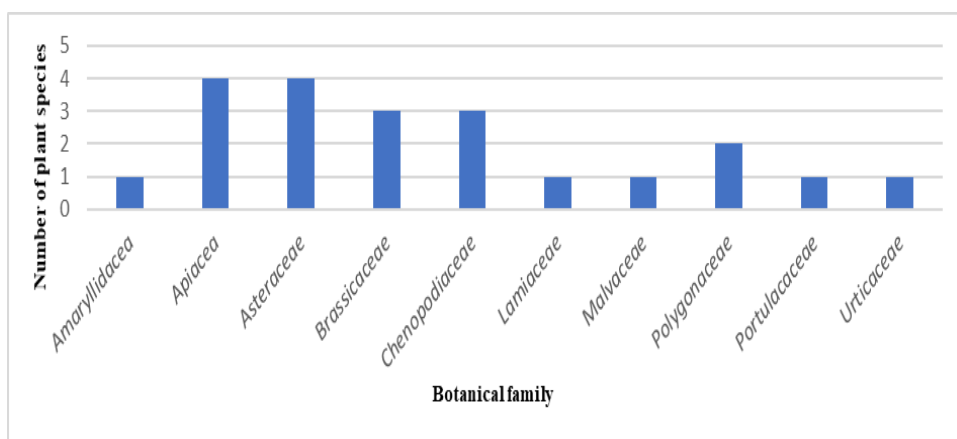


Figure 1. Number of plant species in different families

In the study, *Spinacia oleracea* exhibited the highest relative citation frequency index (0.58), followed by *Petroselinum crispum* (0.52) and *Beta vulgaris* ssp. *cicla* (0.42), (Table 2).

Table 2. List of leafy vegetables used in North Macedonia

No.	Botanical family	Botanical name	Macedonian folk name	Cultivated/ wild	Culinary use	Medicinal use	RFC
1.	Amaryllidaceae (previously in Alliaceae)	<i>Allium ursinum</i> L.	Divorast echki luk, mechkin luk, shumski luk, sremush	Wild	As additive to -salads, -stews, -soups, -juices	Improves the functioning of the heart, gallbladder and liver.	0.06
2.	Apiaceae	<i>Angelica sylvestris</i> L.	Anglica	Wild	-In salads, -In soups, -In stews	Improves food digestion and appetite.	0.01
3.	Apiaceae	<i>Anethum graveolens</i> L.	Kopar	Cultivated	-As spice in salads, tarator, pickled cucumber, soups, fish dishes, vegetable dishes, and sauces	-Improves digestion, -Prevents bloating, -Helps fight infections, -Has a nephroprotective effect.	0.09

4.	Apiaceae	<i>Apium graveolens</i> L.	Celer, Gereviz	Cultivated	-As spice (in stews, salads and soup), -In juices, -In teas, -In smoothies	-Improves heart and lung function, -Helps with asthma, -Reduces stomach acid, -Speeds up metabolism and promotes weight loss, -As a diuretic.	0.31
5.	Apiaceae	<i>Petroselinum crispum</i> (Mill.) Fuss.	Magdons	Cultivated	-As spice in salads, soups, stews, -In smoothies, -In juices, -In teas	-As a diuretic, -For urinary infections, -Improves kidney function, -Supports detoxification, -For reducing high blood pressure, -Promotes weight loss, -Relieves pain (headaches, menstrual cramps), -Helps fight cancer.	0.52
6.	Asteraceae	<i>Calendula arvensis</i> L.	Polski neven	Wild	-Salads, -Teas	-Used for cleaning and rinsing wounds, burns, and chronic ulcers, -For treating liver diseases.	0.01
7.	Asteraceae	<i>Cichorium intybus</i> L.	Cikorija	Wild	-Salads, -Coffee substitute -Tea	-Foods suitable for diabetics -For diarrhea.	0.01
8.	Asteraceae	<i>Lactuca sativa</i> L.	Salata	Cultivated	-Salads	-Strengthens immunity, Improves heart function, -Stimulates metabolism, -Helps eliminate toxins.	0.22

9.	Asteraceae	<i>Taraxacum officinale</i> F.H. Wigg.	Gluvarche, Obicno gluvarche	Wild	-Salads, -Teas, -Syrups, -Juices, -Smoothies -Honey	-Young leaves are used: to regulate blood pressure, a cure for infections, to treat gallbladder and liver problems, to treat bone pain, for rheumatism, -For diabetics it is recommended to chew 5-10 hollow stems, -The root is used as a cure for cancer.	0.15
10.	Brassicaceae	<i>Brassica oleracea</i> var. <i>viridis</i> L. (syn. <i>Brassica oleracea</i> var. <i>acephala</i> DC.)	Lisnata zelka	Cultivated	-Salads	To improve the immune system.	0.01
11.	Brassicaceae	<i>Brassica oleracea</i> var. <i>sabauda</i> L.	Kelj	Cultivated	-Salads, -Soups, -Stews, -Smoothies	/	0.01
12.	Brassicaceae	<i>Eruca vesicaria</i> subsp. <i>sativa</i> (Mill.) Hegi. (syn. <i>Eruca sativa</i> Mill.)	Rukola	Cultivated	-Salads, -Smoothies, -Addition to sandwiches, soups, risotto and butter.	-In the treatment of ulcers, -For the treatment of diabetes, -For breast cancer, -For cardiovascular disease, -For regulating digestion, -For detoxification, -For weight loss, -Against viruses and colds, -For healthy bones.	0.28

13.	Chenopodiaceae	<i>Beta vulgaris</i> ssp. <i>cicla</i> (L.) Schübl. & G. Martens)	Blitva, Mangold	Cultivated	-Salads, -Soups, -Stews, -Pies, -Sarma, -Spice, -Smoothies.	-For anemia, -For better heart function, -For better immunity, -Helps with weight loss, -For stabilizing blood sugar, -Against cancer.	0.42
14.	Chenopodiaceae	<i>Chenopodium album</i> L.	Loboda, Belasamoniknata loboda, Guskina noga	Wild	-Pies, -Salads, -Soups, -Stews	-As a diuretic -For anemia.	0.07
15.	Chenopodiaceae	<i>Spinacia oleracea</i> L.	Spanak	Cultivated	-Pies, -Stews, -Side dish, -Salads, -Soups, -Smoothies	-For anemia, -Prevention of cancer -Prevention of hepatic diseases.	0.58
16.	Lamiaceae	<i>Ocimum basilicum</i> L.	Bosilel	Cultivated	-Salads, -Stews, -Spices, -Pasta additive, -Teas	-Prevention of cancer -Prevents cold symptoms, -Treats cough, sinus, bronchitis and asthma -Has positive effects on mental problems, -Improves stomach and kidney function.	0.18
17.	Malvaceae	<i>Malva sylvestris</i> L.	Crn slez	Wild	-Salads, -Stews, -Syrups, -Soups, -Teas	-Treats gastrointestinal problems, -Treats urological problems, -Treats respiratory problems -For cold.	0.04

18.	Polygonaceae	<i>Rumex patientia</i> L.	Shtavej, Zelje	Cultivated/ Wild	-Salads, -Stews, -Pies, -Sarma, -Teas, -Soups - Smoothies	-As a diuretic -For anemia, -For better digestion.	0.31
19.	Polygonaceae	<i>Rumex acetosa</i> L.	Kiselec, Kiselica, Obichen shtavej	Wild	-Salads, -Soups, - Smoothies	-For diarrhea, -In the treatment of gallstones, -For rashes, -For digestive problems.	0.07
20.	Portulacaceae	<i>Portulaca oleracea</i> L.	Tuchnica	Wild	-Salads	Antiscorvy remedy.	0.01
21.	Urticaceae	<i>Urtica dioica</i> L.	Kopriva	Wild	-Soups, -Pies, -Stews, -Salads, -Puree, - Smoothies -Juices, -Teas, - Syrops	-For anemia, -Improves circulation, -Has an anti-inflammatory effect, -Treats respiratory diseases, -Treats liver diseases.	

Leafy greens are rich in polyphenols and antioxidants, giving them distinctive therapeutic properties. They also exhibit antimicrobial effects and can be incorporated into various food products to prolong shelf life (Randhawa *et al.*, 2015). Research in the northeastern area of North Macedonia highlights several edible and medicinal plants, such as leafy vegetables, used in local dishes or as part of herbal treatments for their nutritional and therapeutic values. Well-known plants studied include *Urtica dioica*, *Cichorium intybus*, *Chenopodium* spp., and *Rumex* spp., recognized for their combined use as food and herbal remedies (Rexhepi, 2018). Respondents in our study also referred to these plants.

Traditional culinary use of leafy vegetables

Based on the results of our research, the leaves of *Allium ursinum* are consumed as an additive in salads, stews, soups, and juices. Nedelcheva (2013) documented the use of *Allium ursinum* consumed raw, in salads, and as a pickle. According to Sobolewska *et al.* (2015), these leaves are incorporated into pesto, soups, risotto, ravioli, and used as a spice to flavor hard cheeses. A previous study (Miskoska-Milevska *et al.*, 2025) documented that this plant is used in the preparation of herbal tea.

According to our research, *Angelica sylvestris* is used in salads, soups and stews. An *Angelica* jam was traditionally prepared using the stems of *Angelica sylvestris* (Koç Alibaşoğlu *et al.*, 2024).

As shown by the results of our study, fresh and dried leaves of *Anethum graveolens* are used as spice in salads, tarator, pickled cucumber, soups, fish dishes, vegetable dishes, and sauces. Similar uses of *Anethum graveolens* to flavor and season various foods are mentioned in the research of Blank and Grosch (1991), and Huopalahti and Linko (1983). Used both raw in salads and as a flavor enhancer in soups and stocks, celery is a versatile ingredient (Malhotra, 2012). Also, in our research, the application of *Apium graveolens* as a spice in stews, soups, and salads, as well as in juices, teas, and smoothies, was mentioned. The essential oil of *Petroselinum crispum* finds application in the food, pharmaceutical, personal care, and chemical industries (Marín *et al.*, 2016). Based on the outcomes of our research, *Petroselinum crispum* is used as a spice in salads, soups, and stews, as well as in smoothies, juices, and teas, while *Calendula arvensis* is used in salads and teas.

The ancient Egyptians cultivated *Cichorium intybus* as a coffee alternative, edible crop, and herbal remedy (Street *et al.*, 2013). In our research, respondents reported using *Cichorium intybus* as a coffee substitute and in salads. The young leaves of *Taraxacum officinale* are used as food in salads, beverages, and vegetable dishes, owing to their nutritional benefits, including high levels of minerals, fiber, vitamins, and essential fatty acids (Escudero *et al.*, 2003). Based on the outcomes of our research, *Taraxacum officinale* is used in the preparation of salads, teas, syrup, juices, smoothies, and honey.

As noted by Šamec *et al.* (2018), the leaves of *Brassica oleracea* var. *viridis* are commonly eaten raw in salads and juices, and prepared in a range of soups, omelets, and stir-fries. In accordance with the results of our study, *Brassica oleracea* var. *viridis*, *Lactuca sativa*, and *Portulaca oleracea* are consumed primarily in salads. According to Masoodi *et al.* (2011), *Portulaca oleracea* has been consumed in salads and utilized as a medicinal herb for centuries. Łuczaj *et al.* (2013b) found that young leaves of *Portulaca oleracea* are used for boiled salad. *Portulaca oleracea* is consumed as a salad, either fresh or mixed with yogurt and garlic, as well as cooked with onion (Dogan *et al.*, 2013). Also, in our research, *Brassica oleracea* var. *sabauda* is consumed in salads, soups, stews, and smoothies.

Beta vulgaris ssp. *cicla* is a perpetually available and affordable provider of essential compounds (Ninfali and Angelino, 2013). According to our research, the leaves of *Beta vulgaris* ssp. *cicla* are used as food in salads, soups, stews, pies, sarma, smoothies, and as a spice.

Species of *Chenopodium*, *Malva*, *Rumex*, and *Urtica*, are commonly eaten as leafy vegetables in Turkey (Dogan *et al.*, 2015). In Italy, *Chenopodium album* is traditionally eaten boiled or featured in salads, soups, and stews (Bianco *et al.*, 1996; Nedelcheva, 2013). Numerous studies highlight that *Chenopodium album* is an essential food source, packed with nutrients and antioxidants (Afolayan and

Jimoh, 2009; Hussain *et al.*, 2009). According to the data from our research, *Chenopodium album* and *Spinacia oleracea* are consumed in pies, salads, soups, and stews.

As noted by Ishtiaq *et al.* (2007), *Malva sylvestris* can be made into soup, but it is most commonly used in salads. As shown by the results of our study, *Malva sylvestris* is consumed in salads, stews, soups, teas, and syrup. The leaves of *Rumex acetosa* are utilized in sauces, soups, and for making sarma (Dogan *et al.*, 2015). According to Nedelcheva (2013), *Rumex acetosa* was consumed raw, in salads, and mashed, whereas *Rumex patientia* was used raw, in salads, pies, and soups. *Rumex acetosa* seeds have been traditionally utilized for making flour (Miskoska-Milevska *et al.*, 2025). In our study, respondents reported using *Rumex acetosa* in salads, soups, and smoothies, while *Rumex patientia* is used in stews, pies, sarma, salads, soups, teas, and smoothies. *Urtica dioica* is a valuable source of vitamin A, C, iron, manganese, potassium, and calcium, and is commonly used to prepare herbal teas, soups, as a filling in burek dough, and in other recipes (Singh and Sengar, 2021). In light of the results from our study, *Urtica dioica* is consumed in soups, pies, stews, salads, puree, smoothies, juices, teas, and syrups. Also, Nedelcheva (2013) mentioned the consumption of *Urtica dioica* raw, in salads, soups, pies, dried, and mashed. According to Miskoska-Milevska *et al.* (2020), the dried leaves of *Urtica dioica* are used as a spice (Figure 2).

According to our research, *Eruca vesicaria* subsp. *sativa* has culinary applications in salads and smoothies, as well as being an addition to sandwiches, soups, risottos, and butter. *Ocimum basilicum* is consumed in salads, stews, teas, as a spice, and as a pasta additive.

Traditional medicinal use of leafy vegetables

The favorable nutritional profile of leafy vegetables is beneficial in reducing the risk of cardiovascular diseases and cancer (Randhawa *et al.*, 2015; Rakašćan *et al.*, 2021; Vasileva *et al.*, 2023; Popović *et al.*, 2012; Popović, 2025). Based on the findings of our research, eight plants have been used as a treatment for addressing cardiovascular issues in traditional medicine (*Allium ursinum*, *Apium graveolens*, *Petroselinum crispum*, *Lactuca sativa*, *Taraxacum officinale*, *Eruca vesicaria* subsp. *sativa*, *Beta vulgaris* ssp. *cicla*, and *Urtica dioica*). This effect is in line with the research of Sobolewska *et al.* (2015), Kooti *et al.* (2014), Agyare *et al.* (2017), and Ninfali and Angelino (2013). According to Rexhepi *et al.* (2013), *Allium ursinum* has a positive effect on the cardiovascular system by improving blood flow. The significant potential of *Allium ursinum* in preventing and treating cardiovascular diseases has been demonstrated through both in vitro and in vivo studies (Sobolewska *et al.*, 2015; Simić *et al.*, 2025). *Lactuca sativa* has been applied in traditional medicine for a range of uses, including enhancing circulatory system health (Oladimeji and Kumar, 2023). It is also thought that the leaves of *Taraxacum officinale* may benefit the cardiovascular system owing to their elevated potassium levels (Gonzalez-Castejo *et al.*, 2012; Gallahera *et al.*,

2006). According to Singh and Sengar (2021), the rich combination of vitamin C and iron in stinging nettle makes it excellent for boosting red blood cell production, which in turn enhances blood circulation (Figure 2).

In this research, six plants are mentioned that possess anticancer activates (*Petroselinum crispum*, *Taraxacum officinale*, *Eruca vesicaria* subsp. *sativa*, *Beta vulgaris* ssp. *cicla*, *Spinacia oleracea*, *Ocimum basilicum*). The anticancer effect is due to the chemical composition and it is in keeping with the research of Fernandes *et al.* (2020), Di Napoli and Zucchetti (2021), Garg and Sharma (2014), Ninfali and Angelino (2013), Maeda *et al.* (2008), and Bhat *et al.* (2020). According to Maeda *et al.* (2008), *Spinacia oleracea* may assist in cancer prevention and serve as a functional food with anticancer properties. Due to its chemical composition, *Eruca vesicaria* subsp. *sativa* exhibits inhibitory effects on melanoma and tumors (Garg and Sharma, 2014).



Figure 2. Collection and sale of *Urtica dioica* (Photograph by E. Miskoska-Milevska)

Green leafy vegetables are also important for individuals with type 2 diabetes due to their high fiber and magnesium content, as well as their low glycemic index (Randhawa *et al.*, 2015). Inulin from *Cichorium intybus* roots is considered a functional food ingredient that promotes better health and reduces the risk of many diseases (Kaur and Gupta, 2002). As indicated by our research findings, four plants are identified as suitable for diabetics: *Cichorium intybus*, *Taraxacum officinale*, *Beta vulgaris* ssp. *cicla*, and *Eruca vesicaria* subsp. *sativa*. This is in accordance with the research of Pushparaj *et al.* (2007), Hardeep and Pandey (2013), Di Napoli and Zucchetti (2021), Ninfali and Angelino (2013), and El-Missiry and El-Gindy (2000) and Trifunović *et al.*, (2015).

Portulaca oleracea is utilized as a remedy for scurvy (Bosi *et al.*, 2009).

Also, in our research, it was found that *Portulaca oleracea* has the same use. The study highlighted the use of *Calendula arvensis* in wound healing and

the treatment of burns. Through maceration, the leaves of *Calendula arvensis* were employed to treat internal ulcers, warts, and skin lesions (Saidi *et al.*, 2015). Fortini *et al.* (2016) discovered that *Calendula arvensis*, whether macerated or in ointment form, is used to treat burns and sunburn. In an ethnobotanical study in Greece, Brussell (2004) reported that the flowers, stems, and leaves of *Calendula arvensis* are applied to reduce swelling, treat acne, and alleviate diaper rash. According to Garg and Sharma (2014), *Eruca vesicaria* subsp. *sativa* exhibits antiulcer activity, which correlates with the findings of our research.

The analgesic effects of *Petroselinum crispum* are attributed to the active components in parsley leaves (Fernandes *et al.*, 2020). Additionally, the use of *Petroselinum crispum* in pain relief is highlighted in our ethnobotanical research.

The results obtained from our research showed that 14 plants have a positive effect on gastrointestinal system (*Allium ursinum*, *Angelica sylvestris*, *Anethum graveolens*, *Apium graveolens*, *Calendula arvensis*, *Cichorium intybus*, *Taraxacum officinale*, *Eruca vesicaria* subsp. *sativa*, *Ocimum basilicum*, *Spinacia oleracea*, *Malva sylvestris*, *Rumex patientia*, *Rumex acetosa* and *Urtica dioica*). Al-Howiriny *et al.* (2010) found that *Apium graveolens* extract significantly protects the gastric mucosa and reduces basal gastric secretion in rats, likely due to its antioxidant properties. Korkmaz *et al.* (2016) reported that the flower of *Calendula arvensis* was utilized due to its liver-protective properties. Hepatoprotective activities of *Taraxacum officinale* was reported by Di Napoli and Zucchetti (2021). According to Bhat *et al.* (2020), *Ocimum basilicum* is used in the treatment of gastrointestinal disorders. The leaves of *Rumex acetosa* are used to treat constipation and diarrhea (Dogan *et al.*, 2015; Miskoska-Milevska *et al.*, 2025). The leaves of *Urtica dioica* are utilized for treating liver disorders (Singh and Sengar, 2021). *Anethum graveolens* is used to relieve colic pain in babies and flatulence in young children (Pulliah, 2002). *Eruca vesicaria* subsp. *sativa* has applications in traditional medicine for its therapeutic properties as a digestive aid (Sharma *et al.*, 2012). *Spinacia oleracea* is used in the prevention of hepatic diseases (Jain and Singhai, 2012; Maximas *et al.*, 2014) and nervous disorders (Das and Guhta, 2008). *Malva sylvestris* has been used as a gentle laxative and a detoxifying liver tonic (Guarrera, 2003; Ishtiaq *et al.*, 2007). In European herbal traditions, *Allium ursinum* has been valued for its digestive benefits, antimicrobial properties, detoxifying effects, and its role in cardiovascular disease prevention (Treben, 1992; Macků and Krejča 1989; Leporatti and Ivancheva 2003; Miskoska-Milevska *et al.*, 2025). *Angelica sylvestris* has traditionally been used for its anti-inflammatory, diuretic, expectorant, and diaphoretic properties. It has served as a remedy for colds, influenza, hepatitis, arthritis, indigestion, coughs, chronic bronchitis, pleurisy, typhoid, headaches, flatulence, fever, colic, motion sickness, rheumatism, bacterial and fungal infections, as well as disorders of the urinary system (Sarker and Nahar, 2004).

According to the research *Angelica sylvestris* has a positive effect on appetite. According to Chiocchio et al., (2024), the boiled flowers of *Angelica sylvestris* have traditionally been used to treat baldness.

According to Vasas et al. (2015), *Rumex acetosa* is used for treating skin and gallbladder disorders, which aligns with our findings.

As per the research, four plants show an influence on weight loss (*Apium graveolens*, *Petroselinum crispum*, *Beta vulgaris* ssp. *cicla*, and *Eruca vesicaria* subsp. *sativa*). According to Ahmad et al. (1998), *Apium graveolens* seed stimulates perspiration which may help with weight loss, especially in cases of water retention. *Beta vulgaris* ssp. *cicla* is widely consumed in the modern diet and serves as a significant source of biologically active compounds, including plant pigments, polyphenolic compounds, and essential elements with protective and immune-enhancing properties (Ivanovic et al. 2019). The anti-obesity effects of *Eruca vesicaria* subsp. *sativa* were mentioned by Markou et al. (2024).

The study found that five plants are used in the treatment of anemia (*Beta vulgaris* ssp. *cicla*, *Chenopodium album*, *Spinacia oleracea*, *Rumex patientia*, and *Urtica dioica*). Mehra et al. (2014) reported that *Chenopodium album* is traditionally used in the treatment of anemia. *Urtica dioica* has a long-standing tradition of use in folk medicine for treating anemia (Mueen Ahmed and Parasuraman, 2016; Miskoska-Milevska et al., 2025). *Beta vulgaris* ssp. *cicla* leaves have the highest content of fiber, sodium, magnesium, flavonoids, and vitamin C, while stems are high in potassium (Gamba et al., 2021). *Spinacia oleracea* has beneficial effects on anemia and constipation due to its content of saponins, dietary fiber, ferric salts, and folic acid (Luka et al., 2014).

In keeping with the research, four plants (*Anethum graveolens*, *Petroselinum crispum*, *Ocimum basilicum*, and *Malva sylvestris*) were mentioned as suitable for the treatment of the urinary system. According to Jana and Shekhawat, (2010), *Anethum graveolens* is incorporated into more than 56 Ayurvedic treatments. Based on our research findings, nephroprotective effects are characteristic of *Anethum graveolens*, aligning with the findings of Srivastava et al. (2018). Our research agrees that *Petroselinum crispum* is used for urinary infections and improving kidney function, which aligns with the findings of Blumenthal et al. (2000). According to Bhat et al. (2020), *Ocimum basilicum* is effective in the management of renal/urinary conditions. *Malva sylvestris* leaves and roots are used to treat urinary disorders (Ouhaddou et al., 2014).

In line with the research, diuretic features were mentioned for four plants: *Apium graveolens*, *Petroselinum crispum*, *Chenopodium album* and *Rumex patientia*. According to Belanger (1998) and Al-Howiriny et al. (2010), *Apium graveolens* demonstrates a protective effect on the gastric mucosa and exhibits a diuretic effect. Due to the active components present in parsley leaves, *Petroselinum crispum* possesses many important pharmacological activities, such as diuretic, antispasmodic, hepatoprotective, anti-inflammatory, antidiabetic, analgesic, antianemic, antihyperlipidemic, antitumor, anticoagulant, antihypertensive, and immunosuppressive effects (Fernandes et al., 2020).

According to Das and Borthakur (2022), *Chenopodium album* has traditionally been used as a diuretic. In this research, *Lactuca sativa* was mentioned as a plant that stimulates metabolism. According to Kim *et al.* (2016), the bioactive compounds in *Lactuca sativa* are responsible for its anti-diabetic, anti-inflammatory, and cholesterol-lowering activities.

Due to the phytochemicals found in its flower, leaf, stem, and roots, *Taraxacum officinale* is rich in medicinal properties. The most commonly discussed activities in the scientific literature are its antioxidant, hepatoprotective, and anticancer effects (Di Napoli and Zucchetti, 2021). In agreement with our research, *Taraxacum officinale* is suitable for the treatment of rheumatism.

According to our research, four plants (*Apium graveolens*, *Ocimum basilicum*, *Malva sylvestris* and *Urtica dioica*) are used in the treatment of respiratory problems. Stinging nettle has been linked to treating various respiratory ailments, such as hay fever, asthma, and seasonal allergies (Singh and Sengar, 2021). *Apium graveolens* has various applications in traditional medicine, including the treatment of bronchial diseases and asthma (Mehraj and Alam, 2024). As stated by Bhat *et al.* (2020), *Ocimum basilicum* is incorporated in the treatment of respiratory ailments. The leaves and roots of *Malva sylvestris* are utilized in treating respiratory ailments (Ouhaddou *et al.*, 2014).

Seven plants (*Anethum graveolens*, *Lactuca sativa*, *Taraxacum officinale*, *Eruca vesicaria* subsp. *sativa*, *Brassica oleracea* var. *viridis*, *Beta vulgaris* ssp. *cicla*, and *Urtica dioica*.) were mentioned as suitable for improving the immune system. *Lactuca sativa* is known for its significant vitamin C content, which supports the prevention of infections (Anasuya *et al.*, 2024). Immunoprotective characteristic of *Taraxacum officinale* was reported in scientific literature (Kim *et al.*, 1998; Lee *et al.*, 2012; Sang *et al.*, 2019; Tan *et al.*, 2017). As reported by Dogan *et al.* (2015), *Urtica* spp. is utilized for its anti-inflammatory and diuretic effects. Based on Ninfali and Angelino (2013), the healing properties of *Beta vulgaris* ssp. *cicla* contribute to enhancing immunological responses. Additionally, *Anethum graveolens* plays a significant role in immunity maintenance (Meena *et al.*, 2019). Sinapic acid and its derivatives are essential in the immune defense against infection (Nićiforović and Abramović, 2014). *Eruca vesicaria* subsp. *sativa* shows antimicrobial activity (Garg and Sharma, 2014).

Based on the findings of our research, *Petroselinum crispum*, *Lactuca sativa*, and *Eruca vesicaria* subsp. *sativa* are used in detoxification. *Lactuca sativa* is applied in Unani medicine as a blood detoxifier (Oladimeji and Kumar, 2023).

As shown by the results of our study, *Taraxacum officinale*, and *Eruca vesicaria* subsp. *sativa* are used because of their benefits on bone health. Malik *et al.* (2011) report that a mixture of boiled leaves of *Taraxacum officinale*, salt, and turmeric is typically used to heal bone fractures.

According to Bhat *et al.*, (2020), *Ocimum basilicum* is used in the treatment of mental health issues, which aligns with our research.

CONCLUSIONS

Based on the findings of our study, twenty-one species from ten plant families were recognized as leafy vegetables. The greater part of the species is classified within the Apiaceae (4) and Asteraceae (4) families. *Spinacia oleracea* recorded the greatest relative citation frequency index (0.58), followed by *Petroselinum crispum* (0.52) and *Beta vulgaris* ssp. *cicla* (0.42). The plant species listed in this study are used not only as food but also for traditional medicinal purposes in the treatment of various health problems. Only for *Brassica oleracea* var. *sabauda* was no medicinal use documented. The intuitive use of these species for treating health problems is further supported by scientific research. However, studies focusing on traditional knowledge related to leafy vegetables in the Republic of North Macedonia remain limited, and more comprehensive investigations are required from social, dietary, and ecological perspectives. To protect the knowledge related to leafy greens and their application in human life, it is crucial to continue conducting similar ethnobotanical research.

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ANALYSIS OF THE EFFECTIVENESS OF AFFORESTATION TECHNOLOGIES IN THE NORTH-EASTERN FOREST-STEPPE OF UKRAINE

SUMMARY

The study is dedicated to the scientific substantiation of the effectiveness of technologies for creating highly productive and biologically stable forest plantations under the critical conditions of the North-Eastern Forest-Steppe of Ukraine, considering the impact of anthropogenic and climatic challenges that necessitate a shift from monocultures to more sustainable mixed stands. The analysis showed that the conditional survival rate of seedlings is consistently high in all studied forest site types and mixing schemes, exceeding 85%. Furthermore, the dispersion analysis demonstrated that the influence of both the forest site type and the mixing scheme on the average survival rate is statistically insignificant. However, the mensurational inventory of mature stands revealed a significant process of natural thinning, where the proportion of suppressed and dying trees (according to Kraft) reaches over 50% for some species. In view of this, the results obtained underscore that the main focus in afforestation should be shifted from ensuring high initial survival rates to developing strategies for managing mature mixed stands to enhance their long-term stability, resistance, and adaptive capacity to changing climatic conditions.

Keywords: afforestation, mixing schemes, survival, sanitary condition, stability

INTRODUCTION

Creating highly productive forest stands is one of the key tasks of Ukraine's forestry complex, especially amid the anthropogenic impact caused by military actions. The North-Eastern Forest-Steppe of Ukraine, and the Slobozhanshchyna region in general, is one of the areas most affected by the full-scale invasion (Kuzyk and Tovarianskyi, 2023; Kovalzhy *et al.*, 2024; Voitovyk

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et al., 2024a). Therefore, the issue of careful selection and substantiation of afforestation and reforestation technologies is extremely relevant now and will remain so for quite a long time in the future.

On the other hand, climate change is also having a detrimental effect on forest stands. Specifically, a study of Vacek *et al.* (2023) analyzed more than 360 publications between 1993 and 2022 and found that climate change has become quite tangible for European forests, particularly through fires, moisture deficit, or the development of pests, which significantly impacted natural wood increment. This is the other side of the coin that also requires a detailed study of afforestation strategies. The ineffective use of technological approaches leads to low seedling survival rates, reduced biological stability of stands, and, consequently, significant economic losses.

Given the challenges mentioned, the scientific community is paying significant attention to the issues of increasing the effectiveness of forest stand establishment. Specifically, the study of Liu *et al.* (2022) discusses that afforesting drylands creates a dilemma due to conflicting results. Namely, increasing forest cover enhances water consumption through a nonlinear increase in evapotranspiration, a trend that will be deepened by climate change.

The research of Pérez-Silos *et al.* (2021), based on a meta-analysis of 75 publications covering 128 afforestation actions in Spain, found that large-scale afforestation programs of the 20th century improved timber provision and carbon sequestration. At the same time, it was established that afforestation programs did not enhance regulating ecosystem services (ES) and biodiversity conservation compared to natural sites, and were only effective compared to highly degraded areas. Many authors conclude that there is a need to shift towards integrated landscape approaches in afforestation that are not limited solely to productivity criteria (Song *et al.*, 2023; Briske *et al.*, 2024; Enescu *et al.*, 2025).

The research of other scientists focuses on the selection of sustainable tree species (Belmega *et al.*, 2024), the optimization of mixing schemes and planting density (Vasilevskyi *et al.*, 2024), as well as the improvement of soil preparation methods (Varnagirytė-Kabašinskienė *et al.*, 2022). However, most existing scientific works typically focus on individual elements of the technological process (for example, only on the types of planting material or only on methods of mechanized soil cultivation).

Currently, there is a critical deficit of comprehensive studies that would provide a comparative assessment of the entire technological chain of forest stand establishment—from site preparation to tending of young growth—directly under the conditions of the North-Eastern Forest-Steppe of Ukraine. This gap becomes even more significant in light of the need for rapid and effective restoration of forests affected by hostilities (Hryhoriv *et al.*, 2024; Voitovyk *et al.*, 2024b; Datsko *et al.*, 2025) and the adaptation of forest stands to changing climatic conditions.

Unlike most previous studies of afforestation in Ukraine and Eastern European countries, which focused mainly on general silvicultural indicators or regional features of plantation cultivation, this work focuses on a comprehensive

analysis of the conditions for growing planting material and its survival in specific forest vegetation conditions. The results obtained allow us to clarify the effectiveness of the applied technologies for creating forest crops and can be used in forestry practice to increase the productivity and stability of artificial plantations. The practical recommendations formulated based on the results of the study are of practical importance for optimizing silvicultural activities in the study region.

In view of the critical deficit of comprehensive regional studies mentioned, this work aims to provide scientific substantiation and a comparative assessment of the effectiveness of the entire technological chain for the establishment of highly productive and biologically stable forest stands under the specific conditions of the North-Eastern Forest-Steppe of Ukraine.

MATERIAL AND METHODS

The research was conducted on the territory of the Sumy Forestry Enterprise, located in the eastern part of the Sumy region, which belongs to the Left-Bank Dnieper Forest-Steppe (according to forest-growing regionalization). The region is situated in the southern wing of the Dnipro-Donetsk Depression on the East European Platform.

The relief is predominantly flat, with elevations ranging from 115 to 230 meters above sea level, marked by the presence of river valleys and ravines (balkas), as well as the floodplains of the Psel River. The soil cover is mainly represented by gray and podzolized soils, as well as dark gray soils and chernozems.

The region's climate is moderately continental, characterized by mild winters and warm summers. The average frost-free period lasts 169 days. The average annual relative air humidity is maintained at about 78%. The prevailing wind direction is south-east, with an average speed ranging from 4.6 m/s (maximum) to 2.4 m/s (minimum). Overall, the natural and climatic conditions of the studied region are favorable for the growth and development of forest vegetation, despite a noticeable trend toward warming.

The objects of the study were artificial forest plantations of various ages, established and grown on the territory of the Sumy Forest District. The research included five inventory sample plots, which were laid out in artificial stands representing the main forest site types of the forestry enterprise (Table 1). To ensure representativeness and assess a wide range of silvicultural approaches and growing conditions, sample plots were selected from the forest inventory and forest plantation records of the forestry unit. Five permanent sample plots were established to assess the condition and productivity of the forest stands. The size of each research plot was determined in strict compliance with current regulatory documents, ensuring a 5% error margin for the forest inventory and the assessment of key forestry indicators (Fig. 1).

Table 1. Detailed Characteristics and Mensurational Indicators of the Research Plots

No. of SP (Sample Plot)	Area, ha	Forest Site Type (FST)	Stand Age, years	Stand Composition	Planting Scheme, m
SP No. 1	2.2	Moist Oak-Pine Undergrowth (V3)	39	8Sc2Qc+B	2.5×0.5
SP No. 2	0.8	Fresh Oak-Pine Undergrowth (B2)	57	10Sc+Bp	2.5×0.7
SP No. 3	1.2	Fresh Pine Forest (B2)	45	9Sc1Bp	2.5×0.5
SP No. 4	3.2	Fresh Conglomerate (C2)	55	7Sc3Qc+Bp	2.5×0.5
SP No. 5	0.6	Fresh Birch Forest (A2)	48	10Sc	2.5×0.6

*Sc – Pine, Qc – Oak, B – Birch, Bp – Silver Birch.

**Figure 1.** Research plots of forest plantations

The trial plots established within the studied forestry enterprise represent typical forest vegetation conditions of the North-Eastern Forest-Steppe of Ukraine (fresh subors and sugruds). At the same time, the obtained results primarily characterize the conditions of forestry management of a particular enterprise and can be extrapolated to other forestry objects of the region with similar soil-climatic and technological conditions.

A comprehensive field description was conducted on each sample plot, which included recording the geographic location (quarter, compartment, and area numbers), characterization of forest growth conditions (FGC), and a detailed description of the understory and ground cover (with identification of dominant species such as lingonberry, heather, rowan, and buckthorn). Furthermore, the current sanitary condition of the stands was assessed, and archival data regarding establishment silviculture (year and season of planting, type of planting material, specifically standard seedlings, and the exact planting scheme) were collected.

Tree inventory was conducted stem-by-stem, classified by main species. Tree diameter was measured at a 2 cm interval. Height was measured multiple times for each diameter category with an accuracy of 0.5 m. Stand quality (site index, or bonitet) was assessed using the M.M. Orlov scale, which is based on the ratio of the stand's average age and average height. The sanitary condition of each tree was classified according to the "Sanitary Rules in the Forests of Ukraine". Calculations for stand fullness (the ratio of the actual sum of the cross-sectional areas of the trunks to the normative value) and the stock of stem wood were performed according to generally accepted forest inventory methods.

The results obtained primarily characterize the conditions of forestry management of a particular enterprise and can be extrapolated to other forestry facilities in the region with similar soil, climatic and technological conditions.

To identify the patterns of growth and productivity of the forest stands, as well as to evaluate the influence of various factors (age, FGC, fullness) on the inventory indicators, the software Statistica 10.0 and Microsoft Excel were used.

RESULTS AND DISCUSSION

Figure 1 illustrates the dependence of the average survival rate of forest stands on the five main forest growth condition types (FGC) that are the subject of the study. The overall analysis indicates that seedling survival is relatively high across all studied FGCs, ranging between 89% and 94%. The highest average survival rate was recorded in the B2 Fresh Oak-Pine Forest (Subir), where it is approximately 91.8%, and in the B3 Moist Oak-Pine Forest (Subir) (91.6%). These two condition types also demonstrate the largest range of potential survival, reaching almost 94%. The lowest average survival rate was noted in the A1 Dry Pine Forest (Bir) around 89.8% while this FGC is also characterized by the least variability. The A2 Fresh Pine Forest (Bir) and C2 Fresh Oak-Pine Grove (Sugrud) types demonstrate intermediate values, indicating the consistently high effectiveness of traditional forest regeneration technologies in fresh and moist subors compared to drier conditions.

The results of the analysis of variance (ANOVA), shown in Table 2, indicate that the influence of forest growth condition type (FGC) on the average seedling survival rate is statistically insignificant. Since the p-value (0.74) significantly exceeds the generally accepted significance level of 0.05, there are insufficient grounds to assert that the difference in survival between the studied FGCs is substantial.

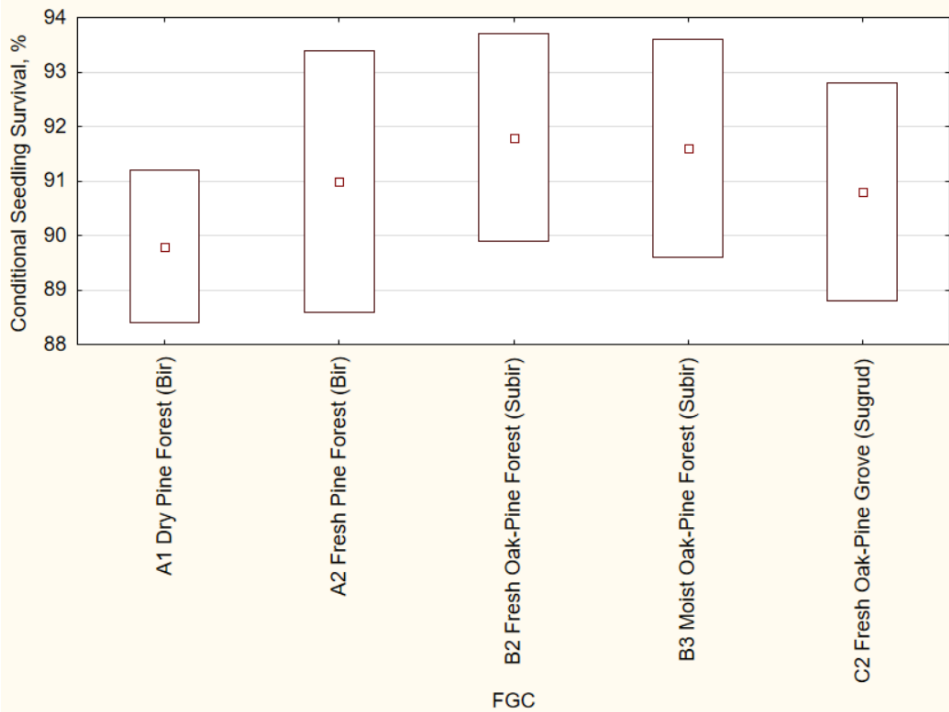


Figure 2. Diagram of the average survival rate of forest stands by forest growth condition types

This implies that the observed differences in average survival rates, as illustrated in Figure 2, can be attributed to randomness rather than the type of growing conditions.

Table 2. Analysis of variance (ANOVA) of the influence of forest growth condition type on conditional seedling survival

Indicator	Sum of Squares – Effect	Degrees of Freedom (df) – Effect	Mean Square – Effect	Sum of Squares – Error	Degrees of Freedom (df) – Error	Mean Square – Error	F-Ratio	p-value
Conditional Seedling Survival, %	7.44	4.0	1.86	38.66	10.0	3.866	0.48	0.74

Figure 3 demonstrates the dependence of the average conditional seedling survival rate on five different forest stand mixing schemes that were the subjects of the study. Overall, all examined schemes ensure high survival rates. The highest average survival value was recorded for the 10Sc+Bp scheme – approximately 91.7% – while the lowest average indicator was observed in the pure pine culture 10Sc – around 89.7%. Furthermore, the 9Sc1Bp scheme demonstrates the greatest variability, reaching a potential maximum of over 93%, and the 7Sc3Qc+Bp and 8Sc2Qc+B schemes have virtually identical average indicators. The consistently high survival in mixed stands may indicate a positive influence of the admixture of deciduous species on the microclimate and soil conditions.

High initial seedling survival (Table 3) characterizes only the establishment stage and does not take into account long-term factors, including competition, climatic stresses, and biotic damage, which manifest themselves at later stages of plantation development and can reduce their stability.

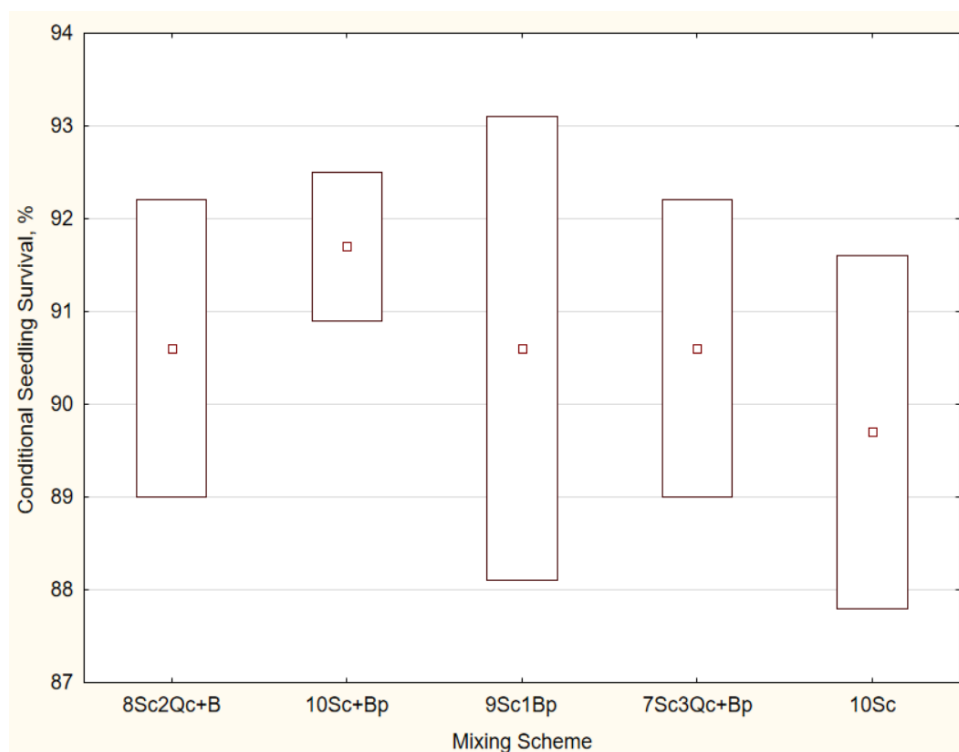


Figure 3. Dependence of the average survival rate of forest stands on the mixing scheme of the plantations

According to the data obtained, the F-ratio is equal to 0.48, and the corresponding p-value is 0.74. Since the obtained p-value (0.74) significantly exceeds the critical significance level of 0.05, it can be concluded that the

difference in average survival rates among the studied mixing schemes is not statistically significant.

Table 3. Results of the analysis of variance (ANOVA) of the influence of mixing scheme on seedling survival

Sum of Squares - Effect	Degrees of Freedom (df) - Effect	Mean Square - Effect	Sum of Squares - Error (Residual)	Degrees of Freedom (df) - Error (Residual)	Mean Square - Error (Residual)	F-Ratio	p-value
Conditional Seedling Survival, %	6.04	4.00	1.51	31.24	3.12	0.48	0.74

Table 4 displays the key forest inventory indicators and growing conditions of five research sample plots established in artificial Scots pine stands. The age of the stands covers a wide range, from 39 to 57 years, which allows for the assessment of their development at various stages. The vast majority of plantations were created using the standard 2.5×0.5 m planting scheme, while the stands are characterized by high productivity and fullness indicators: timber stock ranges from 220 to 290 m³ ha⁻¹, and fullness is between 0.7 and 0.9. It is worth noting that the research plots represent various forest growth condition types and mixing schemes, including both pure pine culture and complex mixed stands with admixtures of oak and birch, which ensures the representativeness of the sample for further analysis.

Table 4. Forest Inventory Characteristics and Growing Conditions of Research Sample Plots

Sample Plot No. (SP)	Age, years	Planting Scheme, m	Mixing Scheme	FGC	Fullness	Site Index (Bonitet)	Stock, m ³ ha ⁻¹
1	39	2.5×0.5	8Sc2Qc+B	B3	0.9	I	220
2	57	2.5×0.7	10Sc+Bp	B2	0.7	II	290
3	45	2.5×0.5	9Sc1Bp	B2	0.8	I	280
4	48	2.5×0.5	7Sc3Qc+Bp	C2	0.9	I	260
5	55	2.5×0.6	10Sc	A2	0.7	II	250

The results of the forest inventory, presented in Table 5, illustrate the structural and sanitary condition of the forest stands on the research sample plots. According to the Kraft classification, the codominant layer (Class II) prevails in most stands, especially in pine, where its share ranges from 37.5% to 64.6% (SP No. 2 and No. 4, respectively). The share of suppressed, dying, and dead trees (Classes III, IV, and snags) is significant; for example, in pine on SP No. 2, it is about 50.0%, and in birch on SP No. 3, it is 85.7%, which indicates an active process of natural thinning at a mature age. Analysis of the sanitary condition confirms this trend: in some areas (e.g., SP No. 5, pine), the share of severely weakened and dying trees (Classes III, IV) is high (43.3%), whereas in birch on

SP No. 3, healthy trees (Class I) constitute 90.5%. This demonstrates that stability depends not only on the species but also on specific forest growth conditions and the mixing scheme.

Table 5. Distribution of Trees by Kraft Classes and Sanitary Condition, %

SP No.	Species	Kraft Classes, %	Sanitary Condition (Living Trees), %	Deadwood (Snags/Logs), %
		Dominant (I)	Codominant (II)	Suppressed (III, IV, Dead)
1	Sc	35.3	47.1	17.6
	Qc	14.3	42.9	42.8
	Bp	-	41.9	58.1
2	Sc	12.5	37.5	50.0
	Bp	-	28.6	71.4
3	Sc	22.6	50.9	26.5
	Bp	-	14.3	85.7
4	Sc	8.3	64.6	27.1
	Qc	26.9	30.1	43.8
	Bp	-	10.2	88.2
5	Sc	16.7	40.0	43.3

The analysis of variance (ANOVA) indicated that the influence of both the forest growth condition type (FGC) and the mixing scheme on seedling survival is statistically insignificant, despite the observed high average survival rates, with a tendency toward better results in fresh subors and mixed schemes. Concurrently, the forest inventory reveals a significant process of natural thinning in mature stands, where the proportion of suppressed and dying trees (Kraft Classes III, IV) can exceed 50% in some species, highlighting the necessity of controlling their sanitary condition. In the study by (Urgoiti Otazua and Paquette, 2018), it is proven that forest plantations are becoming crucial for providing society with both timber and non-timber products amidst a global reduction of natural forests. However, the predominance of monocultures can negatively impact ecosystem functions and reduce resilience to growing ecological threats. Thus, there is an urgent need for mixed-species stands, which, by supporting biodiversity and different recovery mechanisms, ensure greater resistance, adaptive capacity, and stability of productivity compared to monocultures. Based on the review by (Nguyen *et al.*, 2018), recent studies confirm the functional advantages of mixed plantations over traditional monocultures, although practical guidelines for their design and management remain insufficient. Mixed systems have been found to improve productivity and ecosystem services, but full implementation requires additional research into economic feasibility, the development of adapted silvicultural guidelines for various socio-economic conditions, and the assessment of their resistance to pests and disturbances at a mature age. The research by (Blystiv *et al.*, 2021), focused on stand stability, tested a methodology based on calculating stability indicators (stability coefficient, loss of biotic/ecological stability) using forest inventory assessment,

sanitary condition, and natural regeneration, allowing stands to be classified as stable, conditionally stable, or unstable. The resulting indicators (particularly the living space tension indicator) are crucial for planning forestry measures in breeding facilities, including forest genetic reserves and permanent forest seed plots. The study by (Puzrina *et al.*, 2022) confirms the relevance of the pine stand dieback problem in Ukraine, revealing that the main pathological cause is infection by the pathogen *Heterobasidion annosum*, combined with mass infestation by stem pests, predominantly bark beetles of the genus *Tomicus*. The dynamics of observations since 2011 show a deterioration in the sanitary condition of pine stands, especially after the dry growing seasons of 2015-2017, which led to an outbreak of aggressive bark beetle species (*Ips acuminatus*); however, since 2019, a decrease in their numbers has been observed due to climatic factors (increased precipitation and decreased temperature). The results obtained serve as a basis for developing a strategy for managing damaged forest areas.

The results obtained indicate a relatively high level of survival of forest crops under the studied conditions, however, the differences found between individual options were not always statistically significant at the accepted level of significance. This may be due to the limited number of trial plots, intra-plot spatial heterogeneity, and the influence of abiotic and biotic factors that are difficult to fully control in field conditions. At the same time, from an ecological and silvicultural point of view, even statistically insignificant differences in survival are of practical importance, since they affect the initial density of stands, competitive relationships, and subsequent stability of forest crops. These limitations necessitate cautious interpretation of the results and indicate the feasibility of further studies with an expanded sample and longer monitoring.

CONCLUSIONS

The analysis established a high, yet statistically insignificant, influence of both the forest growth condition type and the mixing scheme on seedling survival, indicating the consistently high effectiveness of traditional forest regeneration methods under the studied conditions, irrespective of minor variations in FGC and composition. Simultaneously, the inventory of mature stands revealed a significant process of natural thinning and deterioration of the sanitary condition, where the proportion of suppressed and dying trees reaches up to 85.7% for birch and 50% for pine. These results, consistent with current global trends, confirm that while initial survival is high, the long-term stability of stands against external factors remains a critical issue. Therefore, future silvicultural measures should be directed not so much at increasing initial survival but at developing adapted guidelines for managing mature mixed stands that ensure increased biotic and ecological stability in the face of growing climatic risks.

Forestry recommendations.

1. Orientation of silvicultural measures to the long-term stability of plantations. Given the high initial survival rate of seedlings, further silvicultural

measures should be directed not to intensifying the stage of crop creation, but to ensuring their stable development in the middle and old age periods by regulating the density and species composition.

2. Optimization of maintenance and thinning regimes. It is recommended to carry out the first maintenance felling during the period of crown closure in order to reduce intraspecific and interspecific competition. Further thinning should be carried out in a differentiated manner, taking into account the sanitary condition of trees and the spatial structure of plantations, giving preference to the removal of depressed and weakened individuals.

3. Adjustment of the composition of mixed plantations. According to the results of the research, it is advisable to maintain mixed stands with the dominance of the main species (pine) and the participation of related deciduous species within limits that do not lead to excessive competition at later stages of development. Particular attention should be paid to regulating the participation of fast-growing deciduous species, which in mature cultures demonstrate a high level of suppression and drying.

4. Systematic monitoring of the sanitary condition. It is recommended to implement regular monitoring of the condition of stands with recording indicators of viability, the level of suppression and manifestations of drying, which will allow timely adjustment of silvicultural measures and reduce the risks of degradation of stands.

5. Adaptation of forestry decisions to climate change. When planning maintenance activities, it is advisable to take into account the increase in climatic risks, in particular the frequency of dry periods, by reducing excessive density of plantings and maintaining structural diversity, which will contribute to increasing their biotic and ecological resilience.

Further research should focus on long-term monitoring of forest crops with an assessment of the dynamics of their growth, condition and structure in different age periods. The priority area is the study of the resistance of stands to abiotic and biotic disturbances, in particular droughts, temperature extremes and damage by pests. Special attention should be paid to the analysis of the dynamics of mixed stands with different ratios of tree species, which will allow assessing their potential for increasing ecological stability and productivity in the conditions of the North-Eastern Forest-Steppe of Ukraine.

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ASSESSMENT OF POSITIVE CHANGES IN WINTER WHEAT VARIETIES UNDER MUTAGEN TREATMENT

SUMMARY

The exploration of novel agents capable of inducing practical genetic diversity in modern winter wheat varieties represents a significant avenue for enhancing both commercial wheat breeding and recombination-based selection methods. This study investigated the effectiveness of a relatively novel chemical mutagen, 1,4-bisdiazoacetylbutane (DAB), as an inducer of beneficial variability in grain yield and quality attributes, as well as in other agriculturally significant traits. Eight varieties of winter wheat (Perspektyva Odeska, Sonata Poltavska, Shpalivka, MIP Lada, Farell, NE 12443, Ronin, and Seilor) were exposed to the mutagen DAB at three concentrations: 0.1%, 0.2%, and 0.3%. Among the tested varieties, Farell, Ronin and Seilor exhibited the highest responsiveness, making them particularly favorable candidates for targeted mutagenesis aimed at enhancing beneficial traits. Optimal results, characterized by substantial genetic variability and an increased proportion of agriculturally beneficial forms, were consistently achieved at the highest DAB concentration tested (0.3%). Two particularly promising mutant lines emerged from the study, exhibiting the most favorable combination of high grain productivity, consistent yield stability and desirable grain quality traits suitable for bread-making purposes. Furthermore, several other mutant lines derived in this study exhibit significant potential as valuable parental lines for cross-breeding.

Keywords: cereals; variability; 1,4-bisdiazoacetylbutane; rate of positive changes; nutrition value; site-specific action; mutations; *Triticum aestivum* L.

INTRODUCTION

Changes in environmental conditions increasingly require agricultural crops to adapt to new and sometimes challenging circumstances. Climate change, in particular, has introduced a broad array of stress factors, including altered

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precipitation patterns, rising temperatures, increased incidence of extreme weather events, and shifts in pest and disease dynamics (Bezie *et al.*, 2020)

Among various methods of mutagenesis, chemical mutagenesis has demonstrated significant potential due to its unique ability to induce specific genetic alterations in plant genomes, thereby enhancing variability in beneficial traits. According to Lal *et al.* (2020), chemical mutagens have been notably effective in triggering genome mutations that lead to heritable phenotypic changes, enhancing crop adaptation and resilience. However, mutations induced by chemical mutagens are inherently random, lacking precise predictability regarding their outcomes. As J. Chaudhary *et al.* (2019) have reported, many mutations induced by chemical mutagenesis do not confer immediate advantages, and in fact, some mutations might prove detrimental, negatively impacting plant growth, survival, and productivity. Nevertheless, within these randomly generated genetic changes, breeders can discover and isolate beneficial mutations, leading to varieties characterized by improved yield (Nazarenko, 2016). W. Yali and T. Mitiku (2022) emphasized that while chemical mutagenesis significantly influences the heredity and developmental processes in crops.

Chemical supermutagens, characterized by their pronounced DNA-damaging capacity, are particularly notable within mutation breeding, as they typically induce more extensive and complex genetic alterations. M. Spencer-Lopes *et al.* (2018) highlighted the effectiveness of chemical supermutagens in major crops, demonstrating that they could successfully produce mutant forms with complex and highly valuable trait combinations. These supermutagens are routinely used not only in applied breeding programs but also in fundamental research aiming to elucidate the genetic basis of complex traits (Kartseva *et al.*, 2023).

Specific chemical mutagens exhibit preferential affinities toward particular genetic structures or sequences, resulting in higher mutation frequencies at these loci. G. Abaza *et al.* (2020) emphasized two critical components necessary for effective site-specific mutagenesis: the genetic constitution of the treated variety and the chemical properties of the mutagen itself. The effects induced by high concentrations of chemical mutagens can be challenging to predict accurately, as different crop varieties may respond differently to the same mutagenic agent (Ahumada-Flores *et al.*, 2021). The interaction between genotype and mutagen concentration plays a critical role in the outcome of mutagenesis experiments. Consequently, breeding programs incorporating chemical mutagenesis must carefully consider these factors through comprehensive preliminary studies, as suggested by Ram *et al.* (2019) and Abdel-Hamed *et al.* (2021).

Winter wheat (*Triticum aestivum* L.) represents a critically important crop for Ukraine and countries with comparable agroecological conditions. The changing climate conditions necessitate the urgent development of novel winter wheat genotypes adapted to emerging environmental challenges, either through introducing genetic diversity from different ecological and geographic sources or

by generating new variability directly from local genetic resources (Cann *et al.*, 2022).

To address these challenges, chemical mutagenesis methods are increasingly applied in breeding programs focused on winter wheat. These programs aim to induce genetic variability sufficient to create novel genotypes capable of sustaining high productivity under changing climatic conditions (Nazarenko *et al.*, 2021). Recent research has demonstrated that properly selected mutagenic agents and optimal treatment concentrations can significantly enhance adaptive traits in winter wheat, particularly those related to stress tolerance, disease resistance, and grain quality attributes essential for baking and human nutrition (Jalal *et al.*, 2021).

In summary, the purpose of this study was to comprehensively investigate the specific patterns of induced heritable variability in winter wheat varieties, emphasizing the frequency and range of beneficial mutations arising from genotype-by-mutagen interactions.

MATERIAL AND METHODS

Field experiments were conducted during 2022–2024 (M₄–M₆ generations) at the experimental fields of the Educational and Scientific Center of the Dnipro State Agrarian and Economic University (48°50'98" N, 35°25'63" E), located in the Dniprovskiy district, Dnipropetrovsk region, Ukraine. This region is characterized as a northern warm, moderately arid agro-climatic zone with a hydro-thermal coefficient above 0.9. Total precipitation during the growing season typically ranged from 250 to 280 mm, with an annual accumulation of approximately 450–490 mm. The sum of temperatures above 10°C during the growing period was approximately 2900°C.

Eight winter wheat varieties (*Triticum aestivum* L.), Perspektiva Odeska, Sonata Poltavska, Shpalivka, MIP Lada, Farell, NE 12443, Ronin, and Seilor, were selected as initial plant material. Seeds (1000 grains per treatment variant, 14 % seeds moisture) were treated with the mutagen DAB (1,4-bis(diazoacetyl)butane) in concentrations of 0.1, 0.2 and 0.3% (Sigma-Aldrich, Germany). After treatment, the seeds wrapped in gauze were rinsed under running water; all procedures were performed in a laboratory hood using appropriate personal protective equipment. Sowing was carried out under field conditions immediately after rinsing. Treatments were conducted by soaking seeds for 18 hours, following established protocols for chemical mutagenesis (Spencer-Lopes *et al.*, 2018; Nazarenko, 2021).

In the M₂ and M₃ generations, induced mutations were visually assessed, and inheritance patterns were studied. Seeds were sown by hand in family plots (1–3 rows per plot; row spacing 0.15 m, row length 1.5 m). Mutation rates were calculated as the percentage of mutant occurrences relative to the total number of families.

Mutant lines were evaluated for yield and grain quality parameters from the M₄ to M₆ generations. Plots varied from 5 to 10 m², depending on the year of

the experiment, with replication performed 1–2 times and standard control varieties included every 20 plots. Grain protein content was quantified using a Spektra RT device. Glutenin and gliadin contents were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). The concentration of essential microelements (Mg, Mn, Zn, Mo, Co, Cu) in grains was determined using an Agilent 5110 inductively coupled plasma atomic emission spectrometer (ICP-AES), with wavelength parameters calibrated against Agilent multi-element standard solutions.

Statistical analyses were conducted using Statistica 10.0 software (multivariate module, TIBCO, Palo Alto, USA). The significance of treatment effects was evaluated through analysis of variance (ANOVA), complemented by discriminant and cluster analyses (Euclidean distance, single linkage method). Pairwise comparisons between treatments were performed using Tukey's HSD test, with significance set at $P < 0.05$.

RESULTS AND DISCUSSION

The evaluation of plant material derived from NP-40 treatment began with the assessment of positive phenotypic changes in the second and third generations (M_2 – M_3), followed by analysis of yield and grain quality traits in later generations (M_4 – M_6). All selected lines were successively sub-cultivated to confirm the inheritance of observed changes, thereby ensuring that the modifications were stable mutations rather than transient epigenetic effects. In total, 16,000 families (lines) were evaluated at M_2 – M_3 stages for DAB -induced populations. Out of these, 481 mutant lines were selected as carriers of noticeable trait changes (see Table 1). Among them, 205 lines showed agriculturally valuable traits, and 76 lines were identified as elite material — exhibiting positive traits without any undesirable characteristics, and thus were advanced for breeding purposes. Each treatment variant included 500 families.

Mutant lines were selected based on the following morphological traits (evaluated at M_2 – M_3 stages): thick stem, short-stem, semi-dwarf, dwarf forms, intense epicuticular wax accumulation, long spike and large spike size, large grain size, early ripeness, disease tolerance, tillering capacity, productivity indicators (preliminary M_2 – M_3 , confirmed at M_4 – M_6). Grain quality traits (evaluated during M_4 – M_6 ecological trials), such as higher total protein content, positive shifts in gluten composition: changes in HMW/LMW glutenins and gliadins, enhanced microelement content: Mg, Mn, Zn, Mo, Co, Cu. These traits were used as selection markers to distinguish lines with practical breeding value. The overall mutation efficiency under DAB was moderate promising: nearly 1.2% of families produced valuable breeding material (205/16,000 averaged across variants).

For most varieties and traits, the frequency of induced changes increases as DAB concentration increases from 0.1% to 0.3%. Most responsive traits across varieties were early ripeness, tolerance for diseases, protein-related traits (content

and composition) and productivity. Less responsive traits were thick stem, dwarf types, intense wax accumulation. Other traits had average variability.

Table 1. Parameters of mutagen action, DAB, ($\bar{x} \pm \text{SD}$, $n=500$)

Variety	Variant	RPC, %	PFGR, %	NL, pcs	NPL, pcs	RPL, %
Perspektyva Odeska	water	0.00 ± 0.00^a	0.00	0	0	0.00 ± 0.00^a
	DAB 0.1%	2.00 ± 0.15^b	0.59	9	3	0.60 ± 0.08^b
	DAB 0.2 %	1.60 ± 0.12^c	0.42	7	2	0.40 ± 0.07^b
	DAB 0.3 %	3.40 ± 0.21^d	0.65	12	3	0.60 ± 0.08^b
Sonata Poltavska	water	0.20 ± 0.03^a	0.20	0	0	0.00 ± 0.04^a
	DAB 0.1%	1.60 ± 0.13^b	0.57	7	2	0.40 ± 0.08^b
	DAB 0.2 %	1.40 ± 0.12^b	0.39	5	2	0.40 ± 0.08^b
	DAB 0.3 %	2.80 ± 0.20^c	0.56	10	4	0.80 ± 0.14^c
Shpalivka	water	0.20 ± 0.01^a	0.50	0	0	0.00 ± 0.00^a
	DAB 0.1%	1.40 ± 0.12^b	0.47	5	1	0.20 ± 0.04^b
	DAB 0.2 %	2.20 ± 0.17^c	0.58	7	3	0.60 ± 0.04^c
	DAB 0.3 %	3.40 ± 0.22^d	0.68	10	4	0.80 ± 0.09^c
MIP Lada	water	0.40 ± 0.03^a	0.67	1	1	0.20 ± 0.04^a
	DAB 0.1%	1.60 ± 0.13^b	0.62	7	2	0.40 ± 0.05^a
	DAB 0.2 %	2.20 ± 0.18^c	0.61	8	3	0.60 ± 0.05^{ab}
	DAB 0.3 %	2.80 ± 0.21^d	0.58	10	5	1.00 ± 0.10^c
Farell	water	0.20 ± 0.00^a	0.33	1	0	0.00 ± 0.00^a
	DAB 0.1%	1.40 ± 0.12^b	0.47	6	2	0.40 ± 0.05^b
	DAB 0.2 %	2.40 ± 0.18^c	0.60	9	4	0.80 ± 0.08^c
	DAB 0.3 %	3.40 ± 0.21^d	0.68	12	7	1.40 ± 0.15^d
NE 12443	water	0.00 ± 0.00^a	0.00	0	0	0.00 ± 0.00^a
	DAB 0.1%	1.20 ± 0.10^b	0.40	5	1	0.20 ± 0.03^b
	DAB 0.2 %	1.60 ± 0.14^c	0.38	7	1	0.20 ± 0.03^b
	DAB 0.3 %	3.80 ± 0.23^d	0.79	12	3	0.60 ± 0.08^c
Ronin	water	0.20 ± 0.01^a	0.50	0	0	0.00 ± 0.00^a
	DAB 0.1%	1.80 ± 0.13^b	0.75	8	3	0.60 ± 0.03^b
	DAB 0.2 %	2.20 ± 0.16^c	0.69	8	4	0.80 ± 0.07^c
	DAB 0.3 %	3.80 ± 0.23^d	0.79	14	7	1.40 ± 0.12^d
Seilor	water	0.00 ± 0.00^a	0.00	0	0	0.00 ± 0.00^a
	DAB 0.1%	1.60 ± 0.11^b	0.62	7	3	0.60 ± 0.02^b
	DAB 0.2 %	2.00 ± 0.14^c	0.50	8	3	0.60 ± 0.04^b
	DAB 0.3 %	3.00 ± 0.21^d	0.65	11	4	0.80 ± 0.11^b

Note: indices (a, b, cetc.) indicate that the data belong to different groups with significant differences at $P < 0.05$ by ANOVA-analyze with Bonferroni amendment. Comparison in terms of one variety. RPC - rate of positive changes; PFGR - part from general rate; NL - number of lines; NPL - number of perspective lines; RPL - rate of perspective lines.

As a result recommendations in general by variety based on variability for variety Farell were traits changed broadly and consistently across all DAB levels, ideal for inducing high trait diversity, DAB 0.3% preferred. For variety Ronin DAB was high-effective in early ripeness, productivity and biochemical changes, variety suitable for high-stability breeding in terms of agriculture-value forms produce, DAB at concentrations 0.2–0.3% more optimal. Variety Shpalivka was moderate to response in spike and grain traits changes, good for quality

improvement, 0.2% and 0.3% more effective for practical purpose. Variety Sonata Poltavska focused on spike-related and early ripeness traits, effective DAB 0.2% for spike improvements, 0.3% for protein traits. Seilor was less responsive to protein changes, more for spike morphology and ripeness traits, prefer DAB 0.3%. Variety Perspektyva Odeska, variability limited to a few key traits, may be used in background improvement for specific goals, DAB 0.2–0.3%. NE 12443 was low or moderate by variability, some traits respond at higher concentration, consider only DAB 0.3% for nutritional or disease-resistance traits improvement. Genotype MIP Lada, low in variability, few traits triggered, more stable genome, use for minor trait enrichment only, not suitable for DAB action as initial form.

Summary of varietal responses were for varieties Ronin and Farell were strong concentration-dependent response. Highest rates of positive changes and perspective lines at elevated concentrations. The most effective for creating viable lines with new traits. Varieties NE 12443 and Perspektyva Odeska have good responsiveness in terms of positive changes, but moderate perspective rates, indicating beneficial mutations occurred but fewer resulted in clearly advantageous lines.

Varieties Shpalivka, Sonata Poltavska and Seilor were intermediate responses, balanced between rate of positive changes and perspective line generation. Suitable for mutation breeding but might require more extensive selection. MIP Lada was moderate overall response, though notable improvement at higher concentration (0.3%), indicating usefulness at targeted higher doses for obtaining valuable lines.

Some differences in traits can be identified early ripeness inducted for all varieties from DAB 0.1%; tolerance for diseases for Ronin, NE 12443, Shpalivka, DAB 0.2% and above; for productivity all varieties (except Perspektyva), clearly at DAB 0.3%; for protein content Farell, Ronin, Shpalivka Peak at DAB 0.3%; for positive protein changes Farell, Ronin, Shpalivka, significant from DAB 0.2%; for microelement enrichment Ronin, Farell, Shpalivka, higher at 0.3%; spike architecture, Sonata, Shpalivka, Seilor, modest at DAB 0.2–0.3%; for dwarfism/semi-dwarfism, varieties Ronin, Sonata, Seilor, low frequency, specific.

As observed, the rate of positive trait changes increased with higher mutagen concentrations for all varieties across all tested variants with one exception for Sonata Poltavska. Variety Sonata Poltavska ($F=3.11$; $F_{0.05}=4.23$; $P=0.07$) showed no significant differences between the lower two DAB concentrations (0.1% and 0.2%). Overall variability in positive changes was generally consistent across genotypes (ranging from 2.8% to 3.8% with some significant differences for varieties), less variable were Sonata Poltavska and MIP Lada (3.8%), more NE 12443 and Ronin 12443 (3.8%) ($F=7.89$; $F_{0.05}=4.23$; $P=0.009$), Perspektyva Odeska and Farell (up to 3.4%) ($F=5.14$; $F_{0.05}=4.23$; $P=0.03$). Thus, NE 12443, Ronin, Perspektyva Odeska, Farell exhibited a gradual and concentration-dependent increase in obtaining valuable forms without

introducing additional negative traits, although the rate of complex changes typically increased with higher DAB concentrations.

Factor analysis revealed that the induction rate of positive trait changes was significantly influenced by increased DAB concentrations ($F=42.17$; $F_{0.05}=3.86$; $P=3.41 \times 10^{-5}$) and was significantly affected by varietal differences ($F=36.14$; $F_{0.05}=4.11$; $P=2.42 \times 10^{-4}$). Additionally, the genotype-mutagen interaction was significant ($F=21.01$; $F_{0.05}=4.55$; $P=3.44 \times 10^{-3}$).

The proportion of positive changes relative to the general mutation rate was modest, ranging approximately from 0.5% to 0.7% for most varieties, except NE 12443 and Ronin, which showed somewhat higher values. Other parameters, such as the number of promising lines, were generally unaffected by increasing mutagen concentrations or by varietal differences, except for variety Farell, which showed a greater number of promising lines and to a lesser extent Ronin.

Evaluation of the number of promising lines indicated the ineffectiveness of lower DAB concentrations (0.1% and 0.2%) due to the minimal induction of additional desirable traits. Importantly, unlike classical mutagens, higher mutagen concentrations did not lead to an increased frequency of negative characteristics. Another key parameter for mutation breeding, the proportion of promising lines derived from the total number of families (500 for all variants), did not substantially increase with higher DAB concentrations (less variable indicator overall). Nevertheless, the rate of promising lines was influenced significantly by DAB concentration increases ($F=4.94$; $F_{0.05}=3.86$; $P=0.03$), varietal differences ($F=22.17$; $F_{0.05}=4.11$; $P=1.34 \times 10^{-3}$), and the genotype-mutagen interaction ($F=7.44$; $F_{0.05}=5.20$; $P=0.02$).

For each variety genotype-mutagen interaction was significant in negative sense for varieties NE 12443 ($F=5.34$; $F_{0.05}=3.55$; $P=0.03$) and MIP Lada ($F=5.93$; $F_{0.05}=3.55$; $P=0.02$). Thus, in general, DAB as mutagen in complex with these genotypes not can be used for high mutation rate with any concentration, for the most part of varieties the critical value of the mutagen in terms of valuable changes in the range DAB 0.2 % – DAB 0.3 %. For rate of positive changes for all varieties, the highest concentration (0.3%) consistently induced the greatest positive change, demonstrating a clear concentration-dependent effect. The varieties Ronin and NE 12443 reached the highest values (3.8%), indicating a significant responsiveness to higher mutagen concentrations. For the part from general rate, generally increased with higher mutagen concentrations across all varieties, suggesting a proportionally higher influence of mutagen concentration on the induction of positive traits. For the number of lines clearly increased with increasing concentrations of DAB for all varieties, Sonata Poltavska and Seilor produced slightly fewer promising lines, indicating moderate responsiveness. Number of perspective lines increased with concentration for all varieties, with the strongest response in Farell and Ronin (7 perspective lines at 0.3%).

To assess the complex, site-specific response of different genotypes to the mutagenic action of DAB, a cluster analysis was performed based on the

cumulative trait response across all concentrations and traits (Fig. 1). Each group showed a different response to DAB in terms of mutational variability.

Five distinct genotype groups were identified through cluster analysis based on their response to DAB. The first group included the varieties Farell, Seilor and Ronin, which exhibited similar reactions to DAB. Consequently, the potential for these genotypes to serve as highly effective initial forms for mutagenesis with substances characterized by low damaging potential. The second minor group consisted solely of the variety Perspektiva Odeska, which demonstrated a unique, genotype-specific response to DAB treatment. Given its genetic structure, this variety is likely to yield intermediate effectiveness, contributing partially to enhanced mutation frequencies for beneficial traits.

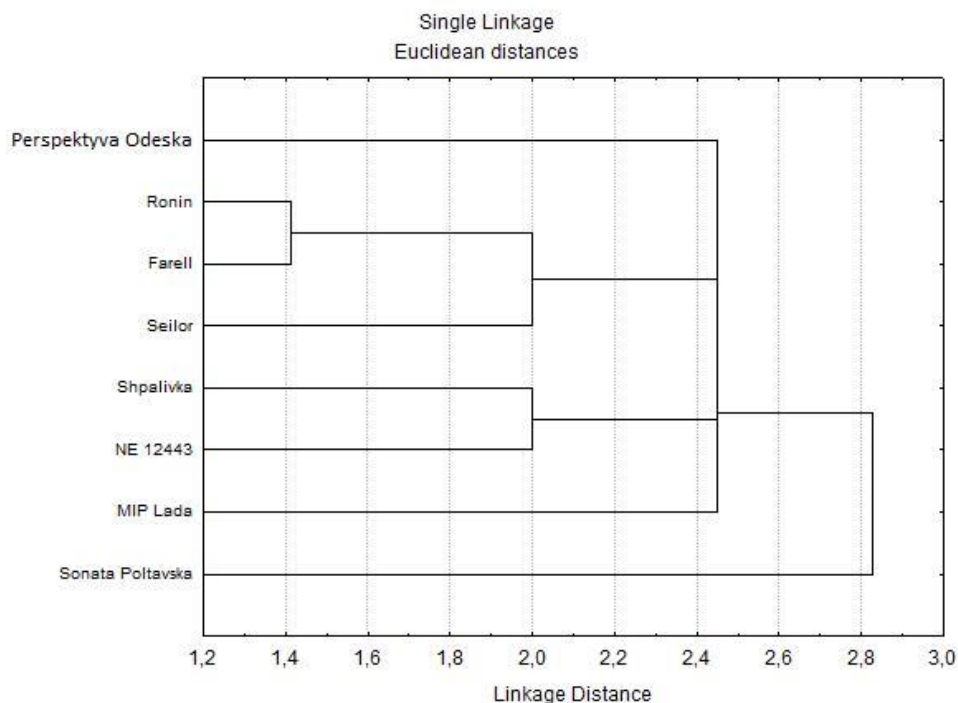


Figure 1. Cluster analysis of winter wheat varieties based on the rate of positive changes under different concentrations of DAB

The third group encompassed two varieties: Shpalivka, NE 12443. These varieties exhibited low-average responsiveness to DAB treatment, suggesting a relatively limited number of positive trait mutations when treated with this agent. The forth minor group encompassed one variety Sonata Poltavska. These varieties exhibited average responsiveness to DAB treatment. The fifth minor group encompassed one variety MIP Lada. These varieties low average responsiveness to DAB treatment.

Discriminant analysis results (Fig. 2) indicated that DAB concentrations of 0.1% and 0.2% had comparable effectiveness across all genotypes. In contrast,

the higher DAB concentration of 0.3% demonstrated increased variability and effectiveness in inducing mutations.

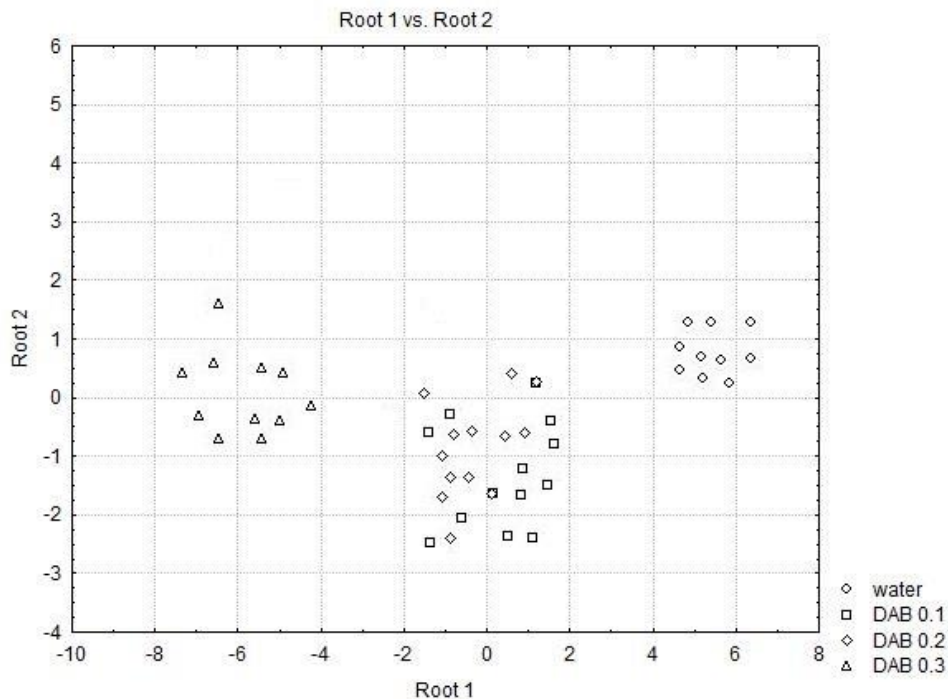


Figure 2. Discriminant analysis of winter wheat varieties based on the rate of positive changes under different concentrations of DAB

In the spectrum of induced changes, traits were classified using discriminant analysis to evaluate their relative mutability depending on the nature of the mutagen. This analysis enabled the identification of traits most susceptible to DAB treatment, such as early ripeness, spike architecture, and biochemical grain quality parameters. Conversely, traits related to tillering capacity and plant height demonstrated lower variability across treatments. A complementary classification analysis was conducted to further characterize the mutation process across genotypes and treatment levels (Table 2). This classification allowed for a more detailed interpretation of the mutation spectra, highlighting genotype-specific responses and the site-specificity of the mutagenic factor. For DAB mutations by so traits as early ripeness, tolerance for diseases, productive are probable with sufficient reliability. Regarding this DAB as mutagen high effective for this initial material (Ronin, Farell). Moderately traits by occurrence were long spike and positive changes in microelement content, positive changes at protein components, higher protein content, semi-dwarf. Traits such as dwarf, short-stem, thick stem, intense epicuticular wax, large-size grain and spike, long spike, tillering capacity accumulation were infrequent or absent, suggesting that these traits are difficult to induce or strongly genotype-dependent. As conclusion,

action of DAB in complex is the most practical suitable variant for breeding process, but strongly dependent from genotype.

Table 2. Positive changed traits. Results of discriminant analyses.

Trait at model	Wilks Lambda λ	Concentration at model	F _{0.05}	P-level
Thick stem	0.467	non	2.01	0.085
Short-stem	0.411	non	2.45	0.065
Semi-dwarf	0.311	DAB 0.3%	4.12	0.047
Dwarf	0.396	non	2.76	0.083
Intense epicuticular wax accumulation	0.490	non	1.72	0.112
Large-size grain	0.463	non	1.88	0.102
Long spike	0.465	non	1.79	0.093
Large-size spike	0.496	non	1.46	0.137
Early ripeness	0.097	DAB 0.2 – 0.3%	22.14	0.001
Tolerance for diseases	0.151	DAB 0.2 – 0.3%	7.45	0.024
Productive	0.159	DAB 0.3%	7.11	0.025
Tillering capacity	0.451	non	2.16	0.072
Higher protein content	0.270	DAB 0.3%	5.01	0.036
Positive changes at protein components	0.254	DAB 0.2 – 0.3%	5.05	0.035
Positive changes at microelement content	0.291	DAB 0.3%	4.92	0.038

Characteristics of lines obtained under the action of the studied mutagen were evaluated in terms of productivity and grain technological quality traits, which influence bread-making parameters (Tables 3 and 4). Among the more productive lines derived, all initial varieties except NE 12443, Sonata Poltavska and MIP Lada contributed positively. Three varieties Perspektivya Odeska, Shpalivka and Seilor were represented by a single form resulting exclusively from DAB 0.2 – 0.3 % treatment. Varieties Farell and Ronin, treated with DAB concentration of 0.3%, each contributed two lines. In 2022, the evaluated lines formed three productivity groups relative to the standard: lines 89, 90, 98, 100 and 105 comprised the most productive group, one form 10 occupied intermediate positions, variety Podolyanka and line 41 alone formed the least productive group. In 2023, productivity differentiation was significantly more pronounced. Lines 89, 100, and 105 again stood out clearly as the highest productivity group. The intermediate groups were statistically distinct, with a clear separation from the top group. In the third testing year, despite conditions favoring semi-intensive rather than highly intensive genotypes, productivity differences were even more distinct. Lines 89, 100 and 105 remained in the top group, while lines 10, 41, 90 and 98 occupied a statistically separate second group with significant yield differences from subsequent groups.

Aggregating results from the three-year testing period, three productivity groups were distinctly identified: lines 89, 100 and 105 formed the most productive and stable group ($F=10.17$; $F_{0.05}=4.23$; $P=0.009$), lines 90, 98 occupied an intermediate productivity level ($F=7.14$; $F_{0.05}=4.23$; $P=0.02$) and line 10

exceeded the standard yield ($F=4.01$; $F_{0.05}=3.71$; $P=0.04$) but significantly trailed the first group ($F=9.11$; $F_{0.05}=4.01$; $P=0.01$). Lines 89, 100 and 105 demonstrated consistent trait stability, whereas other lines exhibited considerable annual variability. Line 41 on the level of variety Podolyanka.

Table 3. Grain yield of mutant lines of winter wheat during the ecological estimation (DSAEU, 2022–2024), t ha⁻¹

Number	Origin	2022	2023	2024	Average
1	Podolyanka, standard	5.48 ± 0.09 ^a	6.12 ± 0.11 ^a	5.55 ± 0.13 ^a	5.72 ± 0.10 ^a
10	Perspektyva Odeska, DAB 0.3 %	5.78 ± 0.10 ^b	6.97 ± 0.17 ^b	6.12 ± 0.15 ^b	6.29 ± 0.12 ^b
41	Shpalivka, DAB 0.2 %	5.56 ± 0.10 ^a	6.67 ± 0.14 ^b	6.04 ± 0.14 ^b	6.09 ± 0.11 ^{ab}
89	Farell, DAB 0.3 %	6.29 ± 0.12 ^c	7.52 ± 0.18 ^c	6.72 ± 0.14 ^c	6.84 ± 0.15 ^c
90	Farell, DAB 0.3 %	6.11 ± 0.12 ^c	7.12 ± 0.16 ^d	6.14 ± 0.13 ^b	6.46 ± 0.17 ^{bc}
98	Ronin, DAB 0.3 %	6.17 ± 0.12 ^c	7.02 ± 0.16 ^{bd}	6.44 ± 0.13 ^{bc}	6.54 ± 0.17 ^{bc}
100	Ronin, DAB 0.3 %	6.34 ± 0.12 ^c	7.44 ± 0.18 ^c	6.98 ± 0.16 ^c	6.92 ± 0.18 ^c
105	Seilor, DAB 0.3 %	6.38 ± 0.12 ^c	7.56 ± 0.18 ^c	6.89 ± 0.15 ^c	6.94 ± 0.18 ^c

Note: indices (a, b, cetc.) indicate that the data belong to different groups with significant differences at $P<0.05$ by ANOVA-analyze with Bonferroni amendment.

Regarding technological grain quality, none of the evaluated lines notably excelled in protein content; however, lines 10, 90, and 100 stood out positively in gluten content, highlighting a close association between these two technological parameters. Overall, the majority of the studied lines exhibited good grain quality, maintaining a satisfactory protein content of around 14%, suitable for bread-making purposes, with the exception of line 105.

Table 4. Technological properties of wheat grain (DSAEU, 2024)

Variety/line	Protein, %	Gluten, %	Glutenins		Gliadins
			HMW	LMW	
Podolyanka	13.97 ^a	25.20 ^a	0.15861 ^a	0.45441 ^a	0.4563 ^a
10	14.02 ^a	26.01 ^b	0.15675 ^a	0.45541 ^a	0.4519 ^a
41	14.05 ^a	25.35 ^a	0.15753 ^a	0.46007 ^a	0.4505 ^a
89	14.10 ^a	25.48 ^a	0.16946 ^b	0.42401 ^b	0.4911 ^b
90	14.01 ^a	26.12 ^b	0.19915 ^c	0.43126 ^b	0.4567 ^a
98	14.04 ^a	25.61 ^a	0.19831 ^c	0.44144 ^c	0.4759 ^c
100	14.05 ^a	26.18 ^b	0.16113 ^a	0.44513 ^{ac}	0.4867 ^{bc}
105	13.45 ^b	23.60 ^c	0.20821 ^c	0.45134 ^a	0.4837 ^{bc}

Note: indices (a, b, cetc.) indicate that the data belong to different groups with significant differences at $P<0.05$ by ANOVA-analyze with Bonferroni amendment. HMW - high molecular weight glutenins, LMW - low molecular weight glutenins

Positive grain-quality characteristics observed among the tested lines included a relatively high concentration of high molecular weight glutenins, a trait exhibiting substantial variability, as well as lower levels of low molecular weight glutenins and moderate variability in gliadin content. Specifically, lines 89, 90, 98, and 105 excelled in the accumulation of high molecular weight glutenins. In addition, lines 89, 90, and 98 presented advantageous reductions in low molecular weight glutenin content. Furthermore, lines 89, 98, 100, and 105 demonstrated elevated gliadin levels, beneficial from a baking-quality perspective. Notably, none of the evaluated lines demonstrated a complete combination of multiple positive quality traits alongside consistently high grain yield. However, lines 89 and 98 exhibited a favorable complex of optimal storage protein structures. Among lines consistently exhibiting higher yields, 89 and 100 represented the best overall balance between productivity and desirable grain quality characteristics. Conversely, line 105 was excluded from further breeding consideration due to suboptimal grain quality attributes. Thus, lines 89 and 100 are recommended as promising candidates for further advancement in breeding programs aiming at combined productivity and grain-quality enhancement.

The systematic exploration and utilization of induced genetic variability have become pivotal strategies in modern agricultural breeding programs. The beneficial mutational processes have been extensively characterized in previous studies (Nazarenko *et al.*, 2021; Lal *et al.*, 2020), facilitating their application in generating novel genetic materials. These newly derived genotypes exhibit enhanced manageability, reliability, and predictability regarding economically valuable traits (Hongjie *et al.*, 2019).

Through targeted mutagenesis, traits typically challenging to improve, such as high-intensity dwarfism, grain-rich elongated spikes (Bayhan *et al.*, 2024), and early maturity (Kartseva *et al.*, 2023; Naserian Khiabani *et al.*, 2025), have been successfully modified, particularly utilizing locally adapted genetic backgrounds.

Our empirical data highlight the suitability and effectiveness of DAB as a mutagen, applicable not only to local but also to international wheat germplasm collections (Chakraborty *et al.*, 2023). DAB treatments predominantly generate genetic material valuable as parental components for recombinant breeding strategies, rather than producing directly deployable commercial cultivars (le Roux *et al.*, 2021; Horshchar and Nazarenko, 2024; Han *et al.*, 2025).

Crucially, our findings indicate that certain traits traditionally considered genetically stable or difficult to manipulate displayed meaningful variation under DAB exposure, with minimal negative side effects (OlaOlorun *et al.*, 2021; le Roux *et al.*, 2021). Significantly, the risk of combining beneficial with negative trait alterations remained low even as concentrations of the mutagen increased, a marked improvement over traditional mutagens (Liu *et al.*, 2025). At the highest tested DAB concentration (0.3%), adverse trait combinations were notably infrequent. This result markedly reduces the frequency of discarding promising mutant lines (Mangi *et al.*, 2021; Hassine *et al.*, 2023; Ergun *et al.*, 2023b).

Varieties exhibiting extreme initial variability, both in terms of mutagenic depression in the first generation and diverse heritable changes in subsequent generations, do not necessarily yield the most agriculturally valuable mutant lines (Nazarenko, 2017). Instead, genotypes demonstrating greater resistance to negative mutational effects produced economically superior lines more reliably (Rebouh *et al.*, 2023; Stearns *et al.*, 2025).

Notably, we observed a stronger correlation between agriculturally significant traits and the overall frequency and spectrum of visually identifiable mutations. This distinctive genotype-specific response was particularly evident under mutagenic treatments, contrasting earlier observations involving classical chemical supermutagens (Yali and Mitiku, 2022; Liu *et al.*, 2025).

While direct selection of commercially viable mutant lines via chemical mutagenesis remains challenging due to inherent genetic randomness, mutational processes provide substantial opportunities for targeted improvement (OlaOlorun *et al.*, 2021; Arumingtyas *et al.*, 2023). The observed biochemical alterations - particularly reductions in low-molecular-weight glutenins and simultaneous increases in essential microelement content, positively impact grain nutritional and commercial value (Spencer-Lopes *et al.*, 2018; Shimelis *et al.*, 2019).

Analysis of our experimental data (Nazarenko *et al.*, 2022) has revealed interesting mutational dynamics. Typically, increased mutagenic potency and dosage correlate negatively with mutational site specificity. However, when applying DAB, the situation differed markedly. Previous research by A. Anter (2021) corroborates that mutations induced by chemical mutagens, including DAB, constitute a reliable and continuous source of valuable local genetic diversity, beneficially impacting traits such as spike morphology, early maturation, and improved disease resistance profiles. Additionally, prior studies by Hussain *et al.* (2021) support the strategic use of chemically-induced mutants primarily as parental genetic resources, contributing to the subsequent enhancement of existing commercial varieties through recombination and cross-breeding approaches (Ergun *et al.*, 2023a; Hassine *et al.*, 2023; Fradgley *et al.*, 2024).

The results reaffirm the importance of careful genotype selection to maximize beneficial outcomes while minimizing the risk of adverse genetic modifications (Farooq *et al.*, 2024). Despite inherent challenges, the experimental results strongly advocate the combined use of responsive genotypes and optimized DAB concentrations to enhance trait-specific genetic variability effectively (Sala and Herbei, 2023).

CONCLUSIONS

The winter wheat varieties Farell, Ronin, and Seilor proved to be strong donors for breeding, showing useful mutation-derived variation in plant architecture, phenology, and grain quality (protein content and microelement profiles). Obtaining breeding-valuable material is substantially more likely when DAB is applied at a concentration of 0.3%, provided that appropriate initial

(source) material is selected. Among the derived material, mutant lines 89 and 100 combined consistently high yield with grain quality at or above target levels, making them priority candidates for inclusion in state varietal testing.

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FOLIAR APPLICATION OF *Moringa oleifera* LEAF EXTRACT IMPROVES MORPHO-PHYSIOLOGICAL TRAITS AND YIELD OF PAKCHOI

SUMMARY

To address the growing global demand for food, the agricultural sector must perform at its highest capacity. *Moringa oleifera* leaf extract (MLE) is a good source of beneficial compounds and nutrients to improve crop yield in an eco-friendly manner. The current study aimed to investigate the effects of different MLE concentrations on the morpho-physiological traits and yield of pakchoi. The designed study followed a randomized complete block design with six replications. Pakchoi cultivar ‘Nauli’ was foliar applied with MLE solutions at concentrations of 1:40, 1:30, and 1:20. A water spray was used as a control. MLE has altered most parameters observed, except for root fresh weight, root dry weight, and root length. Foliar MLE application at 1:30 was found to be more prominent compared to the control regarding net photosynthesis rate (125.93%), stomatal conductance (53.68%), internal CO₂ (22.15%), and transpiration rate (22.35%). A significant enhancement in plant height (21.78%), leaf number (38.50%), leaf area index (59.91%), and chlorophyll content index (22.35%) validated the efficacious effect of MLE 1:30 over control-treated plants. The increments in shoot fresh weight (36.40%) and shoot dry weight (76.06%) were also observed with a similar treatment. This underscores the potential of MLE foliar application at 1:30 for pakchoi cultivar ‘Nauli’ under greenhouse conditions.

Keywords: moringa leaf extract, photosynthesis, pakchoi, biostimulant, foliar spray

INTRODUCTION

Pakchoi (*Brassica rapa* subsp. *Chinensis*) is one of the Brassicaceae vegetables consumed worldwide for many culinary dishes, due to its crisp texture, mild, and peppery flavour (Lin *et al.*, 2024). It is a rich source of minerals, vitamins, and antioxidant compounds (Zou *et al.*, 2021). Further, these

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phytochemicals present in pakchoi may offer protection against cancer and help prevent many chronic diseases (Park *et al.*, 2023).

The global population projected to reach nearly 10 billion by 2050 would create a subsequent increase in food demand (Muñoz-Torres *et al.*, 2025). Given these attributes, a short-season vegetable such as pakchoi is widely cultivated with a high input of chemical fertilizer during its growth period (Wang *et al.*, 2022). However, the overuse of synthetic inputs aggravates ecological and environmental damage, which in turn compromises food safety and reduces crop yield (Wu and Ge, 2019). Addressing the cultivation challenges of pakchoi, there is a growing concern to optimize production toward sustainable agricultural practices.

Biostimulant has received extensive attention as a promising strategy to improve plant growth and yield. It refers to formulations derived from biological materials, comprising essential nutrients, phytohormones, plant protective compounds, and plant growth regulators (Yakhin *et al.*, 2017). *Moringa oleifera* leaf extract (MLE) is considered an ecologically inviting and secure biostimulant since it contains numerous useful nutrients (K, P, N, Mg, Ca, Zn, Cu, and Fe), antioxidants (carotenoids, phenols, ascorbic acid, and proline), phytohormones (indole-3-acetic acid, gibberellins, cytokinins), vitamins, soluble sugars, and amino acids (Shalaby, 2024). The availability of chemical components in MLE might be used as a supplement to standard fertilization on plants (Mehmood *et al.*, 2021).

Moringa oleifera is a member of the Moringaceae family, and it is widely known as the horse radish tree or drumstick (Batool *et al.*, 2020). Due to its high adaptability to various climates, the plant has become extensively distributed across tropical and subtropical regions (Kamanga *et al.*, 2025). The application of MLE in agricultural practices showed great potential for improving vegetative growth and yield of many horticultural crops, including lettuce (Admane *et al.*, 2023), cabbage (Yaseen *et al.*, 2023), and radish baby leaf (Romano *et al.*, 2022). The improvement in gas exchange traits and photosynthetic pigments was also recorded in MLE-treated plants, contributing to the enhancement in the yield (Khan *et al.*, 2022).

One of the most effective ways to provide MLE to plants is through the foliar application method, in which nutrients and beneficial substances from the extract are efficiently adsorbed through the epidermis and stomata, supporting the growth of plants (Karthiga *et al.*, 2022). In addition, the effectiveness of biostimulant is highly dependent on the concentrations applied to the plant, as optimal doses are crucial for maximizing plant growth and yield (Quille *et al.*, 2025). The MLE dilution of approximately 1:30 (v/v) has been frequently adopted in foliar application studies and has proven to be an optimal concentration to improve plant growth performance (Romano *et al.*, 2022; Yuniati *et al.*, 2023). Aslam *et al.* (2016) found that too high MLE concentrations resulted in no significant improvement in plant growth. Keeping in view the literature reported, there is no information revealing the potential of MLE

application in pakchoi. Therefore, the purpose of this study was to investigate the effects of different MLE application concentrations on the morpho-physiological traits and yield of pakchoi. We hypothesized that increasing concentrations of MLE would promote pakchoi growth and yield up to an optimal level, beyond which the stimulatory effect diminishes or remains unchanged.

MATERIAL AND METHODS

Experimental Site

The present experiment was conducted in a screenhouse at the School of Life Sciences and Technology, Institut Teknologi Bandung, Jatinangor, Indonesia (6°55'41.124"S, 107°46'13.62"E) from May to July 2025. The screenhouse temperature and relative humidity were at an average of 22 oC and 70%, respectively. The composition of the experimental soil is shown in Table 1.

Table 1. The characteristics of experimental soil in this study

Soil Characteristics	Value
Total nitrogen	1.01%
Available phosphorus	95.94 ppm
Potassium	67.34 mg 100 g ⁻¹
pH	6.3
Sand	50%
Silt	27%
Clay	23%

Experimental Setup

The experiment was laid out and evaluated in a randomized complete block design with six replicates per treatment. Pakchoi (*Brassica rapa* subsp. *Chinensis* cultivar 'Nauli') plants were foliar sprayed with MLE at different concentrations under the following treatment groups: control (distilled water), MLE (1:40), MLE (1:30), and MLE (1:20).

Pakchoi seeds used in this study were purchased from East West Seed Indonesia company. The sowing was undertaken in a pot tray, filled with a mixture of rice husk charcoal and compost in equal parts. At the four-leaf stage, around 4 weeks after planting, pakchoi seedling was individually transplanted into perforated polybags (20 cm in diameter and 20 cm in height). Each polybag contained 2 kg of soil and chicken manure mixture in a 1:1 ratio. A total of six plots per treatment were arranged in a screenhouse, each containing four plants. Thus, each treatment comprised 24 plant samples, with a planting distance of 20 cm x 20 cm. A water-soluble granular NPK 16:16:16 fertilizer was applied to the plants, with all treatments receiving 250 mL per polybag at a concentration of 5 g L⁻¹. Applications were repeated at 7 d intervals until harvest, beginning at one week after transplanting. After 47 d of growth, plants were harvested to measure both aerial and root parameters.

MLE Preparation and Application

The preparation of MLE followed the procedure described by Yuniati *et al.* (2023). In brief, fresh mature leaves were harvested from moringa plants. A total of 30 g of moringa leaves was mixed with 300 mL of distilled water and crushed using a household blender. The blended leaves were then purified twice using cheesecloth and Whatman No. 1 filter paper. The filtration was then centrifuged at 8000 rpm for 15 m. Finally, the supernatant was diluted with distilled water to 40, 30, and 20 times its original volume, corresponding to the assigned treatments. Fourteen days after transplanting, the plants were foliar-sprayed with the extract to the point of runoff, and reapplication was performed at two-week intervals. The application of MLE in this study was intended solely as a supplementary input to the standard fertilizer. The chemical composition of MLE used is summarized in Table 2.

Table 2. Chemical properties moringa leaf extract used in this experiment

Parameters	Value (mg L ⁻¹)
P	217.63
K	896.80
Ca	156.80
Mg	912.00
Auxin	26.06
Zeatin	12.00
GA ₃	23.00

Photosynthetic Analysis

Net photosynthetic rate, stomatal conductance, intercellular CO₂ concentration, and transpiration rate were measured with the help of a photosynthesis system (CI-340; CID, Biosciences, Camas, WA, USA). Chlorophyll Content Index (CCI) was analysed using a non-destructive hand-held chlorophyll meter (Konica Minolta, Inc., Tokyo, Japan). All photosynthetic traits were evaluated 1 d before harvesting on the third fully expanded leaf.

Plant Growth and Yield Analysis

The analysis of plant growth and yield parameters was carried out during the harvest period. Plant height was measured using a steel ruler, and the number of leaves per plant was recorded. Whole plants were then harvested by carefully excavating the experimental soil. Roots were thoroughly rinsed with tap water to remove soil and dirt. The shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight were assessed, along with root length. Later in the experiment, the gravimetric method was used to determine leaf area index (LAI) according to the method explained by Kumar and Salakinkop (2017).

Statistical Analysis

Collected data were analysed with Analysis of Variance (ANOVA) using SPSS v21 software (SPSS Inc., Chicago, IL, USA). The treatment means were separated using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

RESULTS AND DISCUSSION

This study delineates the dynamics of the plant height of pakchoi as they respond to MLE applications (Figure 1A). Pakchoi supplemented with foliar MLE 1:30 showed the maximum plant height. However, this treatment had no remarkable differences with MLE 1:20. The present results corroborate those of Sardar *et al.* (2021), who reported that MLE application supported the plant height of stevia. Moreover, taller plants were recorded in plots where MLE was sprayed on black cumin during the critical vegetative development phases (Mehmood *et al.*, 2021). This increment might be attributed to the presence of a considerable number of growth-enhancing hormones. Accordingly, auxins and gibberellins present in MLE were likely responsible for promoting cell elongation and positively affected stem growth (El-Serafy *et al.*, 2021).

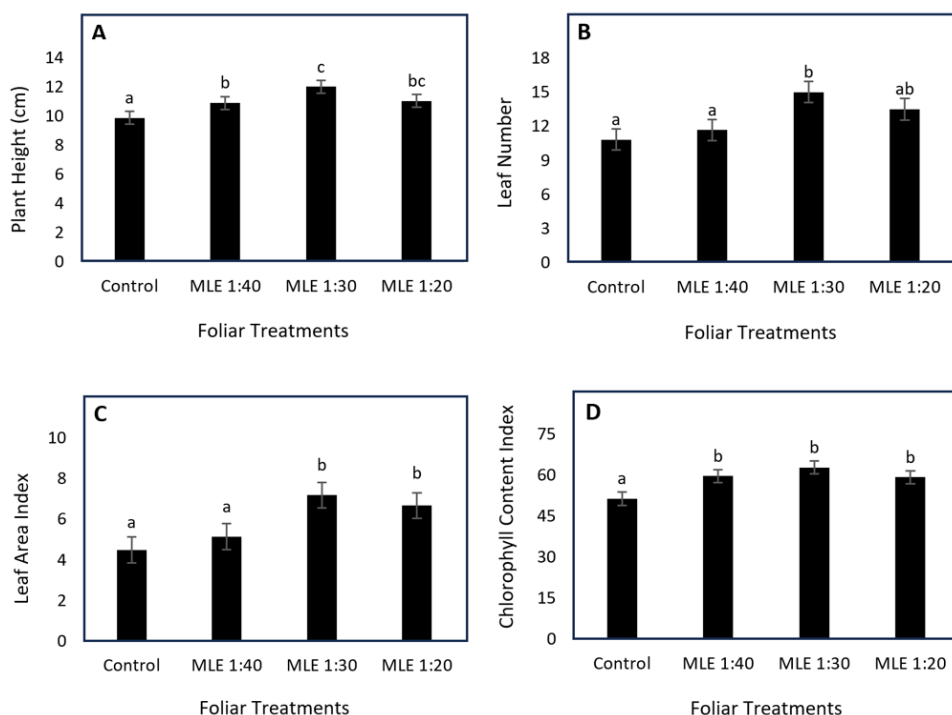


Figure 1. Plant height (A), leaf number (B), leaf area index (C), and chlorophyll content index (D) of pakchoi under different rates of moringa leaf extract application. Different lowercase letters indicate significant differences at $P < 0.05$

Leaf number per plant was significantly affected by treatments, especially when MLE 1:30 was applied (Figure 1B). At this concentration, plants produced an average of 38.50% more leaves than the control, though statistically at par with MLE 1:20. In general, the number of leaves per plant tended to increase as the concentration of MLE was raised (Marie *et al.*, 2025). Exogenous application of MLE showed a significant efficacy on the leaf number of *Silybum marianum* (Yap *et al.*, 2021). Prior research on tomato also indicated the advantageous effect of foliar MLE at 1:32 concentrations on the number of leaves both under stress and non-stress conditions (Bello and Ahmed, 2024). The proper concentrations of nutrients and cytokinin found in MLE may have influenced metabolic activity in a way that increased leaf number (Farooq *et al.*, 2023). Cytokinin facilitated fast cell division, cell multiplication, and cell enlargement, which subsequently improved the meristematic activity in the growth zone (Iqbal *et al.*, 2020).

As one of the primary growth parameters, leaf area index (LAI) is an important attribute that depicts the economic yield of a plant. LAI was notably influenced by MLE treatments (Figure 1C). Compared to the control, MLE 1:30 and MLE 1:20 generated a similar improvement of up to 59.91%. In accordance with these findings, previous studies also validated MLE effects that consequently developed bigger leaves in common bean (Zaki and Rady, 2015). Romano *et al.* (2022) reported faster growth in radish baby leaves by detecting an increase in leaf area and stem diameter using foliar MLE application at 1:30. The maximum leaf area was noted in the plant treated with foliar spray MLE alone due to the cumulative effect of micro and macro nutrients, as well as growth hormones (Khan *et al.*, 2020). The improvement in LAI following MLE treatments also might be associated with the role of phosphorus (P) available in the extract. It was reported that enriching P in plants improved the values of LAI and photosynthetic rate (Zhang *et al.*, 2018).

On the other hand, plants treated with foliar MLE showed significantly better enhancement in chlorophyll content index, ranging from 15.33 - 22.35% relative to the control (Figure 1D). Leaf chlorophyll content determines the visual appearance of most leafy vegetables. Various studies revealed a stimulating effect of MLE on chlorophyll concentration of lettuce (Yaseen and Takacz-Hajos, 2022), sweet basil (Alkuwayti *et al.*, 2020), and wheat (Afzal *et al.*, 2020). These findings were possibly linked to magnesium (Mg) content in the extract (El-Serafy *et al.*, 2021). Mg acts as an essential element of chlorophyll, in which nearly 15–35% of the Mg taken up by plants is allocated to this photosynthetic pigment (Ishfaq *et al.*, 2022). In addition, MLE comprises a substantial amount of specific phytochromes with proven antioxidant properties, including chlorophyll, which ultimately led to greener leaves (Khan *et al.*, 2023).

Referring to the data, the net photosynthesis rate was progressively enhanced by MLE 1:30, accounting for 125.93% more than the control (Table 3). But this treatment was essentially the same as MLE 1:40. The use of MLE has been discovered to be an excellent biostimulant for improving photosynthesis rate

in soybean (Irshad *et al.*, 2025). The observed increase in photosynthetic rate is likely linked to the cytokinin-driven stimulation of chloroplast development and maintenance of chlorophyll content (Howladar, 2020). Likewise, the increase in chlorophyll concentration can explain the higher photosynthetic rate and better plant performance due to MLE application in sword lily (Zulfiqar *et al.*, 2020).

Foliar spraying with MLE displayed greater stomatal conductance compared to the control (Table 3). In this regard, the greatest improvement was recorded in MLE 1:30 treatment, which was statistically equivalent to MLE 1:40. The results from this study are in accordance with those claimed by Lalarukh *et al.* (2022) and Khan *et al.* (2023), who concluded that MLE application stimulated stomatal conductance in wheat and rice, respectively. Potassium (K) content in MLE might be recognized for its crucial role in stomatal regulation. According to Azizah *et al.* (2022), K serves as a key factor in modulating guard cell turgor during stomata movement. They explained that increasing the K⁺ concentration in guard cells elevates the osmotic pressure, which then magnifies guard cell turgidity and results in stomata opening to ensure smooth CO₂ transport.

Additionally, lower stomatal conductance and net photosynthesis rate observed in MLE 1:20 compared to MLE 1:30 in this study suggested that this concentration is still capable of stimulating stomatal opening and photosynthesis more than the control. However, this stimulatory effect appears to diminish due to physiological limitations or saturation of the plant response. This is supported by Ramamoorthy and Munisamy (2025), who stated that biostimulant works at low concentrations and is involved in many plant physiological systems.

Table 3. Net photosynthesis rate, stomatal conductance, internal CO₂, and transpiration rate of pakchoi under different rates of moringa leaf extract foliar application

Treatments	Net Photosynthesis Rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Stomatal Conductance ($\text{mmol m}^{-2} \text{s}^{-1}$)	Internal CO ₂ (ppm)	Transpiration Rate ($\text{mmol m}^{-2} \text{s}^{-1}$)
Control	0.81 a	13.71 a	412.23 a	0.18 a
MLE 1:40	1.42 ab	20.08 bc	489.48 b	0.34 b
MLE 1:30	1.83 b	21.07 c	503.55 b	0.36 b
MLE 1:20	1.17 a	17.14 b	488.76 b	0.33 b

Note: Different lowercase letters on the same column indicate significant differences at $P < 0.05$

For internal CO₂ concentration, the measurements showed that plants exposed to MLE had a higher value than the control by up to 22.15% (Table 3). This aligns with a study showing that MLE enhanced internal CO₂ levels in rice under both stress and non-stress conditions (Saleh *et al.*, 2025). It was reported that cytokinin found in MLE increased biosynthesis of photosynthetic enzymes such as Rubisco, thereby improving carbon assimilation (Irshad *et al.*, 2025). Additionally, the improvement may also be attributed to a higher chlorophyll

accumulation, which translated to an increase in chemical activity in the leaves (Dolorima *et al.*, 2019).

A comparable pattern appeared on the transpiration rate of pakchoi (Table 3). All MLE treatments were more pronounced compared to the control, reaching an increase of up to 22.35%. Toscano *et al.* (2023) reported that in the case of petunia plants grown under normal conditions, the transpiration rate in leaves exhibited a greater value following foliar MLE treatment. Foliar application of MLE in combination with chemical fertilizer also improved this parameter in radish (Raza *et al.*, 2022). The elevated transpiration rate observed under biostimulant treatment was closely associated with the improvement in stomatal conductance (de Farias *et al.*, 2024). In lettuce, a higher transpiration rate was observed in plants receiving biostimulant, but it did not represent a significant decrease in water use efficiency (Atero-Calvo *et al.*, 2024).



Figure 2. Pakchoi under different concentration application of MLE (A) and moringa leaf extract used in the study (B)

Foliar treatment of MLE caused a significant variability in shoot fresh weight (Table 4 and Figure 2A). Pakchoi treated with MLE 1:30 was characterized by the highest shoot fresh weight, although the difference was not significant compared to MLE 1:20. Applying MLE at 1:20 enhanced biomass yield by 34.77% over the untreated plants (Ali *et al.*, 2018). Based on the findings by Noreen *et al.* (2024), a substantial increase in shoot fresh weight was recorded in pea plants following foliar MLE application under non-stress conditions. They stated that plant extracts (fresh and/or dry) have stimulatory impacts by upregulating enzyme activity and metabolic contents. The improvement in mineral content and photosynthetic activity due to MLE application also contributed to increased vegetative growth and biomass production (Hafeez *et al.*, 2022). The MLE used in this study (Figure 2B) is rich in important minerals and growth-promoting compounds like auxin, gibberellin, and cytokinin. These compounds may stimulate the growth of plants by enhancing cell elongation and cell division during the development of overall biomass (Nazir *et al.*, 2025).

Table 4. Shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, and root length of pakchoi under different rates of moringa leaf extract foliar application

Treatments	Shoot Fresh Weight (g)	Shoot Dry Weight (g)	Root Fresh Weight (g)	Root Dry Weight (g)	Root Length (cm)
Control	17.36 a	1.42 a	1.25 a	0.19 a	10.25 a
MLE 1:40	18.81 a	1.89 ab	1.27 a	0.20 a	12.12 a
MLE 1:30	23.68 b	2.50 c	1.39 a	0.29 a	14.05 a
MLE 1:20	20.30 ab	2.07 bc	1.49 a	0.28 a	11.10 a

Note: Different lowercase letters on the same column indicate significant differences at $P < 0.05$

Amongst all the treatments, the heaviest shoot dry weight was noted in plant foliar-sprayed with MLE 1:30 (Table 4). However, it was statistically equal to MLE 1:20. The presence of nutrients, such as Ca, K, Mg, Mn, Fe, and Zn in MLE might also be involved in biomass increment of treated plants (Hafeez *et al.*, 2022). Afzal *et al.* (2020) revealed that supplementing plants with MLE improved assimilation of photosynthates as a result of extended green leaf area. In a previous study by Jain *et al.* (2020), the exogenously applied MLEs effectively increased zeatin (a cytokinin-related hormone) content in stevia plants, which can be linked to the increment in isopentyl amino purine (precursor of zeatin) content. These reports were presumed to be the underlying factor contributing to the increase in shoot dry weight in plants treated with MLE. Further research is needed to quantify endogenous cytokinin and related hormone dynamics in the plant tissues of pakchoi after foliar spraying with MLE.

As shown in Table 4, there was no significant variation in root fresh weight, root dry weight, and root length regarding foliar MLE application. The root fresh weight values of all treatments ranged from 1.25 to 1.49 g, while 0.19–0.29 g for root dry weight. The data pertaining to the root length showed the values ranged from 10.25 to 14.05 cm.

Consistent with previous investigations, the exogenous application of MLE at the vegetative stage did not considerably improve root fresh weight and root dry weight of chickpea (Nazir *et al.*, 2025). In purple nutsedge, no MLE-induced enhancement effect was detected in both the fresh weight and the dry weight of the root. In harmony with the present study, a response of sweet basil's root length to different foliar MLE levels was non-significant (Alkuwayti *et al.*, 2020). Similarly, the root length of tamarind was not notably affected by biostimulant treatment (Dantas *et al.*, 2012). A study comparing foliar and root-based application of biostimulant has shown that root-based application often produces stronger effects on root growth than the foliar method due to direct contact with root tissues, thereby improving nutrient and water uptake and activating root plasma membrane ATPase activity (Atero-Calvo *et al.*, 2024). Therefore, this difference in mode of action likely explains the limited response found in root traits in the present study.

CONCLUSIONS

According to the current study's findings, the treatments had no significant impact on root growth traits. Meanwhile, foliar application of MLE at 1:30 had a substantial improvement on photosynthesis rate (125.93%), stomatal conductance (53.68%), internal CO₂ (22.15%), and transpiration rate (22.35%). This experiment also confirmed that plants sprayed with MLE 1:30 showed better results for all growth and yield attributes as compared to the control, including plant height (21.78%), leaf number (38.50%), leaf area index (59.91%), chlorophyll content index (22.35%), shoot fresh weight (36.40%), and shoot dry weight (76.06%). From a practical agronomic perspective, the application of MLE at 1:30 can serve as an effective supplementary biostimulant to standard chemical fertilization for the pakchoi cultivar 'Nauli' under greenhouse conditions. Moreover, identifying an optimal MLE concentration in this study may help improve the overall efficiency of crop production and avoid unnecessary biostimulant overuse, thereby offering potential economic benefits. Further studies involving different cultivars and open-field conditions are recommended to assess the consistency of the observed responses.

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FORAGE POTENTIAL OF WILD-GROWING POPULATIONS OF *BITUMINARIA BITUMINOSA* (L.) STIRT.

SUMMARY

The aim of the present study includes an analysis of the basic chemical composition and an evaluation of the nutritional value of the aboveground biomass from local populations of *Bituminaria bituminosa* (LP 144, LP 191, LP 375, LP 420), harvested at the early flowering phenophase.

On average for the period (2022–2023), the crude protein content in the dry matter of *B. bituminosa* swards was 130.0 g kg⁻¹ DM (CV=5.05–11.80%). The analysis data indicate a low degree of variability in the values of this indicator depending on environmental factors. The concentrations of crude fiber, neutral detergent fiber, and acid detergent fiber were 354.4, 483.4, and 375.7 g kg⁻¹ DM, respectively. The species recorded high values in terms of the macroelement calcium content (up to 4.79 g kg⁻¹ DM) and very low values for phosphorus (0.03–0.08 g kg⁻¹ DM).

The values of relative feed value (RFV) in the swards ranged from 95.06 to 138.98%, and the dry matter digestibility (DDM) ranged from 55.18% to 62.75%. These results suggest good intake and assimilation of the forage, especially in dry pasture areas, by ruminant animals.

The analysis data confirm that the high presence of *B. bituminosa* improves the overall forage value of degraded xerothermic grasslands. The species' ability for summer and autumn regrowth makes it a valuable resource for pastures in the hilly and foothill regions of Central Northern Bulgaria.

Keywords: *Bituminaria bituminosa*, chemical composition, relative feed value

INTRODUCTION

Locally adapted legume species are an important component of natural grassland communities. Some of them represent a viable economic alternative for addressing the challenges associated with forage production in areas frequently affected by prolonged droughts (Gil *et al.*, 2016). The identification of new wild

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drought tolerant legume species can extend grazing potential on poorly fertile soils, and areas with low rainfall or prone to drought (Real *et al.*, 2014).

Such a species is teder (*Bituminaria bituminosa* (L.) Stirt.) which has shown a growing natural distribution in our country (Glogov and Georgieva, 2021). In Northern Bulgaria, it occurs in dry forest-steppe habitats up to 800 m above sea level. The species is drought- and cold-tolerant, adapted to eroded terrains and soils deficient in the macronutrients nitrogen and phosphorus. Niza Costa *et al.* (2023) also identify *B. bituminosa* as the most common early colonizer of burned areas in Spain and, for this reason, use it as a model species for studying the potential of nitrogen-fixing legumes in the restoration of soils and grassland communities after fire events. Our own studies have also reported an increased presence of *B. bituminosa* in post-fire successions in various regions of Bulgaria (Naydenova *et al.*, 2022). In this context, an important question arises: to what extent can this pioneer species contribute forage value to burned natural pastures in the hilly and foothill regions of Central Northern Bulgaria during the early years of their natural recovery? Therefore, the biochemical characterization of naturally occurring ecotypes of *B. bituminosa* in these areas becomes a subject of research interest.

B. bituminosa is a perennial legume widely distributed across the Mediterranean Basin and Macaronesia. The plants are grazed by small ruminants but are primarily used as hay for feeding dairy goats, particularly in the Canary Islands (Pazos *et al.*, 2013). According to several international studies, *B. bituminosa* exhibits important ecological advantages as a forage species (Zennouhi *et al.*, 2019; Real *et al.*, 2022; Real, 2022; Fernández-Habas *et al.*, 2022; Fernández-Habas *et al.*, 2025a). In very dry and semi-arid regions with poor soils, it is considered a valuable legume component for grazing by sheep and cattle (Raeside *et al.*, 2013).

Following a comparative assessment of the biochemical and physiological responses to prolonged drought, as well as the recovery capacity after such stress, Foster *et al.* (2015) highlight the advantage of *B. bituminosa* over alfalfa. According to a recent pilot study by Thielecke *et al.* (2024), *B. bituminosa* is among the Mediterranean forage grasses that offer potential for increasing the botanical diversity of grasslands in temperate European countries, particularly during dry and hotter periods in the summer months. Several recent studies on quality parameters identify *B. bituminosa* as a good forage source, demonstrating nutritional value for ruminants comparable to that of major forage crops (Zarat *et al.*, 2020; Bayram *et al.*, 2022; Barbera *et al.*, 2025). According to Barbera *et al.* (2019) the species exhibits high productive and nutritional potential and can be used as an alternative source of natural protein in ruminant diets.

According to our previous studies on semi-natural grasslands in poor and eroded lands in Northern Bulgaria, *B. bituminosa* is the legume with the strongest vegetative growth and the highest summer biomass productivity compared to eight other legume species found in the pasture (Naydenova and Bancheva,

2025). Currently, Bulgarian origins of the species have not been evaluated in terms of quality parameters and, consequently, forage potential.

The aim of the present study is to analyze the basic chemical composition and evaluate the nutritional value of dry forage biomass from local wild populations of *Bituminaria bituminosa*.

MATERIAL AND METHODS

The experimental work was conducted during the period 2022–2024. In 2022, permanent pastures with a high presence of *B. bituminosa* in the sward were mapped in the hilly and foothill regions of Central Northern Bulgaria. Four populations (LP) from characteristic habitats were selected. The altitudes of the habitats were used as identifiers for the populations in the study, namely: LP 144, LP 191, LP 375 and LP 420. During the following two years (2023–2024), at the early flowering phenophase, mean plant samples were collected from each population through four randomly selected sample plots (1 m²), evenly distributed across the surveyed areas, for biochemical analysis of the aboveground biomass. The analysis included the determination of the basic chemical composition of the dry fodder mass, using the Weende method:

- Crude protein (CP, g kg⁻¹ DM) – determined by the Kjeldahl method (according to BDS/ISO-5983);

- Crude fiber (CFr, g kg⁻¹ DM);

- Crude fat (CF, g kg⁻¹ DM) – determined using Soxhlet extraction (according to BDS/ISO-6492);

- Ash (g kg⁻¹ DM) – determined by gradual combustion of the organic matter in a muffle furnace at 550°C (according to BDS/ISO-5984);

- Dry matter (DM, g kg⁻¹) – empirically calculated based on moisture content;

- Nitrogen-free extracts (NFE, g kg⁻¹ DM) – calculated as: $NFE (\%) = 100 - (CP \% + CFr \% + CF \% + Ash \% + Moisture \%)$;

- Calcium (Ca, g kg⁻¹ DM) – determined by complexometric titration according to Schottz;

- Phosphorus (P, g kg⁻¹ DM) – determined with vanadate-molybdate reagent using, measured spectrophotometrically with an Agilent 8453 UV–Visible Spectroscopy System at a wavelength of 425 nm.

Evaluation of feeding value on the basis of fiber components (Linn and Martin, 1991):

- Digestible Dry Matter (DDM, $\% = 88.9 - (0.779 \times ADF, \%)$);

- Dry Matter Intake (DMI, $\% = 120/NDF, \%$);

- Relative Feeding Value (RFV, $\% = DDM \times DMI/1.29$).

For the statistical analysis of the obtained data, a two-factor analysis of variance (ANOVA) without replication was performed, with the factors 'habitat/population' and 'year.' Additionally, a multivariate analysis, including principal component analysis (PCA), was conducted, and a biplot model was constructed using singular value decomposition of the first two principal components. All analyses were carried out using the statistical software *Statgraphics 18* and *Excel for Windows XP*.

RESULTS AND DISCUSSION

Chemical composition of forage biomass from wild populations of *Bituminaria bituminosa*

At the early flowering phenological stage, the average crude protein (CP) concentration in the dry forage biomass of the studied *B. bituminosa* populations was 130.0 g kg⁻¹ DM, ranging from 108.8 in LP 191 to 141.1 g kg⁻¹ DM in LP 375 (Table 1).

Table 1. Basic chemical composition and structural fiber components of the cell walls in wild populations of *B. bituminosa* (g kg⁻¹ DM) – by year and average for the period (2023–2024)

Populations	CP	CFr	CP / CFr	CF	Ash	NFE	Ca	P	NDF	ADF	ADL	Hemi.	Cellul.
First Year													
LP 144	138.7	381.1	1/2.7	27.9	69.6	304.4	21.2	1.4	501.1	406.4	65.7	94.7	340.7
LP 191	108.8	386.7	1/3.6	15.7	66.5	344.7	17.2	0.6	540.0	432.8	77.6	107.3	355.2
LP 375	141.1	377.7	1/2.7	22.8	68.5	310.9	21.2	0.7	500.9	406.2	89.8	94.8	316.4
LP 420	122.9	355.1	1/2.9	31.4	64.7	344.9	28.0	0.6	443.6	343.1	114.8	100.5	228.3
Mean	127.9	375.2	1/3.0	24.5	67.3	326.2	21.9	0.8	496.4	397.1	87.0	99.3	310.2
CV	11.80	3.70		27.90	3.20	6.60	20.50	46.80	8.00	9.60	24.10	6.00	18.30
Second Year													
LP 144	125.8	338.0	1/2.7	21.8	58.8	371.2	40.3	1.1	481.9	362.6	62.5	119.3	300.1
LP 191	128.3	340.9	1/2.7	13.6	61.4	373.8	34.0	0.5	500.5	345.1	69.9	155.4	275.2
LP 375	141.0	334.0	1/2.4	20.0	68.0	355.6	47.0	1.0	479.4	374.0	77.4	105.4	296.6
LP 420	132.3	321.7	1/2.4	32.3	66.2	365.8	47.9	0.3	420.0	335.7	84.5	84.3	251.2
Mean	131.9	333.7	1/2.5	21.9	63.6	366.6	42.3	0.7	470.5	354.4	73.6	116.1	280.8
CV	5.05	2.53		35.39	6.67	2.20	15.34	53.27	7.42	4.85	12.90	25.75	8.04
Average for the period													
Mean ± Standard error	129.9 ±3.9	354.4 ±8.7		23.2 ±2.4	65.5 ±1.3	346.4 ±9.3	32.1 ±4.3	0.8±0.1	483.4 ±13.2	375.7 ±12.6	80.3 ±5.9	107.7 ±7.7	295.5 ±15.2
SD	11.0	24.6		6.9	3.7	26.3	12.1	0.4	37.3	35.7	16.7	21.9	43.0
Factorial effect													
Year	NS	**		NS	NS	*	**	NS	*	P<0.1	NS	NS	NS
Populations/Habitats	NS	*		**	NS	NS	P <0.1	P <0.1	**	NS	P <0.1	NS	NS

According to the analysis of variance, differences in climatic conditions between the two experimental years did not significantly affect this key quality parameter. Likewise, variation in the ecological conditions among habitats had no significant effect. The observed crude protein content was significantly lower than that reported for the most widespread local perennial forage species, such as clover and alfalfa (Naydenova *et al.*, 2022; Naydenova and Bozhanska, 2020; Marinova, 2017; Marinova *et al.*, 2024). However, given the high biomass productivity of the studied populations under the strongly limiting conditions of the selected habitats (Naydenova and Ignatova, 2025), the recorded CP values can be considered satisfactory. A synthesis of published data indicates that the protein content of *B. bituminosa* ranges from 159 to 243 g kg⁻¹ DM (Gülümser and Acar, 2012). Leaf protein concentration is reported to be nearly twice as high as that of

stems. As small ruminants (sheep and goats) preferentially consume leaf biomass during grazing, the species represents a valuable protein source in degraded xerothermic grasslands dominated by *Chrysopogon gryllus* and in yellow bluestem pastures (*Bothriochloa ischaemum*), where it naturally occurs. Grasslands dominated by *Chrysopogon gryllus* are characterized by limited nutritional value. According to Iliev *et al.* (2017), crude protein content reaches a maximum of 100.4 g kg⁻¹ DM even under optimal macromineral fertilization (annual application of N₆P₆). The inclusion of *B. bituminosa* in these grassland communities is therefore expected to enhance the overall protein content of the forage mass. This improvement may contribute to higher pasture productivity and increased resilience under drought conditions.

Based on our observations, *B. bituminosa* demonstrates the ability to regrow after seed harvest at the end of July, forming robust leaf rosettes (Figure 1). Thus, the species' presence can significantly increase the yield and quality of semi-natural grasslands when used for grazing in the late summer and autumn periods.



Figure 1. Second regrowth of *B. bituminosa* population (LP 375)

The crude fiber (CFr) content in the dry aboveground biomass of *B. bituminosa* is very high, averaging 354.4g kg⁻¹ DM. It varies between 321.7 and 386.7g kg⁻¹ DM, depending on the yearly ($p < 0.01$) and habitat conditions ($p < 0.05$). The observed values can be linked to the following biological characteristics of the species: a perennial life cycle and a strong growth habit, a slow developmental rate during the growing season, and flowering under very high summer temperatures, which stimulate the accumulation of structural carbohydrates in the plants. Due to the high fiber content, the crude protein to crude fiber ratio is much lower (ranging from 1:2.4 to 1:3.6) than the optimal ratio of 1:1.2.

Similar to crude fiber, the content of macrominerals and crude fat in the biomass of the species is influenced by environmental factors ($p < 0.1$ and $p < 0.01$

respectively). For population LP 420, which develops on a steep, heavily eroded slope under the most adverse soil conditions, the highest calcium (Ca) content ($47.9 \text{ g kg}^{-1} \text{ DM}$) and the lowest phosphorus (P) content ($0.3 \text{ g kg}^{-1} \text{ DM}$) were recorded in the second experimental year. The Ca:P ratio in the dry forage mass from this habitat is 160:1, which is many times higher than the critical value of 6:1 (NRC, 2000). The same population also shows significantly higher crude fat content. In a similar study conducted under conditions in Turkey, Bayram *et al.*, (2022) report considerably lower calcium levels ($12.3\text{--}14.0 \text{ g kg}^{-1} \text{ DM}$) in the dry biomass of the species and much higher phosphorus contents ($3.1\text{--}4.1 \text{ g kg}^{-1} \text{ DM}$). The significant effect of soil and meteorological conditions on the macromineral content of pasture vegetation is well known, with mineral supplementation for animals being essential in dry conditions and regions (Mokolopi, 2019).

The basic forage profile of the species necessitates a comparison between *B. bituminosa* and other naturally occurring legumes in the study region that utilize similar ecological resources. The average contents of crude fat ($23.2 \text{ g kg}^{-1} \text{ DM}$), phosphorus ($0.8 \text{ g kg}^{-1} \text{ DM}$), and ash ($65.5 \text{ g kg}^{-1} \text{ DM}$) in the forage of *B. bituminosa* determined in our study were significantly lower than those recorded for *Medicago falcata* (with corresponding values of $39.7 \text{ g kg}^{-1} \text{ DM}$, $2.1 \text{ g kg}^{-1} \text{ DM}$, and $90.8 \text{ g kg}^{-1} \text{ DM}$), which is the dominant perennial legume species in the dry natural pastures of Central Northern Bulgaria (Naydenova *et al.*, 2022). In contrast, calcium content was higher in *B. bituminosa* – $32.1 \text{ g kg}^{-1} \text{ DM}$ compared to $22.4 \text{ g kg}^{-1} \text{ DM}$ in *Medicago falcata*.

The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) is a key determinant of the relative feed value (RFV) of forage (Kumbasar *et al.*, 2018). The same authors report that ADF and NDF contents in *B. bituminosa* are closely dependent on developmental stage and may range from 198.0 to $471.4 \text{ g kg}^{-1} \text{ DM}$ (ADF) and from 273.7 to $563.8 \text{ g kg}^{-1} \text{ DM}$ (NDF). The average NDF content observed in the biomass of local *B. bituminosa* populations in our study was high – $483.4 \text{ g kg}^{-1} \text{ DM}$, ranging from $420.0 \text{ g kg}^{-1} \text{ DM}$ in LP 420 to $540.0 \text{ g kg}^{-1} \text{ DM}$ in LP 191. Variation across years and habitats was statistically significant ($p < 0.05$ and $p < 0.01$ respectively).

In both experimental years, population LP 420 recorded the lowest NDF values, which correspond to higher forage quality and greater intake potential by ruminants. This population is located at the highest elevation, on a steep, heavily eroded slope under the most restrictive soil fertility and moisture conditions. In Central Northern Bulgaria, *B. bituminosa* naturally occurs in xerothermic grassland communities dominated by *Brachypodium pinnatum* or *Festuca valesiaca*. The studied populations showed significantly lower NDF concentrations compared to the values reported for the dominant grasses in these habitats (610.4 g kg^{-1}), as documented by Bozhanska *et al.* (2022). Therefore, the inclusion of *B. bituminosa* in such pasture swards contributes to the improvement of their overall forage quality.

The concentration of acid detergent fiber (ADF) is directly related to forage digestibility and is used in the calculation of total digestible nutrient (TDN) values. The average ADF content determined in our study was 375.7 g kg⁻¹ DM. This parameter was not significantly influenced by the ecological conditions of the habitats, although a notable trend was observed toward lower ADF concentrations during the second experimental year (average across all populations: 354.4 g kg⁻¹ DM), under conditions of reduced summer precipitation. Based on the observed values, *B. bituminosa* may have significant potential to enhance forage digestibility in the xerothermic pastures where it naturally establishes. This is particularly relevant as the most well-adapted legume species when grazed or cut at early flowering stage, exhibit similar ADF concentrations ranging from 278 to 426 g kg⁻¹ DM (Vasiljević *et al.*, 2022).

The biological value of the analyzed biomass also strongly depends on its content of hemicellulose (a fully digestible polysaccharide) and cellulose, which is only partially digestible. According to the results obtained, the hemicellulose content in the dry biomass of the studied local *B. bituminosa* populations was high, justifying the classification of this legume forage as a high-energy feed. The mean values of this parameter were 99.3 g kg⁻¹ DM and 116.1 g kg⁻¹ DM for the first and second experimental years, respectively. The cellulose fraction in the dry matter was also high, with average values of 310.2 g kg⁻¹ DM and 280.8 g kg⁻¹ DM, respectively. For both indicators, variation across years and habitats was assessed as insignificant according to the results of the ANOVA.

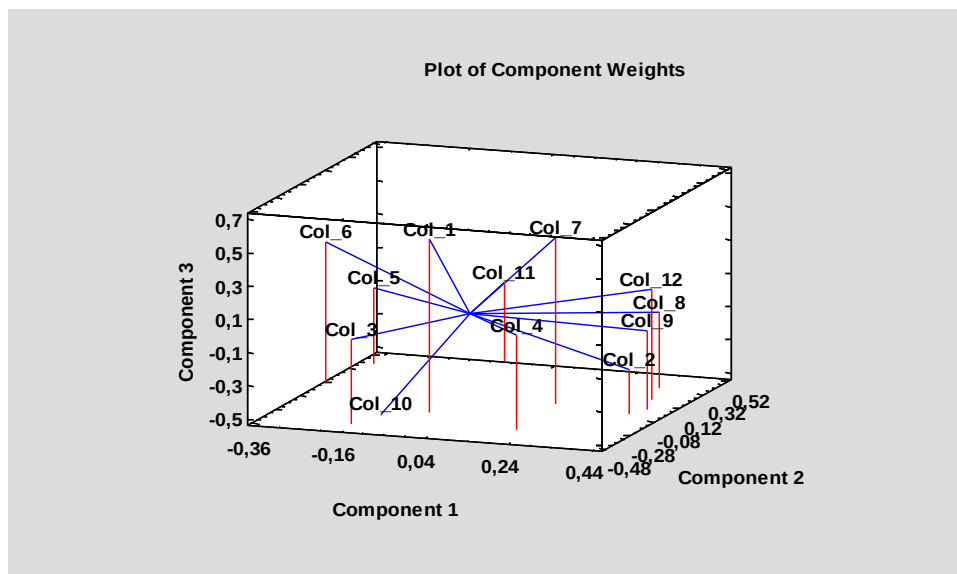
The lignin fraction contributes to the rigidity and structural strength of plant cells but negatively affects forage digestibility (Bonawitz *et al.*, 2010). The quantity of lignified stems in the later stages of *B. bituminosa* development mainly influences the quality of the feed (Fernández-Habas *et al.*, 2025b). At the early flowering stage, the local germplasm of the species recorded relatively low acid detergent lignin (ADL) values, ranging from 62.5 to 114.8 g kg⁻¹ DM, with no significant differences between years. A clear trend was observed whereby populations developing at lower elevations and under more favorable soil conditions exhibited lower ADL content.

To assess the variability of the 12 observed indicators of basic chemical composition, as well as the interrelationships among them, a multivariate analysis was conducted (Table 2 and Figure 2).

Table 2. Principal components analysis

Component Number	Eigenvalue	Percent of	Cumulative
		Variance	Percentage
1	5.26272	43.856	43.856
2	3.24647	27.054	70.910
3	1.82257	15.188	86.098
4	0.684814	5.707	91.805
5	0.659165	5.493	97.298

The analysis was conducted up to the third principal component, which accounted for 86% of the total variation. The variables contributing most significantly to the variation in principal component 1 (PC1), and consequently to the overall variability, were the fiber-related parameters: crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and cellulose.



Legend: Col_1 - CP; Col_2 - CFr; Col_3 - CF; Col_4 - Ash; Col_5 - NFE; Col_6 - Ca; Col_7 - P; Col_8 - NDF; Col_9 - ADF; Col_10 - ADL; Col_11 - Hemicell; Col_12 - Cellulose

Figure 2. Biplot Analysis of the Chemical Parameters in Wild Populations of *Bituminaria bituminosa*

These can be considered as the elements of the basic chemical composition most strongly influenced by habitat conditions and harvest years. For the second principal component (PC2), which accounts for 27% of the total variation, the determining factors are the contents of hemicellulose, crude fat, and ash. Protein and macromineral contents are grouped within the same quadrant and have the highest relative loading on principal component 3 (PC3). The key quality indicator (crude protein content) is positively influenced by the levels of the two macrominerals, calcium (Ca) and phosphorus (P), as well as by hemicellulose content.

The assessment of the relative feed value (RFV), which reflects the potential intake and digestibility of the plant biomass (Fekadu *et al.*, 2017), showed low to moderate variability across the studied *B. bituminosa* populations, with RFV values ranging from 95.06% to 138.98% (Table 3). Year-to-year changes in the forage value of *B. bituminosa* revealed relatively low variation in the average values for potential dry matter intake (DMI), which ranged from 2.43% to 2.56%.

Table 3. Dry Matter Digestibility and Energy Feed Value of Wild Populations of *Bituminaria bituminosa*

Populations	DDM	DMI	RFV	GE	EE	FUM	FUG
First year							
LP 144	57.24	2.39	106.26	17.52	7.26	0.66	0.59
LP 191	55.18	2.22	95.06	17.15	7.21	0.66	0.59
LP 375	57.26	2.40	106.33	17.43	7.27	0.66	0.59
LP 420	62.17	2.71	130.38	17.41	7.42	0.68	0.61
Mean	57.96	2.43	109.51	17.38	7.29	0.67	0.60
Sd	2.97	0.20	14.89	0.16	0.09	0.01	0.01
Second year							
LP 144	60.65	2.49	117.08	17.22	7.51	0.69	0.63
LP 191	62.02	2.40	115.26	17.08	7.47	0.69	0.63
LP 375	59.77	2.50	115.97	17.17	7.47	0.69	0.62
LP 420	62.75	2.86	138.98	17.33	7.59	0.70	0.64
Mean	61.30	2.56	121.82	17.20	7.51	0.69	0.63
Sd	1.34	0.20	11.46	0.10	0.06	0.01	0.01

Legend: DDM (%); DMI (%); RFV-relative (%); GE (MJ/kg DM); EE (MJ/kg DM); FUM (n in kg DM); FUG (n in kg DM)

The energy feed value of forage encompasses both the biological metabolism and the quantitative ratio of nutrients in the dry matter. It is a key criterion in the modern evaluation of biomass quality and is typically expressed through feed units for milk and growth (Todorov, 2010). The gross energy (GE) and energy required to support the basic physiological functions in animals (EE) in the vegetative biomass of *B. bituminosa* correlate with the fiber components of the cell walls, which represent the primary source of nutritional energy for ruminants.

In the present study, the variability in these parameters was low, with GE values ranging from 17.08 MJ/kg DM (LP 191) to 17.52 MJ/kg DM (LP 144), and EE ranging from 7.21 MJ/kg DM (LP 191) to 7.59 MJ/kg DM (LP 420). This trend also persisted in the values of net energy (FUM and FUG). On average over the study period, the number of feed units in the wild populations of *B. bituminosa* ranged from 0.66 to 0.70 per kg of dry matter (FUM), and from 0.59 to 0.64 per kg of dry matter (FUG).

The dry matter digestibility (DDM) of the studied *B. bituminosa* populations was relatively high. The empirically calculated DDM values showed low variability as well, ranging from 55.18% (LP 191) to 62.75% (LP 420).

CONCLUSIONS

Across four habitats located at altitudes between 144 and 420 m, the local germplasm of *B. bituminosa* was evaluated over two growing seasons. The habitats differed in edaphic and climatic conditions. On average, the dry aerial biomass harvested at the beginning of the flowering stage showed the following basic chemical composition: crude protein (CP)–130.0 g kg⁻¹ DM; crude fiber (CFr)–354.4 g kg⁻¹ DM; neutral detergent fiber (NDF)–483.4 g kg⁻¹ DM; acid detergent fiber (ADF)–375.7 g kg⁻¹ DM; hemicellulose–107.7 g kg⁻¹ DM; cellulose–295.5 g kg⁻¹ DM; acid detergent lignin (ADL)–88.6 g kg⁻¹ DM; crude

fat (CF)–23.2 g kg⁻¹ DM; ash–65.5 g kg⁻¹ DM; calcium (Ca)–32.1 g kg⁻¹ DM; and phosphorus (P)–0.8 g kg⁻¹ DM. The contents of crude fiber, macrominerals, and crude fat were significantly influenced by ecological variability. The species showed very high hemicellulose and calcium concentrations. In contrast, phosphorus content was extremely low. Fiber-related parameters were the main drivers of variability in chemical composition. These included crude fiber, neutral and acid detergent fiber, and cellulose. They formed the first principal component in the multivariate analysis. This indicates a strong dependence on habitat characteristics, particularly soil properties, altitude, and harvest year. The relative feed value of the biomass ranged from 95.06 to 138.98%. This indicates that the inclusion of *B. bituminosa* in xerothermic pastures positively affects biomass quality.

Given the proven feed value of the species under limiting environmental conditions, further research should focus on evaluating the potential for its integration into semi-natural grasslands, for example through direct overseeding following fire events. The investigated genetic material may also serve as a valuable initial resource for breeding programs, as it exhibits the key agronomic trait of specific adaptation to local regions, environmental conditions, and targeted production systems.

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EMMER – A BYGONE AND NEWLY FOUND CEREAL: A BIBLIOMETRIC ANALYSIS (1905-2025)

SUMMARY

Emmer has recently garnered significant attention. This bibliometric study analyses a dataset from Scopus covering the period from 1905 through August 18, 2025 and including a total of 2,383 emmer-related documents. Emmer research has experienced a marked increase in both publication output and citation impact over time. Specifically, the cumulative annual percentage growth rate for the number of publications reached 893% (1982–2021), while the average annual citation count grew by 67% between 1970 and 2019. Theoretical and Applied Genetics, Euphytica and Vegetation History and Archaeobotany were found to be the top journals in terms of both number of publications and H-index. China, USA, Israel, Italy, Turkey, Germany, United Kingdom, India and Japan were identified as the most prolific countries, with Israel being the most cited one. The University of Haifa and Sichuan Agricultural University turned out to be the most productive organizations. Nevo E. and Fahima T. were both the greatest contributors and the most cited authors. The collaboration clusters were constructed for countries, institutions and authors. The theme trends and hotspots in emmer research were analysed: thus, genetics, metabolism and phylogeny were detected as Motor Themes, while archaeobotany and related cereal species were Emerging or Declining Themes. Through such structured approach, researchers can better define their problems, identify future challenges and establish collaboration links.

Keywords: *Triticum dicoccum*, *Triticum dicoccoides*, scientific output, knowledge mapping, research trends

INTRODUCTION

Ancient wheat species, i.e. einkorn, emmer and spelt, have been neglected for many years. They were widely cultivated in the ancient world, but later

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became primarily relict crops because of low yields as well as cultivation and processing difficulties. Nowadays, health issues are gaining importance and underutilised wheat species have a number of benefits for health compared to traditional bread wheat (*Triticum aestivum* L.) and pasta wheat (*Triticum durum* Desf.), so they are drawing growing attention of scientists, farmers and consumers. The increasing interest shown towards ancient wheat species is associated with the search for naturalness. Since the 1980s, health-conscious people and gourmets have started to demand ancient cereals, including emmer.

Emmer (*Triticum dicoccum* (Schuebl.) Schrank) is a tetraploid ($2n=48$) wheat species. Emmer was one of the first crops domesticated by humans. Wild emmer (*Triticum dicoccoides* (Schrank) Schuebl.) is the ancestor of domestic emmer. The phylogeny of wild emmer seems to be clear: it is a result of the hybridization between two diploid wild grasses, the wild red einkorn (*Triticum urartu*) and the goatgrass (*Aegilops speltoides* Tausch) (Marcussen *et al.*, 2014). Wild emmer is native to the Fertile Crescent of the Middle East. In modern times, it grows in the grasslands and woodlands of Israel and Iran. The earliest location of emmer domestication is still under debate (Weide, 2015), as several locations were suggested as the earliest sites of emmer domestication during the Early Pre-Pottery Neolithic B. The principal difference between the wild and the domestic species is that ripened spikes of the wild species shatter and spread seeds onto the ground while in the domesticated emmer spikes remain intact, thus making it easier for farmers to harvest grain (Weiss and Zohary, 2011).

Emmer is a hulled wheat species, meaning it has strong glumes that enclose its caryopses and semibrittle rachides. These traits make threshing difficult: upon threshing, emmer spikes break up into spikelets that require great efforts (milling or pounding) to release grains from glumes (Amar, 2015), so it is understandable that producers prefer more easily threshable wheats. However, this underutilized cereal has a great potential to improve nutrition, boost food security and satisfy the increasing demand for traditional products (National Research Council, 1996; Stagnari *et al.*, 2008). Currently, emmer is grown in Armenia, Morocco, Spain, Czechia, Slovakia, Albania, Turkey, Switzerland, Germany, Greece, Italy and the US.

Emmer value lies in its ability to yield a lot in aridity (Krugman *et al.*, 2011; Kuzuoglu-Ozturk *et al.*, 2012; Suneja *et al.*, 2015), in heat (Ullah *et al.*, 2021; Yousif *et al.*, 2022) and on poor soils (Giuliani *et al.*, 2009). Emmer is also noticeable for resistance to fungal diseases (Piarulli *et al.*, 2012; Sharma *et al.*, 2019; Tene *et al.*, 2022; Lhamo *et al.*, 2023) and pests (Liu *et al.*, 2005). Hence, emmer can be successfully grown in organic farming even in areas with lower production potentials (Kovács, 2008; Emódi *et al.*, 2014).

Emmer is considered a healthy food due to increased contents of protein (Osokina *et al.*, 2020; Zaparenko *et al.*, 2020), carotenoids (Somma *et al.*, 2004; Marconi and Cubadda, 2005; Holik *et al.*, 2017), phenolic acids (Lachman *et al.*, 2011; Benincasa *et al.*, 2015), flavonoids (Serpén *et al.*, 2008), alkylresorcinols (Andersson *et al.*, 2008), fibre (D'Antuono *et al.*, 1998; Kulathunga and Simsek,

2022) and trace minerals (Peleg *et al.*, 2008; Chatzav *et al.*, 2010; Lachman *et al.*, 2011).

An emmer-base diet can reduce the diabetes incidence, preventing or delaying diabetes development (Thorup *et al.*, 2014; Gorelick *et al.*, 2017; Christopher *et al.*, 2018). Being non-gluten-free, emmer products may still be suggested to some patients with predisposition to celiac disease or other types of wheat intolerance (Vincentini *et al.*, 2007). All wheat species contain amylase-trypsin inhibitors (ATIs), but emmer showed a lower ATI activity than hexaploid wheat (Zevallos *et al.*, 2017), offering a potential to become a safe cereal for patients with gastroenterological pathologies.

With numerous papers on emmer published globally, efficient synthesis of this abundant information is essential for scientists to gain a comprehensive understanding of the current challenges, research trends and forthcoming prospects. Bibliometric analysis employs statistical methods and visualising approaches to dissect vast arrays of published data (Okubo, 1997; Van Raan *et al.*, 2010). Bibliometric analysis enables researchers to timely identify the most relevant topics, patterns, trends and key findings. Major steps of bibliometric analysis include 1) Data retrieval from databases; 2) Data screening, cleaning, merging and refining; and 3) Processing of data with different bibliometric tools (Passas, 2024). Unlike literature reviews, which summarise and synthesise specific and relatively small datasets and meta-analyses, which summarise evidence from homogeneous studies to uncover new relationships, bibliometric analysis summarizes large amounts of data to pinpoint trends and patterns by operating broad and large (not too specific) datasets, often from heterogeneous studies. In our case, we would say that a traditional literature review analyzes findings on emmer, while a bibliometric analysis review explores emmer research. To date, there are quite a many bibliometric publications devoted to wheat research in general (Giraldo *et al.*, 2019; Pérez-Pérez *et al.*, 2024), bread wheat investigations for a certain period of time (Yuan and Sun, 2022), Turkish studies on pasta wheat (Tekin and Akar, 2024), Indian studies on wheat (Siwach, 2020), ploidy-based analysis (Chandra *et al.*, 2024), research into wheat allergy (Zhan *et al.*, 2024), etc., as well as a comprehensive traditional review on *T. dicoccum* (Biradar *et al.*, 2022); however, to our knowledge, emmer research has not been addressed from a scientometric point of view.

Thus, we applied bibliometric tools to elucidate the knowledge structure within emmer research. Our objectives were to trace time profiles of scientific output in emmer research, to pinpoint core research content, prevailing directions and major patterns, to evaluate connection maps and to identify leaders in this field.

MATERIAL AND METHODS

Data Retrieval and Processing

We used specific keywords to retrieve literature from the Scopus database (data cut-off date was 08/18/2025). The datasets were extracted at the Library of

the Yuriev Plant Production Institute of the National Academy of Agrarian Sciences of Ukraine. The search terms were as follows: "emmer" OR "emmer wheat" OR "wild emmer" OR "*Triticum dicoccum*" OR "*Triticum turgidum* subsp. *dicoccum*" OR "*Triticum turgidum* var. *dicoccum*" OR "*Triticum dicoccon*" OR "*Triticum turgidum* subsp. *dicoccon*" OR "*Triticum turgidum* var. *dicoccon*" OR "*Triticum dicoccoides*" OR "*Triticum turgidum* subsp. *dicoccoides*" OR "*Triticum turgidum* var. *dicoccoides*". In analysis, we included publications in English and in national languages (German, Chinese, Japanese, Ukrainian, etc.) provided they had an English abstract.

Recognizing that the earliest emmer-related literature in the reviewed database dates back to 1905, we selected a total corpus of 3,812 publications covering the period from 1905 to 2025. We used the CSV export option, with all data elements included. To make our analyses more specific, we eliminated duplicates and wrong positive retrievals, which finally left 2,383 documents for analyses. The algorithm of this study is depicted in Figure 1.

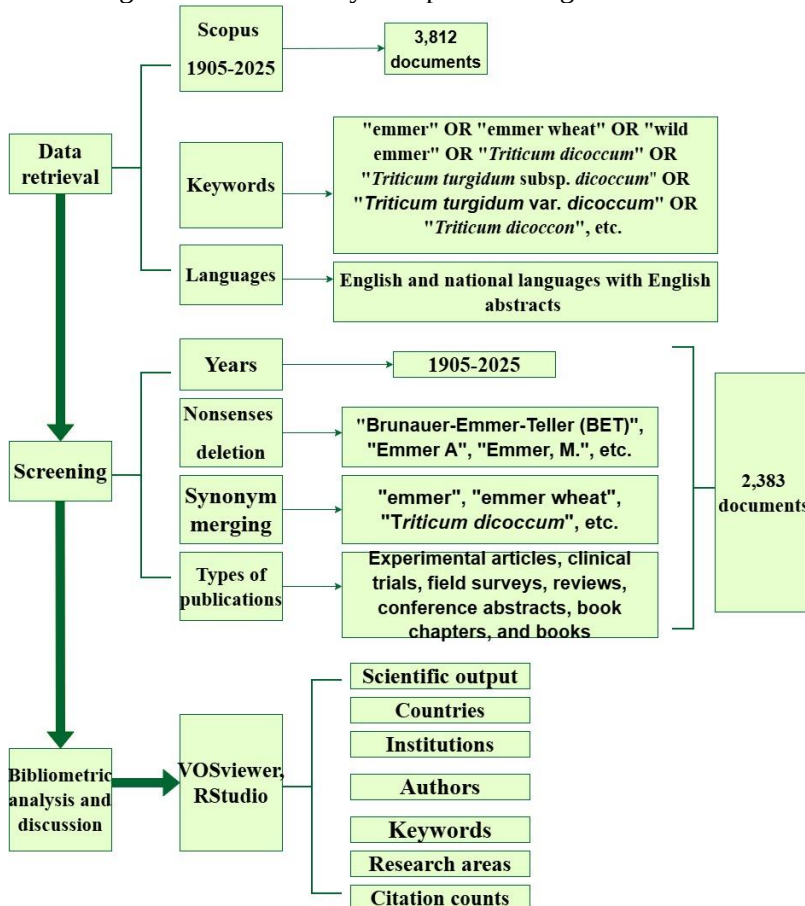


Figure 1. The study flowchart

The Annual Percentage Growth Rate (APGR) was calculated using the following formula: $((\text{Ending Value} - \text{Beginning Value}) / \text{Beginning Value}) \times 100$.

Data Visualization and Analysis

Various metrics such as the number of publications by countries and years, journal contributions, citations, etc., were sourced from the Scopus database. To gain information regarding the top most publishing countries, institutions and journals, most cited documents and most recurrent keywords as well as to visualize the results of metric frequency analysis and interaction analysis based on the Scopus dataset, we employed VOSviewer (version 1.6.20) and RStudio/Biblioshiny (version 2025.05.0-496). To merge name entries such as Nevo Eviatar and Nevo E., we reduced first names of authors to initials and used thesaurus files. We used thesaurus files to merge synonyms, like emmer, emmer wheat, *Triticum dicoccum*, spelling variants, like dietary fibre and dietary fiber, full terms and abbreviations, like single nucleotide polymorphism and snp, and singular and plural forms, like antioxidant and antioxidants. First, plots built in Biblioshiny were downloaded as PNG files; then we constructed their modifiable counterparts as vector images in RStudio, using codes written by Gemini or Claude and extracting plots as PDF files. Connection maps were created VOSviewer and extracted as PNG files. Tables include data calculated in Biblioshiny and extracted as XLSX files. The flowchart was constructed in the free online diagram software diagrams.net (formerly draw.io; JGraph Ltd., 2026).

RESULTS AND DISCUSSION

Time Profiles – Publications and Citations

The temporal evolution of the scientific output of emmer research is illustrated in Figure 2.

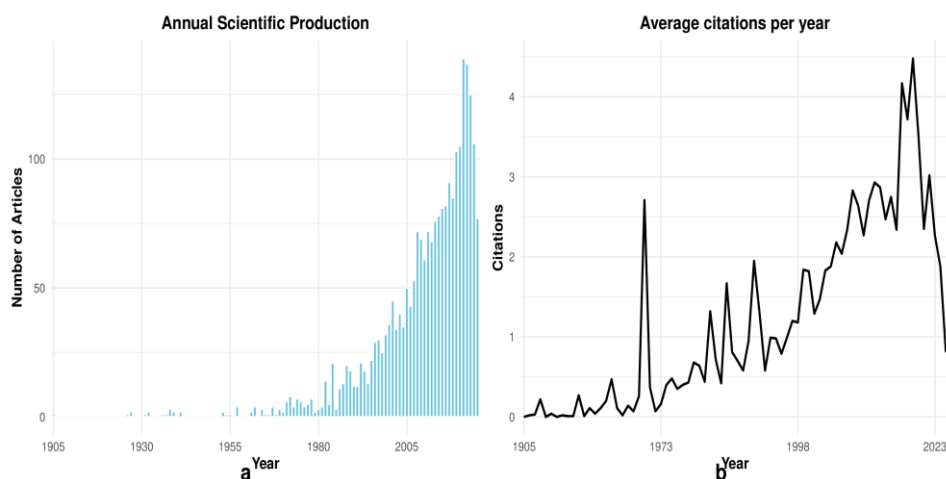


Figure 2. Temporal trends of emmer-related publications. A – Number of publications per year; B – Mean number of citations per year

The number of publications started steadily grow in the early 1980s (Figure 2a), which is understandable because the 1980s saw a renaissance of hulled wheat species (D’Antuono, 1995; Padulosi *et al.*, 1996). The publication peak was reached in 2021. The APGR calculated for the period from 1982 (14 documents) to 2021 (139 documents) was 893%. After 2021, there were somewhat fewer publications in the following years. Does it mean that emmer has arrived at the end of its potential and is starting to seem less promising to researchers? We still think it is too early to make such a conclusion. The 2025 data are too immature and this decline may be another fluctuation, like those seen in 1998-1999 or in 2011-2013.

It should be noted that although there were a few publications on emmer during the period of 1905-1954, they had few citations or were not cited at all (Figure 2b). We see a sharp surge in the average number of citations per year in 1970 (2.7); then this parameter continued to increase, despite rather drastic fluctuations, until 2019 (4.5), indicating a growth both in the interest in emmer and in the publication quality. The APGR for the period from 1970 (the average number of citations per year was 2.7) to 2019 (4.5) was 67%. We believe that such a decline after 2019 does not mean that researchers lost interest in this subject as we can hardly suspect that the publication quality became worse; it is rather a short post-publishing period does not allow seeing the complete picture of citing the literature. Temporal changes in emmer-related publishing activities of top countries, institutions, authors, etc. are summarized in the corresponding sections below.

Sources

Table 1. Ten most relevant journals in emmer research

Publishing activity		Local impact	
Most publishing journals	Number of articles	Most citable journals	H-index*
Theoretical and Applied Genetics	157	Theoretical and Applied Genetics	54
Euphytica	93	Vegetation History and Archaeobotany	33
Vegetation History and Archaeobotany	90	Euphytica	32
Genetic Resources and Crop Evolution	75	Genetic Resources and Crop Evolution	24
Journal of Cereal Science	48	Journal of Cereal Science	24
Frontiers in Plant Science	47	Crop Science	23
Cereal Research Communications	43	Journal of Archaeological Science	20
Japanese Journal of Genetics	36	PLoS ONE	20
Journal of Archaeological Science	35	Plant Breeding	19
PloS ONE	34	Frontiers in Plant Science	18

*Note. The local H-index is calculated based on the analysed emmer research corpus (1905–2025).

Given that articles (experimental articles + review articles + some conference proceedings) constitute a majority in the literature corpus under investigation, we further concentrated our analysis on top journals in this field. Table 1 shows that Theoretical and Applied Genetics is the most popular journal among authors in the field of emmer research. It is followed by Euphytica and Vegetation History and Archaeobotany. Local impact reflects how journals contribute to the academic advancements in their immediate surroundings versus in the global scholar community. Table 1 displays the local impact of the top 10 journals based on their H-index calculated specifically for the studied dataset. It is evident that articles published in Theoretical and Applied Genetics, Vegetation History and Archaeobotany and Euphytica are the most cited ones at present, so these journals are the leaders not only by quantity of emmer-related publications but also by citation count in this field.

The temporal progression of the journals' activities in this field is illustrated in Figure 3. Theoretical and Applied Genetics took the lead in 1988 and Vegetation History and Archaeobotany in 2007. At the dawn of emmer research, The Japanese Journal of Genetics was the leader for many years, but after 1995 its number of publications ceased increasing. The relatively recent dash of Vegetation History and Archaeobotany means that the significance of emmer in wheat evolution and the role of emmer in ancient cultures have gained rising interest in the academic community.

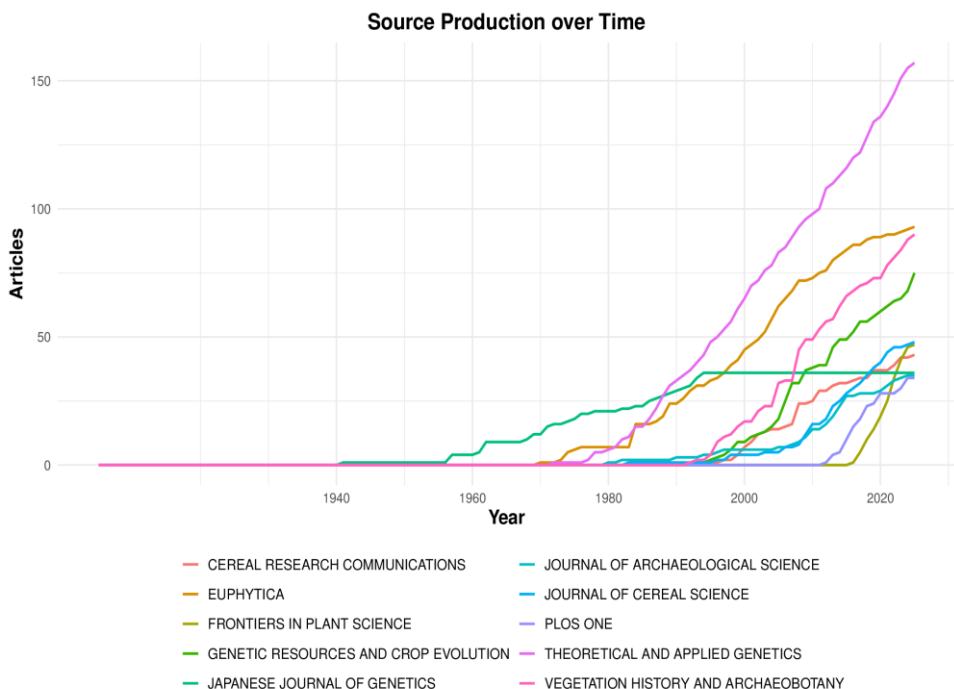


Figure 3. Temporal progression of the publishing activity of ten most relevant journals in emmer research

Top countries

We also analysed the scientific output of emmer-studying countries. Table 2 indicates that China, USA, Israel, Italy, Turkey, Germany, United Kingdom, India and Japan are the leaders in the emmer-related publishing activities. While China is the most prolific contributor, Israel remained the most cited one. In general, the lists of the most productive and the most cited countries are very similar.

Table 2. Ten most relevant countries in emmer research

Greatest contributors		Most citable countries	
Country	Number of documents	Country	Total Citation Count
China	1,966	Israel	4,588
Italy	1,210	China	3,342
USA	938	USA	3,318
Israel	897	Italy	3,128
India	604	Germany	2,482
Germany	551	Turkey	2,266
Turkey	402	United Kingdom	1,509
UK	402	France	1,109
Japan	307	Japan	771
Czech Republic	263	Czech Republic	588

For many years, Israel ranked first in the number of publications, with the USA and Italy alternately ranking second and third (Figure 4). From the beginning of this millennium, China showed a rapid rise, surpassing Italy, the USA and Israel by 2020.

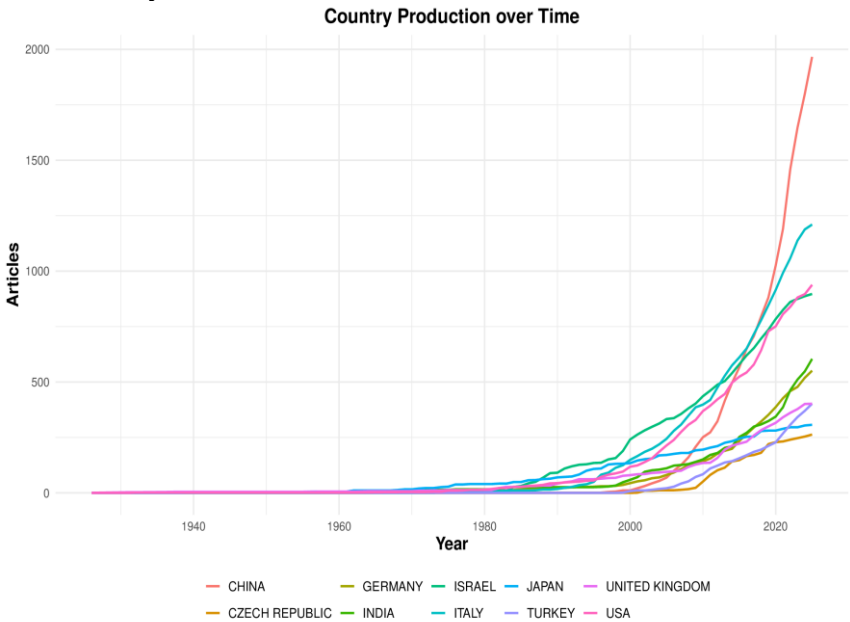


Figure 4. Publication productivity of the most relevant countries in emmer research over time

It is anticipated that Israel, Turkey, and Italy are among the most relevant countries in emmer research. The former two countries harbor rich populations of wild emmer. This abundance of genetic resources has historically driven intensive research in these regions (Nevo and Beiles, 1989; Özkan *et al.*, 2011). Meanwhile, Italy's significant contribution is driven by its long-standing focus on wheat species suitable for high-quality pasta production. USA, Japan, Germany, France and other European countries are science-oriented countries in general; in addition, many of these countries have turned their attention to ancient wheat species since the 1980s. China's rapid advancement in this field can be explained by great interest in the whole genus *Triticum* (Xiao *et al.*, 2022; Zhuang *et al.*, 2024).

We have also summarized intra- and inter-country cooperation in the emmer-related publishing activities of the top countries. According to the multi-country to single-country publication ratio, the countries were ranked as follows: Israel (61.4%) > United Kingdom (60.7%) > China (39.3%) > USA (38.4%) > Germany (36.7%) > Italy (28.7%) > Czech Republic and India (27.3%) > Japan (26.7%) > Turkey (18.2%), showing quite high percentages of inter-country collaboration for the major contributors.

The connection map (Figure 5) illustrates distribution and collaboration among countries engaged in emmer research.

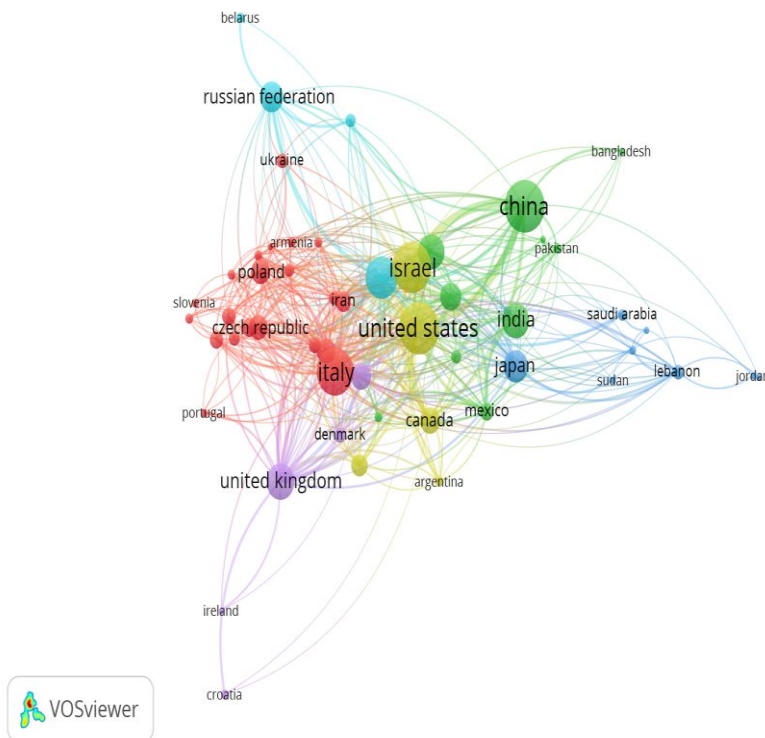


Figure 5. Co-authorship map for countries (frequency ≥ 5 ; total link strength)

Analysis of the Scopus dataset in VOSviewer grouped 54 countries in 6 clusters. The yellow cluster contains two of the greatest contributors, Israel and the USA, with China being in the green cluster. Other publishing-active countries, such as Germany, United Kingdom, Turkey, Japan, Italy, fall into different clusters: Italy is in the red cluster, United Kingdom – in the lilac cluster, Japan – in the dark-blue cluster, Turkey – in the green cluster, Germany – in the light-blue one, etc. The most prolific contributors, China and Israel, have established collaborative ties with 24 and 29 countries, respectively. Lines between countries show link strength. For example, the total link strength between the USA and Israel is 80 and it is the strongest connection. For comparison, the total link strength between the USA and Ukraine is 2.

Institutions

Further, institutions were evaluated for scientific output. Our analysis identified the ten most prolific affiliations, which are ranked in descending order as follows: University of Haifa (499 articles) > Sichuan Agricultural University (463) > Northwest A&F University (266) > North Dakota State University (174) > China Agricultural University (166) > Università degli Studi di Bari Aldo Moro (142) > University of California (140) > Hebrew University of Jerusalem (137) > Institute of Cytology and Genetics (100) > (Tel Aviv University (99)).

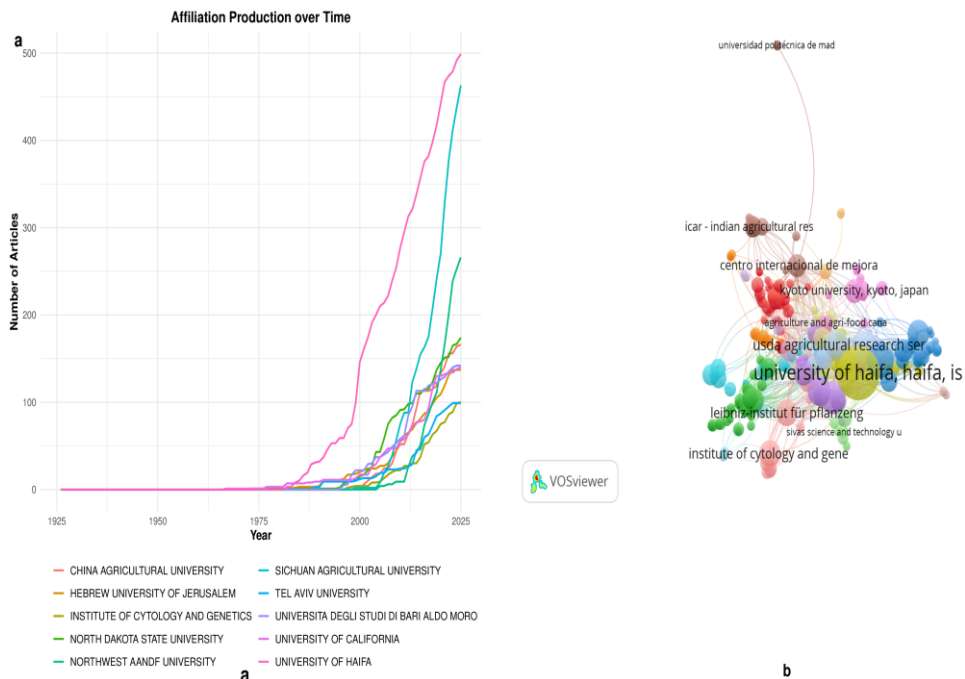


Figure 6. Most relevant institutions in emmer research. a – Institutions' production over time; b – Collaboration network (frequency ≥ 5 ; total link strength)

Figure 6a demonstrates that the University of Haifa has been the leader in the emmer related publishing activities for the entire period of analysis. Sichuan Agricultural University moved ahead of the other leading organizations in 2013, nearly equaling the output of the University of Haifa. Of 1,703 affiliations, VOSviewer identified 234 organizations that met the clustering threshold and grouped them in 17 clusters (Figure 6b). The yellow cluster with University of Haifa and University of California (Davis) stands out. The second ranking Sichuan Agricultural University is in the dark-blue cluster. University of Haifa has established 78 collaboration links with the total strength of 369 and published 199 documents. For comparison, Peking University (dark-blue cluster), for example, has established 8 collaboration links with the total strength of 13 and published 7 documents.

Leading Authors and Research Topics

A total of 5,517 authors contributed to emmer-related literature. The scientific outputs of the leading researchers, i.e. their publication counts and H-indices, are summarised in Table 3. Nevo Eviatar and Fahima Tzion, who rank first and second, are undeniably the most publishing authors in emmer research, outpacing the other top-ranking authors. Nevo E. and Fahima T. have the highest values of H-index, meaning that they are not only the most prolific authors but also the most cited ones.

Table 3. Most relevant authors in emmer research

Authors	Number of articles	Author	H-index
Nevo, Eviatar	113	Fahima, Tzion	45
Fahima, Tzion	80	Nevo, Eviatar	44
Beiles, Avigdor	36	Korol, Abraham B.	26
Korol, Abraham B.	36	Beiles, Avigdor	23
Krugman, Tamar	34	Peleg, Zvi	22
Peleg, Zvi	31	Röder, Marion S.	21
Zheng, Youliang	31	Saranga, Yehoshua	21
Saranga, Yehoshua	30	Blanco, Antonio	20
Faris, Justin D.	28	Krugman, Tamar	20
Xu, Steven S.	27	Faris, Justin D.	19

From Figure 7a, it is clear that the most prolific author, Nevo E., started publishing emmer-related articles as early as in 1984 and continues his publishing activity till now. He works at the Institute of Evolution, University of Haifa. It appears that his work has made the University of Haifa the main contributor to emmer-related literature and Israel the most influential country in this field. The other top authors have much shorter periods of emmer-related publishing activities, except for Beiles A. (Institute of Evolution, University of Haifa; 1984-

2019) and Krugman T. (Institute of Evolution, University of Haifa; 1986-nowadays), who have been publishing emmer-related articles for a long time. As to citation counts for the entire analyzed period (Figure 7a), Nevo E. and Fahima T. (also working at the Institute of Evolution, University of Haifa) had the highest total count per year: Nevo E. reached over 30 citations per year in 2010-2011 and Fahima T. had over 30 citations per year in 2003 and 2009. VOSviewer analysis of the Scopus dataset found that 425 authors met the clustering threshold and grouped them in 17 clusters in the connection map (Figure 7b). Nevo E. (pink cluster) and Fahima T. (yellow cluster) rank the first in this analysis: they have established 69 and 62 collaboration ties with the total link strength of 316 and 370, respectively. Nevo E. has published 113 documents with the total citation count of 6,227 and Fahima T. has published 80 documents with the total citation count of 5,853.

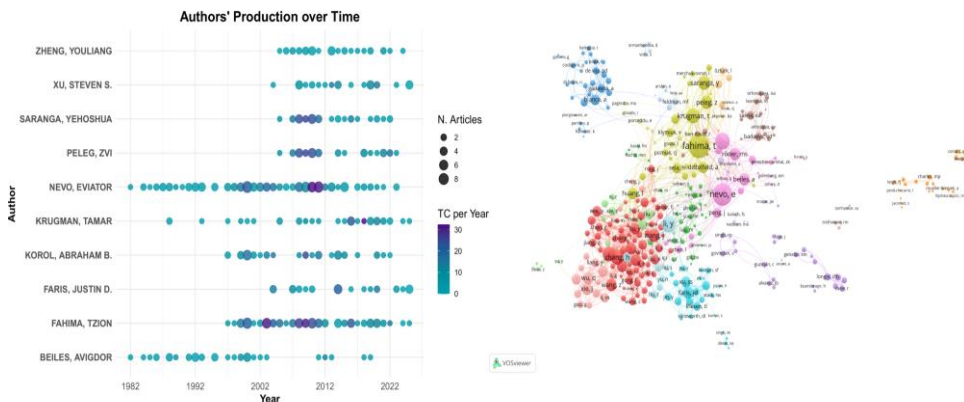


Figure 7. Most relevant authors in emmer research. a – Production over time (the size of circles corresponds number of publications and the tinting strength means citation count); b – Collaboration network (frequency ≥ 5 ; total link strength).

We selected the 30 most cited articles (over 200 citations), which are listed Table 4. Having reviewed these articles, we distinguished major directions in emmer research: 1) Wheat origin, evolution and domestication. It seems that these issues make up the greatest mainstream in emmer research. Anvi *et al.* (2017) reported about a 10.1-gigabase assembly of the 14 chromosomes of wild emmer and findings on gene content, genome architecture and genetic diversity. The authors revealed “genomic regions bearing the signature of selection under domestication.” Maccaferri *et al.* (2019) also described regions exhibiting conspicuous signatures of domestication-associated genetic divergence. Haudry *et al.* (2007) estimated “the intensity of the bottlenecks associated with domestication and subsequent selection.” According to their estimate, a domesticated emmer population size was about one third of a wild emmer population size. Studying such traits as brittle rachis, heading date, plant height,

grain size, yield and yield components, Peng J. *et al.* (2003) mapped domestication-related quantitative trait loci in *T. dicoccoides*. Research often addresses the place of emmer in the general picture of wheat evolution and domestication (Evans and Dunstone, 1970; Matsuoka, 2011; Peng *et al.*, 2011). 2) Grain quality. Specifically, mineral micronutrient levels are a hotspot. Emmer grain was shown to contain significantly more selenium than common or durum wheat (Zhao *et al.*, 2009). Cakmak *et al.* (2004) and Peleg *et al.* (2009) demonstrated that wild emmer was a valuable genetic resource for increasing zinc and iron contents in modern cultivars. Distelfeld *et al.* (2007) obtained similar results. In addition, they demonstrated that emmer was also richer in manganese and protein than modern durum wheat. In a durum wheat line carrying *T. dicoccoides* chromosomes, Joppa *et al.* (1997) identified markers that could be used in marker-assisted breeding for high grain protein content in durum wheat. High grain protein content is a frequent target of wheat breeding programmes; hence the grain protein content locus has been intensively investigated, including its pleiotropic effects (Uauy *et al.*, 2006). Dietary fibre is a valued ingredient of grain, hence the study where a set of wheat accessions had been screened for contents of dietary fibre and components thereof was highly cited (Gebruers *et al.*, 2008). This set was also screened for levels of tocopherols, sterols, phenolic acids, folates and alkylresorcinols (Ward *et al.*, 2008). Bioactivity of amylase-trypsin inhibitors (ATIs) is another indicator of grain quality that has been a frequent subject of studies. Emmer products were shown to have lower ATI bioactivity than modern hexaploid wheat (Zevallos *et al.*, 2017). Arzani and Ashraf (2017) summarized benefits of ancient wheat species, including emmer, as potential sources of healthy foods. However, Shewry and Hey (2015) rebutted the opinion that ancient wheats were healthier than modern wheats. 3) Biology of pathogens and resistance to diseases. Biology of wheat pathogens (especially fungi) has been a research mainstream for a long time (Bolton *et al.*, 2008). Emmer is noticeable for resistance to fungal pathogens. Wild emmer carries several genes for resistance to yellow rust: Yr15, Yr35 and Yr36 (Uauy *et al.*, 2005; Chen *et al.*, 2014; Klymiuk *et al.*, 2018). 4) Tolerance to abiotic stresses. It is another advantage of this ancient wheat. Nevo and Chen (2010) identified drought- and salt-tolerant genes and quantitative trait loci (QTLs) in *T. dicoccoides* and highlighted a great potential for wheat improvement. Turkish scientists also studied response of wild emmer to drought stress and discovered 13 microRNAs that were differentially regulated in response to drought (Kantar *et al.*, 2011). 5) Molecular breeding approaches. It is often difficult to separate this research direction from others, as molecular genetic methods are widely used to improve grain quality or to enhance resistance to diseases, etc. However, some influential publications were devoted specifically to approaches and tools. For example, Maccaferri *et al.* (2015) assembled a high-density tetraploid wheat consensus map that could be used for wheat breeding purposes. 6) Nucleic acid sequence polymorphism. This direction is also difficult to separate from others because the sequence polymorphism studies often deal with grain quality traits, or agronomic

characteristics, or resistance to unfavourable conditions, etc. Nevertheless, there are influential articles devoted nucleic acid sequence polymorphisms per se (Appels and Dvořák, 1982). We should, nonetheless, point out that such findings often carry evolutionary or breeding implications. 7) Early cultivation. Studies on the roles of emmer and other wheat species in early cultures stand apart in the corpus of biological and agricultural research. Nonetheless, we cannot ignore this theme as it is among top ones and publications on pre-domestication cultivation in the pre-Neolithic and Neolithic era are on the list of the most cited articles (Boardman and Jones, 1990; Willcox *et al.*, 2008; Riehl *et al.*, 2013). It should be noted that the paleobotany and archaeobotany theme is closely intertwined with the domestication theme (Willcox, 2005).

Table 4. Most cited emmer-related articles in the Scopus dataset

No	Publication	Total citation count	Major directions
1	Avni <i>et al.</i> (2017)	627	Wheat origin, evolution and domestication
2	Maccaferri <i>et al.</i> (2019)	551	Wheat origin, evolution and domestication
3	Zhao <i>et al.</i> (2009)	427	Grain quality
4	Chen <i>et al.</i> (2014).	393	Biology of pathogens and resistance to diseases
5	Bolton <i>et al.</i> (2008)	373	Biology of pathogens and resistance to diseases
6	Nevo and Chen (2010).	364	Tolerance to abiotic stresses
7	Kantar <i>et al.</i> (2011)	341	Tolerance to abiotic stresses
8	Cakmak <i>et al.</i> (2004)	320	Grain quality
9	Haudry <i>et al.</i> (2007)	312	Wheat origin, evolution and domestication
10	Matsuoka (2011)	296	Wheat origin, evolution and domestication
11	Zevallos <i>et al.</i> (2017)	281	Grain quality
12	Peng <i>et al.</i> (2003)	280	Wheat origin, evolution and domestication
13	Klymiuk <i>et al.</i> (2018).	259	Biology of pathogens and resistance to diseases
14	Arzani and Ashraf (2017)	259	Grain quality
15	Distelfeld <i>et al.</i> (2007)	258	Grain quality
16	Peng <i>et al.</i> (2011)	249	Wheat origin, evolution and domestication
17	Joppa <i>et al.</i> (1997)	247	Grain quality
18	Peleg <i>et al.</i> (2009)	245	Grain quality
19	Willcox <i>et al.</i> (2008)	244	Early cultivation
20	Uauy <i>et al.</i> (2006)	239	Grain quality
21	Evans and Dunstone (1970)	238	Wheat origin, evolution and domestication
22	Gebruers <i>et al.</i> (2008)	236	Grain quality
23	Maccaferri <i>et al.</i> (2015)	234	Molecular breeding approaches.
24	Boardman and Jones (1990)	228	Early cultivation
25	Ward <i>et al.</i> (2008)	224	Grain quality
26	Uauy <i>et al.</i> (2005)	219	Biology of pathogens and resistance to diseases
27	Shewry and Hey (2015)	215	Grain quality
28	Riehl <i>et al.</i> (2013)	215	Early cultivation
29	Willcox (2005).	213	Early cultivation; wheat evolution and domestication
30	Appels and Dvořák (1982)	212	Nucleic acid sequence polymorphism

Here we can also trace some regional peculiarities in emmer research. For example, Turkish researchers are more focused on drought tolerance (Budak *et al.*, 2013), which can be explained by the country's climatic conditions, while Israeli triticologists have made use of their (and Turkish) populations of wild emmer (Nevo and Beiles, 1989; Peng *et al.*, 2003; Cakmak *et al.*, 2004).

Research Hotspots and Evolution Trends

Since there is a notion that author keywords are highly subjective and lack statistical uniformity (Qin *et al.*, 2021), at first we used Keywords Plus in RStudio and Index Keywords in VOSviewer to achieve greater relevance in this study (data are not presented). However, we found that Keywords Plus- or Index Keywords-based clusters poorly reflect the actual state in emmer-related literature and in some cases do not corroborate with each other. Zhang *et al.* (2016) pointed out that Authors' Keywords and Keywords Plus were equally effective in terms of bibliometric analysis to investigate the knowledge structure, but Authors' Keywords better represented contents of publications. Hence, we decided on using authors' keywords.

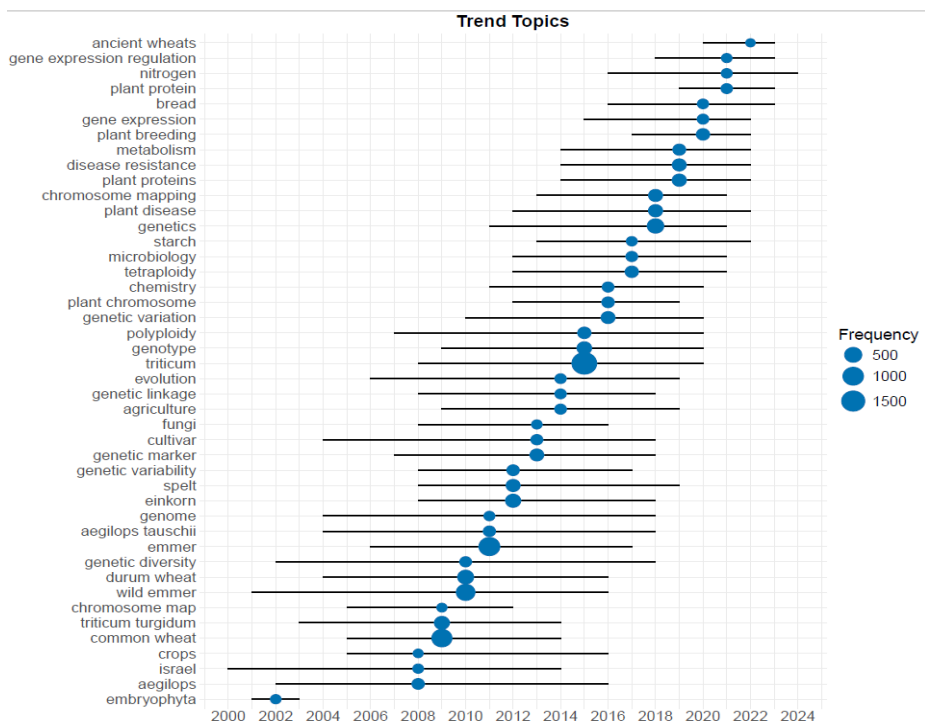


Figure 8. Keywords with the strongest occurrence bursts (word minimum frequency – 50; number of words per year – 3)

Here, ten the most frequent keywords are listed in the descending order: *Triticum* (1,483 occurrences) > common wheat (807) > emmer (612) > genetics (423) > wild emmer (350) > durum wheat (266) > *Triticum turgidum* (257) > genotype (247) > plant disease (216) > einkorn (212). This confirms that resistance to diseases, improvement of widely cultivated wheat species, wheat genetics and related ancient wheat species are the most frequent subjects in emmer research.

Trend Topics plots provide visualization of research focal points over time. They help assess which themes arouse persistent research interests, what themes are likely to become future research directions and that themes can be considered as exhausted or currently irrelevant. Circles denote medians of term frequencies. The biggest circles for the keywords ‘*Triticum*’, ‘common wheat’, ‘wild emmer’ and ‘emmer’ reflect the significant attention devoted to these topics (Figure 8). The themes, such as ‘genetic diversity’, ‘wild emmer’, ‘*Aegilops*’, appear to be subjects of rather persistent research interests, as they emerged with high frequencies for 16, 15 and 14 years with the medians in 2010, 2010, 2016 and 2008, respectively. The themes ‘ancient wheats’, ‘gene expression regulation’ and some others, which have appeared recently, may become future research directions. The term ‘embryophyta’ disappeared from emmer-related literature in 2003, meaning this topic is not linked with emmer research hotspots any longer.

Keyword clustering elucidates relationships between keywords, allowing one to pinpoint research hotspots. The visualization of the Scopus dataset in VOSviewer identified 5 rather heterogeneous clusters (of 9,817 keywords, 447 met the clustering threshold; Figure 9a). The largest red cluster in the connection map focuses on domestication, organic farming, breeding, grain quality, related cereal species, yield, germination and other issues. The second largest, green cluster, is concentrated around genetics and disease resistance: plant chromosomes and genes, phenotype, quantitative trait loci, genetic maps and linkage, biomarkers, immunology and fungal pathogens. The blue cluster is significantly smaller, focusing on evolution, genetic variability, ploidy levels, plant genome and wild related species. The yellow cluster includes more biochemical and physiological issues: metabolism, gene expression regulation, transcriptome, plant proteins, resistance to abiotic stresses and many minor issues. The smallest, lilac cluster includes classification, phylogeny and molecular evolution. It is noteworthy that such topic as plant genetics or evolution are so broad and comprise so many multifarious aspects that it is impossible to place them in one cluster. Figure 9b elucidates the categorization of themes in emmer research. In such maps, each cluster named after the most common words is represented as a bubble. The bubble size is determined by word frequency in the cluster. The X-axis measures the cluster’s centrality (relevance) in the network, i.e., the degree of interaction with other clusters in the map, evaluating the importance of research themes (Bastazini, 2025) The Y-axis measures development (density), i.e. the strength of work within the cluster and the theme growth. Basic themes (lower right quadrant) are usually characterized by high centrality and low density. They are often essential for transdisciplinary

investigations and may serve as foundational topics for multiple themes, but these themes have few connections. This plot contains one big blue bubble right between this quadrant and the Motor Themes. We would describe it as the “emmer and related species bubble”, as it contains keywords ‘emmer’, ‘wild emmer’, ‘durum wheat’, ‘common wheat’, ‘*Triticum turgidum*’ and ‘*Aegilops*’, but it also includes themes ‘genetic variability’ and ‘genotype’. Motor Themes (upper right quadrant) are characterized by high centrality and density, indicating that they are well-developed and crucial for structuring the research field. The Motor Themes in our study encompass keywords ‘genetics’, ‘plant chromosomes’, ‘plant genes’, ‘alleles’, ‘plant proteins’, ‘metabolism’ and ‘phylogeny’ (green bubble in the upper right corner). In addition, half of the yellow bubble is in the upper right quadrant, too. This bubble covers such topics as ‘chromosome mapping’, ‘plant diseases’, ‘disease resistance’, ‘fungi’, ‘genetic markers’ and ‘microbiology’. The other half of the orange bubble belongs to the Niche Themes. Niche Themes (upper left quadrant) are highly specialized and well-developed in terms of internal research but also have limited interactions with other research themes. In our analysis, the Niche Theme quadrant contains no bubbles of themes that could be classified as purely niche. Peripheral themes or Emerging or Declining Themes (lower left quadrant) are themes with low centrality and low density, suggesting that they are underdeveloped and marginal, i.e. either emerging or declining in the literature. One “archaeobotanical” bubble (the small lilac bubble) represents such themes in the lower left corner of the plot in Figure 9b. The Emerging or Declining Themes in this analysis also contain the red bubble, which is somewhat similar to the blue bubble, as it also includes keywords designating related cereal species; however, the list of species is different: the red bubble has keywords ‘einkorn’, ‘spelt’, ‘barley’ and ‘rye’. The red bubble also contains the general terms ‘cultivation’ and ‘agriculture’.



Figure 9. Keywords co-occurrence network in emmer research. a – VOSviewer map (frequency ≥ 10); b – thematic keywords (minimal cluster frequency per 1,000 documents – 5)

CONCLUSIONS

The bibliometric analysis indicates a transformative shift in emmer research from sporadic historical observations to a dynamic, multifaceted field of modern cereal science. While traditional studies focused on wheat origin and domestication, the current landscape is increasingly dominated by molecular breeding and the exploration of nucleic acid sequence polymorphism. The transition of leadership in publication output to China, alongside the sustained high contributions of Israeli and American institutions to the total scientific output, reflects a global strategic interest in diversifying wheat genetic resources.

The core research mainstreams—ranging from grain quality to biotic and abiotic stress resistance—underscore the growing recognition of emmer as a critical reservoir for crop improvement. Future scientific endeavours are expected to prioritize the physiological and genetic mechanisms of stress tolerance. Specifically, the introgression of beneficial traits from wild and cultivated emmer accessions into advanced common and durum cultivars remains a vital frontier for ensuring global food security in the face of climate change. These findings highlight emmer not only as a subject of evolutionary interest but as a valuable starting material for sustainable wheat breeding.

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DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, we used AI tools (Gemini and Claude) for technical assistance. Specifically, these tools were employed to generate R-codes for creating high-resolution vector visualizations in RStudio (as detailed in the Materials and Methods section), to improve our English, and to format the bibliographic references according to the journal's style. Following the use of these tools, we all reviewed and edited the manuscript, taking full responsibility for the final scientific content and the accuracy of the reference list.

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SUSTAINABLE FOREST ECOSYSTEM ASSESSMENT: PLANT DIVERSITY, BIOMASS, AND CARBON SEQUESTRATION POTENTIAL

SUMMARY

Understanding the dynamics of forest ecosystems is essential for achieving sustainability and climate-resilient development. This study aimed to assess plant diversity, biomass, and carbon sequestration potential within the dry evergreen and dry dipterocarp forests of Tat Ton National Park, northeastern Thailand. Field inventories were conducted across a 320-ha area encompassing 22 sampling plots, where 4,047 trees were measured with an average density of 12.65 trees ha⁻¹. Structural parameters and vegetation composition were analyzed to evaluate forest heterogeneity and carbon distribution. Results revealed rich species diversity and a wide variation in carbon storage, with total carbon stock estimated at 25,383.90 tC and an overall mean of 79.95 tC ha⁻¹, ranging from 59.72 to 110.24 tC ha⁻¹ among plots. The coexistence of dry evergreen and dipterocarp ecosystems enhances ecological stability and strengthens the park's role as a substantial carbon sink. These findings underscore the ecological importance of

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conserving mixed forest landscapes through sustainable forest management. The study provides scientific evidence supporting climate adaptation, carbon-credit development, and Thailand's long-term pathway toward a Net Zero future.

Keywords: plant diversity, carbon sequestration, dry evergreen forest, dry dipterocarp forest, carbon credit, green economy

INTRODUCTION

Climate change has become one of the most important environmental problems affecting all regions of the world (Uttaruk and Laosuwan, 2020; Čadro *et al.*, 2024; Uttaruk and Laosuwan, 2026). Its development is driven mainly by the increase of greenhouse gases in the atmosphere, resulting in negative changes to Earth's climate system. These gases are released by human activity (such as industrial processes, use of motor vehicles, oil or gas burning and farming). Further, large scale deforestation and land use conversions have also contributed climate destabilization at local, regional and global scales (Prudil *et al.*, 2023; Dagiliūtė and Kazanavičiūtė, 2024). Of different GHG, CO₂ dominates with around 77% and is followed by CH₄ with approximately 14%, N₂O at about 8%. Less prevalent greenhouse gases, such as hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulfur hexafluoride (SF₆), collectively account for approximately 1% of the global emissions (Tian *et al.*, 2019; Laosuwan *et al.*, 2023). Fossil fuel CO₂ emissions are expected to rise to an unprecedented level of 37.4 billion tons in 2024, an increase by 0.8% from the year before, according to Global Carbon Project (Thai PBS, 2025). That increase is evidence that the world remains nowhere near on track to deliver on its climate reduction pledges. The findings were released on (UNFCCC, 2024). While the peak of emissions might well arrive within this decade - propelled in particular by the swift spread of renewables as solar, wind or electric mobility – such solutions presently cater for just a small fraction of global energy need. As a result, fossil fuels remain the primary energy sources in the world (Carlsen, 2025).

Decreasing atmospheric CO₂ levels can be achieved by incorporating a combination of complementary methods, including enhancing the efficiency of clean production processes, promoting the use of low-carbon and renewable energy sources as well as attempting carbon capture and storage (CCS) technology (Saxena *et al.*, 2024). CO₂ is considered among these as one of the most powerful means to keep CO₂ out of the atmosphere (Kongkuah *et al.*, 2025). One of the most cost-effective and environmentally sound forms of sequestration is storing carbon in trees and wood products. Trees sequester CO₂ from the atmosphere through photosynthesis and store it as carbon in their biomass spread over above- (stem, branch, leaf) and below-ground (mostly root) organs (Albrecht and Kandji, 2003; Brockerhoff *et al.*, 2017; Nunes *et al.*, 2020; Uttaruk *et al.*, 2024a; Angkahad *et al.*, 2025; Laosuwan *et al.*, 2025a). The sequestered carbon is stored in the tree and remains until that tree is harvested; increased photosynthetic activity increases CO₂ uptake and accumulation (Plybour, *et al.*, 2025). To estimate the biomass, tree anatomical characteristics can be analyzed through

allometric equations that express the mathematical relation between dimensions of a tree and its biomass volume (Pati *et al.*, 2022; Oumasst *et al.*, 2024). Strategies like afforestation (establishment of a forest where there was no forest), reforestation (replanting trees in areas that were cut down), restoration of forests, conversion of agricultural land to agroforestry and the planting or setting aside land for commercial purposes as a plantation help to expand the forest cover (Uttaruk *et al.*, 2024b). These practices increase the storage of carbon and decrease CO₂ emissions into atmosphere supporting equilibrium in the global carbon cycle, thus influencing climate stability (Bussotti and Pollastrini, 2025). However, the quantity of carbon sequestered in forests significantly differs depending on many factors such as forest type, species composition, stand density, terrain features and site-specific environmental conditions (Dixon *et al.*, 1994; Van Con *et al.*, 2013). Furthermore, carbon stocks are determined by species-specific traits, tree age, local climate conditions and soil characteristics (Spittlehouse and Stewart, 2003; Wattanasuksakul *et al.*, 2012; Chen *et al.*, 2022; Zhang *et al.*, 2023; Laosuwan *et al.*, 2025b) as well as silvicultural management events occurring in the forest ecosystem.

This research is based on the hypothesis that both dry evergreen and dry dipterocarp forests are characterized by high levels of plant species diversity, which determine the accumulation of aboveground biomass (AGB) and enhance the overall carbon storage capacity of these ecosystems. Species diversity is assumed to be positively correlated with the potential of forest ecosystems to sequester and maintain carbon, reflecting the intricate relationship between biodiversity and ecosystem functionality (Xu *et al.*, 2020). In other words, greater species richness is expected to promote higher AGB and more efficient carbon storage, thereby mitigating greenhouse gas emissions (Khan *et al.*, 2024).

Given this ecological significance, the main objective of the present study is to evaluate plant species diversity, aboveground biomass, and carbon stock across two dominant forest types—dry evergreen and dry dipterocarp forests—in Tat Ton National Park. These ecosystems serve as essential natural resources that contribute to climate change mitigation, biodiversity conservation, and ecological stability. The findings from this research are anticipated to provide valuable insights for ecosystem restoration, carbon accounting, and sustainable forest management strategies that support climate resilience and long-term environmental sustainability.

MATERIAL AND METHODS

Study Area

Tat Ton National Park (Figure 1) is located in Nafai Sub-district, Mueang District, Chaiyaphum Province, northeastern Thailand, covering approximately 320 ha. Established on December 31, 1980 as Thailand's 23rd national park, it lies within the Phu Laen Kha mountain range at elevations of 200–945 m a.s.l. The landscape features undulating plateaus and shallow valleys with lateritic and

sandy-loam soils derived from sandstone, forming the headwaters of the Lam Pathao and Tat Ton creeks that support surrounding ecosystems and agriculture.

The park's vegetation is dominated by dry dipterocarp forest, with smaller portions of dry evergreen forest, distributed along distinct topographic and moisture gradients—dipterocarp forests occupying lower, drier slopes and evergreen forests occurring at higher, more humid elevations. The area has a tropical monsoon climate with three seasons: hot (April–May), rainy (June–October), and cool (November–March), with an average annual temperature of 27.6 °C and rainfall of 1,154 mm. These environmental conditions shape the park's vegetation composition, species diversity, and carbon sequestration potential, making it an ideal site for assessing plant diversity, above-ground biomass, and carbon storage within Thailand's characteristic dry forest ecosystems.

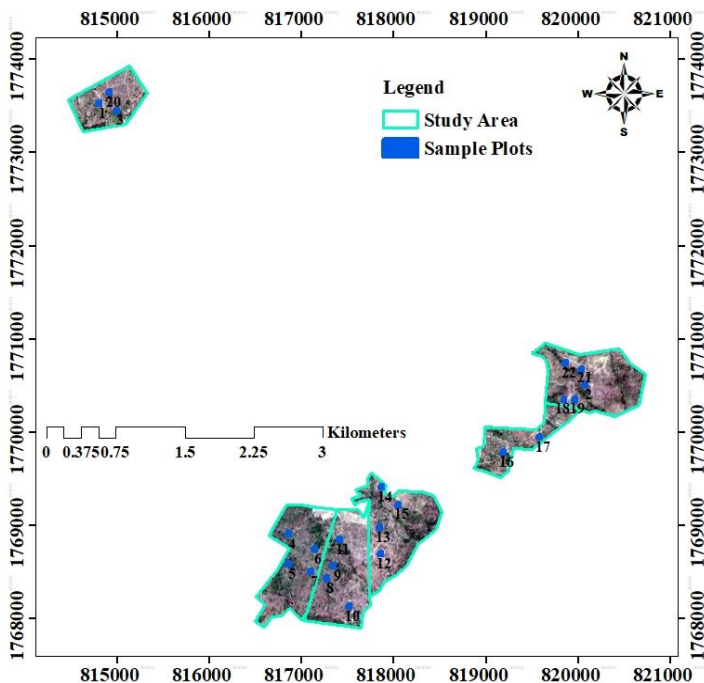


Figure 1. Study area and spatial distribution of the six main plots and twenty-two subplots in Tat Ton National Park, northeastern Thailand

Field Measurement and Data Analysis

A stratified random sampling approach was used to represent forest conditions across the 320-ha study area. Six main plots were selected in areas representing the dominant forest types of Tat Ton National Park, including dry evergreen forest (DEF) and dry dipterocarp forest (DDF). Each plot measured 40×40 m (0.16 ha), which follows common practice in forest carbon studies in

Thailand. The six plots were divided into 22 subplots distributed across the study area to improve spatial coverage. Subplot locations were selected using a random approach while considering field accessibility and vegetation differences. In each subplot, all trees with a diameter at breast height (DBH) greater than 4.5 cm were recorded. The distribution of sampling plots is shown in Figure 1.

For every recorded tree, species identification and classification were performed to describe the overall composition of the forest community. The Importance Value Index (IVI) was subsequently calculated to evaluate vegetation structure and species dominance. IVI combines key ecological parameters—density (D), frequency (F), and dominance (Do)—together with their relative values: relative density (RD), relative frequency (RF), and relative dominance (RDo). The formulas and calculation procedures for these variables are summarized in equation 1-7.

$$IVI = RDi + RFi + RDoi \quad (1)$$

Relative density (RDi)

$$RDi = \frac{\text{Density of a species } (Di)}{\text{Total density of all species}} \times 100 \quad (2)$$

Density (Di)

$$Di = \frac{\text{Total number of tree a species } i}{\text{Total sampled area}} \quad (3)$$

Relative frequency (RFi)

$$RFi = \frac{\text{Frequency value of a species } i \text{ } (Fi)}{\text{Total frequency value of a species}} \times 100 \quad (4)$$

Frequency (Fi)

$$Fi = \frac{\text{Number of plots where a species } i \text{ occurs}}{\text{Total number of sampled plots}} \quad (5)$$

Relative dominance ($RDoi$)

$$Fi = \frac{\text{Number of plots where a species } i \text{ occurs}}{\text{Total number of sampled plots}} \times 100 \quad (6)$$

Dominance (Doi)

$$Doi = \frac{\text{Total basal area of a species } i}{\text{Total sampled area}} \quad (7)$$

Furthermore, species diversity was quantified using the Shannon–Wiener diversity index (Shannon and Weaver, 1949), while species evenness was determined using Pielou’s evenness index (Pielou, 1975). Both indices were calculated following the formulas presented in equations (8) and (9)

$$H' = \sum_{i=1}^s (Pi) \ln(Pi) \quad (8)$$

The Shannon diversity index, denoted as H' , is calculated using P_i , which represents the proportion of the total population made up of species I , and S , the total number of species observed.

$$E = H' \ln S \quad (9)$$

H' represents the Shannon diversity index, while S denotes the total count of distinct species.

We tested the relationship between biodiversity and carbon storage using Pearson correlation. Biodiversity was represented by species richness (number of species) and the Shannon–Wiener index (H') for each sampling plot ($n=22$). Carbon storage was represented by carbon density (tC ha^{-1}). Correlation results are reported with r and p values.

The biomass of tree stems, branches, and leaves was calculated using the allometric equation (equation 10) for dry dipterocarp forests developed by Ogawa (Ogawa *et al.*, 1965). The carbon sequestration of individual species and the entire forest area was estimated based on the carbon proportion in woody biomass, which for dry dipterocarp forests is approximately 47% (IPCC, 2006).

$$\begin{aligned} W_s &= 0.0396 D^2 H^{0.9326} \\ W_b &= 0.003487 D^2 H^{1.0270} \\ W_l &= (28.0 / w_{tc} + 0.025)^{-1} \\ W_t &= W_s + W_b + W_l \end{aligned} \quad (10)$$

In these equations, D denotes the diameter at breast height (cm), and H represents the total tree height (m). The variables W_s , W_b , and W_l correspond to the dry weights of the stem, branches, and leaves, respectively, expressed in kilograms (kg).

The below-ground biomass (BGB) was estimated from above-ground biomass (AGB) using a root-to-shoot ratio (R) of 0.26, following the 2006 IPCC Guidelines for National Greenhouse Gas Inventories (IPCC, 2006). This ratio represents the default value for tropical dry forests as specified in Volume 4, Chapter 4 (Forest Land). Accordingly, BGB was computed as $\text{BGB} = \text{AGB} \times 0.26$, and the total biomass (TB) was obtained by summing AGB and BGB. The carbon stock (C) was then derived by multiplying TB by a carbon fraction of 0.47, consistent with the IPCC default values.

RESULTS AND DISCUSSION

Plant Species Survey

A total of 4,047 individual trees were recorded across the 320-ha study area, corresponding to an average density of approximately $12.65 \text{ trees ha}^{-1}$. This structure reflects a diverse and well-balanced forest community characteristic of dry dipterocarp ecosystems in Tat Ton National Park, where both dominant and

co-dominant species contribute to stand stability, ecological resilience, and the overall carbon accumulation potential of the forest.

Dominant species such as *Syzygium cumini*, *Pterocarpus macrocarpus*, and *Microcos paniculata* were widely distributed, suggesting strong adaptability to semi-arid soils and temperature fluctuations. In contrast, less abundant species tend to be ecological specialists that occupy narrower niches. Collectively, this structural and functional diversity enhances the forest's capacity for biomass accumulation, nutrient cycling, and carbon sequestration. The ten most abundant species are summarized in Table 1, representing the dominant taxa that shape the forest composition and maintain community balance.

Among the recorded species, *Syzygium cumini* (695 individuals) was clearly the most abundant, followed by *Pterocarpus macrocarpus* (304) and *Microcos paniculata* (269). Together, these three taxa represent nearly one-third of all trees recorded, confirming their ecological dominance and strong adaptability to the semi-arid conditions of the region. *Cratoxylum formosum* (169 individuals) and *Nephelium hypoleucum* (167) were also found frequently throughout the plots. Both species are well-known for their tolerance to dry, nutrient-poor soils and are common constituents of open deciduous formations, where they play important roles in canopy regeneration and understory shading.

The remaining five species—*Tectona grandis* (144), *Grewia eriocarpa* (139), *Peltophorum dasyrrhachis* (118), *Syzygium claviflorum* (97), and *Wrightia religiosa* (96)—were less abundant but still form integral parts of the forest community. These species contribute significantly to the heterogeneity and multi-layered structure of the stand, particularly by occupying different canopy levels and microhabitats. Their coexistence with dominant trees helps stabilize nutrient cycling and promotes species turnover, which are key ecological processes supporting long-term forest persistence.

Table 1. Dominant Tree Species and Number of Individuals in the Study Area

Rank	Species (1–5)	Number of Trees	Rank	Species (6–10)	Number of Trees
1	<i>Syzygium cumini</i> (L.) Skeels	695	6	<i>Tectona grandis</i> Linn.f.	144
2	<i>Pterocarpus macrocarpus</i> Kurz.	304	7	<i>Grewia eriocarpa</i> Juss.	139
3	<i>Microcos paniculata</i> L.	269	8	<i>Peltophorum dasyrrhachis</i> (Miq.) Kurz.	118
4	<i>Cratoxylum formosum</i> (Jacq.) Benth. & Hook.f. ex Dyer	169	9	<i>Syzygium claviflorum</i> (Roxb.) A.M.Cowan & Cowan	97
5	<i>Nephelium hypoleucum</i> Kurz.	167	10	<i>Wrightia religiosa</i> Benth.	96

Overall, the quantitative composition presented in Table 1 highlights that a small group of dominant species largely determines the forest structure, while a wide variety of less common taxa enriches species diversity and ensures

ecological balance. This coexistence of dominant and subordinate species reflects the natural dynamics of dry dipterocarp forests, where both environmental stress and species adaptation shape the composition and stability of the ecosystem.

Plant Species Diversity

In Table 2, the details of tree species recorded in Tat Ton National Park are presented, providing a comprehensive overview of the various families, scientific names, total numbers of trees, and mean densities for each species. An examination of these data reveals clear patterns and notable variations, offering valuable insights into the forest composition and the structural distribution of trees in terms of abundance and density. The dominance of several species such as *Syzygium cumini*, *Pterocarpus macrocarpus*, and *Microcos paniculata* highlights their adaptability to local environmental conditions, particularly the dry dipterocarp forest type. Meanwhile, the presence of numerous species with lower densities reflects a diverse and balanced ecosystem, indicating stable regeneration processes and ecological resilience within the park's forest community.

Table 2. Tree Species Composition, Number of Individuals, and Density

Family Name	Scientific Name	Number of Trees	Density (tree ha ⁻¹)
ANACARDIACEAE	<i>Anacardium occidentale</i> L.	1	0.003
	<i>Buchanania lanzan</i> Spreng.	8	0.026
	<i>Gluta usitata</i> (Wall.) Ding Hou.	2	0.007
	<i>Lannea coromandelica</i> (Houtt.) Merr.	92	0.304
	<i>Mangifera caloneura</i> Kurz.	10	0.033
	<i>Spondias pinnata</i> (L.f.) Kurz	1	0.003
ANNONACEAE	<i>Diospyros ehretioides</i> Wall. ex G.Don	21	0.069
	<i>Goniotalamus laoticus</i> (Finet & Gagnep.) Ban	4	0.013
	<i>Melodorum fruticosum</i> Lour.	15	0.050
APOCYNACEAE	<i>Holarrhena pubescens</i> Wall. ex G.Don	10	0.033
	<i>Wrightia pubescens</i> R.Br.	99	0.327
BIGNONIACEAE	<i>Dolichandrone serrulata</i> (Wall. ex DC.) Seem.	8	0.026
	<i>Fernandoa adenophylla</i> (Wall. ex G.Don) Steenis	46	0.152
	<i>Millingtonia hortensis</i> L.f.	6	0.020
	<i>Stereospermum neuranthum</i> Kurz.	2	0.007
BURSERACEAE	<i>Canarium subulatum</i> Guill.	5	0.017
CELASTRACEAE	<i>Salacia chinensis</i> L.	8	0.026
CHRYSOBALANACEAE	<i>Parinari anamense</i> Hance	9	0.030
COMBRETACEAE	<i>Terminalia bellirica</i> (Gaertn.) Roxb.	1	0.003
	<i>Terminalia corticosa</i> Pierre ex Laness.	5	0.017
CONNARACEAE	<i>Ellipanthus tomentosus</i> Kurz.	60	0.198
CRYPTERONIACEAE	<i>Crypteronia paniculata</i> Blume	9	0.030

Family Name	Scientific Name	Number of Trees	Density (tree ha ⁻¹)
DILLENIACEAE	<i>Dillenia ovata</i> Wall. ex Hook.f. & Thomson	11	0.036
DIPTEROCARPACEAE	<i>Dipterocarpus alatus</i> Roxb.	12	0.040
	<i>D. intricatus</i>	1	0.003
	<i>D. obtusifolius</i> Teijsm. ex Miq.	18	0.060
	<i>Shorea obtusa</i> Wall. ex Blume	2	0.007
	<i>Shorea roxburghii</i> G.Don	9	0.030
	<i>Shorea siamensis</i> Miq.	2	0.007
	<i>Vatica harmamdiana</i> Pierre	4	0.013
	<i>Diospyros ferrea</i> (Willd.) Bak.	6	0.020
EBENACEAE	<i>D. glandulosa</i> Lace.	2	0.007
EUPHORBIACEAE	<i>Aporosa villosa</i> Lindl. Baill.	120	0.397
	<i>Croton oblongifolius</i> Roxb.	3	0.010
	<i>Suregada multiflorum</i> (A.Juss.) Baill.	27	0.089
FABACEAE	<i>Acacia catechu</i> (L.f.) Willd.	19	0.063
	<i>Afzelia xylocarpa</i> (Kurz) Craib	18	0.060
	<i>Albizia lucidior</i> (Steud.) I.C.Nielsen	16	0.053
	<i>Cassia bakeriana</i> Craib	68	0.225
	<i>Dalbergia cochinchinensis</i> Pierre	43	0.142
	<i>Dalbergia oliveri</i> Prain	12	0.040
	<i>Peltophorum dasyrrhachis</i> (Miq.) Kurz.	124	0.410
	<i>Pterocarpus macrocarpus</i> Kurz	304	1.015
	<i>Senna siamea</i> (Lam.) H.S.Irwin & Barneby	91	0.301
	<i>Sindora siamensis</i> Teijsm. ex Miq.	88	0.291
	<i>Castanopsis cerebrina</i> (Hickel & A.Camus) Barnett	24	0.079
FAGACEAE	<i>Cratoxylum formosum</i> (Jacq.) Benth. & Hook.f. ex Dyer	189	0.625
IRVINGIACEAE	<i>Irvingia malayana</i> Oliv. ex A.W.Benn.	106	0.350
LAURACEAE	<i>Beilschmiedia roxburghiana</i> Nees	39	0.129
LYTHRACEAE	<i>Lagerstroemia floribunda</i> Jack	25	0.083
MORACEAE	<i>Ficus hispida</i> L.f.	126	0.417
MYRTACEAE	<i>Syzygium cumini</i> (L.) Skeels	695	2.261
SAPINDACEAE	<i>Nephelium hypoleucum</i> Kurz	234	0.774
TILIACEAE	<i>Grewia eriocarpa</i> Juss.	142	0.469
	<i>Microcos paniculata</i> L.	269	0.906
VERBENACEAE	<i>Tectona grandis</i> Linn.f.	144	0.476
Total		4,047	12.650

As shown in Table 2, the tree community in Tat Ton National Park includes a wide range of species and families, reflecting the structural complexity of both dry evergreen forest (DEF) and dry dipterocarp forest (DDF). The distribution of species among families indicates that several taxa contribute strongly to the overall stand structure. Species such as *Syzygium cumini*,

Pterocarpus macrocarpus, and *Microcos paniculata* were among the most abundant, confirming their importance in the forest community.

Among the recorded families, Myrtaceae, Fabaceae, and Tiliaceae were well represented. *Syzygium cumini* showed the highest number of individuals and density, indicating its ecological dominance. Several Fabaceae species, including *Pterocarpus macrocarpus* and *Peltophorum dasyrrhachis*, also occurred in relatively high numbers. Other families, such as Irvingiaceae, Euphorbiaceae, and Moraceae, were less abundant but still contributed to species diversity and forest structure. In addition, dipterocarp species were present at lower densities, representing characteristic elements of this forest type.

A total of 4,047 individual trees were recorded across the 320-ha study area, with an average density of 12.65 trees ha⁻¹. The coexistence of DEF and DDF creates a structurally diverse landscape with varying canopy conditions and species composition. The data presented in Table 2 provide the basis for evaluating species importance using the Importance Value Index (IVI), which is discussed in the following section.

To further examine species dominance, the Importance Value Index (IVI) was calculated by combining relative density, relative frequency, and relative dominance. This index reflects the overall contribution of each species to the forest structure. Higher IVI values indicate more dominant species, while lower values represent less abundant taxa. The calculated IVI values are summarized in Table 3.

Table 3 presents the calculated Importance Value Index (IVI) for all tree species recorded in Tat Ton National Park, derived from relative density (RD), relative dominance (RDo), and relative frequency (RF). The IVI serves as a composite measure reflecting the ecological significance and structural contribution of each species within the forest community. The results reveal distinct patterns of dominance and coexistence among species typical of a dry dipterocarp forest ecosystem.

The analysis indicates that *Syzygium gratum* had the highest IVI (53.951), attributed primarily to its exceptionally high relative dominance (RDo=53.697). This suggests that although the species occurs in low numbers, it consists of large, mature individuals with substantial basal area, making it a key structural component in the canopy layer. In contrast, *Syzygium cumini* recorded the second highest IVI (20.385), driven mainly by its very high relative density (RD=16.901) and frequency (RF=3.484), confirming it as the most abundant and widely distributed species in the study plot. Together, these two *Syzygium* species define the upper canopy dynamics and play a crucial role in maintaining carbon storage and canopy closure.

Following them, *Microcos paniculata* (IVI=16.823) and *Pterocarpus macrocarpus* (IVI=12.837) showed balanced contributions across RD, RDo, and RF, suggesting they are structurally important and well-adapted to local soil and microclimatic conditions. *Dalbergia oliveri* (IVI=10.205) had a relatively moderate density but extremely high RDo (9.294), indicating a few large-

diameter individuals dominating the basal area. Other co-dominant species such as *Nephelium hypoleucum* (IVI=8.651), *Irvingia malayana* (IVI=8.178), and *Cratoxylum formosum* (IVI=7.745) also contributed substantially to the stand's biomass and vertical structure, reflecting their ecological adaptability to dry and nutrient-poor soils.

Species including *Grewia eriocarpa* (IVI=7.170) and *Tectona grandis* (IVI=6.005) exhibited moderate IVI values, representing co-dominant taxa that help maintain canopy heterogeneity and support successional regeneration. The coexistence of dominant, co-dominant, and subordinate species across varying IVI ranges illustrates a balanced forest composition that enhances ecosystem resilience and stability.

Table 3. Importance Value Index (IVI) of Tree Species Recorded

Scientific Name	RD (%)	RDo (%)	RF (%)	IVI
<i>Anacardium occidentale</i> L.	0.025	1.636	0.205	1.865
<i>Buchanania lanzan</i> Spreng.	0.198	0.000	0.615	0.813
<i>Gluta usitata</i> (Wall.) Ding Hou.	0.049	0.064	0.205	0.318
<i>Lannea coromandelica</i> (Houtt.) Merr.	2.273	0.017	2.869	5.159
<i>Mangifera caloneura</i> Kurz.	0.247	0.000	1.025	1.272
<i>Spondias pinnata</i> (L.f.) Kurz	0.025	0.003	0.205	0.232
<i>Diospyros ehretioides</i> Wall. ex G.Don	0.519	0.002	0.615	1.135
<i>Goniothalamus laoticus</i> (Finet & Gagnep.) Ban	0.099	0.015	0.205	0.319
<i>Melodorum fruticosum</i> Lour.	0.371	0.116	0.820	1.306
<i>Holarrhena pubescens</i> Wall. ex G.Don	0.247	0.922	1.025	2.194
<i>Wrightia pubescens</i> R.Br.	2.446	0.025	3.484	5.955
<i>Fernandoa adenophylla</i> (Wall. ex G.Don) Steenis	1.137	0.011	0.820	1.967
<i>Ellipanthus tomentosus</i> Kurz.	1.483	0.429	2.459	4.370
<i>Dipterocarpus obtusifolius</i> Teijsm. ex Miq.	0.445	0.001	0.820	1.265
<i>Aporosa villosa</i> Lindl. Baill.	2.965	0.125	2.664	5.755
<i>Suregada multiflorum</i> (A.Juss.) Baill.	0.667	1.408	1.434	3.510
<i>Azelia xylocarpa</i> (Kurz) Craib	0.445	1.478	0.820	2.743
<i>Peltophorum dasyrrhachis</i> (Miq.) Kurz.	3.064	0.008	2.459	5.531
<i>Pterocarpus macrocarpus</i> Kurz	7.586	0.948	4.303	12.837
<i>Cratoxylum formosum</i> (Jacq.) Benth. & Hook.f. ex Dyer	4.670	0.001	3.074	7.745
<i>Irvingia malayana</i> Oliv. ex A.W.Benn.	2.619	2.280	3.279	8.178
<i>Ficus hispida</i> L.f.	3.113	0.040	2.664	5.817
<i>Syzygium cumini</i> (L.) Skeels	16.901	0.000	3.484	20.385
<i>Syzygium gratum</i> (Wight) S.N.Mitra var. <i>gratum</i>	0.049	53.697	0.205	53.951
<i>Nephelium hypoleucum</i> Kurz	5.782	0.000	2.869	8.651
<i>Grewia eriocarpa</i> Juss.	3.509	2.022	1.639	7.170
<i>Microcos paniculata</i> L.	6.770	5.749	4.303	16.823
<i>Tectona grandis</i> Linn.f.	3.558	1.217	1.230	6.005

Figure 2 graphically summarizes these results, highlighting the Top 10 species by IVI. The bar chart clearly visualizes the sharp dominance gap between *Syzygium gratum* and the rest of the species, followed by a gradual decline among co-dominant taxa. This pattern typifies the “uneven dominance structure” characteristic of mature dry dipterocarp forests, where a small number of species exert strong structural control while numerous others contribute to ecological diversity and regeneration potential. Overall, the IVI distribution underscores that Tat Ton National Park supports a floristically diverse yet structurally hierarchical forest community, which underpins its high ecological stability, long-term productivity, and significant capacity for carbon sequestration.

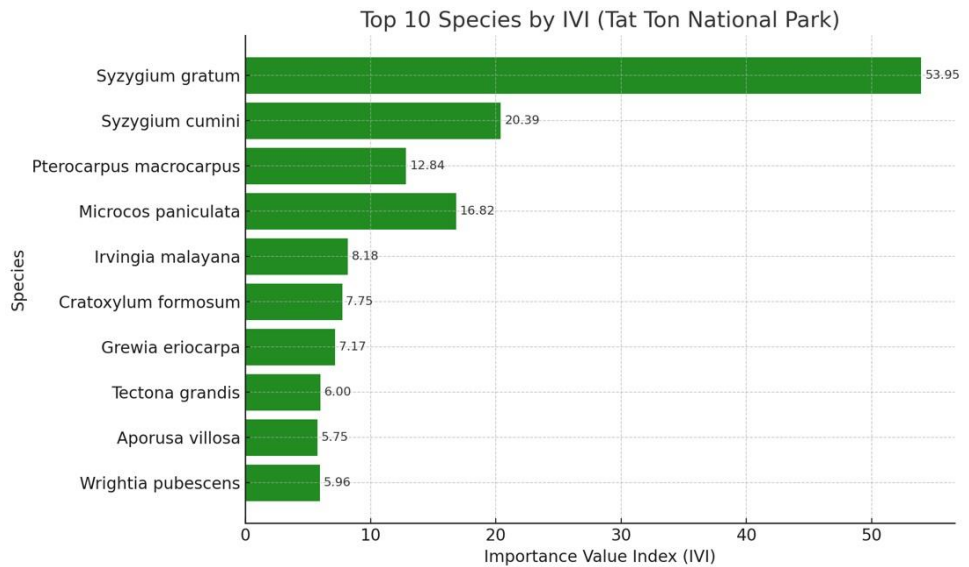


Figure 2. Top 10 species by IVI

Biomass and Carbon Stock

To summarize the overall findings from the field plots, the aboveground and belowground biomass as well as the corresponding carbon stock values from main plot 1–6 were aggregated to represent the total biomass and carbon sequestration potential within the study area. This integrated analysis provides a comprehensive overview of carbon distribution between above- and belowground components, allowing for an understanding of how different forest structures contribute to total carbon storage. The results demonstrate clear variations in carbon accumulation among plots due to differences in stand density, canopy coverage, and species composition. The combined biomass and carbon stock values for plots 1–6 are presented in Table 4.

Table 4 presents the total above- and below-ground carbon stock across six major forest plots covering 320 ha in the study area. Each record includes forest type, area, number of samples, and measured carbon content of above-ground

biomass (AGB) and below-ground biomass (BGB), expressed in metric tons of carbon (tC). The calculations follow the IPCC (2006) methodology, where biomass was converted to carbon using a factor of 0.47, and BGB was estimated from AGB using a root-to-shoot ratio consistent with tropical dry forests.

Table 4. Total Aboveground and Belowground Biomass and Carbon Stock

Main Plot	Forest Type	Area (ha)	No. of Samples	AGB (tC)	BGB (tC)	Total (tC)	Average (tC/ha)
1	Dry evergreen and dry dipterocarp forest	35.20	3	2,018.88	545.10	2,563.98	72.82
2	Dry dipterocarp forest	62.40	4	4,197.23	1,133.25	5,260.41	84.30
3	Dry dipterocarp forest	62.40	4	3,805.16	1,027.39	4,832.55	77.37
4	Dry dipterocarp forest	62.40	4	2,935.82	792.67	3,728.49	59.72
5	Dry dipterocarp forest	32.00	2	2,777.64	749.96	3,527.61	110.24
6	Dry dipterocarp forest	64.00	5	4,399.40	1,187.84	5,587.24	87.30
Total Project	—	320.00	22	20,042.50	5,411.48	25,383.90	79.95

Overall, the 320 ha study area stores 25,383.90 tC, with an average of 79.95 tC ha⁻¹. Of this total, 20,042.50 tC occurs above ground and 5,411.48 tC below ground, giving an AGB:BGB ratio of about 79:21. All carbon values in this study are reported in metric tons of carbon (tC). When converted to CO₂ equivalents using the factor 44/12, the total is approximately 93,000 tCO₂e (about 291 tCO₂e ha⁻¹). These results indicate a high carbon storage potential in the forest ecosystem. Carbon density showed a positive relationship with species richness across the sampling plots ($r=0.77$, $p<0.001$, $n=22$). However, the relationship between carbon density and the Shannon diversity index (H') was weak and not statistically significant ($r=0.29$, $p=0.18$, $n=22$).

Carbon density varies among the six main plots, ranging from 59.72 to 110.24 tC ha⁻¹, mainly due to differences in stand structure, canopy closure, and species composition. Plot 5 (32 ha) exhibits the highest carbon density (110.24 tC ha⁻¹) despite its smaller area, suggesting a mature canopy structure and dominance of high wood-density species, representing a high-carbon patch that should be prioritized for long-term conservation and monitoring. Plot 6 (64 ha) has the highest total carbon (5,587.24 tC) and accounts for about 22% of the project's total, making it the principal contributor to overall carbon sequestration; maintaining its ecological stability through fire prevention and minimizing disturbance is therefore essential. Plots 2 and 3 (62.4 ha each) contain moderate-to-high carbon densities (84.30 and 77.37 tC ha⁻¹, respectively), jointly contributing nearly 40% of the total carbon stock and representing stable, well-developed stands. Plot 1 (35.2 ha), representing a transitional zone between dry evergreen and dry dipterocarp forests, shows 72.82 tC ha⁻¹, slightly below the project mean, indicating a moderately developed canopy that could increase carbon storage through selective enrichment. In contrast, Plot 4 (62.4 ha) has the

lowest mean (59.72 tC ha⁻¹), likely influenced by recurring fires or previous disturbances, suggesting that targeted restoration measures such as fuel-load reduction and gap enrichment planting would enhance biomass recovery.

From a sustainable forest management (SFM) perspective, these results highlight three functional zones within the study area: high-carbon hotspots such as Plot 5 that should be strictly protected to ensure long-term carbon stability; key leverage areas such as Plot 6 that determine the majority of project-scale carbon sequestration and thus require careful maintenance of ecological integrity; and restoration zones such as Plot 4, where ecological enhancement can generate substantial additional carbon benefits over time.

The carbon density observed in this study (59.72–110.24 tC ha⁻¹, mean 79.95 tC ha⁻¹) falls within the range reported for tropical dry forest ecosystems. Previous studies in Thailand have reported carbon densities of approximately 60–78 tC ha⁻¹ for dry dipterocarp and dry evergreen forests, indicating comparable biomass accumulation patterns under similar climatic and soil conditions. In some cases, higher values exceeding 150 tC ha⁻¹ have been observed in mature stands with greater basal area and stand age. These comparisons suggest that the forest ecosystems in Tat Ton National Park represent moderately developed carbon stocks typical of regional dry forest systems.

The positive relationship between carbon density and species richness found in this study is consistent with several studies indicating that higher tree diversity can enhance biomass accumulation through complementary resource use and structural complexity. However, the weak relationship observed with the Shannon diversity index also agrees with previous findings showing that biodiversity–carbon relationships may vary depending on forest structure, environmental conditions, and disturbance history. Some studies suggest that structural attributes such as basal area and canopy architecture may play a stronger role in determining carbon storage than species diversity alone.

Overall, the results confirm that mixed dry evergreen and dry dipterocarp forests can function as important carbon sinks while maintaining relatively high biodiversity, supporting the concept that conservation of heterogeneous forest landscapes contributes simultaneously to climate mitigation and ecosystem stability.

CONCLUSIONS

This study provides an assessment of forest ecosystems in Tat Ton National Park, focusing on plant diversity, above- and below-ground biomass, and carbon sequestration across two main forest types. A total of six main plots and twenty-two subplots recorded 142 species and 4,047 individual trees, indicating a highly diverse dry forest ecosystem. The combined total biomass and carbon stock reached 25,383.90 tC (≈93,000 tCO₂e), highlighting the important role of these forests as long-term carbon sinks.

The results underscore that biodiversity and carbon storage are mutually reinforcing components of sustainable forest ecosystems. Forests with greater

species diversity exhibited more stable biomass accumulation and higher carbon retention, confirming their essential role in climate-change mitigation and ecosystem resilience. These findings reinforce the importance of protecting and restoring mixed dry forest landscapes as a nature-based approach to enhance Thailand's carbon balance.

From a broader perspective, this assessment contributes valuable baseline data for carbon-credit development and sustainable forest management under the national Net Zero framework. By quantifying both ecological diversity and carbon sequestration potential, the study aligns with the principles of the Green Economy, offering scientific evidence to support the integration of biodiversity conservation into carbon market mechanisms such as T-VER and VERRA.

In conclusion, the forests of Tat Ton National Park not only preserve ecological integrity but also provide measurable climate benefits. Promoting their protection and sustainable utilization will enhance both environmental sustainability and socio-economic value, advancing Thailand's transition toward a low-carbon, Net Zero future. Moreover, integrating community-based forest monitoring, local participation, and continuous satellite-based carbon assessment could ensure long-term transparency and accountability in carbon credit projects. This holistic approach will bridge science, policy, and practice, turning Tat Ton National Park into a living model of sustainable forest governance and contributing to the achievement of the UN Sustainable Development Goals (SDGs), particularly Goals 13 (Climate Action) and 15 (Life on Land).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SPATIAL DISTRIBUTION OF COPPER IN THE SOILS UNDER ORCHARDS IN THE KUMANOVO REGION

SUMMARY

A total of 22 soil samples from 11 locations under fruit orchards in the Kumanovo region were analyzed to determine total copper content. Samples were collected at depth of 0 - 30 cm and 30 - 60 cm, corresponding the main root zone of fruit trees. The orchards are in their productive phase and regularly treated with copper-containing pesticides, as well as mineral and organic fertilizers. The study aimed to assess whether copper accumulation originates from anthropogenic activities or lithological composition. To interpret copper distribution, soil texture and chemical properties (pH, carbonate content, humus, electrical conductivity, total nitrogen, available P₂O₅ and K₂O) were analyzed. Results show heterogeneous soil texture, with physical sand fractions dominating the topsoil and physical clay fractions prevailing at deeper layers. Copper concentrations were consistently higher in the topsoil (average 45.33 mg/kg, median 41.6 mg/kg) compared to the subsoil (average 36.31 mg/kg, median 37.7 mg/kg), indicating anthropogenic accumulation. Statistical analyses (correlation, factor analysis, PCA, and cluster analysis) revealed strong associations between copper and clay fractions, phosphorus, humus, and nitrogen, while negative correlations were observed with sand fractions and soil pH. PCA Biplots confirmed that copper distribution is primarily controlled by anthropogenic inputs, with soil texture and chemical properties modulating its bioavailability. Although copper levels remain below the contamination threshold of 100 mg/kg, they exceed WHO's recommended limit (20-36 mg/kg) in several orchards. These findings highlight the need for careful management of copper-containing agrochemicals to ensure sustainable fruit production and protect soil health.

Keywords: copper (Cu), soil texture, chemical properties, orchards, copper distribution maps, PCA, cluster analysis.

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INTRODUCTION

Copper (Cu) is an essential micronutrient for plants, animals, and humans, playing a crucial role in numerous physiological and biochemical processes, including enzymatic reactions, photosynthesis, and oxidative stress regulation. However, its accumulation in agricultural soils has become an increasing environmental concern, particularly in areas subjected to long-term intensive farming practices. In orchard systems, elevated copper concentrations are most commonly associated with prolonged and repeated application of copper-based fungicides and mineral fertilizers, which represent the dominant anthropogenic sources of Cu in cultivated soils (Komárek *et al.*, 2021; Markoski *et al.*, 2025; Kos *et al.* 2026).

Modern fruit production relies on intensive management practices, including the use of high-yield cultivars, mechanized soil operations, irrigation, and frequent application of plant protection products. Due to its broad-spectrum antimicrobial activity, copper is widely applied in fruit orchards in various formulations, such as Bordeaux mixture, copper hydroxide, and copper oxychloride, to control fungal and bacterial diseases (La Torre *et al.*, 2018; Lamichhane *et al.*, 2018). Although copper-containing agrochemicals are considered effective and relatively persistent, their repeated use often results in gradual Cu accumulation in the topsoil, particularly in perennial cropping systems such as orchards and vineyards (Komárek *et al.*, 2021; Ballabio *et al.*, 2018).

The behavior, mobility, and bioavailability of copper in soils are strongly controlled by a range of physicochemical properties. Soil pH is one of the most influential factors, as copper solubility generally decreases with increasing pH due to enhanced adsorption and precipitation processes (Kabata-Pendias, 2011; Chuancheng *et al.*, 2020). Organic matter plays a dual role by forming stable organo-metal complexes that reduce Cu mobility while simultaneously increasing its retention in surface horizons (Jeon *et al.*, 2024; Hou *et al.*, 2020). Soil texture, particularly the content of fine fractions such as clay, significantly affects copper accumulation due to the high specific surface area and abundance of reactive adsorption sites. In addition, cation exchange capacity (CEC) is closely linked to Cu retention, as soils with higher CEC exhibit a greater capacity to bind copper ions. The presence of carbonates further influences copper behavior by promoting immobilization through co-precipitation and surface complexation mechanisms, especially in calcareous soils (Wang *et al.*, 2009).

Excessive copper accumulation in soils may lead to phytotoxic effects, altered microbial activity, and increased transfer of Cu along the soil-plant-human continuum. While copper deficiency in fruit trees can impair pollen development, reduce fertilization efficiency, and lower yields, excessive copper concentrations may inhibit root growth, disrupt nutrient uptake, and result in elevated Cu levels in edible plant parts (Yruela, 2021; Adrees *et al.*, 2015). Furthermore, long-term copper inputs may negatively affect soil microbial diversity and enzymatic activity, thereby reducing soil biological fertility (Fagnani, *et al.*, 2020).

In recent decades, multivariate statistical techniques have become indispensable tools in pedological and ecotoxicological studies aimed at disentangling the complex interactions between soil properties and trace elements. Among them, Principal Component Analysis (PCA) is widely used to identify dominant factors controlling metal distribution, distinguish between natural (lithogenic) and anthropogenic sources, and reduce data dimensionality while preserving the main variance structure (Reimann *et al.*, 2018; Filzmoser *et al.*, 2018). When combined with correlation analysis, factor analysis, and cluster analysis, PCA provides a robust framework for interpreting spatial variability of trace metals in soils and for supporting environmentally sound land management decisions. Despite numerous studies addressing copper accumulation in orchard soils and intensively cultivated regions, quantitative assessments of the relative influence of agricultural practices and intrinsic soil properties on copper distribution in orchard soils of Southeast Europe remain limited. This lack of region-specific evidence constrains the development of targeted soil management strategies and risk assessment frameworks under conditions of increasing agricultural intensification and climate variability. The Kumanovo region, located in the northern part of North Macedonia, represents one of the important agricultural areas characterized by intensive crop production and diversified soil types developed on heterogeneous geological substrates. The region has a long tradition of fruit cultivation, where plant protection practices, including the application of copper-based fungicides, are commonly used to control fungal and bacterial diseases. In addition, the area is characterized by pronounced spatial variability of soil properties, including texture, organic matter content, and carbonate presence, which may significantly influence the mobility and accumulation of trace elements. These characteristics make the Kumanovo region a suitable case study for investigating the spatial distribution and controlling factors of copper in orchard soils under conditions typical for Southeast European agricultural landscapes.

The hypothesis of this study is that the spatial distribution of copper in orchard soils is primarily controlled by soil physicochemical properties—particularly organic matter content, texture, and cation exchange capacity—while anthropogenic inputs from plant protection practices act as an additional, but spatially heterogeneous, enrichment factor. To test this hypothesis, the present study investigates the spatial distribution of copper in orchard soils of the Kumanovo region by integrating soil physicochemical analyses with multivariate statistical methods. The results are expected to improve the understanding of copper dynamics in orchard ecosystems and to support evidence-based soil management practices consistent with the objectives of sustainable agriculture and the EU Soil Strategy for 2030 (European Commission, 2021).

MATERIAL AND METHODS

Study area

The Kumanovo region is located in the northeastern part of the Republic of North Macedonia and covers an area of 1293.81 km², representing approximately 4.71% of the national territory. The investigated fruit orchards are distributed

across the central and northern parts of the region, as presented in Table 1, while the spatial position of the study area is shown in Figure 1.

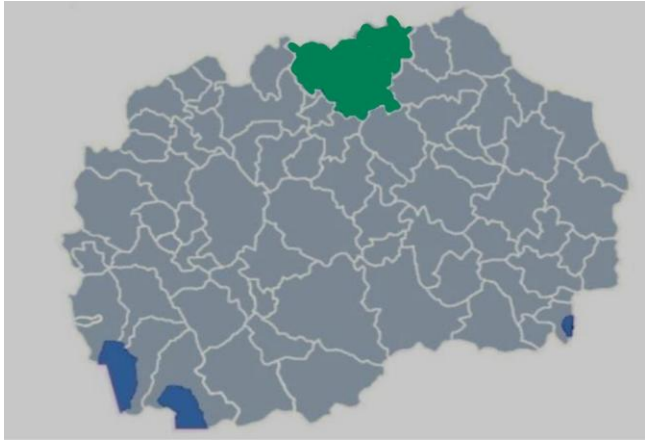


Figure 1. Spatial distribution of the Kumanovo region-North Macedonia

From a pedogenetic perspective, the region is characterized by a heterogeneous landscape composed of undulating hills, plains, and slope terrains formed on diverse geological substrates, including metamorphic, magmatic, and sedimentary rocks. Quaternary deposits of lacustrine, deluvial, and alluvial origin are widespread and contribute to considerable variability in soil texture and chemical properties.

The climate of the region is classified as sub-Mediterranean–continental, with a moderately characteristics and local influence of Mediterranean air masses along the Pčinja River valley, while more continental conditions prevail in elevated areas (Markoski *et al.*, 2023).

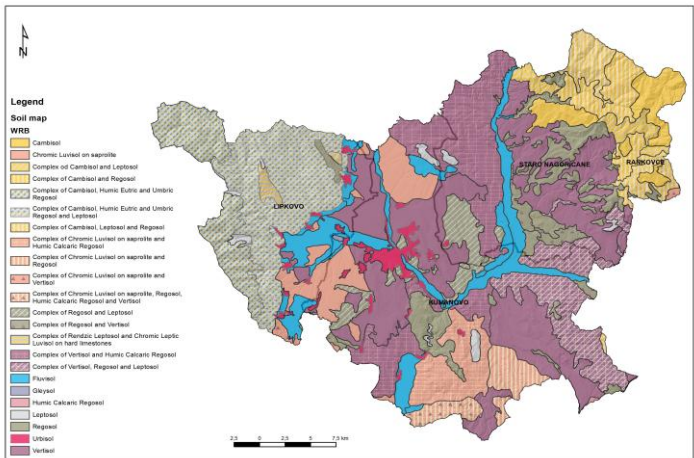


Figure 2. Pedological map of the Kumanovo region

Within the framework of the regional pedological assessment, the spatial distribution and taxonomic classification of soils in the Kumanovo region are presented in the pedological map (Fig. 2). The dominant soil types in the region include Leptosols, Regosols, Cambisols, Vertisols, Fluvisols, and Luvisols, developed on limestone, clays, and heterogeneous Quaternary deposits. Soils under the investigated orchards are predominantly classified as Aric Regosols, characterized by a developed anthropogenic plough (P) horizon formed as a result of long-term cultivation and trenching practices.

Field, laboratory and data processing workflow

The study was conducted through three main phases: field sampling, laboratory analyses, and data processing.

During the field phase, a total of 22 soil samples were collected from 11 fruit orchard locations in the Kumanovo region (Fig. 1; Table 1). At each location, soil samples were taken from two depths: 0-30 cm and 30-60 cm, corresponding to the main root zone of fruit trees. The orchards are in their productive phase and are regularly treated with pesticides and copper-based fertilizers as part of standard fruit protection and fertilization practices.

Table 1. Geographical location of fruit orchards

N ^o	Location	Land ha	Type of plantation	Plantation age (years)	GPS Plantation coordinates	Preceding crop
1	Mlado Nagorichane	6	Nuts	10	42°10'NL 21°48'EL	Vineyard
2	Klechovce	0.6	Apples	>20	42°06'NL 21°51'EL	Orchard
3	Staro Nagorichane	1.3	Pears	6	42°11'NL 21°49'EL	Field
4	Kokoshinje	4	Apricots	>20	42°00'NL 21°55'EL	Field
5	Gabresh	0.9	Hazelnuts	7	42°01'NL 21°50'EL	Pasture
6	Rechica	0.7	Peaches	11	42°10'NL 21°41'EL	Meadow
7	Vaksince	2	Nuts	10	42°12'NL 21°39'EL	Pasture
8	Cherkeze	2	Hazelnuts	8	42°07'NL 21°40'EL	Field
9	Cherkeze	2	Nuts	10	42°07'NL 21°40'EL	Field
10	Chelopek	2	Plums	13	42°23'NL 21°28'EL	Field
11	Tabanovce	2	Apricots	10	42°12'NL 21°41'EL	Meadow

Field sampling was carried out in accordance with the national soil survey methodology (Mitrikeski and Mitkova, 2013) and the requirements of ISO/IEC 17025:2006. 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 and the procedures described in MKS ISO 11464:2015.

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.

Laboratory analyses

Laboratory analyses included the determination of selected physical and chemical properties relevant to copper behavior and mobility. Soil texture was determined using standard sedimentation methods. Soil reaction (pH) was measured according to ISO 10390:2022, calcium carbonate (CaCO_3) equivalent volumetrically (ISO 10693), humus content by standard wet oxidation procedures, total nitrogen according to ISO 11261:199 and available phosphorus (P_2O_5) and potassium (K_2O) (ammonium lactate method).

All analyses were performed using accredited and standardized procedures in the Laboratory of Soils and Fertilizers (L-04), Faculty of Agricultural Sciences and Food, which operates under ISO/IEC 17025 accreditation.

Determination of total copper content

Total copper (Cu) content in soil samples was determined following aqua regia digestion. Soil samples were air-dried at room temperature for approximately two weeks, crushed, and sieved through a 2 mm mesh. A representative subsample was further ground to a particle size below 0.125 mm to ensure homogeneity.

Copper extraction was carried out using aqua regia in accordance with ISO 11466:1995. The concentration of copper in the digested solutions was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Agilent 715, USA) in the accredited laboratory of RZ "Technical Control" - AD Skopje. Calibration standards were prepared by serial dilution from a certified stock solution with a concentration of 1000 mg/L.

Statistical data analysis

Statistical analysis of the data was performed to evaluate the distribution, variability, and controlling factors of copper in the studied soils. Descriptive statistical parameters, including minimum, maximum, mean, median, and standard deviation, were calculated for all analyzed soil properties.

Correlation analysis was applied to assess relationships between copper concentrations and selected physical and chemical soil parameters. Multivariate statistical techniques, including factor analysis and Principal Component Analysis (PCA), were used to identify dominant factors controlling copper distribution and to distinguish between anthropogenic and lithogenic influences. Cluster analysis was applied to group soil samples based on similarities in copper content and soil properties.

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). Spatial distribution maps of copper concentrations were generated using inverse distance weighting (IDW) interpolation in ArcGIS version 10.8. Separate maps were produced for the 0-30 cm and 30-60 cm soil layers.

RESULTS AND DISCUSSION

According to the obtained data, the soils 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.

From the analyzed soil samples (Table 2 and 3), it can be seen that at the first depth (0-30 cm), the soil is predominantly composed of the physical sand fraction (coarse sand + fine sand), with an average value of 50.68%, compared to the physical clay fraction (clay + silt), which averages 49.32%. The median for the total sand fraction is 54.00%. In the physical clay fraction, the clay fraction with the highest average value is 32.42%, and ranges from 13.80 to 59.40% in relation to the silt fraction, which averages 16.90%. The median for the clay fraction is 32.90% higher than the median for the silt fraction 16.90%.

At the second depth (30-60 cm), the physical clay fraction becomes dominant, with an average of 55.20%, while the sand fraction accounts for 44.80%. The median for the total sand fraction is 46.60%. In the physical clay fraction, the clay fraction with the highest average value is 31.58%, and ranges from 14.10 to 53.40% in relation to the silt fraction, which averages 23.58%. The median for the clay fraction is 29.10% higher than the median for the silt fraction 21.70%.

Based on the USDA soil texture classification triangle (Mitkova and Markoski, 2022), the analyzed soils are classified into the following textural classes: sandy clay loam (SCL), clay (C), clay loam (CL), sandy loam (SL), loam (L), and sandy clay (SC).

The results obtained from investigations of the Cu content and some chemical properties of the soil samples are presented in Table 2 and 3.

Copper (Cu) is an important micronutrient, essential for fruit crops, required in small quantities, crucial for proper growth and development. Depending on its concentration, copper can act as a necessary micronutrient, but it can also become toxic to fruit crops and lead to reduced yields. On the one hand, its deficiency in organisms leads to various disorders (Xu *et al* 2024; Sharma and Sehkon, 2009), while on the other hand, in higher concentrations it causes harmful effects.

Table 2. Descriptive analysis of soil parameters in samples (N=11) at a depth of 0-30 cm

Parameter	Measurement values	Min	Max	H	SD	Median
Coarse sand	[%]	5.42	28.12	16.03	7.18	14.40
Fine sand	[%]	16.95	51.48	34.65	10.20	31.64
Physical sand	[%]	23.70	71.30	50.68	12.91	54.00
Silt	[%]	2.30	27.90	16.90	6.50	16.90
Clay	[%]	13.80	59.40	32.42	11.83	32.90
Physical clay	[%]	28.70	76.30	49.32	12.91	46.00
Cu	[mg/kg]	31.1	69.9	45.33	12.09	41.6
Humus	[%]	1.90	2.75	2.29	0.28	2.18
Total N	[%]	0.114	0.165	0.137	0.031	0.166
pH in H ₂ O	/	7.31	8.61	8.05	0.50	8.21
EC	[dS/m ⁻¹]	6.28	19.35	10.54	3.24	10.41
CaCO ₃	[%]	0.00	19.99	11.51	7.93	15.02
P ₂ O ₅	[mg/100 g]	13.87	91.29	36.78	21.11	37.63
K ₂ O	[mg/100 g]	30.44	77.90	50.34	13.51	52.06

Table 3. Descriptive analysis of soil parameters in samples (N=11) at a depth of 30-60 cm

Parameter	Measurement values	Min	Max	H	SD	Median
Coarse sand	[%]	4.34	26.80	13.10	7.62	13.37
Fine sand	[%]	7.24	47.05	31.70	10.81	33.23
Physical sand	[%]	13.50	65.80	44.80	15.50	46.60
Silt	[%]	9.90	39.90	23.58	8.84	21.70
Clay	[%]	14.10	53.40	31.58	12.09	29.10
Physical clay	[%]	34.20	86.50	55.20	15.50	53.40
Cu	[mg/kg]	26.3	49.3	36.31	6.44	37.7
Humus	[%]	1.10	2.01	1.42	0.29	1.37
Total N	[%]	0.060	0.121	0.085	0.017	0.082
pH in H ₂ O	/	7.22	8.57	8.07	0.49	8.32
EC	[dS/m ⁻¹]	7.14	24.05	11.19	4.949	9.31
CaCO ₃	[%]	0.00	20.41	10.86	7.58	14.16
P ₂ O ₅	[mg/100 g]	7.95	55.37	31.41	16.31	32.30
K ₂ O	[mg/100 g]	23.43	77.30	42.76	13.92	41.85

Copper content in soils is influenced by complex interactions between the mineralogical composition of the parent material, soil texture, chemical

properties, and potential anthropogenic inputs such as the use of mineral and organic fertilizers and copper-containing pesticides.

(Chen *et al.*, 2024), in their book on copper in soils, state in Chapter 5 that copper (Cu) is the 25th most abundant component of the Earth's crust and represents an essential microelement for the growth and development of plants, animals, and humans. However, excessive concentrations of Cu in soils are potentially hazardous and may be highly toxic to the microflora, flora, fauna, and humans (Nannipieri *et al.*, 2003).

In the lithosphere, copper concentration is approximately 55 ppm. In arable soils, copper levels vary widely from 1 to 390 ppm, most commonly ranging between 5 and 50 ppm. The total copper content in soil depends largely on the chemical composition of the parent material. For example: acidic rocks contain around 30 ppm, sedimentary rocks around 57 ppm and basic rocks up to 140 ppm.

Copper in soil exists in various forms: in the soil solution, within oxides, embedded in the crystal lattice of primary and secondary minerals, and bound to organic matter. Soil colloids tend to adsorb copper, reducing its availability to plants. The most important available form is water-soluble copper, although its concentration in the soil solution is very low less than 1% of the total copper content in the soil. The bioaccumulation of copper in soils and its bioavailability to fruit crops depends on the physical and chemical properties of the soil, such as mechanical composition (texture), soil sorption capacity, pH reaction, electrical conductivity (EC), humus content, carbonate content, and other factors (Vlcek and Pohanka, 2018; Vukadinović and Vukadinović, 2011, 2013; Alloway, 2013; Mitkova and Markoski, 2022; Kiprijanovski, 2020; Markoski *et al.*, 2025). The reaction of the soil solution (pH) influences the availability of copper to plants. With increasing soil pH, copper availability decreases (Mitkova *et al.*, 2009; Vukadinovic and Vukadinovic, 2016; Markoski *et al.*, 2011; Mitkova and Markoski, 2022; Kiprijanovski, 2020). This explains the deficiency of copper in carbonate and alkaline soils. Below pH 6, copper availability increases. An exception occurs in strongly acidic soils (pH<4.5), where the intensive uptake of other ions may reduce copper absorption. Furthermore, in such soils, aluminium-silicate, phosphate, and other ions can lower the level of water-soluble copper. In acidic soils, due to higher solubility, leaching of available copper may occur, as well as greater removal of copper from the soil through crop yields. The examined soil samples, according to the pH results, are neutral, slightly alkaline, moderately alkaline and strongly alkaline. Only two soils samples at both depths are non-carbonate, while the others are slightly, moderately and highly calcareous (classification by Penkov, 1996). Fruit trees grow best in slightly carbonate soils (CaCO₃ up to 5%). The presence of CaCO₃ in the soil prevents acidification (buffer effect), stabilizes organic matter, and improves soil structure. In our research, the average value of the total copper content at a depth of 0 to 30 cm is 45.33 mg/kg and the median is 41.6 mg/kg, and at a depth of 30 to 60 cm, the average value is 36.31 mg/kg and the median is 37.7 mg/kg. Copper has a

high potential for accumulation in agricultural soils due to the long-term use of fertilizers and copper-based pesticides. Depending on soil properties, excessive concentrations may become toxic to fruit crops and reduce yields. The recommended maximum level of copper in plants is 10 mg/kg, while the permissible concentration in soils, according to the World Health Organization (1996), ranges from 20 to 36 mg/kg. Concentrations above this threshold can negatively affect plant growth and soil functioning (Ballabio *et al.*, 2018; Mitkova *et al.*, 2022; Kiprijanovski, 2020; Dube *et al.*, 2000; Artiolaet *et al.*, 2004; Kabata-Pendias *et al.*, 2007; Kabata-Pendias *et al.*, 2001; Kabata-Pendias, 2011; Kopittke *et al.*, 2011; Fagnani *et al.*, 2020; Neaman *et al.*, 2024). In orchard soils of the Kumanovo region, average copper concentrations were 45.33 mg/kg at 0-30 cm and 36.31 mg/kg at 30-60 cm. These values fall within the global range for arable soils (5-50 ppm), but several exceed WHO's recommended limit, placing them in a borderline to potentially risky category. Although levels remain below the contamination threshold of 100 mg/kg (Decree 214/2007 MEF), continued accumulation from anthropogenic inputs may pose risks to soil quality and fruit production. Copper concentrations are consistently higher in the topsoil compared to the subsoil, confirming that accumulation is primarily due to agricultural practices rather than lithological origin. Overall, the orchards are adequately to well supplied with copper, but careful management of copper-containing agrochemicals is needed to prevent long-term risks.

The comparative assessment of copper (Cu) concentrations in soils from the Kumanovo region reveals notable differences when juxtaposed with data from Skopje (Stafilov *et al.*, 2017), the broader territory of North Macedonia (Stafilov and Šajn, 2016), the Prespa region (Markoski *et al.*, 2025), and Europe (Salminen *et al.*, 2005). The median copper content in Kumanovo soils was found to be 37.7 mg/kg, with values ranging between 26.3 and 49.3 mg/kg. In contrast, soils from Skopje exhibited a slightly lower median of 33 mg/kg, though with a wider variability, spanning from 7.3 to 59.0 mg/kg.

Soils from the Prespa region showed comparable copper levels, with concentrations ranging from 14.90 to 53.50 mg/kg, an average value of 32.13 mg/kg, and a median of 30.85 mg/kg, indicating moderate enrichment likely associated with long-term agricultural practices, particularly intensive orchard management. At the national level, the median copper concentration was considerably lower, amounting to 16 mg/kg, with a range between 1.7 and 73.0 mg/kg. European soils, on average, demonstrated the lowest median value of 13 mg/kg, albeit with a much broader distribution, extending from 0.81 to 256 mg/kg.

This comparison highlights that the soils of Kumanovo contain relatively elevated copper levels compared to both national and continental averages. While these concentrations fall within the lower to medium spectrum of European soils, they surpass the thresholds recommended by the World Health Organization (WHO) in several instances. This indicates a potential risk to the environment and

human health, particularly in orchards where soil contamination may directly affect both the land and agricultural produce.

Figure 3 illustrates the spatial distribution of total copper in soils under fruit orchards in this region.

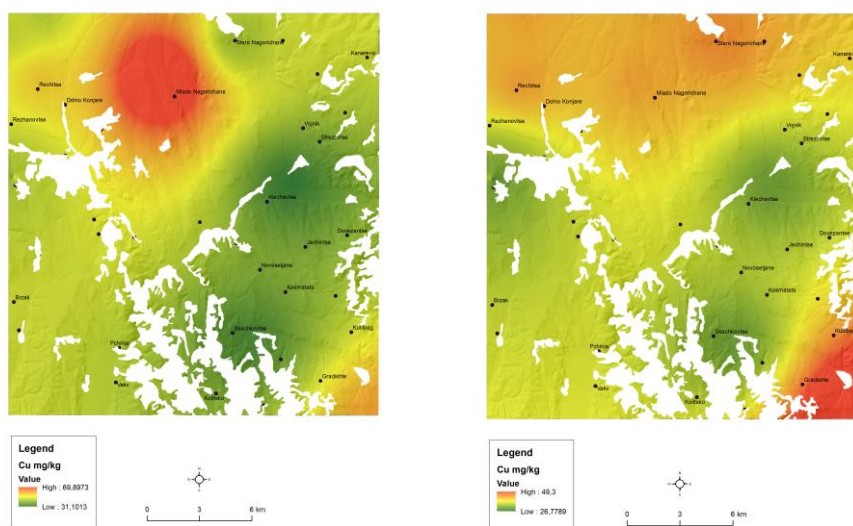


Figure 3. Spatial distribution of total copper content in soil samples at depths of 0-30 cm and 30-60 cm

Tables 4 and 5 present the factor analysis, i.e., the loading matrix of dominant rotated factors for soil samples taken at depths of 0-30 cm and 30-60 cm. Through factor analysis, a large number of variables are reduced to a smaller set of variables called factors, which are part of the principal component analysis (PCA). The factors contain significant information about the original variables and typically have a defined meaning. They are obtained by standardizing the data to a zero mean and a unit standard deviation.

In the topsoil layer (0-30 cm), five principal components with eigenvalues greater than 1 were extracted, explaining 93.51% of the total variance, with the first three components accounting for 74.98% of the variability (Table 4). This high explained variance indicates that the selected soil parameters adequately capture the dominant processes controlling copper distribution in the topsoil.

Principal Component 1 (PC1) explains 32.65% of the total variance and represents a texture-controlled sorption and accumulation factor. The strong positive loadings of physical clay, clay fraction, and copper, combined with strong negative loadings of physical sand fractions, clearly indicate that copper accumulation is preferentially associated with fine-textured soils. This

relationship reflects the higher specific surface area and cation exchange capacity of clay minerals, which promote copper adsorption and retention.

Table 4. Matrix of loading coefficients with dominant rotated factors at a depth of 0-30 cm

Parameters	PC1	PC2	PC3	PC4	PC5
Humus %	0.087	0.61	0.749	-0.02	-0.20
Total N %	0.080	0.60	0.757	-0.03	-0.20
pH in H ₂ O	0.095	-0.86	0.425	-0.08	-0.001
CaCO ₃ %	0.102	-0.86	0.408	-0.10	-0.01
EC	-0.050	0.65	-0.544	-0.23	0.01
P ₂ O ₅ mg/100g	0.175	0.45	0.084	0.48	0.73
K ₂ O mg/100g	-0.17	0.40	-0.31	0.50	-0.61
Coarse sand %	-0.68	0.14	-0.38	-0.43	0.19
Fine sand %	-0.77	-0.11	0.40	0.43	-0.02
Physical sand %	-0.98	-0.01	0.11	0.10	0.09
Clay %	0.85	-0.29	-0.35	0.20	-0.15
Silt %	0.41	0.53	0.42	-0.56	0.09
Physical clay %	0.98	0.01	-0.11	-0.10	-0.09
Cu mg/kg	0.79	0.15	0.12	0.20	0.24
Eigenvalue	4.57	3.41	2.51	1.46	1.13
% of Variance	32.65	24.38	17.95	10.45	8.08
Cumulative (%)	32.65	57.03	74.98	85.42	93.51

Table 5. Matrix of loading coefficients with dominant rotated factors at a depth of 30-60 cm

Parameters	PC1	PC2	PC3	PC4	PC5
Humus %	0.15	0.87	0.42	-0.02	0.15
Total N %	0.12	0.88	0.41	-0.06	0.12
pH in H ₂ O	-0.63	-0.45	0.45	0.41	-0.63
CaCO ₃ %	-0.49	-0.46	0.46	0.56	-0.49
EC	-0.33	0.58	0.56	-0.61	-0.33
P ₂ O ₅ mg/100g	0.43	0.50	-0.33	0.61	0.43
K ₂ O mg/100g	0.69	0.47	-0.28	0.22	0.69
Coarse sand %	0.87	-0.20	-0.43	0.09	0.87
Fine sand %	0.73	-0.20	0.63	-0.07	0.73
Physical sand %	0.94	-0.23	0.23	-0.01	0.94
Clay %	-0.75	-0.10	-0.62	-0.10	-0.75
Silt %	-0.62	0.55	0.45	0.16	-0.62
Physical clay %	-0.94	0.23	-0.23	0.01	-0.94
Cu mg/kg	-0.11	0.69	-0.31	0.51	-0.11
Eigenvalue	5.46	3.74	2.31	1.59	5.46
% of Variance	38.97	26.74	16.53	11.35	38.97
Cumulative (%)	38.97	65.72	82.25	93.60	38.97

Principal Component 2 (PC2) accounts for 24.38% of the variance and is dominated by strong negative loadings of soil pH and CaCO_3 , indicating a carbonate-alkalinity control factor. This component reflects the buffering capacity of calcareous soils, where elevated pH and carbonate content reduce copper solubility and bioavailability through precipitation and surface complexation mechanisms. The weak association of copper with PC2 confirms that, although alkalinity influences copper mobility, it does not represent the primary driver of copper accumulation in the studied orchards.

Principal Component 3 (PC3) explains 17.95% of the variance and is characterized by strong positive loadings of humus content and total nitrogen. This component represents an organic matter–biological activity factor, highlighting the role of organic matter in copper complexation and stabilization. The association between humus, nitrogen, and copper reflects the strong affinity of copper for organic ligands, which can immobilize copper in the topsoil while simultaneously increasing its persistence over time.

Together, these components indicate that copper distribution in the topsoil is primarily controlled by anthropogenic inputs, with soil texture and organic matter acting as key modulators of copper retention and spatial variability.

In the subsoil layer (30-60) cm depth, four principal components with eigenvalues greater than 1 were extracted, explaining 93.60% of the total variance, with the first three components accounting for 82.25% (Table 5).

Principal Component 1 (PC1) explains 38.97% of the variance and is dominated by strong positive loadings of physical sand fractions and available K_2O , coupled with strong negative loadings of clay, physical clay, and soil pH. This component represents a textural differentiation and eluviation factor, reflecting downward redistribution processes and reduced influence of direct anthropogenic inputs compared to the surface layer. The weak loading of copper on PC1 suggests that copper is less controlled by simple textural contrasts at this depth.

Principal Component 2 (PC2) accounts for 26.74% of the variance and shows strong positive loadings of humus, total nitrogen, and copper. This indicates that, even at greater depths, organic matter remains a critical control on copper retention, likely due to downward transport of dissolved organic complexes and bioturbation effects.

Principal Component 3 (PC3) reflects secondary geochemical controls related to nutrient dynamics and minor textural effects, but its influence on copper distribution is comparatively limited.

Overall, the PCA results demonstrate a vertical differentiation in copper-controlling mechanisms, with strong anthropogenic and surface-related controls dominating the topsoil and more pedogenically driven processes influencing the subsoil.

The cluster analysis supports and validates the PCA interpretation by grouping soil parameters according to their degree of similarity. At both investigated depths, copper clusters with humus, total nitrogen, and fine soil

fractions rather than with pH or CaCO_3 , confirming that organic matter and clay fractions are the primary sinks for copper in orchard soils. The separation of electrical conductivity into an independent cluster further indicates that salinity-related processes play a negligible role in copper distribution.

Thus, cluster analysis does not introduce new controlling factors but reinforces the robustness of the PCA-derived conceptual model. These findings are consistent with recent studies indicating that copper accumulation in orchard soils is primarily controlled by soil texture, organic matter, and long-term agricultural inputs (Reimann *et al.*, 2018; Filzmoser *et al.*, 2018).

Figures 4 and 5 present PCA Biplots, which provide a graphical representation of the results obtained from principal component analysis (PCA). They help visualize the relationships between different parameters and the principal components. A PCA Biplot is a two-dimensional diagram that displays the principal components (PC1 and PC2) as axes, while the analyzed parameters are represented as vectors or arrows.

In PCA Biplot 1, the parameters from the analyzed soil samples under fruit orchards at a depth of 0-30 cm are grouped into three clusters. The first group consists of humus content, total nitrogen (N), CaCO_3 , and the fine sand fraction, which explain most of the variance in PC1. The soil solution pH, electrical conductivity (EC), and clay content influence PC2 and form the second group, while all remaining parameters constitute the third group, having little influence on PC1 and PC2 but contributing to the other components.

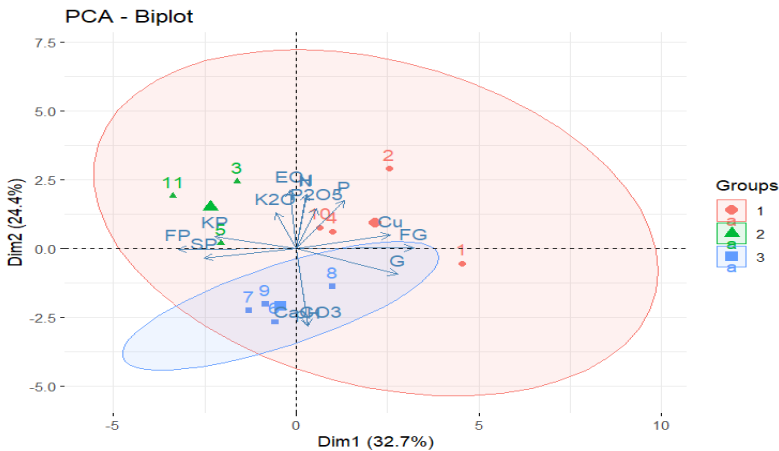


Figure 4. PCA Biplot 1 (at a depth of 0-30 cm)

In PCA Biplot 2, the first group consists of soil solution pH, electrical conductivity (EC), available K_2O , and the fractions of physical sand and clay, which strongly influence the variance in PC1 and PC2. These parameters show either strong positive or strong negative correlations with PC1. Soil solution pH and clay content are strongly negatively correlated with PC1, whereas available K_2O and the physical sand fraction are strongly positively correlated with PC1.

Electrical conductivity (EC) and available K_2O are strongly positively correlated with PC2, meaning they explain most of the variance in this component. The second group consists of humus content, available P_2O_5 , coarse sand, and fine sand fractions, which are most strongly associated with PC3 and PC4, while all other parameters fall into the third group.

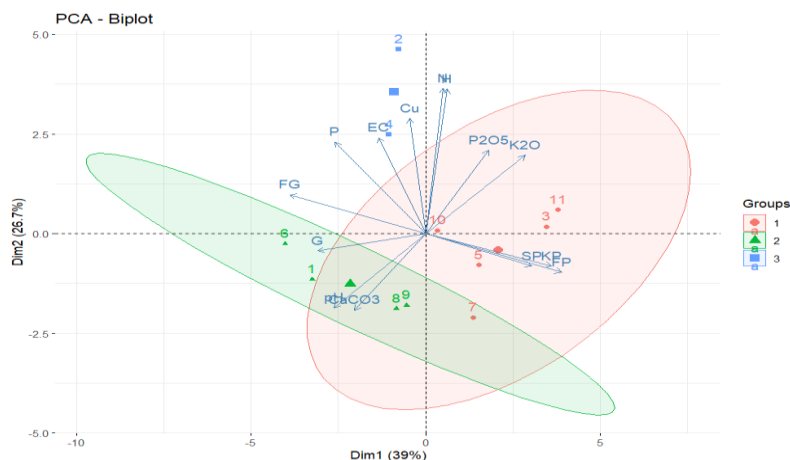


Figure 5. PCA Biplot 2 (at a depth of 30-60 cm)

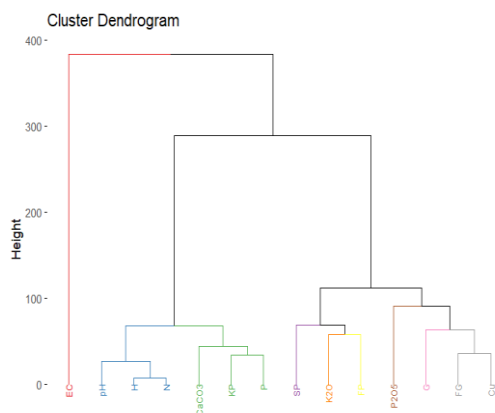


Figure 6. Dendrogram 1 from the cluster analysis of the soil samples (0-30 cm)

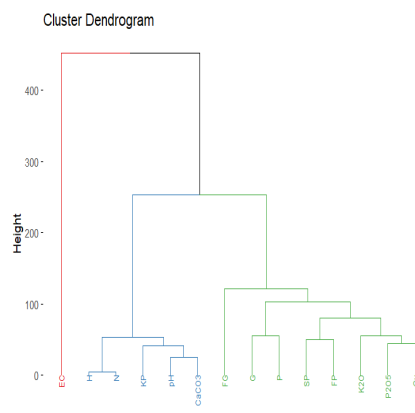


Figure 7. Dendrogram 2 from the cluster analysis of the soil samples (30-60 cm)

A dendrogram is obtained through cluster analysis, in which the results for the elements are grouped into clusters according to their degree of correlation. In Dendrogram 1 (Fig. 6), the elements from the analyzed soil samples at a depth of

0-30 cm are clustered, while in Dendrogram 2 (Fig. 7), the elements from the soil samples at a depth of 30-60 cm are clustered.

In dendrogram 1, the data are divided into three cluster groups. The first cluster includes electrical conductivity (EC), which is negatively correlated with PC1. The second cluster includes soil solution pH, humus content, total nitrogen (N), CaCO₃, coarse sand and silt fractions, which are correlated with PC2. The third cluster includes all other variables that are strongly correlated with PC1.

In Dendrogram 2, the data are also divided into three clusters. The first cluster includes electrical conductivity (EC), which is negatively correlated with PC1. The second cluster consists of humus content, total nitrogen (N), CaCO₃, coarse sand fraction, and soil solution pH, which have the greatest influence on PC2. The third cluster includes all other parameters that are in strong positive and strong negative correlation with PC1.

CONCLUSIONS

The results of this study show that copper distribution in orchard soils of the Kumanovo region is primarily controlled by anthropogenic inputs, with soil texture and organic matter acting as key factors regulating copper retention and spatial variability. Multivariate statistical analyses, particularly PCA, proved effective in identifying the dominant mechanisms controlling copper accumulation.

Copper enrichment is most pronounced in the topsoil (0-30 cm), where it is associated with clay fractions, humus, nitrogen, and available phosphorus, indicating the prevailing influence of long-term agricultural practices. In the subsoil (30-60 cm), copper distribution is more strongly influenced by pedogenic processes and organic matter dynamics, reflecting reduced direct anthropogenic impact with depth. Soil pH and carbonate content act mainly as secondary factors affecting copper mobility rather than accumulation.

Average copper concentrations in orchard soils were 45.33 mg/kg in the 0-30 cm layer and 36.31 mg/kg in the 30-60 cm layer. Although these values generally fall within the typical global range for agricultural soils, several locations approach guideline limits, indicating a potential long-term risk under continued intensive management.

Overall, the findings confirm that rational use of copper-based agrochemicals and regular soil monitoring are necessary to prevent excessive accumulation and to ensure sustainable fruit production and long-term soil health.

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RELATIONSHIP BETWEEN REGENERATION OF BLACK ALDER (*ALNUS GLUTINOSA*) AND ANTHROPOGENIC FACTORS IN RIPARIAN FORESTS AT ITS LIMIT RANGE

SUMMARY

Natural regeneration is crucial for maintaining evolutionary processes in plant species. Understanding the factors that influence recruitment dynamics could shed light on the key processes governing this critical biological process. However, the relationship between anthropogenic factors and the regeneration of *Alnus glutinosa* in Morocco remains unexplored, despite significant habitat reduction due to fragmentation and human disturbance. Twelve representative populations were selected, and ten plots were established in each population. The slope of a linear regression and histograms were used to determine the status of regeneration. Principal component analysis and a linear mixed-effects model (LME) were then employed to evaluate the impact of tree cutting, agricultural practices, cover, slope, and altitude on the natural regeneration of *Alnus glutinosa* across ontogenetic stages (seedlings, young individuals, shrubs, and adult trees) within its populations. Three regeneration patterns were observed: a reversed J-shape, a J-shape, and an irregular shape. The main causes of this species' decline were agricultural activities and tree-cutting. Seedling recruitment was positively associated with slope variation. Additionally, average vegetative cover supported recruitment, and there was no significant correlation between natural regeneration and altitude. Further artificial intervention measures should be considered to promote plantation regeneration. These findings provide a solid foundation for future forest restoration and sustainable management initiatives.

Keywords: Natural regeneration, black alder, anthropogenic factors, conservation

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INTRODUCTION

Natural regeneration is a critical mechanism for generational succession in forest ecosystems (Dech *et al.* 2008; Tinya *et al.* 2009). It is essential for preserving forest sustainability in the long term (Lewis *et al.* 2019). It also enables ecosystems to recover following disturbance (Johnson *et al.* 2021; Pham *et al.* 2022). Compared to artificial regeneration, the natural regeneration of stands from seeds is a more cost-effective and ecologically beneficial approach (Zhao *et al.* 2023). However, human-induced disturbances strongly influence the regeneration success of woody species, controlling the vegetation structure and composition of forests (Tadele *et al.* 2014). This issue has been highlighted in various studies (Winter *et al.* 2017; Kidane *et al.* 2018). The overuse of resources has recently led to significant ecological changes worldwide, largely due to misguided economic policies (Mutia, 2010). Furthermore, population growth and associated factors, such as deforestation for agricultural expansion, fire, and wood collection for charcoal production, have accelerated the decline in forest resources in developing countries (Yakob and Fekadu 2016; Kidane *et al.* 2018). In addition, forest regeneration may be very challenging in areas where the natural forests are degraded and isolated. Riparian forest ecosystems are an important source of multiple ecosystem services, including ecological, socio-economic, and cultural functions (Naiman *et al.* 2005; Riis *et al.* 2020). These services are particularly important at the local level, significantly influencing the physical and chemical processes of aquatic and terrestrial ecosystems (Naiman *et al.* 2005). Unfortunately, all riparian habitats, including *A. glutinosa*, are subject to numerous anthropogenic pressures in the Mediterranean basin. These include the riverbeds cleaning, water abstraction for irrigation and industry, and agro-pastoral activities (Cooper *et al.* 2013; Fois *et al.* 2021).

The Moroccan populations of *A. glutinosa* have remained isolated since the last glacial maximum (Douda *et al.* 2014; Mandák *et al.* 2016). The distribution of these populations is scattered and patchy, and they are geographically isolated from the main distribution of continental Europe (Claessens *et al.* 2010). This represents an exceptional biogeographical situation, as isolated populations (Lepais *et al.* 2013). The range of *A. glutinosa* in Morocco has been significantly reduced due to habitat fragmentation and disturbance caused by human activities (Ennoui *et al.* 2021). Regarding the status of alder in the regional Red List (Fennane, 2021), it is clear that the current status is near threatened (NT) or least concern (LC). However, this status should be revised and replaced by the Endangered category (/EN/ B1ab+B2ab) (Ennoui *et al.* 2021). The IUCN (2012) has classified this species as endangered in Morocco, and the threat is being exacerbated by climate change (Pachauri *et al.* 2014). Therefore, restoration work should be carried out in multiple regions, including Morocco, through various efforts such as trees replanting (enrichment planting and natural regeneration). To date, no study has examined the anthropogenic effects on the natural regeneration of *Alnus glutinosa* (*A. glutinosa*) in Morocco.

To address this research gap, the present study examined the impact of environmental factors and anthropogenic drivers on natural regeneration across different ontogenetic stages (seedlings, young individuals, shrubs, and trees) in *A. glutinosa* populations. The research objectives were threefold: (i) evaluating the influence of altitude, slope, and cover factors on *A. glutinosa* regeneration dynamics; (ii) evaluating the anthropogenic factors (tree cutting and agricultural practices) on regeneration success across developmental stages; and (iii) proposing some management strategies to enhance the efficacy of natural regeneration. These findings are expected to provide critical insights into optimising *A. glutinosa* population restoration and informing sustainable forest regeneration practices in Mediterranean ecosystems.

MATERIAL AND METHODS

Study area

The populations of *A. glutinosa* selected for this study are located in the central-western Rif region of northern Morocco (Sahli *et al.* 2023; Sahli *et al.* 2025a). This species inhabits riparian habitats within a range of bioclimatic environments, from subhumid to perhumid (Table 1).

Table 1. The main characteristics of the study sites. Altitude (ALT). The average maximum temperature during the warmest month of the year (M°C). The average minimum temperature during the coldest month of the year (m°C), and the average annual precipitation (P)

Population	Code	ALT- max (m)	Latitude (°)	Longitude (°)	P mm	M °C	m°C	Bioclimats
Ain Korra	AK	1377	35.16546	-5.31642	1772,13	27	2,1	PH-F
Amlay	AM	1412	35.10789	-5.22525	1005,20	33,5	5,5	H-T
Ben Idder	BN	1377	35.22106	-5.323	1108,00	33,9	6,3	H-T
Boubiyine	BO	1412	35.12983	-5.23099	1005,20	33,5	5,5	H-T
Bouztate	BZ	1505	35.00803	-5.11503	1482,20	32,5	2,5	H-F
Khezana	KH	1562	35.03445	-5.14031	1482,20	32,5	2,5	H-F
Oued Lakhmis	OL	240	35.28634	-5.15291	464,50	28,9	8,6	SH-C
Siouana	SO	596	35.28816	-5.46806	816,80	32,0	6,1	H-T
Tanghaya	TN	1477	34.972778	-5.15222	1482,20	32,5	2,5	H-F
Tayenza	TY	1603	35.16082	-5.25836	1772,13	27	2,1	PH-F
Tifouzal	TF	390	35.06139	-5.16553	1005,20	33,5	5,5	H-T
Ketama	KT	1 308	34.878610	-4.619722	1 616,00	28	1	PH-F

Note: Bioclimates: PH=Perhumid; H=Humid; SH=Subhumid. Bioclimatic variants: Winter: F=Cool; T=Temperate; C=Warm. M°C: average of the maximum temperatures in the warmest month of the year. m °C: average of the minimum temperatures in the coldest month of the year. P: average annual precipitation in mm

From an edaphic perspective, the species settles on schistous substrates in the central Rif and on sandstone substrates in the western Rif (Table 1). The distribution of this taxon's populations of this taxon demonstrates that they are situated along an altitudinal gradient (Sahli *et al.* 2025b). The highest altitudes were observed in the Tayenza populations (1.603 m), while the lowest altitudes were observed at the Oued Lakhemis site (240 m) (Table 1) (Sahli *et al.* 2025c). Observed precipitation levels ranged from 464 mm to 1.770 mm. In terms of stand typology, most stands are classified as high forest (97.73%), with coppice stands being relatively uncommon and predominantly found in heavily disturbed sites. These stands were primarily observed in three locations: The locations in question are Tayenza, Bouztate, and Amlay. (Ennoui *et al.* 2021). Conversely, an examination of the composition of these populations revealed that they are characterized primarily by the dominance of *A. glutinosa* individuals. In these stands, the tree layer dominated by *A. glutinosa* is rarely mixed with *Prunus lusitanica*. This mixture only occurs at the upper limit of the black alder population, corresponding to the base of the *Prunus lusitanica* populations, and marking the transition zone between the two populations (Ennoui *et al.* 2021).

Sampling

Riparian vegetation sampling was conducted during the summer months. Twelve sites were selected for the study (Figure 1). A total of 120 plots were set up. The different types of *A. glutinosa* individuals were classified according to their height.

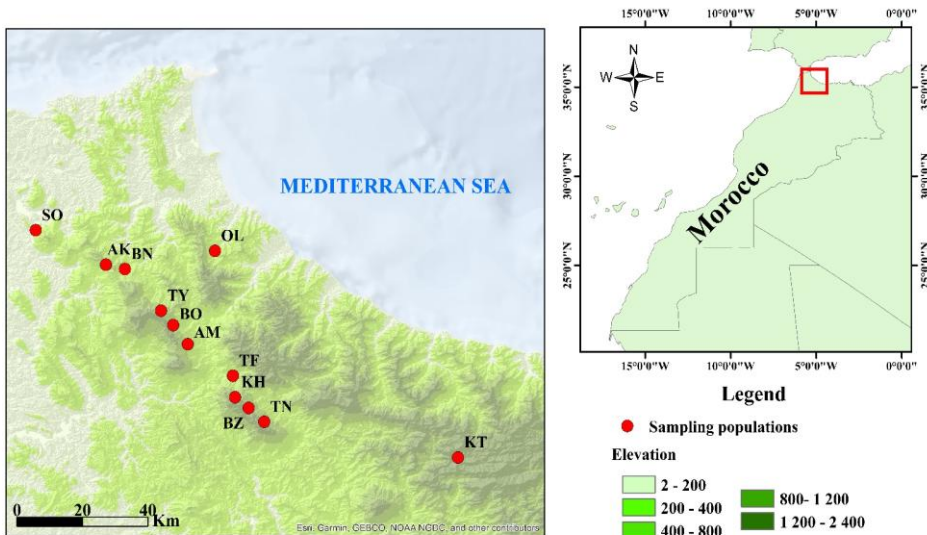


Figure 1. Geographical location of the populations studied in the central-western Rif. Acronyms of populations: SO: Siouana; AK: Ain Korra; AM: Amlay; BN: Benidder; BO: Bobiyine; BZ: Bouztate; KH: Khezana; OL: Oued Lakhmis; TN: Tanghaya; TY: Tayenza; TF: Tifouzal; KT: Ketama

Seedlings were defined as individuals with a height of 0-50 cm, young individuals (50-100 cm), shrubs (100-200 cm and 200-400 cm), and trees (height >400 cm) (Setshogo and Venter 2003; Burton *et al.* 2005; Tsheboeng *et al.* 2017). The height of *A. glutinosa* individuals identified was estimated in a 10 randomly selected 10×10 m² quadrat at each site. Additionally, the number of *A. glutinosa* individuals in each height class within each quadrat was recorded.

Density was standardized and expressed as the number of individuals per 10³ m².

Statistical Analyses

The measured variables are presented with the following parameters: the arithmetic mean (M) and confidence intervals (CI). The nested analysis of variance (ANOVA) was performed to show the level of difference in density and height. The Tukey post-hoc test was used to determine whether there was a statistically significant difference in the density of the various types of individuals of this species across the different sites.

The graphs were conducted for this species at each site to facilitate a comparison of the regeneration structure between the studied populations (Tsheboeng *et al.* 2017). The principal parameters that define the sites, including bank slope, altitude (Debruxelle *et al.* 2004), and cover, were estimated based on available data. Additionally, the intensity of anthropogenic disturbances was assessed following the methods proposed by Debruxelle *et al.* (2004). These disturbances included the cutting of adult trees (i.e., the number of trees cut in each plot) and intensified agricultural practices (i.e., the area of land occupied by agricultural practices around the riparian zone of the species).

The regeneration status of different woody individuals was determined using Shackleton's (1993) procedures by analyzing the slope of a linear regression between height classes and the mean number of individuals per 10³ m² in each class. A positive slope indicates a population with a greater number of individuals in the larger size classes than in the smaller ones. Conversely, a negative slope suggests ongoing recruitment, where there is a greater number of individuals in the smaller size classes than in the larger ones (Tsheboeng *et al.* 2017).

To quantify the direct impact of anthropogenic and environmental factors on the density of seedlings, young individuals, shrubs, and trees, we applied linear mixed-effects models (LMEs). This approach was chosen to explicitly account for the hierarchical structure of the data, whereby observations are nested within sites, by including site as a random effect. Using LMEs allowed us to separate local-scale variability from broader environmental and anthropogenic influences, thus improving parameter estimation and inference (Salazar Zarzosa *et al.* 2021). Linear regressions were also applied where data aggregation permitted (Sahli *et al.* 2005a). Furthermore, as many of the anthropogenic and environmental variables were expected to be correlated, we conducted a principal component analysis (PCA). This was employed to cluster correlated variables,

identify the dominant factors shaping site variations, and simplify the interpretation of multivariate relationships within and between populations. Using PCA alongside LME enables both an exploratory assessment of the structure of the variables and a formal evaluation of their effects on density. Data statistics were analyzed using the R 4.1.3 software program (R Core Team 2024).

In this study, individuals with a height between 200 and 400 cm were defined as shrubs, while trees were considered to be individuals with a height greater than 400 cm (Setshogo and Venter 2003; Tsheboeng *et al.* 2017).

RESULTS AND DISCUSSION

The results illustrate a significant difference in the density between the various categories of individuals within *A. glutinosa* populations. The results of the ANOVA analysis and Tukey test corroborate the assertion that there is a significant difference in density between the various categories of individuals, which include seedlings, young individuals, shrubs, and mature trees (Table 2).

Table 2. Density for the different types of individuals in each population. Anova one-way and different letters Post-hoc test. significant (***) $p < 0.001$) between sites

Sites	Code Sites	Seedling	Young individuals	Shrubs	Trees
Ain korra	AK	20 ^a	7 ^{ab}	21 ^{ab}	11 ^{ab}
Amlay	AM	0 ^a	0 ^a	0 ^a	21 ^b
Beni Idder	BN	63 ^b	17 ^b	4 ^a	34 ^b
Bobiyine	BO	40 ^b	1 ^a	5 ^a	24 ^b
Bouztate	BZ	0 ^a	0 ^a	11 ^b	21 ^b
Ketama	KT	0 ^a	0 ^a	0 ^a	2 ^a
Khezana	KH	0 ^a	0 ^a	1 ^a	17 ^b
Oued Lakhemis	OL	0 ^a	0 ^a	0 ^a	20 ^b
Siouana	SO	31 ^{ab}	21 ^a	35 ^{bc}	50 ^b
Tanghaya	TN	177 ^c	4 ^a	8 ^a	39 ^b
Tayenza	TY	3 ^a	9 ^{ab}	83 ^c	5 ^{ab}
Tifouzal	TF	1 ^a	1 ^{ab}	0 ^a	14 ^{ab}
ANOVA inter-populations		7.01***	3.03 ***	7.37***	5.88***

Furthermore, a highly significant height difference was observed between populations for each category of individual. For example, considerable variation in height was observed among seedlings, ranging from 8.06 ± 0.86 cm in the Tanghaya population to 37.33 ± 7.53 cm in the Tayenza population. The results of

the ANOVA analysis corroborate the significant height differences observed between the populations of the different types of individuals (Table 3).

Table 3. Mean height (Mean±CI) for the different types of individuals in each population. Anova one-way and different letters Post-hoc test. significant (***) $p < 0.001$) between sites

Sites	Seedling	Young individuals	Shrubs	Trees
Ain Korra	26.45±5.69 ^b	77.14±0.00 ^b	159.11±12.4 ^{bcd}	5.25±0.45 ^a
Amlay	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	8.88±0.20 ^a
Ben Idder	28.87±3.18 ^b	65.71±0.00 ^b	0.00±0.00 ^a	7.26±0.13 ^a
Bobiyyine	9.65±1.30 ^b	55.00±0.00 ^b	165.60±0.00 ^{bcd}	11.11±0.20 ^{ab}
Bouztate	0.00±0.00 ^a	0.00±0.00 ^a	106.98±2.88 ^{bcd}	15.98±0.21 ^{ab}
Ketama	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	13.50±2.09 ^{ab}
Khezana	0.00±0.00 ^a	0.00±0.00 ^a	40.00±0.00 ^b	11.85±0.29 ^{ab}
Oued Lakhemis	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	9.00±1.39 ^a
Siouana	22.97±4.96 ^b	72.48±23.33 ^b	221.44±9.89 ^d	6.93±0.12 ^a
Tanghaya	8.06±0.86 ^a	55.00±19.60 ^b	224.80±4.3 ^d	12.54±0.13 ^{ab}
Tayenza	37.33±7.53 ^b	70.00±8.12 ^b	227.82±6.09 ^d	9.09±0.87 ^a
Tifouzal	35.00±0.00 ^b	65.00±0.00 ^b	0.00±0.00 ^a	8.12±0.32 ^{ab}
ANOVA inter-populations	58.818***	2.579*	5.555**	5.582***

A comparison of the regeneration structures among populations of this species studied reveals that the population of Tayenza exhibits an irregular structure. The populations of Ain Korra, Beni Idder, Bobiyyine, and Tanghaya display a reversed J-shape, indicating relatively stable regeneration (Figure 2). The remaining populations are characterized by unstable regeneration. The populations of Amlay, Bouztate, Ketama, Khezana, and Oued Lakhemis exhibit an absence of classes between 0 and 50 cm, 50 and 100 cm, and 100 and 200 cm. The populations of Siouana and Tifouzal display a J-shaped structure, indicating a disproportionate abundance of individuals in the tallest height classes (100-400 cm and >400 cm) compared to the shortest (0-50 cm and 50-100 cm) (Figure 2). This result was corroborated by the regeneration slope coefficient, which exhibited negative values, indicating that the regeneration of *A. glutinosa* in the populations of Ain Korra, Beni Idder, Bobiyyine, and Tanghaya is relatively unimportant. Nevertheless, no evidence of regeneration was observed in the remaining populations (Table 4).

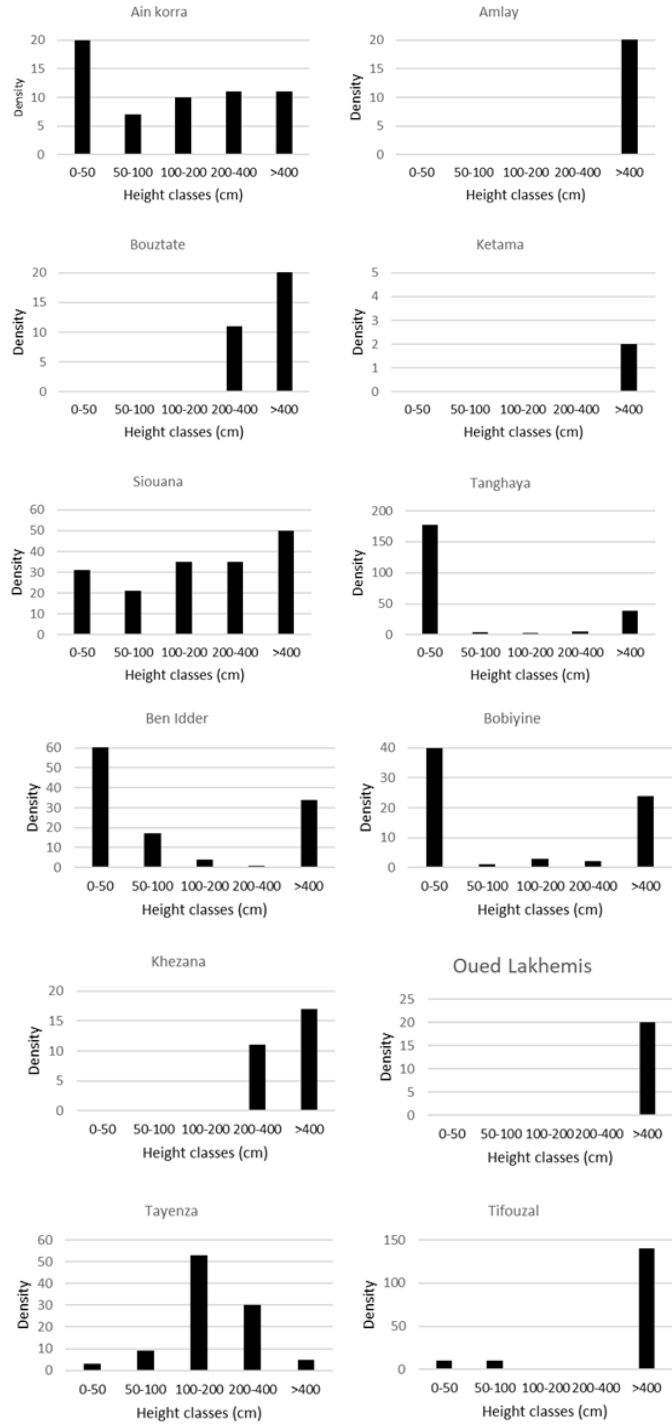


Figure 2. Regeneration structure of *Alnus glutinosa* in its representative populations

Table 4. Regression analysis slope coefficients for *A. glutinosa* in its populations

Sites	Slope coefficient
Ain korra	-0.06
Amlay	0
Beni Idder	-0.23
Bobiyyine	-0.23
Bouztate	0.48**
Ketama	0
Khezana	0.96*
Oued Lakhemis	0
Siouana	0.52
Tanghaya	-0.17
Tayenza	0.46
Tifouzal	0.35**

The Principal Component Analysis (PCA) analysis confirmed the structure of the populations and showed that this disparity in the density of the four categories (seedlings, young individuals, shrubs, and trees) could be attributed to environmental and anthropogenic factors (Figure 3). The analysis revealed that three groups within the studied populations: the first group comprises the Tayenza and Ain Korra populations, and is characterized by a high density of shrubs; the second group comprises the Siouana, Beni Idder, Bobiyine, and Tanghaya populations, and is characterized by a high density of young individuals, trees, and seedlings, respectively; and the third group comprises the remaining populations, and exhibited a level of disturbance (Figure 3).

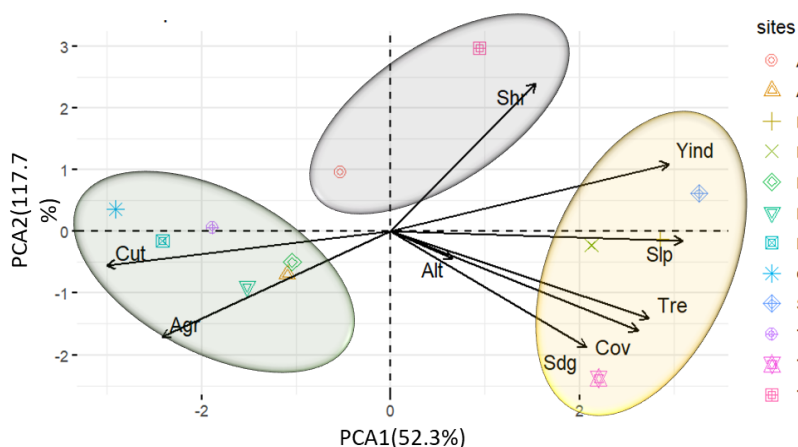


Figure 3. Principal component analysis (PCA) of density and independent factors. PCA1 and PCA2: Principal component analysis axes 1 and 2. Slp: salop. Alt: altitude. Agr: agriculture. Cut: Cutting. Cov. Cover. Sdg: seedling. Yind: young individual. Shr: Shrubs and Tre: trees. Acronyms of sites see Table 1

In addition, the PCA analysis showed that the density of seedlings, young individuals, shrubs, and trees correlates positively with the slope and cover. However, it correlates negatively with tree cutting and agricultural activities. This result was confirmed by the linear mixed model analysis (LME), which showed that tree cutting negatively affects the density of young individuals and the density of trees (Table 5, Figure 4). The slope has a positive effect on the density of seedlings, young individuals, and trees (Table 5, Figure 4). The agricultural practice negatively affects young individuals (Table 5, Figure 4). However, the altitude variation does not affect the density of the four categories (seedlings, young individuals, shrubs, and trees) (Table 5).

Table 5. Results of the LME models explaining the variation of density with anthropogenic and environmental factors. T value for each independent variable and significant correlations is indicated in bold (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) and nearly significant ($0.1 > P > 0.05$) in bold.

Factors	Variables	Estimate	Std. error	t value
Cutting	Seedling	-0.10	0.08	-1.26
	Yong individual	-0.03	0.00	-4.17**
	Shrubs	-0.06	0.04	-1.49
	Trees	-0.05	0.02	-2.63*
Cover	Seedling	0.12	0.06	1.95a
	Yong individual	0.01	0.00	2.11a
	Shrubs	0.03	0.04	0.08
	Trees	0.05	0.01	5.93***
Slope	Seedling	0.22	0.07	2.92*
	Yong individual	0.03	0.01	3.22**
	Shrubs	0.06	0.05	1.14
	Trees	0.06	0.02	2.63*
Agriculture	Seedling	-0.07	0.08	-0.87
	Yong individual	-0.02	0.00	-3.24**
	Shrubs	-0.07	0.04	-1.81a
	Trees	-0.01	0.02	-0.64
Altitude	Seedling	0.00	0.00	0.71
	Yong individual	-0.00	0.00	-0.24
	Shrubs	0.00	0.00	0.43
	Trees	0.00	0.00	0.04

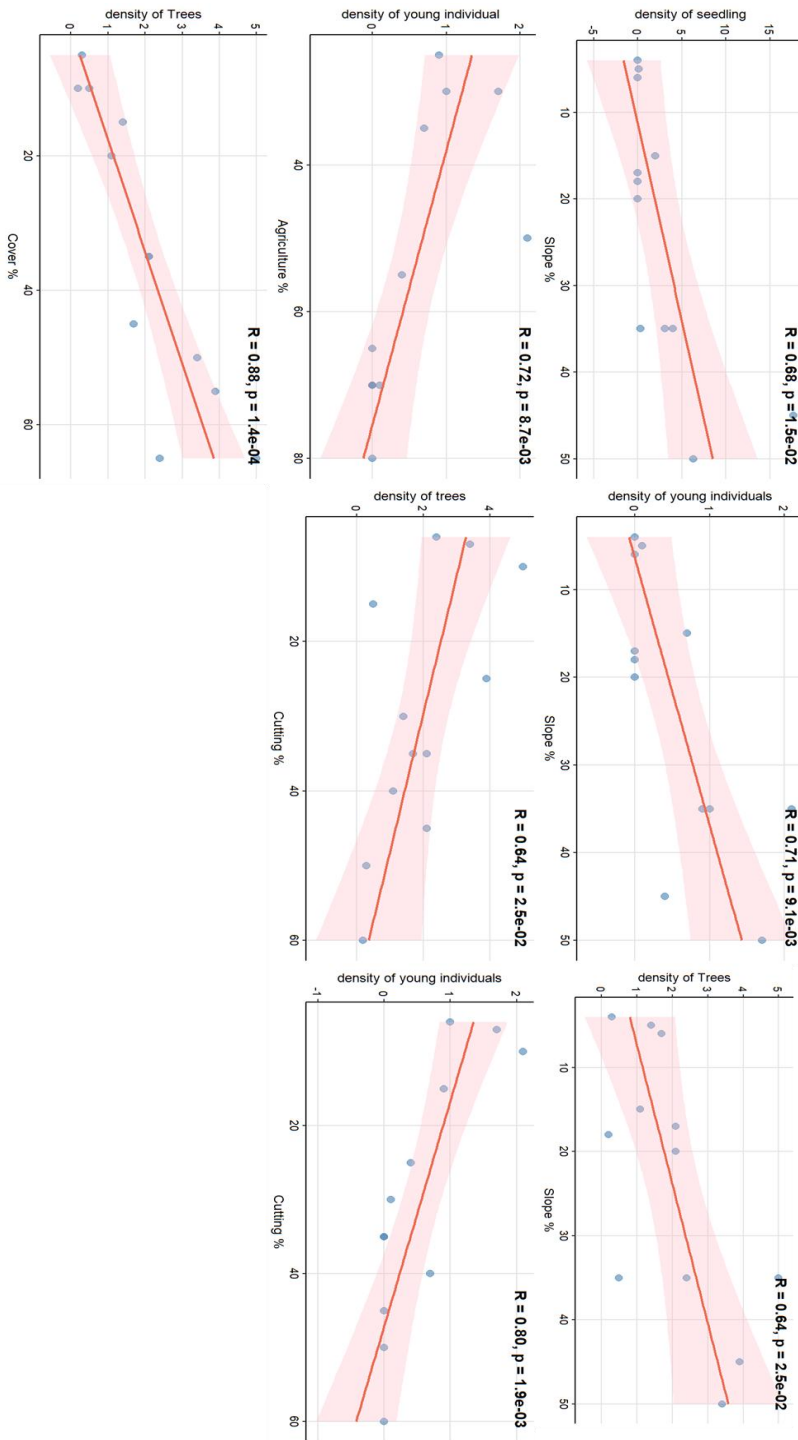


Figure 4. Linear regression analysis between population characteristics and the density of different types of individuals

DISCUSSION

Natural regeneration is a key mechanism in the generational succession of forest ecosystems (Qin *et al.* 2025). This ecological process is essential for ensuring the long-term sustainability of forests (Lewis *et al.* 2019) and helps ecosystems to recover after disturbance (Johnson *et al.* 2021; Pham *et al.* 2022). Compared to artificial regeneration, seed-based natural regeneration is a more economical and ecologically efficient method of stand restoration (Zhao *et al.* 2023). However, the overall regeneration status of the woody species at the study site was unsatisfactory at the population level, with poor regeneration observed in the Amlay, Bouztate, Ketama, Khezana, and Oued Lakhemis populations. This was evident through the absence or low recruitment of *A. glutinosa* seedlings within their natural populations, which may indicate a correlation between seedling density and population characteristics. Good regeneration of this woody species was observed at Ain Korra, Beni Idder, Bobiyine, and Tanghaya, characterized by a reverse J-shape. The linear mixed-model analysis revealed that agricultural activities such as overgrazing and tree cutting negatively impact the density of young individuals. Intensive trampling by livestock while browsing and grazing in the forest also compacts the soil, thereby reducing the germination of seeds from the soil seed bank (Gebirehiwot *et al.* 2023). This could explain the poor regeneration of the *A. glutinosa* species.

In addition, the positive correlation between seedling density and tree density (Figure 3) suggests that the cutting of trees will reduce the quantity of seeds produced and, consequently, lead to a decline in seedling density. Furthermore, well-established that *A. glutinosa* seeds have a low germination capacity (De Atrip and O'Reilly 2005; De Atrip and O'Reilly 2007; Gosling *et al.* 2009; Kaliniewicz *et al.* 2018; Gomes Marques *et al.* 2021). This is because most of the seeds produced by this species are devoid of any viable contents (McVean, 1953). The findings also indicate that an increase in tree cover is associated with an elevated tree density and moderate increases in seedling density and young individuals (Table 5). Moderate cover has been shown to reduce evapotranspiration and protect young seedlings (Gómez-Aparicio *et al.* 2005). Conversely, high canopy cover, in conjunction with a light-poor understorey, markedly restricts photosynthetic activity, thereby rendering regeneration untenable for more than one year (La Mela Veca *et al.* 2013). Additionally, low cover can impede the impact of radiation intensity reaching the soil, which may negatively affect seedling growth (Gómez-Aparicio *et al.* 2005, 2008). This is exemplified by the Amlay, Bouztate, Ketama, Khezana, Oued Lakhemis, and Tifouzal populations, which demonstrate minimal or no seedling growth. The low or zero density of height classes 0.5 to 1 m (young individuals) in the populations of Bouztate, Ketama, Khezana, Oued Lakhemis, and Tifouzal (Figure 2), can be attributed to the impact of agricultural activities and the cutting of adult trees (Table 5, Figure 5). These are the primary direct causes of the decline in young individuals. Furthermore, these populations are also subject to impact from wild herbivores (Figure 5). Their proximity to human communities has resulted in the

overexploitation of water resources by riverside communities, and the diversion of watercourses for the irrigation of food crops (Ennoui *et al.* 2021). Additionally, the presence of goat herds has been observed to intensify the impact of human activities (Benamar 2005). Additionally, the disposal of garbage and the discharge of wastewater containing chemicals (Figure 5) have the potential to impede growth and cause the death of young individuals due to the toxicity of these substances. This low density could indicate a local extinction in the future, as the dying trees will not be replaced.



Figure 5. Intensive impacts on *A. glutinosa* in some populations. a: Cutting of trees (Tanghaya site). b: Grazing (Bouztate site). c: Pollution (Oued Lakhemis site) and c: Water capture for agricultural activities (Bobiyyine site)

The low density of *A. glutinosa* individuals in the medium height classes (shrubs) in Amlay, Beni Idder, Ketama, Khezana, Oued Lakhemis, Tanghaya, and Tifouzal populations may be attributed to agricultural activities, as illustrated in Figure 5. For instance, raising goats along the riverbanks has been observed to result in the consumption of the leaves and young shoots of *A. glutinosa*. Furthermore, the weakening of the herbaceous layer during the summer period exerts additional pressure on the young *A. glutinosa*, not to mention the impact of overgrazing (Ennoui *et al.* 2021). The low density of shrubs may also be due to the selective removal of individuals before the onset of the next growth phase begins (Gurmessa *et al.* 2012), a process that can be facilitated by herbivory

(Bendaanoum, 1998). Herbivory can disrupt the soil structure through compaction (Hulme and Borelli, 1999; Smit *et al.* 2006), and also modify the vegetation and soil structure (Addar *et al.* 2016). Conversely, the findings indicate that the slope is conducive to the successful establishment of young individuals (Table 5). Populations characterized by a low density of young individuals are typically situated on gentle slopes. This is exemplified by the populations of Amlay, Bobiyine, Ketama, Oued Lakhemis, and Tanghaya. Indeed, a moderate slope facilitates the accumulation of nutrients in minor watercourses (Berg ers 2004). The watercourses of this species are typically narrow and characterized by weak banks. (Ennoui *et al.* 2021). However, Chhetri *et al.* (2023) posited that slope exerts no significant influence on species regeneration.

In terms of regeneration structure, populations of *A. glutinosa* Tayenza are characterized by a higher number of low-height individuals (seedlings) than medium-size individuals (young individuals and shrubs). This may be indicative of episodic recruitment (Helm and Witkowski 2012; Traor   *et al.* 2013). In episodic recruitment, there is variation in the growth of individuals in different size classes over time (Helm and Witkowski 2012), indicating that in the past, individuals from lower size classes were previously recruited. This episodic recruitment may result from the selective removal of individuals from the medium-sized classes before they are recruited into the subsequent growth stage (Gurmessa *et al.* 2012). In our study, the elimination of individuals from these classes in Beni Idder and Bobiyine may result from herbivory (Ennoui *et al.* 2021), and agricultural activities.

This species exhibits an irregular regeneration pattern in the Tayenza population, characterized by the absence of height classes of 0-50 cm, 50-100 cm, and 100-200 cm. In contrast, the populations of Siouana and Tifouzal exhibit a J-shaped regeneration structure. The presence of these two regeneration shapes suggests that the regeneration process in these populations is inherently unstable. This result was corroborated by the positive slope coefficient. The low reproduction can be attributed to the low pollen viability of this species (Sahli *et al.* 2025d). Sahli *et al.* (2026) demonstrated that the pollen development of this species is primarily influenced by the climatic factors, particularly temperature, which impacts the starch content in the pollen. Additionally, regeneration in these populations may be attributed to herbivory (Benamar 2005; Ennoui *et al.* 2021). The potential for extinction of this species is exacerbated by the scarcity of individuals in the smaller size classes, hindering the replacement of deceased older individuals. The J-shaped regeneration structure observed in riparian species may indicate local extinction in the future due to the lack of replacement for older and dying stands (Helm and Witkowski 2012).

In the Tanghaya, Beni Idder, Bobiyine, and Ain Korra populations, the *A. glutinosa* is designated as a reverse-J shape. This suggests relatively stable regeneration (Figure 2). Reverse-J shape regeneration is considered a healthy phenomenon because it enables deceased individuals from lower size classes to

be replaced (Mwavu *et al.* 2009). This regeneration structure also demonstrates that individuals are subject to selective elimination in subsequent height classes (Senbeta *et al.* 2003; Gurmessa *et al.* 2012). As observed in these two populations, there is a scarcity of individuals within the 50-100, 200-400 cm, and >400 cm size classes. The removal *A. glutinosa* individuals may have had a detrimental impact on its regeneration structure, as indicated by a low negative regression coefficient (Table 5). Generally, a negative regression slope of height classes indicates that there is ongoing recruitment (Shackleton, 1993). The wood of *A. glutinosa* is generally used by riparian populations for various purposes, including as an energy source, fodder, and services (Ennoui *et al.* 2021).

Other factors that could influence the density of riparian species include, in particular, the construction of dams in watersheds, hydrological and climatic changes (Parolin and Wittmann 2010), and prolonged drought, which mainly affects seedlings that have not yet developed roots to reach underground depths (Pettit *et al.* 2001). The impact of other factors, such as fires, floods, pathogens, competition, light, water regime (de Oliveira *et al.* 2014; Tsheboeng *et al.* 2017), and seed predators (Mwavu *et al.* 2009), must also be considered.

CONCLUSIONS

A better understanding of anthropogenic factors affecting the density of seedlings, young individuals, shrubs, and trees is essential for achieving the regeneration of *A. glutinosa*. The slope and cover favor the seedling recruitment and young individuals' establishment. However, the altitude was not associated with the natural regeneration. Based on PCA analysis and LME analysis, our findings indicate that the natural regeneration of *A. glutinosa* is significantly influenced by agricultural practices and the cutting of trees. This leads to a reduction in the habitat of *A. glutinosa* in its rear-edge populations in Morocco. In addition, this human impact will be added to the loss of their genetic diversity of these isolated populations due to inbreeding and genetic drift, which subsequently leads to the accumulation of deleterious mutations. Protection and conservation of this habitat in the range of *A. glutinosa* in Morocco from fragmentation and disturbance caused by human activities should be in the purview of conservation programs. Further studies on seed germination and control of grazing, protection of seed trees may prove valuable in elucidating the status of this species and in formulating conservation strategies for its populations in Morocco.

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A COMPREHENSIVE MULTISENSORY LEARNING APPROACH FOR OYSTER MUSHROOM MATURITY DETECTION IN GREENHOUSES

SUMMARY

In the mushroom industry, analysing growth and detecting maturity during cultivation is crucial for smart mushroom farming. This study introduces a comprehensive multisensory approach employing diverse machine learning algorithms to automate the classification of oyster mushroom (*Pleurotus ostreatus*) maturity classes in a greenhouse setting. Three unique datasets were created, comprising manually extracted features of oyster mushroom node sizes (height and width) alongside size features derived from bounding boxes automatically identified by trained Detectron2 and YOLOv8 object detectors utilising annotated mushroom images. The manual and automatic datasets were used to train classification algorithms separately, their performance was evaluated and compared. The impact of environmental setting within the mushroom greenhouse on mushroom growth was examined by integrating environmental variables as features for classification algorithms. The results indicated a significant enhancement in classification accuracy, especially incorporating the temperature and humidity values. The findings indicate that the HistGBDT classifier achieved the highest testing accuracies of 91.2%, 90.1%, and 92.7% on the automatic YOLOv8, Detectron2, and manual datasets, respectively, employing extracted mushroom node's width and height, along with temperature and humidity features in the greenhouse. The results of other classifiers demonstrated minor differences between manual and automatic datasets, suggesting that the YOLOv8 object detector generated mushroom bounding boxes significantly more aligned with those drawn by humans compared to the Detectron2 framework. These results demonstrate an invaluable multisensory approach for detecting mushroom maturity classes in greenhouse settings, surpassing existing camera-based systems.

Keywords: Oyster mushroom, YOLOv8, Detectron2, agriculture, classification, object detection

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INTRODUCTION

Mushroom cultivation has become a critical component of contemporary agriculture due to its numerous economic and environmental benefits, which contribute to the sustainability and diversity of the agricultural industry (Vinci *et al.*, 2023, Jayaraman *et al.*, 2024, Ben Mansour *et al.*, 2025). Smart mushroom cultivation integrates sensing, automation, and data-driven analysis to support controlled growth monitoring and decision-making processes (Elewi *et al.*, 2024, Sangeeta *et al.*, 2024, Badoni and Siddiqui, 2025). Recent progress in indoor environmental studies of mushroom cultivation facilities has focused on the development of environmental monitoring and control systems, the application of computer modelling to address indoor environmental issues, and the refinement of mushroom facility design (Chen *et al.*, 2022). These advancements have led to the development of smart farming systems that enable real-time recording of environmental parameters and automatic regulation of equipment, as well as the establishment of growth models for specific mushroom species. One of the key advantages of smart mushroom cultivation is the ability to control and optimise various parameters that affect mushroom growth and yield (Moysiadis *et al.*, 2023). Parameters such as temperature, relative humidity, and air quality can be remotely monitored and maintained at optimal levels using state-of-the-art technologies (Elewi *et al.*, 2024). This level of control and optimization is not possible in conventional methods of mushroom cultivation, leading to lower yields (Rahman *et al.*, 2022). By implementing smart farming techniques, farmers can improve the yield of mushrooms and achieve higher productivity. In addition to improving productivity, smart mushroom cultivation also offers benefits in terms of standardization and safety. This is particularly important for the commercialization of mushroom cultivation, as it allows consistent quality and safety standards. Furthermore, smart mushroom farming has the potential to transform the mushroom industry into a high-technology industry with high levels of mechanization and automation (Raut, 2019; Yin *et al.*, 2022).

Several studies have investigated different aspects of smart mushroom farming, which includes the application of Internet of Things (IoT), computer vision (CV), artificial intelligence (AI), machine learning (ML), and automation technologies to improve efficiency and decision-making processes. A recent investigation (Yin *et al.*, 2022) looked into how cutting-edge technologies are being used in the mushroom sector. It looked at several CV and ML technologies used for growing, collecting, and grading mushrooms. Using machine learning algorithms to classify mushrooms is a data-driven way that makes the process more accurate while minimising human work (Elewi *et al.*, 2024, Adebayo *et al.*, 2025, Singh *et al.*, 2025). Researchers have looked into using machine learning algorithms for a number of tasks, including finding mushrooms and measuring mushroom caps (Lu and Liaw, 2020), classifying the quality of mushroom spawn (Tongcham *et al.*, 2020), classifying the quality of shiitake mushrooms (Zhu *et al.*, 2023), and identifying the difference between poisonous and edible mushrooms (Rahman *et al.*, 2022). Paudel and Bhatta (2022) employed random

forest and representative (REP) tree classifiers for the classification of oyster mushroom growth.

Few studies in the literature have addressed the growth analysis and maturity detection of mushrooms. Lu *et al.* (2019) developed an IoT-based mushroom measurement system utilising YOLOv3 to identify button mushrooms in a controlled experimental setting, with the objective of quantifying mushrooms and estimating their cap sizes and optimal harvest times. Wang *et al.* (2022) conducted a similar study that created a version of YOLOv5 to accurately find and locate shiitake mushrooms using a web-based app. However, this study did not observe mushrooms growth or maturity. Moysiadis *et al.* (2023) conducted one of the most relevant research studies. The authors monitored the growth of oyster mushrooms and classified their growth stages using YOLOv5, achieving an accuracy of 70% with a dataset of 4,271 annotated mushrooms. They also used Detectron2 to show the size of mushrooms on their substrate grow bags. Based on another dataset of 453 annotated mushrooms, they figured that mushrooms grow at an average rate of 5.22 days. But their images were taken in a small mushroom cultivation greenhouse, and they do not clarify where they obtained their data. Sujatanagarjuna *et al.* (2023) conducted a related study that created a scalable growing and harvesting system called MushR, which serves as a digital twin for gourmet mushroom production. They used Mask R-CNN in Detectron2 to find out how mature oyster mushrooms were, and they achieved an average accuracy 80% for large ones using a dataset of 3300 annotated mushrooms. They created a prototype for a mushroom harvesting system and made all of the models, images, and datasets available to the public. They carried out their work in a basic mushroom growing setting, using plastic buckets or pods for the harvesting system prototype and classifying maturity for each mushroom cap instead of for whole mushroom nodes. This limits the practicality and verifiability of their proposed system. Another research presents an innovative deep learning-based instance segmentation tracking pipeline in order to continuously monitor the growth of oyster mushroom nodes in a series of images taken at various times. The findings indicated that Mask R-CNN utilising ConvNeXt as a backbone attained superior performance in mushroom node instance segmentation (mAP=0.876, recall=0.858, F1-score=0.867) and tracking (MOTA=0.967). In this study, only images were used for monitoring and growth tracking, but environmental values should also be taken into account for mushroom growth and monitoring (Charisis *et al.*, 2025).

This study introduces a unique method for detecting the maturity of oyster mushrooms by utilising a combination of environmental data and images to train diverse machine learning algorithms, providing practical applications that surpass conventional camera-based systems. By combining sensor environmental data with mushroom images, the presented method in this paper becomes more flexible and useful for more than just growth analysis and maturity detection. This integrated method could also be used in robotic systems to accurately

identify mushrooms, which would allow for damage-free harvesting and keep stable the quality of the mushrooms.

The main contributions of the study are:

- The development of a multisensory approach to determine oyster mushrooms maturity stage in greenhouse conditions by combining sensor environmental data with camera inputs. This makes the system more flexible and effective than traditional camera-based systems.

- Three distinct datasets of mushroom features were created and utilised for the classification of oyster mushroom maturity: one with manually extracted size features and two with automatically extracted size features using YOLOv8 and Detectron2 object detectors.

- Various classification algorithms, including Decision Trees (DT), Random Forest (RF), Multi-Layer Perceptron (MLP), Histogram-based Gradient Boosting Decision Trees (HistGBDT), and K-Nearest Neighbours (KNN), were trained, tested, and compared for maturity classification.

MATERIAL AND METHODS

This paper introduces a new multisensory approach for detecting the maturity of oyster mushrooms by utilising a combination of environmental data and images to train diverse machine learning algorithms. As shown in Figure 1, the methodology of the proposed system includes; building three new datasets which include the width, height, maturity classes of the mushroom nodes, as well as the temperature, humidity of the environments where the mushrooms are located, and the internal temperature of the mushroom bags at the same date and time. The difference between the datasets called manual and automatic; in the manual dataset, the height and width of the mushroom nodes are determined manually, and in the automatic datasets, these features are determined by the Detectron2 framework and YOLOv8 separately, by detecting the mushroom nodes automatically.

Data Processing and Preparation

Building upon a previously developed smart mushroom cultivation infrastructure (Elewi *et al.*, 2024), the present study focuses on multisensory maturity classification using combined visual and environmental features. Additionally, the environmental and visual data collected from multiple sensors and cameras were timestamped, compiled into a comprehensive dataset, and made publicly available (Duman *et al.* 2024). The dataset comprised 555 raw camera images from the greenhouse (see Figure 2). Getting only one image per day has the disadvantage that the growing days may not be counted precisely in some mushrooms (Moysiadis *et al.*, 2023). In this paper, this issue was addressed by capturing hourly images, which can introduce challenges in accurately tracking the growth of mushrooms.

For instance, in some cases, mushrooms may appear just a few hours after the image was taken, resulting in a shorter number of days of growth than the

actual growth time. Similarly, mushroom mushrooms may have been harvested shortly before the image was taken, resulting in an underestimation of the days of growth.

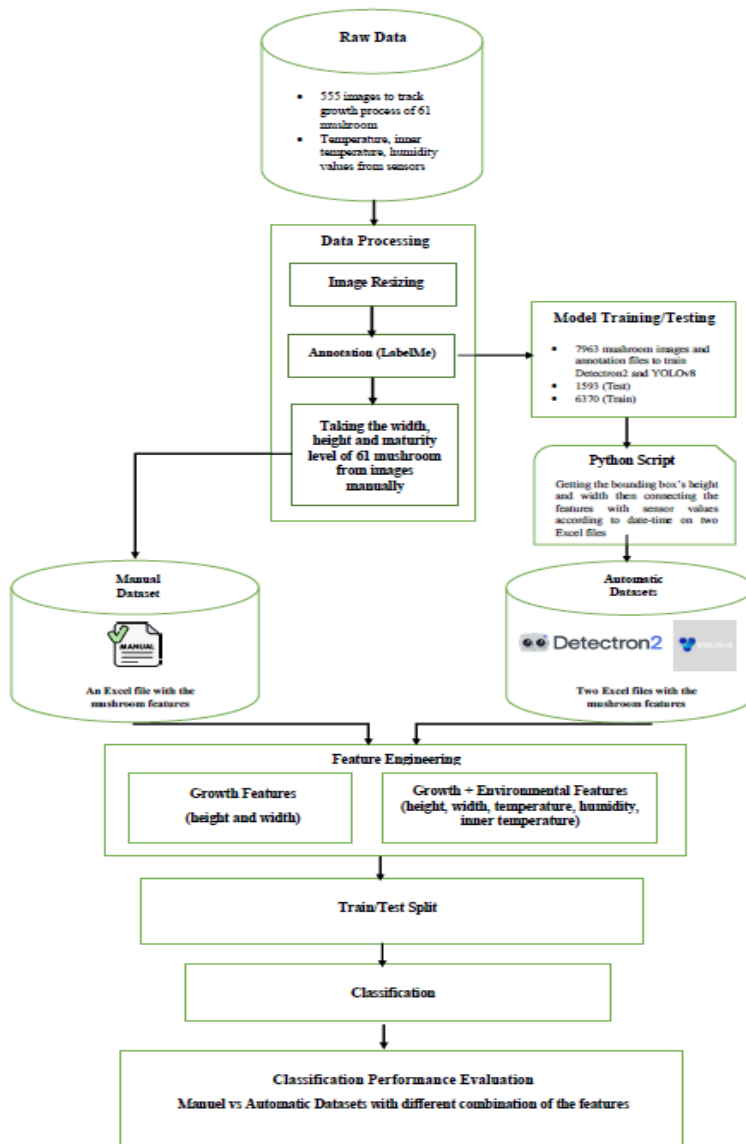


Figure 1. The Methodology of the Proposed Mushroom Maturity Classification System

Therefore, the importance was recognized of taking more frequent images of the substrate bags to yield improved results, offering a more precise assessment of mushroom growth rates and optimal harvesting times. After

collecting the raw images, then 8,282 mushroom node images were extracted and annotated as “mature” and “immature” using LabelMe software (Russell *et al.*, 2007), offering their annotation files in three different (JSON, TXT and XML) formats to be used for training of YOLOv8 and Detectron2 separately in this study (Duman *et al.* 2024).



Figure 2. Sample Camera Raw Image from the Greenhouse

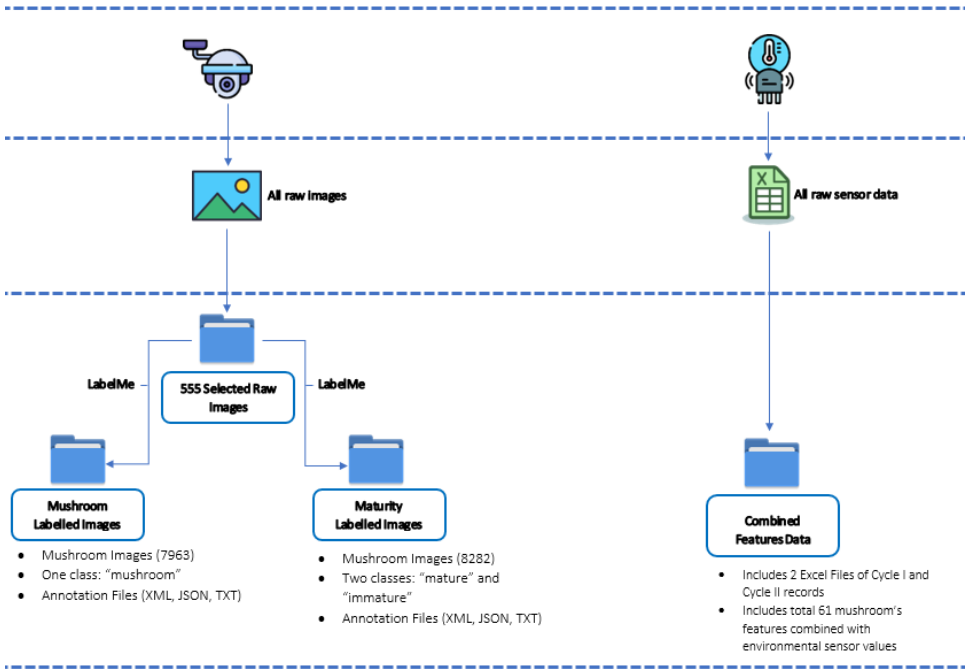


Figure 3. Data Collection Process

In sensor side, the data was collected from the smart mushroom farming system that designed and built with different types of cost-aware sensing nodes (Elewi *et al.*, 2024). Two cameras and a variety of sensors with different prices and functions were used to make it possible to monitor the farm remotely. The DHT21 sensor, which is relatively inexpensive and the SHT20 sensor, which is more reliable, were used to measure the temperature and humidity in the mushroom greenhouse. The CSS811 and SGP30 sensors were used to measure the air quality. The DS18B20 sensors were used to measure the temperature inside the mushroom composite bag. The data collection process of both images and sensor values can be shown as in Figure 3.

Manual Feature Extraction and Manual Dataset Building

In this phase, a manual dataset which includes size and environmental features of 61 mushrooms has built by using 1,703 images. Mushroom size features (width and height) were included manually from annotation files of these images, then a Python script connected these features to associated environmental features as humidity (hum), temperature (temp), and inner temperature (temp In) according to date/time. The main purpose here is to compare the maturity detection made using the manually extracted height and width features and the detection using automatically extracted height and width features via YOLOv8 and Detectron2 detectors together with the sensor data. This manual detection process serves as a valuable benchmark, allowing to compare and contrast the results obtained through the presented methodology with those produced by YOLOv8 and Detectron2. Figure 4 shows the manual extraction of a mushroom node from an image to get width and height of it.



Figure 4. Manual Extraction of a Mushroom Node from an Image

The immature class encompassed mushrooms from their initial appearance up to one day before harvesting, while the mature class included mushrooms that were fully mature and ready for harvesting. In the dataset mature class represented as 1 and immature class represented as 0 corresponding to their respective growth stages. The maturity labels in this study were established based on harvesting time, representing practical cultivation choices typically employed by cultivators rather than direct physiological assessments. This operational definition was selected because harvest readiness represents the primary real-world criterion for mushroom maturity in commercial production.

Table 1 shows a sample of the dataset that was produced manually.

Table 1. Sample view of the manual dataset (the height and width features taken from bounding boxes which drawn manually)

mushroom id	width	height	date-time	maturity	temp	hum	temp in
1	38	47	2023-01-15 01:09:23	0	90.1057	9.76035	10.11
1	41	39	2023-01-15 08:32:36	0	77.0878	11.0136	12.0086
....							
1	60	83	2023-01-17 04:23:02	1	92.2167	11.4935	12.2599
1	70	81	2023-01-17 08:28:19	1	90.5286	11.4191	11.9801

It is important to note that the dataset created manually was not used to train the model. Instead, it was only used as an independent benchmark dataset to compare the consistency of features extracted manually with those automatically extracted by the YOLOv8 and Detectron2 frameworks.

Automatic Dataset Building and Mushroom Detection with Detectron2

Detectron2 is an advanced platform which produced by Facebook AI Research (FAIR) for object detection and segmentation algorithms (Wu *et al.*, 2019). The Detectron2 framework's ability to handle different types of data and annotations, along with its impressive performance in object detection and segmentation tasks, makes it a valuable tool in the field of computer vision. It needs images and annotated databases that utilise the same format as the COCO dataset. Backbone is an important part of getting features from the input image with different architectures as ResNet, ResNeXt, and MobileNet. The Detectron2 framework is built upon convolutional neural networks, and the feature map of a single output for the convolution operation can be represented as Equation 1. Where the output feature map at position (i, j) for the k-th filter, x is input image, w is convolution filter, M and N are height and width of the filter.

$$y_{i,j,k} = \sum_{m=1}^M \sum_{n=1}^N x_{i+m-1,j+n-1} \cdot w_{m,n,k} \quad (1)$$

In this study, after the dataset preparation and training pipeline configuration, the custom oyster mushroom dataset which includes 8,282 images were used to train the Detectron2 framework. The trained Detectron2 was tested on 1703 images to get essential features (mushroom height, width and maturity class from images) automatically. The Detectron2 framework detected mushrooms by drawing bounding boxes around them (see Figure 5). The features were extracted and added to dataset by a Python script that takes the width, height and maturity class of the mushroom node.

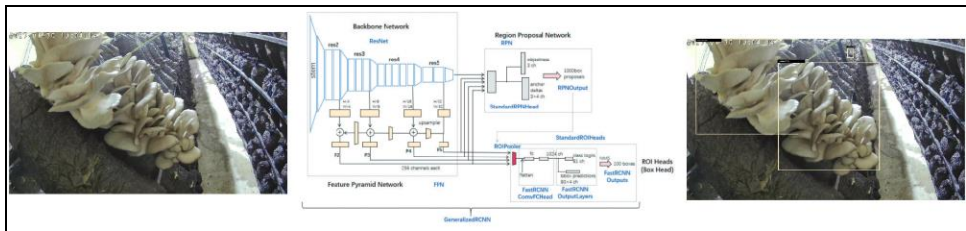


Figure 5. Detectron2 Architecture and Detection on an Image (Modified from Wu *et al.*, 2019)

The testing performance of the Detectron2 (model: Faster RCNN-50) on mushroom detection was evaluated by using some evaluation metrics. The Detectron2 framework provides a range of metrics to evaluate object detection performance. By considering precision at various recall levels and focusing on objects of different sizes, these metrics provide a comprehensive evaluation of the model's performance across different scenarios. These metrics (Zhang and Zhang, 2009); AP75, Average Precision at 75% overlap, which assesses precision at various recall levels by considering a detection as true positive (TP) if the Intersection over Union (IoU) overlap is at least 75%. AP_L, Average Precision (large), focusing on precision at different recall levels for objects with large sizes (area>962). AP_M, Average Precision (medium), measuring precision at different recall levels for objects with medium sizes (322<area<962). AP_S, Average Precision (small), which calculates precision at different recall levels for objects with small sizes (area<322).

The Faster RCNN-50 that was used as Detectron2 model to detect mushroom nodes performs a successful detection with an AP75 score of 43.4, AP_L 51.2, AP_M 43, AP_S 21.1. These metrics show that the model performs best on large-sized mushroom nodes, with moderate success on medium ones, but struggles with small nodes. The height, width and maturity features values were added to the other dataset to compare results with the manual extraction. Table 2 shows the sample view of the dataset that was created automatically by getting width, height and maturity features from Detectron2.

Table 2. Sample view of the automatic dataset (the height, width and maturity features taken from bounding boxes which drawn by Detectron2)

mushroom id	width	height	date-time	maturity	temp	hum	temp in
18	213	206	2023-04-07 15:26:44	0	15.6588	99.11502	16.36695
18	233	226	2023-04-07 18:25:57	0	15.51356	99.37797	16.69209
....							
18	733	729	2023-04-11 15:15:20	1	17.09244 4	100	17.995555
18	748	734	2023-04-11 17:30:53	1	16.38518 5	100	17.818518

Automatic Dataset Building and Mushroom Detection with YOLOv8

In January 2023, Ultralytics introduced their latest YOLO model, YOLOv8, within the Ultralytics repository (Jocher *et al.*, 2023). In addition to pre-trained models, YOLOv8 enables users to create custom models adapted for their particular object detection requirements. In this process, users select and label the desirable object types in their training dataset, which is a component of data preparation. Training a custom model enhances accuracy and precision for object detection tasks specific to the user's application domain.

This work involved training YOLOv8 using a custom mushroom dataset. The dataset descriptor file's path was designated, outlining the dataset's location and format, and the "train" method was employed to train the YOLOv8 model. After training the YOLOv8 model, it was executed to classify mushrooms by drawing bounding boxes around them. Bounding boxes are crucial in this study as they mark the size of mushroom within the images. Figure 6 shows the YOLOv8 architecture by applying it on mushroom images.

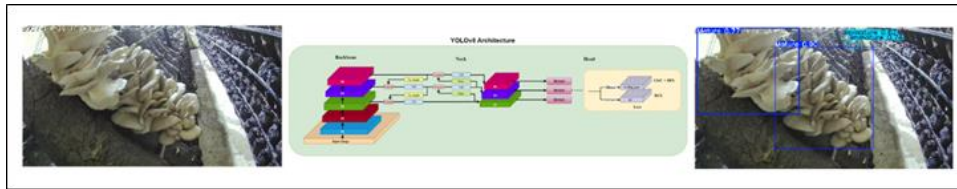


Figure 6. YOLOv8 Architecture and Detection on an Image (Modified from Jocher *et al.*, 2023)

Table 3. Sample view of the automatic dataset (the height, width and maturity features taken from bounding boxes which drawn by YOLOv8)

mushroom id	width	height	date-time	maturity	temp	hum	temp in
18	213	205	2023-04-07 15:26:44	0	15.6588	99.11502	16.36695
18	234	226	2023-04-07 18:25:57	0	15.51356	99.37797	16.69209
....							
18	748	734	2023-04-11 17:30:53	1	16.38518519	100	17.81851852
18	757	739	2023-04-11 21:15:54	1	15.56327434	100	17.2079646

YOLOv8 model provided accurate classification with 81.9% mean average precision (mAP) value at IoU 0.5, this indicates high detection accuracy when evaluated with a moderate overlap threshold. Also, the model achieved a strong detection performance, with a mAP@(0.5:0.95) of 56.7%, reflecting its consistent performance across stricter IoU thresholds. The results demonstrated that the model shows reliability not just at moderate overlap thresholds but also under harder evaluation settings.

The higher mAP@0.5 indicates better bounding box localization and object confidence, making YOLOv8 highly appropriate for real-time mushroom node detection where precision and speed are important. Table 3 presents a sample representation of the height, width, and maturity features extracted from bounding boxes created by YOLOv8.

Classification of Mushroom Maturity Class by Adding Environmental Features with Machine Learning Algorithms

In this study five different machine learning algorithms which include the Decision Tree (DT), Hist Gradient Boosting Classifier (HistGBDT), K-nearest Neighbor (KNN), Multilayer Perceptron (MLP), and Random Forest (RF) were used for classification. The Decision Tree (DT) is an algorithm which based on a tree-like data structure. The internal nodes in a Decision Tree show the dataset's attributes, the branches show the decision criteria, and the leaf nodes show the final outcomes. This tree structure can efficiently encode Boolean values, covering both true and false states (Fratello and Tagliaferri, 2019). K Nearest Neighbour (KNN) is a machine learning algorithm works by comparing the new data to the old data to see how similar they are. It puts the new data into the category that is most similar to the ones that are already there (Sun and Huang, 2010). The Hist Gradient Boosting Classifier (HistGBDT) is a version of the standard gradient boosting decision tree (GBDT) method, designed to accelerate the learning process and enhance the model's predictive performance (Tamim Kashifi and Ahmad, 2022). It comes from applying gradient boosting to decision trees and using a better base learner called the piecewise linear tree, which uses linear functions to make predictions at the leaves. This method improves the weak classifier's loss function by using a learning rate, which decreases the loss for the next classifier. The Multilayer Perceptron (MLP) classifier is an important part of neural networks because it has a multi-layered structure. The hidden layers of a neural network give it the ability to learn complex patterns and representations in data. This allows it capture detailed features and relationships in the incoming data (Rumelhart *et al.*, 1986). Random Forest (RF) is a machine learning technique used for classification and regression. Because it uses more than one decision tree model, it is often called an ensemble classifier. This classifier combines several decision trees made from different parts of the dataset and averages their results to make predictions more accurate (Breiman, 2001).

As mentioned in the previous sections, the three different datasets were created as one manual and two automatic datasets. These datasets were used to classify the mushroom maturity class by the height, width, humidity, temperature and inner temperature features. Model evaluation was performed using a consistent randomized train–test split while preserving class balance across maturity categories. The same data partitioning strategy was applied to all classifiers and datasets to ensure fair comparison. This procedure was repeated 200 times using different random seeds to reduce sampling bias and improve robustness of performance estimation. Classification metrics were computed for

each iteration, and the final reported results correspond to the mean performance values across all repetitions. The algorithms that were used to make classification were evaluated with precision, recall, F-1 Score, and accuracy metrics which are key metrics for evaluating classification models. Precision as shown in Equation 2 is the ratio of true positives(TP) to true positives plus false positives(FP), showing how few false positives the model makes (Ferrer, 2022). Recall (sensitivity) is the ratio of true positives to true positives plus false negatives(FN), reflecting how well the model identifies actual positives. Equation 3 represents the calculation formula of the recall (Ferrer, 2022). Accuracy as shown in Equation 4 is the ratio of true positives plus true negatives(TN) to total instances, indicating overall prediction correctness (Ferrer, 2022). F-1 Score which represented in Equation 5 is the harmonic mean of precision and recall, providing a balanced measure between them (Ferrer, 2022).

$$Precision = \frac{TP}{TP+FP} \quad (2)$$

$$Recall = \frac{TP}{TP+FN} \quad (3)$$

$$Accuracy = \frac{TP+TN}{TP+TN+FP+FN} \quad (4)$$

$$F1\ Score = 2 \times \frac{Precision \times Recall}{Precision+Recall} \quad (5)$$

The classification algorithms were applied on the datasets separately and evaluated with the mentioned evaluation metrics. The results will be discussed in the following section.

RESULTS AND DISCUSSION

The main purpose of the study is to classify the maturity class of mushroom nodes by using these three datasets separately. After the classification, the results between manual and automatic datasets which includes the features detected by Detectron2 and YOLOv8 were compared. During the classification, environment values were included in mushroom node features with different combinations. One of the classification algorithms used is the HistGBT classifier. With this classifier, maturity classes were first tried to be classified by using only the height and width of the mushroom nodes. Afterwards, temperature and humidity values, which are the most important environmental values, were included among the features and reclassified. As seen in Table 4 and 5 the HistGBT algorithm, which works with an accuracy rate of 81% on the manual dataset, increases the success rate to 92.8% when these two features are added. Then, the inner temperature value of the bags was added as the last feature. As seen in Table 6, this feature reduced the classification success of the algorithm. The reduction in classification performance following the inclusion of inner bag temperature as a feature can be explained through various supplementary hypotheses. First, measuring the temperature inside the bags could add noise to the sensors because the micro-environment inside the bags changes a lot and the

sensors are sensitive to where they are placed. Unlike sensors that measure the environment around them, sensors that measure the inside of an object may give very different readings even at the same growth stage. This makes it harder for machine learning models to get consistent signals. Second, the inner temperature information may not be very useful because it is already included in the feature set along with the temperature and humidity of the surrounding area.

Table 4. Evaluation results of the mushroom maturity classification of the Manual and Automatic Datasets (Note. Using only width and height features of mushroom)

Classifier	Accuracy	Precision	Recall	F1 Score
HistGBDT (Manual)	0.84	0.84	0.84	0.84
HistGBDT (Detectron2)	0.83	0.82	0.82	0.82
HistGBDT (YOLOv8)	0.83	0.82	0.84	0.83
RF (Manual)	0.83	0.83	0.83	0.83
RF (Detectron2)	0.81	0.80	0.80	0.80
RF (YOLOv8)	0.82	0.83	0.82	0.82
DT (Manual)	0.82	0.83	0.82	0.82
DT (Detectron2)	0.80	0.81	0.81	0.82
DT (YOLOv8)	0.82	0.82	0.82	0.82
KNN (Manual)	0.81	0.79	0.81	0.80
KNN (Detectron2)	0.80	0.79	0.80	0.79
KNN (YOLOv8)	0.81	0.79	0.81	0.80
MLP (Manual)	0.83	0.82	0.83	0.83
MLP (Detectron2)	0.81	0.81	0.80	0.80
MLP (YOLOv8)	0.82	0.82	0.83	0.82

Random Forest classifier was used as another classification algorithm. As seen in Table 4, the random forest classifier's success in classifying mushroom maturity using only the width and height of mushroom nodes has an accuracy rate of 83% in the manual dataset and 81% in the Detectron2 automatic dataset and 82% in the YOLOv8 automatic dataset. Afterwards, reclassification was made with adding temperature and humidity features from the environmental values which increase the success of the algorithm (see Table 5). Then, the inner temperature feature was added to these features, the classification success

decreased to a certain extent (see Table 6). Decision tree classifier, which is one of the tree classifier models, was used as another classification algorithm.

Similarly, the success increased when temperature and humidity features were added to the environmental values (see Table 5). However, the inner temperature value again reduced the model success (see Table 6).

Table 5. Evaluation results of the mushroom maturity classification of the Manual and Automatic Datasets (Note. Using width, height, temperature and humidity features of mushroom)

Classifier	Accuracy	Precision	Recall	F1 Score
HistGBDT (Manual)	0.92	0.91	0.91	0.91
HistGBDT (Detectron2)	0.91	0.90	0.90	0.90
HistGBDT (YOLOv8)	0.91	0.91	0.91	0.91
RF (Manual)	0.88	0.87	0.87	0.87
RF (Detectron2)	0.85	0.84	0.85	0.85
RF (YOLOv8)	0.87	0.86	0.86	0.86
DT (Manual)	0.83	0.83	0.83	0.83
DT (Detectron2)	0.82	0.82	0.82	0.82
DT (YOLOv8)	0.83	0.82	0.82	0.82
KNN (Manual)	0.84	0.83	0.83	0.83
KNN (Detectron2)	0.82	0.82	0.82	0.82
KNN (YOLOv8)	0.83	0.82	0.82	0.82
MLP (Manual)	0.85	0.83	0.85	0.84
MLP (Detectron2)	0.83	0.82	0.83	0.82
MLP (YOLOv8)	0.84	0.83	0.85	0.84

Unlike other algorithms, KNN gave more successful results when all features were used. A possible reason of this that KNN relies heavily on the similarity of instances. KNN may perform well in classification when the features exhibit significant relevance in proximity or similarity. Alternative classifiers such as Random Forest, Decision Trees, and Gradient Boosting may not properly detect this resemblance, particularly when the interactions between features and the target variable are complex. Finally, MLP classifier, an artificial neural

network-based algorithm, was used as a classifier. The success of the MLP classifier is given in the Table 4, 5, and 6. Unlike KNN, MLP also produced a similar result to the other three classifiers.

Table 6. Evaluation results of the mushroom maturity classification of the Manual and Automatic Datasets (Using width, height, temperature, inner temperature and humidity features of mushroom)

Classifier	Accuracy	Precision	Recall	F1 Score
HistGBDT (Manual)	0.88	0.87	0.88	0.87
HistGBDT (Detectron2)	0.87	0.86	0.86	0.86
HistGBDT (YOLOv8)	0.87	0.87	0.86	0.86
RF (Manual)	0.87	0.86	0.87	0.86
RF (Detectron2)	0.85	0.83	0.83	0.83
RF (YOLOv8)	0.87	0.86	0.86	0.86
DT (Manual)	0.82	0.82	0.81	0.82
DT (Detectron2)	0.79	0.80	0.79	0.79
DT (YOLOv8)	0.82	0.82	0.81	0.82
KNN (Manual)	0.86	0.85	0.85	0.85
KNN (Detectron2)	0.84	0.84	0.83	0.84
KNN (YOLOv8)	0.85	0.85	0.84	0.85
MLP (Manual)	0.84	0.84	0.83	0.84
MLP (Detectron2)	0.82	0.82	0.81	0.82
MLP (YOLOv8)	0.82	0.82	0.82	0.82

According to the results, the HistGBDT classifier showed higher success than the other four classifiers. This can be explained as it adeptly captures complex non-linear relationships between features and the target variable through its sequential ensemble of decision trees, correcting errors iteratively. This capability enables it to model intricate decision boundaries better than RF or DT. When the results obtained are examined, it can be said that the environmental temperature and humidity, as well as the height and width features of the mushroom node, are important in classifying the maturity class of a mushroom node.

CONCLUSIONS

This study introduced a comprehensive approach that aims to automate the classification of mushroom nodes using different features. By creating three different datasets containing features extracted by human and detected by Detectron2, YOLOv8 object recognition frameworks, it was examined how useful these datasets were for mushroom maturity classification. The manual dataset consisted of 61 mushroom instances and was intentionally designed as an evaluation benchmark rather than a training dataset. Therefore, its limited size does not affect model learning capacity but may constrain the statistical representativeness of human–automatic feature comparisons. In addition, it was revealed how the classification success changes when environmental temperature, humidity and inner temperature values are added as features which greatly affect mushroom growth processes. Compared with all classifiers, the overall classification success of the HistGBDT classifier was high on manual and automatic datasets. Also, the results show that especially temperature and humidity values significantly increased the classification success. This indicates that environmental factors also have a high impact on maturity classification. In addition to this, it was observed that the classification results using the automatic datasets are very close to the classification made with the manual dataset. This shows that YOLOv8 and Detectron2 are successful in object detection and bounding box drawing.

In conclusion, this proposed multisensory approach for detecting mushroom maturity class in greenhouses, offering practical applications that transcend conventional camera-based systems. By integrating sensor data alongside cameras, the approach enhances its adaptability and efficiency. Future work may incorporate physiological maturity indicators to evaluate cross-greenhouse transferability and reduce potential temporal bias. A notable application lies in yield prediction, where the relation between cameras and sensor data enables close monitoring of greenhouse conditions, enabling yield forecasts days prior to harvest. Moreover, this integrated methodology can be integrated into robotics systems to accurately locate mushroom nodes, thereby facilitating damage-free harvesting and ensuring the preservation of mushroom quality in the future.

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INVESTIGATION OF BIRTH TYPE IN AWASSI SHEEP AND THEIR CORRELATION TO REPRODUCTIVE HORMONES AND OXIDATIVE/ANTIOXIDANT INDICES

SUMMARY

Many factors affect reproductive performance, including litter size, season, nutrition, management, oxidative stress, and genetics. Therefore, this study investigated the relationship between number of lambs and oxidative status in Awassi ewes. The present study was conducted between July and September 2022 and included 130 sexually mature, healthy Awassi ewes (57 singletons and 73 twins). The ewes weighed 45-50 kg and were between 3 and 4 years old. The blood samples (5 mL) were collected from the sheep's jugular vein in the one day of birth and continued until the weaning period and were centrifuged for 15 minutes at 2,000 g to separate the serum, which was subsequently stored at -20°C for hormone analysis. Hormonal analysis was performed for measurement of estradiol, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH). Statistical analysis were employing the general linear model and correlation was analyzed using the Pearson correlation coefficient, and significance was set at $P \leq 0.05$. The results revealed that Awassi ewes at birth and weaning showed a significant higher levels ($P \leq 0.05$) in estrogen (1.37 ± 0.10 and 1.25 ± 0.16 (pg/ml)), progesterone (0.68 ± 0.07 (ng/ml)), FSH (1.13 ± 0.11 and 0.96 ± 0.12 (ng/ml)), LH (0.89 ± 0.12 (ng/ml)), SOD (0.61 ± 0.06 (nmol/L)), glutathione (0.56 ± 0.07 (nmol/L)), and MDA (0.63 ± 0.12 (nmol/L)) in twins birth type. The highest correlations between reproductive hormones and oxidative/antioxidant indices were observed at birth and weaning in Awassi ewes. In conclusion, Awassi ewes exhibit a significant difference in concentrations of reproductive hormones and antioxidant levels at birth and weaning.

Keywords: Antioxidant, Awassi ewes, litter size, pregnancy, reproductive hormones, weaning

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INTRODUCTION

A sheep is a small ruminant that significantly contributes to the protein content of human diets worldwide (Al-Thuwaini and Al-Hadi 2022; Kadhem and Al-Thuwaini 2022). Higher ewe productivity and reproductive performance are profitable for sheep producers (Habtegiorgis *et al.* 2022; Mohammed *et al.* 2022). Reproductive performance is influenced by many factors, including litter size, season, nutrition, management, and genetics (Ajafar *et al.* 2022). Moreover, the interaction between reproduction and oxidative stress affects pregnancy in goats (Celi *et al.* 2010) and sheep (Soriano *et al.* 2015). Oxidative stress happens when the body cannot neutralize the excessive production of pro-oxidants (free radicals) through antioxidant defenses (Lykkesfeldt and Svendsen 2007). The antioxidant defense mechanisms in aerobic biological systems neutralize or minimize the damaging effects of free radicals and reactive oxygen species (Majlesi *et al.* 2021). Catalase, superoxide dismutase (SOD), and glutathione peroxidase (GSH) enzymes are the most studied antioxidants in sheep during pregnancy (Soriano *et al.* 2015).

During pregnancy, changes in the body make mothers more susceptible to oxidative stress (Aköz *et al.* 2017). Physiological regulation in the uterus during pregnancy involves the increased generation of reactive oxygen species (ROS) linked to oocyte maturation, luteolysis, and embryo implantation, all occurring during pregnancy. Placental functions are negatively affected by oxidative stress, which can lead to an adverse pregnancy outcome (Mutinati *et al.* 2013). Oxidative stress may also develop in late pregnancy due to a negative energy balance (Nawito *et al.* 2016). Lipid peroxidation may increase, while antioxidant activity may decrease, contributing to complications (Rejitha and Karthiayini 2014). The peroxidation of lipids could produce various aldehydes, including MDA (Ayala *et al.* 2014). Elevated oxidative stress with increased metabolic demands during pregnancy and lactation correlates with higher blood levels of MDA, which may increase the risk of ewes and lambs mortality (Bhale *et al.* 2014; Fassah *et al.* 2015). Both ewes and lambs are likely to be affected by oxidative stress during pregnancy (Mutinati *et al.* 2013). Consequently, overall antioxidant status prevent reactive oxygen species concentrations from reaching harmful levels that can cause oxidative damage to follicles and oocytes (Gonzalez-Maldonado *et al.* 2018). Antioxidants may also improve endometrial receptivity, decrease levels of ROS, and relieve uterine inflammation (Zheng *et al.* 2022). An experiment was conducted to evaluate the effect of antioxidants on goat reproductive performance in early pregnancy (Fu *et al.* 2022).

Based on the above literature, no publications have investigated the association between oxidative status and litter size in Awassi ewes. The research was conducted to determine the association between the number of lambs born in one birth (twins and singletons) and oxidative status in Awassi ewes, emphasizing the significant role of the oxidative factor on reproductive performance in Awassi ewes.

MATERIAL AND METHODS

Sheep population and phenotypic measurements

An ethics committee approved the study at Al-Qasim Green University in July and September 2022 (Agri, No. 01, 7, 22). This study involved 130 Awassi ewes (57 singletons and 73 twins) that were sexually mature and healthy. The ewes weighed 45-50 kg and were between 3 and 4 years old. The study involved ewes in their fifth month of pregnancy, which gave birth between October and early November. On day 40 of gestation, ultrasounds were used to identify whether the ewe was carrying a single fetus or twins. A real-time B-mode ultrasound scanner equipped with a 3.5 MHz probe (EDAN DUS 6 Vet, China) was used to diagnose pregnancy. Food and water were withheld for 12 hours overnight before scanning early in the morning. Scanning was performed on the fleece-free inguinal region of the animal. The animals were fed 2.5% of their body weight in a grain concentrate consisting of 59% barley, 40% bran, and 1% salt at the Karbala station. Each animal also consumed three kilograms of alfalfa and one kilogram of straw daily, along with grazing once per day. Fresh water was available to all animals at all times. Their veterinary care included vaccination against foot-and-mouth disease, ivermectin treatment to combat parasites, and periodic treatments (Bayraktar *et al.* 2024).

Five milliliters of blood were collected by puncturing the sheep's jugular vein with an 18-gauge disposable needle. The blood samples were drawn at two time points postpartum (three times within a month) before the morning meal administration, and at three time points within a month after weaning the lambs. Hormone level measurements were conducted after centrifuging the blood for 15 minutes at 2,000 g and storing the serum at -20°C. An ELISA kits provided by Biotech Co., Ltd. (Shanghai, Korain) was used to measure the estradiol, progesterone, FSH, LH, SOD, MDA, and GSH (EA0016sh, EA0004sh, E0166sh, E0106sh, E0119Sh, E0039sh, and EA0011sh, respectively). For assessing the precision of an ELISA kit, it was important to consider the coefficients of variation, which were 8% for inter-assay precision and 10% for intra-assay precision (Kinn Rød *et al.* 2017).

Data analysis

This study was conducted using SPSS version 23.0, employing the general linear model:

$$Y_{ij} = \mu + L_i + e_{ij}$$

Y_{ij} = characteristics phenotypically, μ = overall mean, L_i = fixed effect of i th birth type ($i=1, 2$), and e_{ij} = random error associated with Y_{ij} observation and assumed to be NID (0, σ^2_e). A significant difference was determined by the Tukey-Kramer test at the (0.05, 0.01) level. Pearson's correlation coefficient (r) was calculated to quantify the degree of association among various phenotypic traits, indicating the extent to which two traits vary together, with significance set

at $P \leq 0.05$. Statistical analyses of preliminary data suggest that seasonality, live body weight, parity, and factor interactions do not significantly affect phenotypic traits. Therefore, these variables were excluded from the general linear model.

RESULTS AND DISCUSSION

The effect of birth type on the concentrations of reproductive hormones and oxidative/antioxidant indices for Awassi ewes is shown in Table 1. We observed a significant higher levels ($P \leq 0.01$) in twins' birth type for FSH level at birth (1.13 ± 0.11 (ng/ml), and in estrogen (1.25 ± 0.16 (pg/ml)), progesterone (0.68 ± 0.07 (ng/ml)), MDA (0.63 ± 0.12 (nmol/L)), and glutathione (0.56 ± 0.07 (nmol/L)) at weaning. At the same time, there were significant higher levels ($P \leq 0.05$) in estrogen (1.37 ± 0.10 (pg/ml)) and SOD (0.61 ± 0.06 (nmol/L)) at birth, and at weaning in FSH (0.96 ± 0.12 (ng/ml)) and LH (0.89 ± 0.12 (ng/ml)) in twin birth type. Whereas no significant difference was observed ($P \geq 0.05$) in other indices.

Table 1. Effect of birth type on reproductive hormone and oxidative/antioxidant indices in Awassi ewes

Indices	LSM $\pm$ SE		P-value
	Twins	Single	
Estrogen at birth (pg/ml)	1.37 ± 0.10	1.13 ± 0.12	0.04
Progesterone at birth (ng/ml)	0.67 ± 0.04	0.64 ± 0.09	0.41
FSH at birth (ng/ml)	1.13 ± 0.11	0.85 ± 0.05	0.001
LH at birth (ng/ml)	0.94 ± 0.08	0.81 ± 0.09	0.53
MDA at birth (nmol/L)	0.51 ± 0.04	0.41 ± 0.03	0.15
SOD at birth (nmol/L)	0.61 ± 0.06	0.47 ± 0.02	0.04
Glutathione at birth (nmol/L)	0.58 ± 0.05	0.51 ± 0.07	0.23
Estrogen at weaning (pg/ml)	1.25 ± 0.16	0.92 ± 0.11	0.003
Progesterone at weaning (ng/ml)	0.68 ± 0.07	0.47 ± 0.03	0.001
FSH at weaning (ng/ml)	0.96 ± 0.12	0.73 ± 0.11	0.02
LH at weaning (ng/ml)	0.89 ± 0.12	0.68 ± 0.11	0.01
MDA at weaning (nmol/L)	0.63 ± 0.12	0.31 ± 0.06	0.003
SOD at weaning (nmol/L)	0.62 ± 0.11	0.50 ± 0.08	0.35
Glutathione at weaning (nmol/L)	0.56 ± 0.07	0.39 ± 0.03	0.001

LSM $\pm$ SE, Least square means $\pm$ Standard error. The P value with statistical significance is indicated in bold numbers. FSH, follicle-stimulating hormone; LH, luteinizing hormone; MDA, malondialdehyde; SOD, superoxide dismutase.

As shown in Table 2, the estimation of the correlation coefficient between reproductive hormones and oxidative/antioxidant indices at birth and weaning was recorded in Awassi ewes. At birth, there was a significant ($P \leq 0.01$) positive

correlation coefficient between estrogen and progesterone with glutathione, and between FSH and LH with MDA and SOD. On the other hand, estrogen, FSH, and LH had significant correlation ($P \leq 0.05$) with MDA, SOD, and glutathione at weaning. Whereas progesterone level had significant ($P \leq 0.05$) correlation with glutathione and MDA.

Table 2. Correlation coefficients between reproductive hormones and oxidative/antioxidant indices at birth and weaning

	Variables	MDA (nmol/L)		SOD (nmol/L)		Glutathione (nmol/L)	
		r	P-value	r	P-value	r	P-value
Birth	Estrogen (pg/ml)	0.23	0.45	0.13	0.16	0.82	0.002**
	Progesterone (ng/ml)	0.06	0.21	-0.04	0.62	0.88	0.001**
	FSH (ng/ml)	0.73	0.002**	0.79	0.001**	0.26	0.18
	LH (ng/ml)	0.65	0.006**	0.75	0.001**	0.16	0.43
Weaning	Estrogen (pg/ml)	0.39	0.001**	0.38	0.004**	0.81	0.003**
	Progesterone (ng/ml)	0.30	0.04*	0.21	0.37	0.93	0.001**
	FSH (ng/ml)	0.85	0.001**	0.91	0.001**	0.31	0.04*
	LH (ng/ml)	0.79	0.006**	0.89	0.003**	0.28	0.004**

*($P \leq 0.05$), **($P \leq 0.01$), the P value with statistical significance is indicated in bold numbers. FSH, follicle-stimulating hormone; LH, luteinizing hormone; MDA, malondialdehyde; SOD, superoxide dismutase.

DISCUSSION

Awassi sheep are shown in Table 1 exhibit different reproductive hormones and oxidative/antioxidant indices depending on their litter size. Animal reproductive performance is strongly influenced by litter size (Nowak *et al.* 2020). Ewes capable of producing larger litter sizes tend to have better reproductive performance (Atoui *et al.* 2018). Among the most important economic traits in sheep production is litter size, which is influenced by reproductive hormones (Tesema *et al.* 2020). Additionally, the reproductive hormone of livestock can be used to assess reproductive performance (El Tahawy and Sharkawy 2014). The development and maturation of ovarian follicles are influenced by elevated reproductive hormone levels, leading to larger litter sizes (Luo *et al.* 2018). The results of this study align with the findings of Mohammed *et al.* (2022), which indicated that Awassi ewes with twin lambs exhibited higher levels of sex hormones compared to those with a single lamb. This is also consistent with the research conducted by Kartika *et al.* (2025), which found that Boer goats with multiple births had elevated levels of FSH and LH hormones.

Regarding oxidative status, oxidative stress plays a crucial role in key reproductive functions, including follicular development, oocyte maturation, ovulation, fertilization, and embryo implantation and development (Hardy *et al.* 2021). Spirulina supplementation at 15 g per ewe per day improved the oxidative status of the ewes' bodies and milk (Christodoulou *et al.* 2023). Dietary supplementation with Res enhanced the quality of colostrum in ewes and the growth of lambs, stimulated ovarian steroidogenesis, and improved antioxidant and immune indices across various reproductive stages (Li *et al.* 2024). According to Abd El-Hameed *et al.* (2018), multiple-bearing ewes and does exhibit higher malondialdehyde levels compared to singletons and non-pregnant animals. A study by Abdel-Ghani *et al.* (2016) concluded that elevated MDA levels indicate increased lipid peroxidation, particularly in animals with multiple births. Multiple-bearing animals also showed a significant increase in MDA levels alongside a decrease in total antioxidant activity, superoxide dismutase, and catalase activity, suggesting they may have experienced oxidative stress and lipid peroxidation (Al-Qudah, 2011). Twin pregnancies generate more reactive oxygen species than singleton pregnancies due to increased oxygen demand and heightened lipid peroxidation during gestation, combined with insufficient antioxidant defenses (Abd El-Hameed *et al.* 2018). Oxidative stress in the ovaries restricts the maturation of follicles and oocytes, resulting in fewer and less competent oocytes, as well as abnormally increased levels of follicle-stimulating hormone (Ra *et al.* 2023). Furthermore, higher ROS levels in follicular fluid can cause DNA damage, disruption of cellular scaffolding, and cell membrane damage, leading to abnormal oocyte development and infertility (Chen *et al.* 2023). These findings align with the research conducted by Lee *et al.* (2023) and Lee *et al.* (2024), which indicates that larger litter sizes elevate oxidative stress in sows during late gestation and lactation. However, no publications have examined the effects of reproductive hormones and oxidative/ antioxidant indices on litter size in Awassi ewes.

In terms of correlation analysis, the estimation of the correlation coefficient between hormones and oxidative/antioxidant indices at birth and weaning in Awassi ewes showed significant differences. The result of the current study agreed with the previous study results that explain the close relationship between estrogen and progesterone with the activity of the antioxidant enzymes (SOD, CAT, glutathione peroxidase (GSH-px), glutathione transferase (GSH-tr) and glutathione (GSH)) of cows (Khudhair *et al.* 2021). Additionally, correlations between reproductive hormones and antioxidants revealed positive relationships found between FSH and GSH (0.88; $P < 0.05$), and E2 and GSH (0.90; $P = 0.04$) in camel females (Zeitoun *et al.* 2022). In contrast, the correlation analysis of study by Cetin *et al.* (2021) indicated that there were no positive or negative correlations between hormones (P4 or E2) and oxidants (MDA) or antioxidants (SOD, GSH-Px, and total GSH activity) during different phases of pregnancy in hairy goats.

Although this study is the first to demonstrate a relationship between lambs born to ewes and oxidative status, it has several limitations. Oxidative status levels were not measured during early pregnancy or lactation, which restricts the scope of the findings. Additionally, various factors such as environmental stressors, feed quality, and genetic variation within the sample population may have influenced the results. The small sample size further limits the generalizability of the conclusions. Therefore, further research involving a larger population of Awassi ewes is necessary to better understand the relationship between oxidative status and litter size.

CONCLUSIONS

Awassi ewes bearing twins are subject to higher oxidative stress and hormonal fluctuations compared to those with single births. The study highlights the importance of antioxidant support during pregnancy, particularly in multiple-bearing ewes, to reduce oxidative damage and support better reproductive outcomes. Providing antioxidant supplements can help mitigate these effects by reducing oxidative stress and supporting hormonal balance. This approach may improve fetal development, increase lamb survival rates, and enhance the overall health of the ewes. Additionally, monitoring stress levels and adjusting nutrition can further optimize reproductive performance in Awassi sheep.

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BIOLOGICAL CHARACTERISTICS AND ECONOMIC PRODUCTIVE POTENTIAL OF FORAGE PEA GENOTYPES

SUMMARY

The biological characteristics and productive potential of forage pea genotypes were evaluated under field conditions. Seven genotypes were tested, including five hybrid lines (№115, №29, №9A, №8, and №11) and two control varieties, Kristal and Kerpo. Biometric analyses were performed on the main quantitative traits and phenological stages. Through multiple individual selection within a hybrid population obtained from the cross between Harkovskii Etalon and Harkovskii 376 (LN29), the new spring forage pea variety Ivkos was developed. The vegetation period ranged from 78 to 90 days, similar to that of the control variety Kerpo. The plants were medium tall (average height 74 cm), well-leafy, and resistant to lodging, with 6–8 pods and about 21 seeds per plant. The 1000-seed weight averaged 209.9 g. The variety demonstrated very good drought tolerance, stable yields, and a high productive potential. In competitive variety trials at the Institute of Forage Crops, Pleven, Ivkos (№29) produced 338 kg/ha grain, 20% higher than Kerpo. In 2022, by decision of the Expert Commission at the Executive Agency for Variety Testing, Field Inspection and Seed Control, Bulgaria (appointed by Order RD-10-1/14.01.2022) and Order RD-12-1 of the

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Minister of Agriculture of the Republic of Bulgaria (dated 02.03.2022), Ivkos was officially recognized as a new and original variety.

Keywords: pea, variety and line, yield, genotype $\times$ environment interaction, economic productivity

INTRODUCTION

In many countries worldwide, peas occupy a significant share of the total area sown with leguminous crops. They are cultivated both for the food industry and for animal husbandry purposes (Zotikov *et al.* 2014. Field peas have exceptional forage value and can be grown for grain, hay, and green forage. Established pea cultivars can be successfully grown on poor and sandy soils, have a wide geographic distribution, and are adaptable to cooler northern climates (Kosolapov *et al.* 2009; Chekmarov, 2014).

The cultivation of legumes is essential to the implementation of more environmentally friendly farming methods (Clark and Lenaghan, 2020). A major global legume crop, peas (*Pisum sativum* L.) are vital for bioenergy, animal feed, and human consumption. The pea is a diploid ($2n=14$) legume crop that is self-pollinating and a member of the Leguminosae (Fabaceae) family. *Pisum sativum* that is high in micronutrients, fibre, protein, and bioactive substances that can improve human health. Because of these characteristics, legume products are a strong candidate for biofortification. With 12.4 million tonnes of dry peas and 20.5 million tonnes of green peas produced in 2021, peas are presently the third most important pulse crop produced worldwide, behind common beans and chickpeas (FAO, 2025). The production of peas is seriously threatened by drought, which has gotten worse due to global warming. Therefore, in order to guarantee global food security and the sustainable development of agriculture, there are pressing needs and a lot of interest in finding or creating drought-tolerant pea variety using biotechnological methods.

In light of climate change, drought is becoming more common and is thought to be a significant crop productivity limitation. Water scarcity can diminish water use efficiency, disrupt plant water interactions, and hinder growth. In reducing pea yield because of drought, the intensity and duration of stress are critical. By using techniques like breeding, screening, and marker-assisted selection, pea drought tolerance can be controlled. Because their cultivation significantly affects soil microbial diversity, soil water protection, and soil organic matter enrichment, peas are a crucial rotation crop (Knight, 2012; Liu *et al.*, 2017). Due to peas' capacity, less fertilizer is needed during production, which lowers cultivation costs.

Boosting the production of legumes like peas also contributes to the reduction of poverty. Pea root nodules can fix atmospheric nitrogen (N₂) by forming a symbiotic relationship with soil bacteria (*Rhizobium leguminosarum*). Sustainable agriculture greatly benefits from symbiotic nitrogen fixation (SNF), which lowers the need for fertilizers and enhances soil quality (Knight, 2012). Up to 15–25 kg of shoot nitrogen for every tonne of aboveground biomass is the ideal nitrogen fixation rate for legume crops. Therefore, it is possible to lower these emissions as well as the nitrate and ammonium levels in agricultural soils by utilising pulses like peas, either in rotation or as intercrops (Peoples *et al.*, 2019).

Varieties intended for green mass production are usually characterized by longer stems, late maturity, a higher tendency to lodge, and lower seed yield. In most grain-type varieties, these limitations have been largely overcome (Kondykov *et al.* 2010; Bagheri *et al.*, 2023).

The development of new pea varieties that meet the modern requirements of agricultural production is impossible without the extensive use of the plant's genetic resources. A key factor in breeding such varieties is the choice of initial material, the main source of which is plant genetic collections. The use of a broader gene pool significantly increases the efficiency of breeding programs (Vishniakova 2017; Ashiev *et al.* 2018).

Finding advantageous alleles in the primary gene pool of crops and introducing them to elite cultivars is the major goal of plant breeding science. When these alleles are not found in the primary gene pool, they are moved to the secondary gene pool. A limited genetic base of cultivated pea germplasm results from the majority of pea breeding programs using a small number of genotypes as parents with strong relatedness. Per Castañeda-Alvarez *et al.* (2016), Rubiales *et al.* (2011), and Smýkal *et al.* (2012), pea germplasm collections, including wild relatives, might enhance the peas' larger genetic foundation to improve biotic and abiotic stress resistance and boost grain yield and quality.

According to Naim-Feil *et al.* (2017), certain characteristics of wild pea cousins, such as waxy leaves (in *P. sativum*) and root system architecture (RSA) and rhizome associations (in *P. fulvum*), show promising potentials to withstand drought stress. This wild material still exists in a variety of forms (Smýkal, *et al.*, 2011; Lakić *et al.*, 2019a; 2019b;) that may be helpful in upcoming breeding initiatives (Rakašćan *et al.*, 2021; Milunović *et al.*, 2022; Ljubičić *et al.*, 2023; Popović *et al.*, 2024). Agricultural research has advanced to a new level thanks to biotechnology and engineering advancements, thus creating "smart" goods is now feasible. The technology known as next-generation sequencing (NGS), as an a sophisticated and economical molecular instrument that has

transformed agriculture breeding initiatives, allowing us to understand the characteristics of intricate characteristics including yield-related features and drought tolerance (Singh *et al.*, 2020).

The strategic crossing of complimentary genotypes is encouraged by the high diversity of important features found in both domesticated and wild peas. In order to improve drought adaptation and yield improvements in other pea crops, this diversity is a crucial source of desired alleles in the agronomic, physiological, and biochemical parameters (Rispaal *et al.*, 2023; Smýkal *et al.*, 2018; Yang *et al.*, 2022).

Improving resistance to biotic and abiotic stress, as well as increasing grain yield and seed nutritional profile, are the most important goals for pea breeding (Tayeh *et al.*, 2015). The reservoir of resistance to pests and diseases, drought adaptability, and other agronomic, physiological, and biochemical desired traits required by pea breeders may be expanded through interspecific crosses between *P. sativum* and wild pea species (Smýkal *et al.*, 2018; Rubiales *et al.*, 2021). Numerous physiological and biochemical alterations are part of plants' drought adaptation methods, and these mechanisms also involve a large number of genes linked to stress. While many plant species experience most changes, some have unique responses to dryness. As a result, it is difficult to comprehend how various plants react to drought (Carvalho *et al.*, 2019). It is well known that the broader the genetic base of the breeding material, the greater its adaptive potential. Productivity breeding remains one of the most complex areas of genetic crop improvement since this multi-component trait is largely influenced by prevailing environmental conditions (Sobolev and Shtetinina 2008).

By deducing the phenotypic variation as the sum of interactions influencing performances in the tested genotypes (G) across the various environments (E), plant breeders meticulously assess their material in several places and over several seasons. The assessment of G, E, and G×E interactions (also known as GEI) guarantees reliable suggestions for appropriate varieties that can withstand the strain brought on by changing circumstances. The identification of GEI variables aids geneticists in their breeding operations by directing selection toward cultivars that are niche-specific or suitable for a variety of conditions (Egea-Gilabert *et al.*, 2021).

The aim of this study was to evaluate spring forage pea lines in order to develop new varieties with enhanced grain productivity potential.

MATERIAL AND METHODS

The field trial was conducted during the period 2016–2018 at the experimental field of the Institute of Forage Crops, Pleven, Bulgaria. Seeds of individual spring pea samples were sown at a spacing of 0.20 m between rows and 0.05 m within the row, in three replications, with a plot size of 10 m². Sowing was carried out at the optimal time according to the crop's cultivation technology.

Five spring forage pea lines (№115, №29, №9A, №8, and №11) were evaluated, and the varieties Kristal and Kerpo were used as controls. The following traits were recorded at the stage of technical maturity: plant height (cm), height of the first pod (cm), number of pods per plant, number of seeds per plant, number of seeds per pod, seed weight per plant (g), and 1000-seed weight (g).

Phenological observations included the beginning of flowering (days), full flowering (days), and duration of the growing season (days). Biometric measurements were taken from 10 plants per sample.

The data were processed using a two-factor analysis of variance (ANOVA) for each trait to determine the effects of genotype and environment. The degree of earliness was determined according to Kuzmova (2002). The date of beginning of flowering was used as the criterion for earliness, and the coefficient of earliness served as a quantitative measure. According to this coefficient, ultra-early varieties have values between 1.00 and 1.17; early varieties 1.17–1.33; medium-early 1.34–1.66; and late varieties > 1.66. The coefficient of variation (CV, %) was calculated following the formula of Dimova and Marinkov (1999).

All experimental data were statistically processed using the software GENES 2009.7.0 for Windows XP (Cruz, 2009) and STATGRAPHICS Plus for Windows, Version 2.1.

RESULTS AND DISCUSSION

Agrometeorological characteristic

Agrometeorological conditions during the study period are presented in Figures 1-3. Precipitation in the late autumn and winter months plays a key role in realizing the biological potential of spring pea varieties and lines.

The average amount of precipitation showed a pronounced maximum in May 2017 (155.9 mm) and June 2018 (155.2 mm), and a minimum in July (7.8 mm) and December (14.4 mm) of 2016, as well as in April (19.6 mm) and September (15.4 mm) of 2018. In 2016, the lowest average monthly air temperatures were recorded in December (−0.5 °C) and January (8.7 °C), while the highest was in July (24.6 °C), Figure 1. The combination of high average daily temperatures and low rainfall during the active vegetation period negatively affected plant growth and development. During 2017, average daily temperatures in April, May, and June ranged from 12.2 °C to 23 °C, Figure 2. The lowest average monthly air temperatures occurred in January (−4.4 °C) and December (1.1 °C), and the highest again in July (23 °C). The climatic conditions during the 2016–2017 growing seasons were favorable for the growth and development of spring pea genotypes, supporting normal overwintering and the realization of productive potential. In the third year (2018), plant development began under favorable temperature and moisture conditions, Figure 3. October 2017 was characterized by high precipitation (108.9 mm) and relatively high temperatures. In February and March, precipitation exceeded the norm, while April and May were extremely dry.

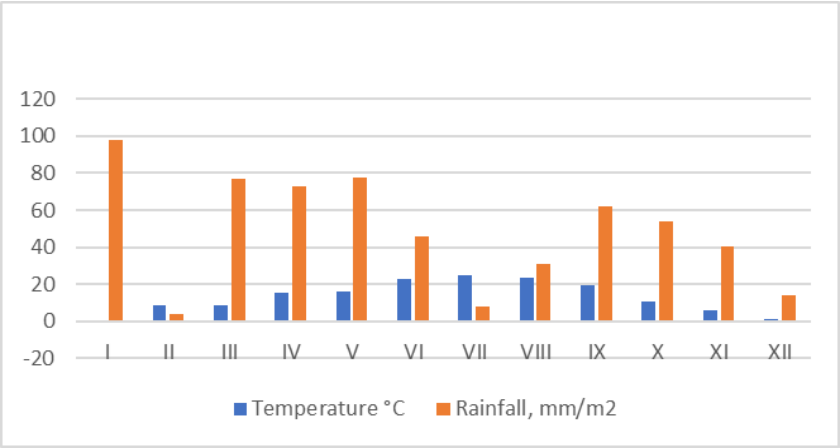


Figure 1. Agrometeorological conditions for 2016

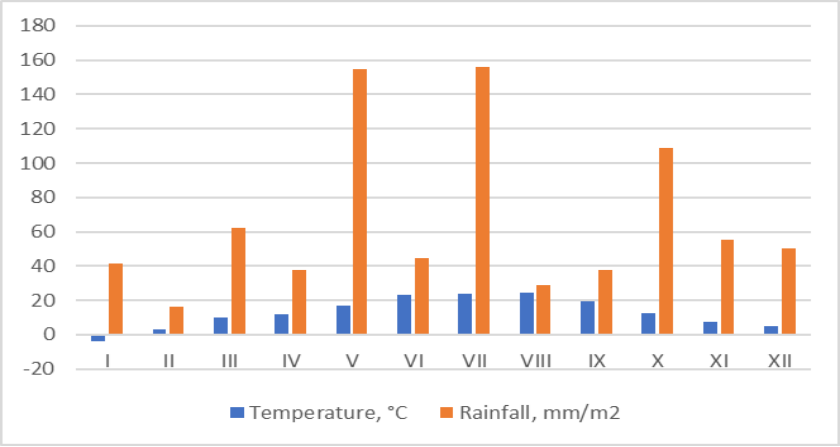


Figure 2. Agrometeorological conditions for 2017

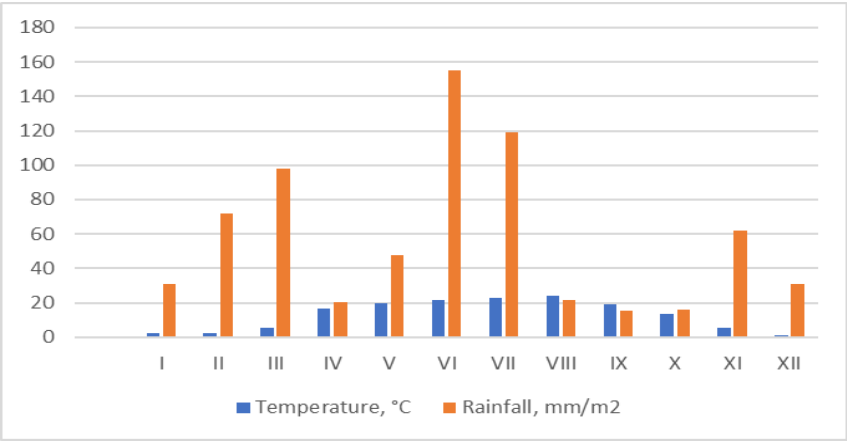


Figure 3. Agrometeorological conditions for 2018

Insufficient moisture and high temperature sums during these months negatively affected seed germination and crop establishment. In May, high temperatures combined with low and unevenly distributed rainfall (20.2–47.7 mm). The abundant rains at the end of the growing season (June–July) did not significantly affect productivity or yield structure.

Some differences were observed among the spring pea lines and varieties regarding flowering rate, growth, and time to technical maturity. The earliest flowering was recorded for the varieties Kristal, Kerpo, and line №29 (12–28 May) (Table 1).

Table 1. Phenological development of the studied spring pea samples (2016–2018)

Phenological stages	Year	Sowing date	Beginning of flowering	Technical maturity	Vegetation period (days)
Kerpo	2016	31.3.2016	25.5.2016	24.6.2016	90
	2017	06.3.2017	12.5.2017	23.6.2017	81
	2018	05.4.2018	28.5.2018	22.6.2018	78
Kristal	2016	31.3.2016	25.5.2016	24.6.2016	90
	2017	06.3.2017	12.5.2017	23.6.2017	81
	2018	05.4.2018	28.5.2018	22.6.2018	78
№115	2016	31.3.2016	31.5.2016	30.6.2016	96
	2017	06.3.2017	23.5.2017	30.6.2017	96
	2018	05.4.2018	06.6.2018	29.6.2018	85
№29	2016	31.3.2016	25.5.2016	24.6.2016	90
	2017	06.3.2017	12.5.2017	23.6.2017	81
	2018	05.4.2018	28.5.2018	22.6.2018	78
№9A	2016	31.3.2016	30.5.2016	27.6.2016	93
	2017	06.3.2017	22.5.2017	30.6.2017	91
	2018	05.4.2018	05.6.2018	28.6.2018	84
№8	2016	31.3.2016	30.5.2016	29.6.2016	95
	2017	06.3.2017	22.5.2017	29.6.2017	91
	2018	05.4.2018	05.6.2018	29.6.2018	85
№11	2016	31.3.2016	30.5.2016	29.6.2016	95
	2017	06.3.2017	22.5.2017	29.6.2017	91
	2018	05.4.2018	05.6.2018	29.6.2018	85

Lines №115 and №9A began flowering 10–12 days later, while lines №8 and №11 were the latest.

Early-flowering varieties and lines (Kristal, Kerpo, and №29) reached technical maturity within 78–90 days, while the remaining lines matured 7–8 days later. Lines №11, №8, and №115 had the longest growing periods (85–96 days), associated with slower growth and development.

In some years, an extension of the growing season was observed, likely due to prolonged rainfall in the second half of plant development. Based on climatic conditions and genetic predisposition, the spring pea genotypes were classified by earliness as follows: Kerpo, Kristal, and №29 – ultra-early ($K=1.00$); №9A, №8, and №11 – late ($K=1.83$ – 1.91); №115 – the latest ($K=2.00$).

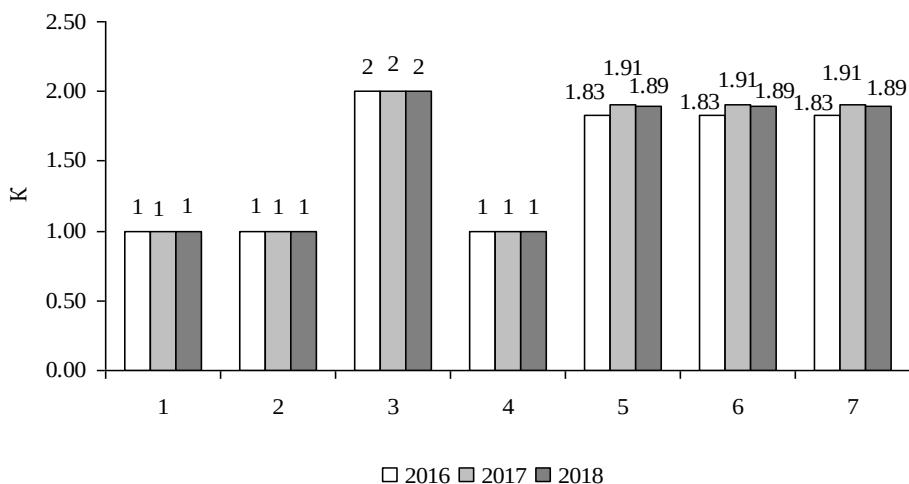


Figure 4. Coefficient of earliness (K) of forage pea varieties and lines (1-Kerpo; 2- Kristal; 3-№115; 4- №29; 5-№9A; 6-№8; 7- №11)

Significant differences were observed among genotypes for most quantitative traits (Table 2; Figure 4). Plant height, an important structural and agronomic characteristic, varied considerably. Stem height largely determines lodging resistance and suitability for mechanized harvesting. The average plant height was 48 cm for Kristal and 51 cm for Kerpo.

Considering the climatic conditions, Kerpo showed greater responsiveness to improved growing conditions, followed by line №115 (62 cm). Lines №11 and №8 formed the tallest plants (91–101 cm), while №9A and №29 were intermediate (71–74 cm).

Similar trends were found for the height of the first pod. Kristal set the lowest first pods (32 cm), followed by Kerpo (39 cm), №115 (43 cm), №9A (48 cm), №29 (56 cm), and the tallest lines №8 and №11 (≈ 61 cm).

The study of the main productivity components, viz. number of pods per plant, number of seeds per pod, and 1000-seed weight is essential for identifying

high-performing genotypes. Lines №11 and №8 produced the highest number of pods (8 per plant), followed by Kristal (7). Lines №29 and №9A formed six pods per plant, while Kerpo produced the fewest (5).

Table 2. Morphological characteristics of the studied spring pea samples (2016-2018)

Variety (line)	Plant height, cm	Height on first pod, cm	No pods per plant	No seeds per plant	No seeds per pod	Seed weight per plant, g	1000 seeds weight, g
Kerpo	51ab	39ab	5a	17ab	3a	3.37c	196.28cd
Kristal	48a	32a	7ab	20abc	3a	4.47d	206.33d
№115	62abc	43ab	5a	16a	3a	3.15bc	162.00bc
№29	74cd	56bc	6ab	21abc	3a	4.88d	209.88d
№9A	71bcd	48cd	6ab	24abc	4b	2.14a	105.02a
№8	91de	61d	8b	26bc	3a	2.45ab	129.33ab
№11	101e	61d	8b	28c	3a	1.93a	120.05a

a, b, c, d, e – statistically significant differences at $P=0.05$

Regarding seed number per plant, the genotypes with fewer pods compensated through more seeds per pod. Line №11 produced the highest seed number (28), followed by №8 (26) and №9A (24). Kristal and №29 had 20–21 seeds per plant, while №115 and Kerpo had 16–17.

All genotypes produced approximately three seeds per pod on average, except №9A (4 seeds per pod).

Seed weight per plant - trait closely correlated with grain yield was highest in Kristal (11.0 g) and №29 (10.9 g), followed by №8 (7.0 g) and №11 (7.4 g), with №9A showing the lowest (6.6 g).

The 1000-seed weight was highest for №29 (260 g) and Kristal (252 g), classifying them as large-seeded genotypes. Kerpo (230 g), №9A (215 g), and №115 (203 g) were medium-seeded, while №8 (129 g) and №11 (121 g) belonged to the small-seeded group.

Analysis of variance

The two-factor analysis of variance (Table 3) showed significant differences among genotypes for plant height, height of the first pod, number of seeds per pod, seed weight per plant, and 1000-seed weight. No significant differences were found for the number of pods and seeds per plant.

The environmental factor (years) significantly affected plant height, height of the first pod, number of pods per plant, seed weight per plant, and 1000-seed weight. Thus, longer-term studies are recommended for these traits.

Environmental conditions explained most of the total variation for the number of pods and seeds per plant, while genotype had a greater effect on plant height, first pod height, seeds per pod, seed weight per plant, and 1000-seed weight.

The coefficients of variation (CV, %) indicated that the number of pods (24.23%) and seeds per plant (25.08%) were highly dependent on environmental conditions. Medium variability was observed for plant height (17.75%), first pod height (13.95%), 1000-seed weight (13.57%), and seed weight per plant (11.77%), while the number of seeds per pod showed low variability (8.70%).

Table 3. Dispersion analysis of the investigated characters by genotypes (samples) and years (2016-2018)

Source of variation	DF	Mean square						
		Plant height, cm	Height on first pod, cm	Number of pods per plant	Number of seeds per plant	Number of seeds per pod	Seed weight per plant, g	1000 seeds weight, g
Years	2	1039.92*	237.94*	6.33*	96.37 ^{ns}	0.15 ^{ns}	0.74*	466.92 ^{ns}
Varieties (lines)	6	1174.10**	378.91**	5.60 ^{ns}	63.53 ^{ns}	0.466**	3.17**	5751.08**
Residuo	12	160.2956	46.1733	2.3544	29.6267	0.0827	0.1511	479.2082
CV (%)		17.75	13.95	24.23	25.08	8.70	11.77	13.57

Productivity breeding is complex, as it requires combining multiple valuable traits within a single genotype. In peas, key yield-related traits include the number of productive nodes, number of pods per plant, number of seeds per pod, and 1000-seed weight (Belyaeva and Naumkina, 2017; Likhacheva *et al.*, 2017). The investigated spring pea cultivars and lines were evaluated for grain yield (Figure 5).

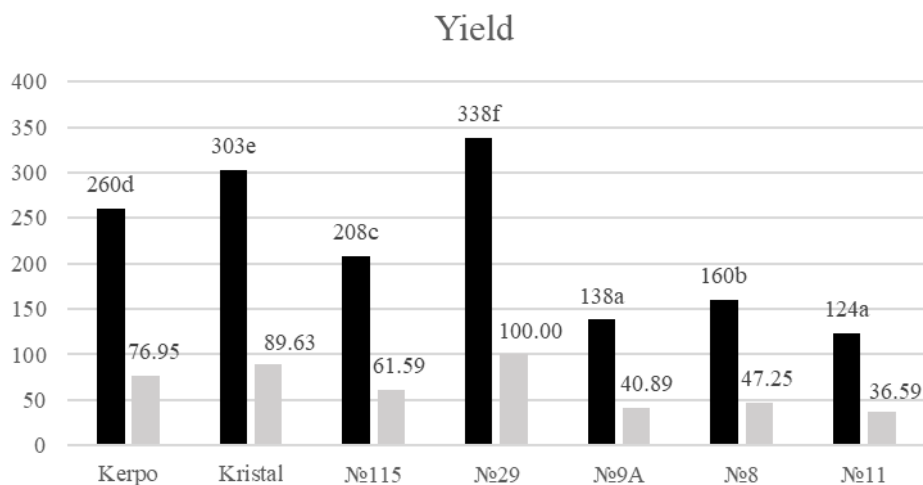


Figure 5. Grain yield (kg/ha) of the investigated pea varieties and lines (2016-2018); a, b, c, d, e, f – statistically significant differences at P=0.001

The investigated spring pea cultivars and lines were evaluated for grain yield (Figure 5). Line №29 consistently demonstrated the highest yield across all years. In the competitive variety trial (2016–2018), line №29 produced 338 kg/ha of grain - 20% higher than Kerpo and about 10% higher than Kristal. In most yield-related traits, except plant height and first pod height, line №29 was comparable only to Kristal.

The findings of the present study regarding the duration of the growing season are consistent with results reported by other researchers. Gul *et al.* (2005) observed a wide variability in this trait, ranging from 143 to 167 days, while Hussain *et al.* (2002) reported a range of 94 to 150 days among pea cultivars. These differences are primarily attributed to variations in environmental conditions affecting the phenological development of plants.

Similarly, variations in plant height observed in this study align with the reports of Hussain *et al.* (2005) and Bozoglu *et al.* (2007), who noted that although plant height is genetically determined, it is strongly influenced by climatic factors.

The number of seeds per pod, considered a relatively stable trait, also showed comparable results. Singh *et al.* (2015) reported a range of 4–5 seeds per pod, which is in agreement with our findings.

Our results regarding the number of pods per plant support the observations of Qasim *et al.* (2001) and Chadha *et al.* (2010), who suggested that smaller pods tend to produce more per plant due to lower nutrient requirements per pod.

Finally, as emphasized by Lysenko and Korobova (2019), detailed characterization of genotypes is essential for future breeding programs. Accumulated specific information facilitates the selection of forms not only with high productivity but also with optimal values of individual yield components, ensuring a more efficient breeding process.

CONCLUSIONS

Line №29 demonstrated the highest grain productivity among the studied genotypes. It is of medium height and has a short vegetation period (78–90 days), exceeding the control varieties in 1000-seed weight (209.88 g) and seed weight per plant (4.88 g). The varieties Kristal and Kerpo showed favorable characteristics in structural elements of seed productivity, making them suitable as parental components in combinatorial breeding for improving grain yield potential.

During the study period, line №29 produced 338 kg/ha of grain, which is 20% higher than Kerpo and 10% higher than Kristal. Lines №8 and №11 developed the tallest plants (91–101 cm), with well-developed foliage, making them suitable for forage purposes.

The coefficient of variation (CV%) indicated high variability for the number of pods per plant (24.23%) and number of seeds per plant (25.08%), moderate variability for plant height (17.75%), first pod height (13.95%), 1000-

seed weight (13.57%), and seed weight per plant (11.77%), and low variability for the number of seeds per pod (8.70%).

These findings highlight the potential of line №29 as a high-yielding genotype for grain production, while lines №8 and №11 may be recommended for forage purposes. The results provide valuable information for future breeding programs aimed at improving productivity and agronomic performance in spring forage peas.

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INCLUSION OF HIGH-PROTEIN SUNFLOWER CONCENTRATE IN DIETS OF GROWING PIGS

SUMMARY

The search for alternative plant sources of protein for pig feeding is an urgent task of modern animal husbandry, due to economic factors and the need to optimise protein nutrition. High-protein sunflower concentrate (HPSC) is considered a promising component of compound feeds, however, its effect on growth, metabolic and haematological status of young pigs at different levels of inclusion requires further study. The study aimed to assess changes in live weight, biochemical and haematological blood parameters of piglets under the conditions of adding high-protein sunflower concentrate to the diet in amounts of 0, 4, 8 and 12% and to determine the optimal levels of such introduction. The study was conducted in the conditions of a production experiment using generally accepted methods of clinical, biochemical and haematological blood analysis. The results obtained indicate that the inclusion of high-protein sunflower concentrate causes dose-dependent changes in the metabolic profile of piglets. The most balanced adaptive reactions of the organism were observed at the level of addition to the piglet diet of 8%, which was characterised by the intensification of protein and nitrogen metabolism without signs of metabolic overload, preservation of the stability of the lipid profile and a decrease in the activity of enzymes associated with tissue stress. Haematological indicators at this level of replacement demonstrated physiological activation of erythropoiesis and adaptive shifts in the leukocyte formula, without exceeding reference values. Based on a comprehensive assessment of growth, biochemical and haematological indicators, it was established that the level of introduction of high-protein sunflower concentrate into the diet of young pigs at the level of 8% is the most appropriate for feeding piglets, as it ensures metabolic efficiency and physiological safety of the diet.

Keywords: piglets, high-protein sunflower concentrate, alternative protein sources, live weight, biochemical blood parameters, haematology

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INTRODUCTION

The search for alternative protein sources for feeding farm animals is a relevant direction in modern animal husbandry, driven by economic factors, limited availability of traditional protein raw materials, and the need to optimise protein nutrition (Plyska *et al.*, 2021). In this context, high-protein sunflower processing products, in particular high-protein sunflower concentrate, are considered as a promising component of compound feeds and protein supplements (Sychov *et al.*, 2025; Pitera and Pitera 2025; Shkarban and Sychov 2025).

Studies show that sunflower meal can be added to the diet of pigs in amounts of 10–20% without negative effects on performance, and in some cases, the level of introduction can reach 25–50% (Cortamira *et al.*, 2000; Bonos *et al.*, 2017). Recent studies demonstrate that the combined replacement of soybean meal with sunflower and pea meal up to 56% allows maintaining optimal growth and health indicators in pigs during fattening, particularly under conditions of reduced crude protein levels in finishing period rations (Graziosi *et al.*, 2024). At the same time, the literature reveals considerable variability in the results, which complicates the formulation of unified recommendations regarding the use of sunflower products in pig nutrition.

A number of studies report a decrease in average daily gains and a deterioration in feed conversion with high levels of sunflower meal inclusion, especially when its share exceeds 20–30% in the diet (Povod *et al.*, 2022). At the same time, other authors do not find significant differences between diets based on sunflower and soybean meal or even record improvements in digestibility and growth, especially in the growth phase of pigs (Kim *et al.*, 2020; He *et al.*, 2024). Such data inconsistency is largely explained by the variability in the chemical composition of sunflower products, which depends on the seed variety, degree of hulling, extraction method, and heat treatment parameters.

From a nutritional point of view, sunflower meal is characterised by a higher fibre content and lower lysine levels compared to soybean meal, which is traditionally considered a limiting factor for its use (Petik *et al.*, 2023). At the same time, sunflower products have a high content of methionine and other sulfur-containing amino acids, which can contribute to the optimisation of the amino acid profile of the diet, provided that lysine is corrected with synthetic analogues (Florou-Paneri *et al.*, 2014; Ryvak, 2021). In addition, the presence of fibre may play not only a restrictive but also a functional role, especially for pregnant sows and animals in the finishing phase, affecting intestinal motility and the feeling of satiety.

The environmental aspect of using sunflower products deserves special attention. Feed rations are recognised as one of the main factors affecting pig farming environment, and the finishing period is critical in terms of nitrogen and greenhouse gas emissions (Berton *et al.*, 2024). It has been proven that reducing the level of crude protein in diets with compensation with synthetic amino acids allows reducing greenhouse gas emissions by up to 20% and reducing the load on

the manure disposal system (Ogino *et al.*, 2013; McAuley *et al.*, 2022). In this context, the use of local protein resources, such as sunflower meal, in low-protein diets is considered a promising tool for increasing the environmental efficiency of pig farming (Graziosi *et al.*, 2024).

However, reducing crude protein and modifying protein sources may affect not only performance but also behaviour, physiology, and the gut microbiota in pigs. Some studies indicate increased aggressiveness and a higher risk of behavioural disorders under conditions of essential amino acid deficiency in low-protein diets (McAuley *et al.*, 2022; Minussi *et al.*, 2023). At the same time, experimental data indicate that the inclusion of sunflower meal only slightly affects the behavioural responses of pigs and is not accompanied by an increase in the level of stress hormones, in particular cortisol and dehydroepiandrosterone, which are considered sensitive biomarkers of animal welfare (Pollock *et al.*, 2021; Graziosi *et al.*, 2024). This confirms the potential safety of such diets, provided that the amino acid balance is properly balanced.

Despite a significant amount of research, the role of sunflower meal and high-protein sunflower concentrates in pig nutrition remains poorly defined. In particular, there is a lack of systematic data on optimal inclusion levels for different growth phases, the effect of the degree of concentrate purification on nutrient digestibility, the interaction of fibre with the intestinal microbiota, and the long-term consequences for animal performance and welfare. This necessitates further comprehensive studies aimed at assessing the effectiveness of introducing high-protein sunflower concentrates into the diet of young pigs, taking into account productive, metabolic, behavioural and environmental aspects.

MATERIAL AND METHODS

The research was carried out in the April-May of 2025 at the experimental facilities of the pig farm “Ternopil Bacon” LLC (Ternopil Oblast, Ukraine) and at the Problem Research Laboratory of Feed Additives, the P.D. Pshenychnyy Department of Animal Nutrition and Feed Technology, National University of Life and Environmental Sciences of Ukraine. Danish-bred weaned piglets were used as the experimental animals. All experimental procedures involving animals were performed in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Union, 2010).

Experimental design

From young pigs of the industrial herd, Danish selection (♀ Landrace/Large White X ♂ Dyurek) aged 24 days, 4 groups of piglets were formed (all animals were either half-brothers or half-sisters), which were kept in 10 m² pens on a slatted floor. Each pen was equipped with a 2.5 m² cover with a heating lamp, which was used in the first week of life after weaning.

The experimental pigs had ad libitum access to water, provided via stainless steel bowl drinkers at a rate of three units per pen. The piglets were fed manually using a TR-4 ROTECNA feeding device. 1 feeding device per 1 group, type of feeding - dry. Feeding principle: every morning from 9.00 to 10.00 - the remaining feed from the previous day was taken out, weighed, and a new amount of feed was poured.

The chemical composition of a high-protein sunflower concentrate used in piglet nutrition were studied and fully described in previous research (Shkarban and Sychov, 2025).

The experiment lasted 30 days in total. The experimental design is presented in Table 1.

Table 1. Experimental design

Groups	Characteristics of feed	The level of introduction of high-protein sunflower concentrate into the diet, %
1 (control)	Basic feed (BF) based on soybean meal, HPSC 0	0
Experimental 2	Basic feed (BF) based on soybean meal with the addition of high-protein sunflower concentrate, HPSC 4	4
Experimental 3	Basic feed (BF) based on soybean meal with the addition of high-protein sunflower concentrate, HPSC 8	8
Experimental 4	Basic feed (BF) based on soybean meal with the addition of high-protein sunflower concentrate, HPSC 12	12

Zootechnical records.

Throughout the rearing period, the number of piglets removed from the experiment (due to mortality and culling) was recorded. Also, every seven days, the live weight of pigs was determined on the AXIS BDU300C-0608X scales, which has weighing range - 1 -300 kg and measurement accuracy - 0,05 kg (Ukraine).

Blood sampling. Blood sampling was performed from 8 pigs (♀4 & ♂4) of each group at the end of the experiment. The sampling was performed from the axillary vein, and 1.0–1.5 ml of blood was taken into a K2 EDTA tube. Blood sampling, as well as the selection of randomised individuals, was performed according to generally accepted methods.

Haematological methods. Haematological parameters of pig blood were determined on the IDEXX ProCyte Dx haematological analyser (IDEXX Europe

B.V.) at the LLC "Veterinary Diagnostics Centre". The following parameters were determined: erythrocyte, leukocyte, and platelet counts—by impedance change (conductometric method); haemoglobin concentration—by the spectrophotometric method.

Biochemical parameters and enzyme activity of piglet blood serum were determined on an automatic Beckman Coulter biochemical analyser (USA) in the laboratory of LLC "Centre for Veterinary Diagnostics" using the manufacturer's original test systems and commercial diagnostic kits High Technology Inc. (USA). Determination of the main serum metabolites (High Technology Inc., USA). The following reagent sets were used to determine the concentrations of metabolites: glucose – set HT-G242 (method: glucose oxidase, endpoint; $\lambda=500$ nm); total protein – HT-T251 (biuret reaction, endpoint; $\lambda=540$ nm); albumin – HT-A203 (reaction with bromocresol green; $\lambda=630$ nm); creatinine – HT-C225 (Jaffe method, kinetic; $\lambda=510$ nm); urea – HT-U254 (Trinder/uricase method, endpoint; $\lambda=520$ nm); inorganic phosphorus – HT-P244 (ammonium molybdate method; $\lambda=340$ nm). calcium – HT-C216 (orthocresolphthalein method, endpoint; $\lambda=570$ nm); ALT (alanine aminotransferase) – HT-A206 (IFCC method, kinetic; $\lambda=340$ nm). The methods were performed according to the instructions of the kit manufacturer.

Statistical analysis

Biometric data processing was carried out on a PC using Statistica 10 software.

The significance of differences between groups was assessed using one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparison test as a post hoc procedure. The normality of the sample data distribution was tested using the Shapiro-Wilk test and P-plot. If the data distribution was significantly different from normal, the nonparametric Mann-Whitney U-test was used. Differences between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The experimental piglets were fed complete feed, balanced for all nutrients according to the recommended norms (CVB, 2023). The composition of the feed used for feeding young pigs is given in Tables 2–4.

Live weight indicators changed depending on the addition of high-protein sunflower concentrate to the diet (Table 5): with 8% introduction, a significant increase in live weight of piglets at 75 days of age by 1.2 kg or 3.6% ($p<0.05$) was noted compared to the control group, while the introduction levels of 4 and 12% did not cause statistically significant differences. Such a moderate but significant advantage at the intermediate introduction level is consistent with the data of studies in which high-protein sunflower meal or concentrate or its fractions provided improved gains during the growing and fattening period of pigs due to the sufficient content of digestible protein and energy (Povod *et al.*, 2022; (Kropiwić *et al.*, 2025).

Table 2. Composition of rations, %

Indicator	Age, days				
	24–44	45–75			
	Group of pigs / % introduction of high-protein sunflower concentrate				
	1–4 HPSC 0	HPSC 0	HPSC4	HPSC8	HPSC12
Wheat	36.02	40.00	40.00	40.00	40.00
Barley	25.00	27.42	26.06	24.92	23.67
Sunflower concentrate 44 CP	0.00	0.00	4.00	8.00	12.00
Soybean meal 45 CP	16.59	22.04	19.18	16.29	13.31
Wheat bran	1.00	2.00	2.00	2.00	2.00
Soybean oil	4.00	3.85	4.10	4.30	4.55
Whey (dry)	3.84	0.00	0.00	0.00	0.00
Whey fat concentrate	3.58	0.00	0.00	0.00	0.00
Source of functional fibre	2.00	0.00	0.00	0.00	0.00
Animal proteins	2.87	0.00	0.00	0.00	0.00
Limestone flour Ca 35%	0.85	1.26	1.24	1.08	1.06
Salt	0.50	0.66	0.66	0.66	0.66
Monocalcium phosphate	0.76	0.61	0.62	0.63	0.64
DL-Methionine 99.5%	0.28	0.18	0.16	0.14	0.10
L-Lysine hydrochloride	0.49	0.43	0.46	0.49	0.52
L-Threonine 99%	0.28	0.19	0.19	0.18	0.17
L-Tryptophan	0.08	0.06	0.05	0.05	0.05
L-Valine	0.04	0.04	0.03	0.01	0.00
Premix (acidifier, absorbent, trace elements, vitamins, enzymes)	1.82	1.20	1.25	1.25	1.27
Total	100.00	100.00	100.00	100.00	100.00

In the initial period of the study (24–44 days), the absolute body weight gains of piglets in all groups remained at the same level (Fig. 1). At the same time, in the period of 45–75 days, differences due to the composition of the diet were recorded. In particular, in animals of the 3rd group, which were fed a diet with the introduction of 8% high-protein sunflower concentrate, the absolute body weight gain exceeded the indicators of the control group by 1.17 kg or 5.9% ($p < 0.05$). In general, for the entire period of growing in this group of young animals, the absolute gain was higher by 1.11 kg or 4.2% ($p < 0.05$). In contrast, the use of diets with the introduction of 4 and 12% high-protein sunflower concentrate did not cause statistically significant differences in body weight gains.

Table 3. Chemical composition of rations

Indicator	Age, days				
	24–44	45–75			
	Group of pigs / % introduction of high-protein sunflower concentrate				
	1–4 HPSC 0	HPSC 0	HPSC4	HPSC8	HPSC12
Net energy, MJ	9.8	9.8	9.8	9.8	9.8
Crude protein, g	165.000	160.527	164.453	168.434	172.000
Crude fat, g	70.000	52.586	55.248	57.369	59.942
Crude fiber, g	41.80	34.17	35.53	36.95	38.30
Calcium, g	5.5	6.5	6.5	6	6
Available Phosphorus, g	4.0	3.5	3.5	3.5	3.5
Sodium, g	3.0	2.5	2.5	2.5	2.5
SID Lysine, g	11.6	10.1	10.1	10.1	10.1
SID Methionine+Cystine, g	6.96	6.06	6.06	6.06	6.06
SID Threonine, g	7.540	6.565	6.565	6.565	6.565
SID Tryptophan, g	2.55	2.22	2.22	2.22	2.22
SID Valine, g	7.54	6.565	6.565	6.565	6.565

Table 4. Dietary content of vitamins and minerals

Indicator	Age, days				
	24–44	45–75			
	Group of pigs / % introduction of high-protein sunflower concentrate				
	1–4 HPSC 0	HPSC 0	HPSC4	HPSC8	HPSC12
Vitamins:					
A, IU	12000				
D, IU	2000				
E, mg	100				
K, mg	2				
Minerals:					
Iron, mg	80				
Copper, mg	177				
Zinc, mg	120				
Manganese, mg	72				
Iodine, mg	0.8				
Selenium, mg	0.39				

Table 5. Live weight of piglets, g

Age, days	Group of pigs			
	HPSC 0	HPSC4	HPSC8	HPSC12
	n=25	n=25	n=28	n=28
24	6.4±0.15 ^a	6.5±0.15 ^a	6.6±0.14 ^a	6.4±0.16 ^a
44	13.0±0.29 ^a	13.3±0.32 ^a	13.1±0.17 ^a	13.2±0.19 ^a
75	33.1±0.34 ^a	33.0±0.42 ^a	34.3±0.36 ^b	32.1±0.43 ^a

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$)

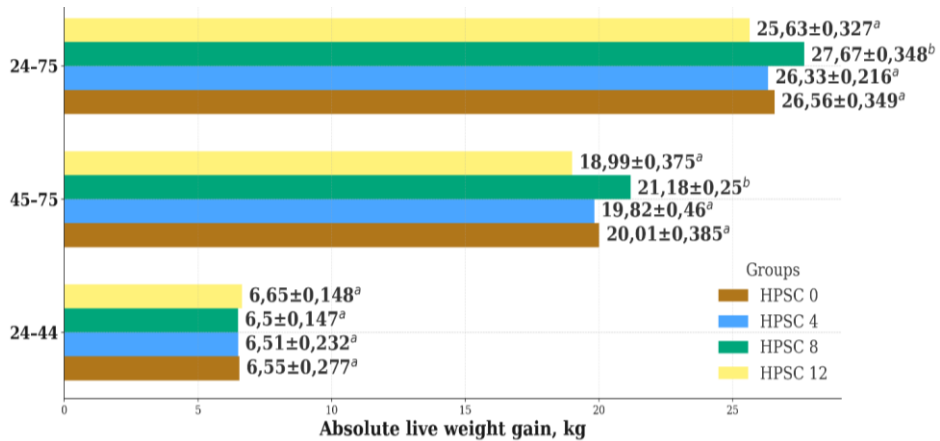


Fig. 1. Absolute body weight gains of piglets, kg

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$)

Regarding average daily body weight gain in piglets, a similar pattern was observed (Fig. 2).

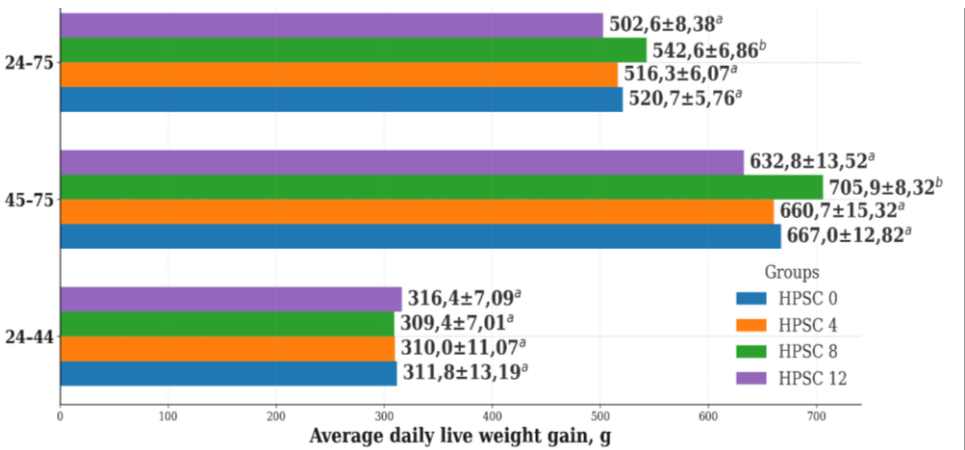


Fig. 2. Average daily body weight gains of piglets depending on the level of introduction of high-protein sunflower concentrate into the diet, kg

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$)

During the 45–75th day of growth, statistically significant differences were observed due to diet composition. In particular, in animals of Group 3, which were fed a diet containing 8% high-protein sunflower concentrate, the average daily body weight gain exceeded that of the control group by 39.9 g, or 5.8% ($p < 0.05$). As a result, over the entire period of growth, the average daily body weight gain in this group was higher by 21.9 g or 4.2% ($p < 0.05$) compared to the control. At the same time, the use of diets with the introduction of 4 and 12% of the specified concentrate did not cause significant changes.

At the same time, the productive indicators varied with the level of introduction of high-protein sunflower concentrate into the main diet. In particular, the introduction of 8% of high-protein sunflower concentrate into the diet contributed to an increase in the live weight of piglets at 75 days of age by 3.6% ($p < 0.05$), absolute gains - by 5.9% for the period 45–75 days ($p < 0.05$) and by 4.2% for the entire growing period ($p < 0.05$). The average daily gains in this group exceeded the control values by 5.8% ($p < 0.05$) at the age of 45–75 days and by 4.2% ($p < 0.05$) on average for the entire growing period.

Therefore, an inclusion level of 8% high-protein sunflower concentrate proved to be optimal, as it resulted in a significant increase in body weight and growth rate of piglets. However, according to Cheng *et al.* (2024), the inclusion level of high-protein sunflower concentrate can reach up to 50% without adversely affecting performance.

The effect of including high-protein sunflower concentrate in the diet of piglets on the biochemical parameters of their organism.

Biochemical blood parameters are an important indicator of the physiological state of the animal body and reflect the level of metabolic processes occurring under the influence of feeding. The study of changes in the biochemical composition of the blood of pigs makes it possible to assess not only their general health, but also the efficiency of using nutrients in the diet (Sheptukha and Masiuk, 2025; Osadcha *et al.*, 2023). In this context, the study of the effect of adding high-protein sunflower concentrate to the diet is of particular interest, since such a correction of feeding can affect the course of protein, lipid and carbohydrate metabolism, and therefore - on animal productivity. Therefore, at the subsequent stage, the biochemical parameters of piglet blood serum were analysed according to diet composition (Table 6). It was found that the introduction of high-protein sunflower concentrate into the diet of young pigs is accompanied by an increase in total protein and urea in their blood serum. In particular, at the level of introduction of 8%, an increase in the level of total protein by 17.8 % ($p < 0.05$) was observed. While the addition of 12% is accompanied by an increasing increase in serum total protein by 22.9% ($p < 0.05$).

The observed increase in total protein levels resulting from the inclusion of high-protein sunflower concentrate in the diet can be explained by the fact that this concentrate is characterised by an enhanced content of available protein and amino acids, due to a reduced crude fiber level. Therefore, when fed with it, piglets receive more protein compounds, which stimulate the synthesis of serum

proteins (albumins and globulins). This leads to an increase in the level of total protein in the blood serum (Binev *et al.*, 2025).

Table 6. Biochemical parameters of blood serum of young pigs, n=8/group

Indicator	Group of pigs				RI ¹
	HPSC 0	HPSC4	HPSC8	HPSC12	
Total protein, g/l	48.4±1.29 ^a	51.8±2.14 ^a	57.0±3.67 ^b	59.5±3.98 ^b	33–63
Albumin, g/l	33.3±0.83 ^a	33.3±0.98 ^a	32.5±0.63 ^a	33.3±0.73 ^a	20–39
Glucose, mmol/l	4.7±0.09 ^a	5.1±0.39 ^a	4.8±0.11 ^a	4.7±0.06 ^a	5.1–8.3
Creatinine, µmol/l	98.8±2.99 ^a	112.3±9.39 ^a	102.3±1.65 ^a	103.8±2.81 ^a	73–111
Urea, mmol/l	1.8±0.36 ^a	2.3±0.31 ^a	3.2±0.56 ^b	3.5±0.59 ^b	0.5–3.8
Uric acid, µmol/L	2.0±0.27 ^a	1.8±1.16 ^a	2.0±0.27 ^a	1.8±0.16	–
Calcium, mmol/l:					
- general	2.8±0.07 ^a	2.8±0.05 ^a	2.7±0.03 ^a	2.9±0.04 ^a	2.4–3.3
- ionised	1.3±0.01 ^a	1.3±0.03 ^a	1.3±0.03 ^a	1.4±0.02 ^a	–
Total phosphorus, mmol/l	5.6±0.11 ^a	5.5±0.04 ^a	5.9±0.07 ^a	5.8±0.03 ^a	2.6–4.0

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$); IRI – reference intervals according to Egeli *et al.* 1998

In addition, an increase in serum urea levels was observed. In particular, when high-protein sunflower concentrate was introduced into the diet in an amount of 8%, an increase in urea levels was observed by 77.8% ($p<0.05$), and when 12% was introduced - by 94.4% ($p<0.05$). This may be due to an increase in the level of available protein entering the body, which leads to an increased formation of amino acid catabolites. Part of the nitrogen that is not used for tissue protein synthesis is transformed into urea (the main end product of nitrogen metabolism in the body of pigs). Therefore, an increase in urea levels is recorded in serum (Park *et al.*, 2021).

The observed rise in serum urea likely reflects intensified nitrogen turnover and metabolic adaptation to increased protein supply, while at the same time potentially signaling a higher renal and metabolic nitrogen burden. Importantly, urea values remained within established physiological ranges and were not accompanied by adverse changes in liver enzyme activity or growth performance at the 8% inclusion level, suggesting that nitrogen utilisation was physiologically acceptable. However, the further increase in urea at the 12% inclusion level, without additional productive benefits, may indicate reduced efficiency of protein utilisation and supports the conclusion that 8% represents the metabolically optimal inclusion level.

The introduction of high-protein sunflower concentrate into the diet of young pigs also affected the activity of blood serum enzymes, namely AST (Table 7). Thus, at the level of introduction of high-protein sunflower concentrate in the amount of 8%, a decrease in AST activity was observed by 13.4% ($p<0.05$), and at the introduction of 12% - by 15.8% ($p<0.05$).

Table 7. Serum enzyme activity of young pigs, n=8/group

Enzyme, U/l	Group of pigs				RI ¹
	HPSC 0	HPSC4	HPSC8	HPSC12	
ALT	53.8±3.18 ^a	55.5±1.71 ^a	54.3±6.85 ^a	55.8±6.91 ^a	15–108
AST	75.5±3.28 ^a	72.5±3.25 ^a	65.4±3.79 ^b	63.6±3.94 ^b	8–84

Notes: a, b – express traits fundamentally different in one table row ($P < 0.05$); 1RI - reference intervals according to (Egelie *et al.* 1998)

AST is an enzyme involved in the transamination of amino acids, and its activity in the blood is frequently associated with the condition of the liver and muscle tissues. An increase in AST levels usually signals cell damage or excessive load on protein metabolism. Therefore, a decrease in its activity may indicate a more balanced amino acid supply and less stress on cells. When feeding high-protein sunflower concentrate, the body receives a sufficient amount of readily available amino acids (Roshanay *et al.*, 2024). This ensures a more efficient course of protein synthesis without the need for excessive activation of transamination enzyme systems, which leads to a decrease in AST activity. In addition, a decrease in AST activity may reflect a positive adaptive effect of the diet - stabilisation of nitrogen metabolism and a decrease in the intensity of catabolic processes in tissues (Xinlan *et al.*, 2025). Therefore, the decrease in AST activity in the blood serum of pigs due to the addition of high-protein sunflower concentrate to the diet is explained by the optimisation of protein metabolism and a decrease in the metabolic load on the liver and muscles, which indicates the beneficial effect of this feed component.

The effect of supplementing the diet of piglets with high-protein sunflower concentrate on the haematological parameters of their organism

Haematological parameters are sensitive indicators of the physiological state and metabolic processes in the animal body. They reflect the intensity of hematopoiesis, the level of oxygen-transport function of the blood and the state of the immune system. In this regard, the study of the effect of introducing high-protein sunflower concentrate into the diet of young pigs on the haematological parameters of piglets is important for assessing the adaptive capabilities of the organism and determining the optimal structure of diets (Nseabasi *et al.*, 2013). This study showed that the introduction of high-protein sunflower concentrate into the diet of piglets affected the concentration of erythrocytes and hemoglobin in their blood (Fig. 3). In particular, the introduction of the concentrate in an amount of 8% was accompanied by an increase in the concentration of hemoglobin in the peripheral blood of piglets by 9.5 g/l or 8.5% ($p < 0.05$), and 12% by 11.8 g/l or 10.6% ($p < 0.05$). In addition, an increase in the concentration of erythrocytes in the blood of piglets was detected. Thus, the introduction of high-protein sunflower concentrate in the diet in the amount of 8% was accompanied by an increase in the concentration of erythrocytes in the blood of

piglets by 1.3 10¹²/l or 23.2% ($p<0.05$), and by 12% by 1.5 10¹²/l or 26.8% ($p<0.05$).

The described changes in the erythrocyte chain of piglets can be explained by increased intake of protein and amino acids (especially methionine and arginine) into their bodies, which contributes to more active haemoglobin synthesis (Prudyus, 2024). This can lead to an increase in the number of erythrocytes and an increase in haemoglobin concentration. The result of this process is an improvement in the oxygen-transport function of the blood.

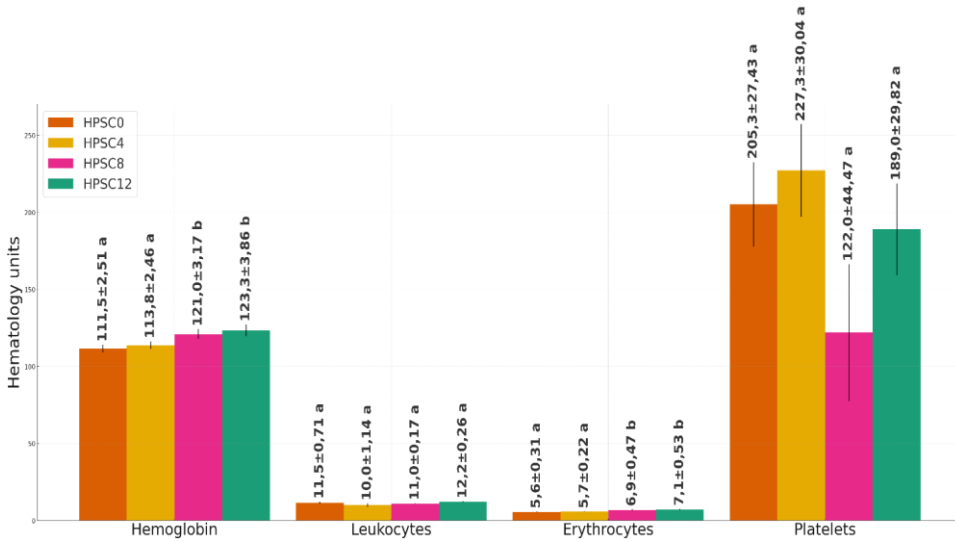


Fig. 3. Haematological blood parameters of young pigs, n=8/group

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$)

When using diets with a high-protein sunflower concentrate of 8% in the diet of piglets, an increase was observed, within the physiological norm, in the concentration of neutrophils in their peripheral blood by 7.5% ($p<0.05$), and 12% - by 8.5% ($p<0.05$) (Table 8). The indicated increase in the number of neutrophils occurred due to an increase in the concentration of the segmented granulocyte fraction, namely: by adding high-protein sunflower concentrate to the diet in the amount of 8% by 8.5% ($p<0.05$), and 12% - by 9.0% ($p<0.05$). Overall, the increase in neutrophil levels following the addition of varying amounts of high-protein sunflower concentrate occurred at the expense of a decrease in lymphocyte concentration. In particular, with the introduction of the concentrate in the amount of 8%, a decrease in the concentration of lymphocytes was observed by 7.3% ($p<0.05$), and with 12% - by 8.1% ($p<0.05$). In addition, the inclusion of the high-protein sunflower concentrate at 8% in the diet decreased monocyte concentration by 4.4% ($P<0.05$).

Table 8. Leukocyte formula of peripheral blood of piglets, n=8/group

Indicator	Group of pigs / % introduction of high-protein sunflower concentrate				RI
	HPSC 0	HPSC4	HPSC8	HPSC12	
Eosinophils	2.0±0.27 ^a	2.3±0.42 ^a	2.0±0.26 ^a	1.5±0.19 ^a	0.02–3.08
Neutrophils	37.8±2.39 ^a	38.8±2.42 ^a	45.3±2.86 ^b	46.3±3.26 ^b	19.50–48.24
including: – segmented granulocytes	34.3±2.41 ^a	35.8±2.54 ^a	42.8±2.96 ^b	43.3±2.95 ^b	–
– band granulocytes	3.5±0.63 ^a	3.0±0.46 ^a	2.5±0.33 ^b	3.0±0.26 ^a	–
Lymphocytes	55.7±1.88	54.6±1.95 ^a	48.4±2.83 ^b	47.6±2.94 ^b	46.75–76.63
Monocytes	4.5±0.19 ^a	4.3±0.16 ^a	4.3±0.16 ^b	4.6±0.17 ^a	3.67–11.50
Basophils	0.0	0.0	0.0	0.0	0.00–0.15

Notes: a, b – express traits fundamentally different in one table row ($P<0.05$); RI - reference intervals according to Præstegaard *et al.* 2025

Changes in the leukocyte count in pigs fed high-protein sunflower concentrate can be explained by several interrelated factors. The high content of available protein and the balanced amino acid composition of the concentrate stimulate protein synthesis in the bone marrow and liver, where immune system cells are formed and regulated. This may stimulate leukopoiesis and affect the relative proportions of specific leukocyte populations (neutrophils, lymphocytes, and monocytes) (Osadcha and Pavlovych 2023; Binev *et al.* 2025). Another process that explains these changes is the adaptive immune response. The transition to a new diet composition – even without pathogenic factors – is a kind of stress for the piglet organism and a stimulus for the immune system. In response, neutrophil or lymphocyte counts may increase slightly, reflecting the activation of the nonspecific and cellular branches of the immune system (Saco and Bassols, 2022; Osadcha *et al.* 2023). However, the detected quantitative changes occurred within the physiological norm, which indicates normal adaptation, and not inflammatory or infectious processes. Therefore, changes in the leukocyte formula when feeding high-protein sunflower concentrate are a consequence of more active protein metabolism, stimulation of the immune system and adaptive processes of the body to an increased supply of available protein, and provided that the indicators remain within physiological limits, they are regarded as a normal protective and adaptive reaction.

CONCLUSIONS

The introduction of 8% high-protein sunflower concentrate into the diet contributed to an increase in the live weight of piglets at 75 days of age by 3.6% ($p<0.05$), absolute gains by 5.9% for the period 45–75 days ($p<0.05$) and by 4.2% for the entire growing period ($p<0.05$). Average daily gains in this group exceeded the control values by 5.8% ($p<0.05$) at the age of 45–75 days and by 4.2% ($p<0.05$) on average for the entire growing period.

At this level of concentrate addition (8%), a significant increase ($p < 0.05$) in the concentration of total protein by 17.8% and urea by 77.8% was established. At the same time, a significant decrease ($p < 0.05$) in the activity of aspartate aminotransferase by 13.4% was noted. The haematological profile under the conditions of adding high-protein sunflower concentrate in an amount of 8% to the diet is characterised by a significant increase ($p < 0.05$) in the concentration of haemoglobin by 8.5% and the number of erythrocytes by 23.2%. In the leukocyte formula, a significant increase ($p < 0.05$) in the proportion of neutrophils by 7.5% was noted, mainly due to segmented forms, with a corresponding significant decrease ($p < 0.05$) in the proportion of lymphocytes by 7.3%.

The results obtained provide a scientific basis for recommending the inclusion of high-protein sunflower concentrate at a level of 8% in piglet diets as a metabolically safe and physiologically appropriate means of partially replacing soybean meal.

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MEDICINAL PLANTS FROM BJELASICA MOUNTAIN, USED IN TRADITIONAL MEDICINE

SUMMARY

Bjelasica Mountain in Montenegro is characterized by remarkable biological diversity. A variety of climates, silicate and limestone soil substrates, relief, aquatic resources and preserved nature have conditioned an abundance of different floral elements, including numerous medicinal and endemic plant species. Additionally, medicinal plants have socio-economic significance for the local population. Plants collected from the uncontaminated terrains are used to effectively prevent or treat various diseases. In the area of Bjelasica Mountain, 102 species within 44 families have been recorded for the use in traditional medicine. The most frequent are the representatives of the family *Lamiaceae* (13) and then *Asteraceae* (12). On the basis of results obtained by this study, the largest number of informants (79.01%) collects the two following species: *Ononis spinosa* L. and *Taraxacum officinalis* Weber. The active ingredients, to which the healing effects are attributed, can be found in various plant species and their organs. The highest content of active substances is determined at the beginning of flowering stage. The most exploited part was herb as it is used from 32 plant species, while the least exploited are the spores of *Lycopodium clavatum* L. From 102 plant species recorded for the use in traditional medicine, 40 are also used in conventional medicine and pharmacy. Some plants used in traditional medicine, contain poisonous substances, so the special attention should be paid to possible consequences due to unprofessional processing and use.

Keywords: Bjelasica, medicinal plants, traditional medicine, biodiversity, Montenegro

INTRODUCTION

Mountain regions are among the most important centres of biological diversity in Europe, owing to their pronounced environmental heterogeneity, complex relief, diverse geological substrates, and wide range of climatic conditions. These factors create favourable conditions for the development of rich

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and highly differentiated floras, including numerous medicinal and endemic plant species. In addition to their biological value, mountain ecosystems represent important repositories of traditional ecological knowledge, where long-term interaction between local communities and natural resources has shaped detailed practices of plant use, harvesting, and preparation.

Within the Balkan Peninsula, Montenegro occupies a distinctive position due to its exceptional geomorphological diversity and relatively well-preserved natural landscapes. One of the most floristically complex mountain massifs in northern Montenegro is Bjelasica Mountain, whose biodiversity is conditioned by a combination of silicate and limestone substrates, abundant water resources, and pronounced altitudinal gradients. The relief and climatic diversity of Bjelasica have resulted in the formation of multiple vegetation belts, including an exceptionally complex upper forest limit composed of several ecosystem types, a feature rarely observed elsewhere in the Balkans or Europe (Lakušić, D. *et al.*, 1991; Lakušić, R. *et al.*, 1991).

The central part of the Bjelasica massif includes Biogradska Gora National Park, one of the last remaining primeval forests in Europe and a recognized hotspot of European biodiversity (Blečić and Lakušić, 1970; Čurović *et al.*, 2011; Milošević *et al.*, 2019; Motta *et al.*, 2024). Floristic research in this region has a long tradition, beginning in the late nineteenth century with the work of Baldacci (1892) and continuing throughout the twentieth century with contributions by Novak (1932), Muravjov (1940), Rohlena (1942), Šilić (1983, 1984), Lakušić (1990), Stešević and Petrović (2003). These studies have provided a solid foundation for understanding the taxonomic and ecological complexity of the flora of Bjelasica and surrounding mountain systems.

The first written records of medicinal plant use in Montenegro date back to the early nineteenth century, when numerous species were documented as being collected and traded by local populations (Bojović and Dostjan, 1992). More systematic research on medicinal plants intensified during the second half of the twentieth century (Pulević, 1965; Lakušić and Milojević, 1972; Pulević, 1980; Sarić, 1989), and was later complemented by pharmacognostic and phytochemical studies (Menković, 1997; Jovančević *et al.*, 2011). Ethnobotanical investigations conducted in various mountainous regions of Montenegro and the wider Balkans have confirmed the importance of medicinal plants for both traditional healthcare and rural livelihoods (Pieroni, 2008; Menković *et al.*, 2011; Balijagić *et al.*, 2012; Šavikin *et al.*, 2013; Mullačija *et al.*, 2021).

Despite this long research tradition, comprehensive ethnobotanical studies focused specifically on the Bjelasica Mountain remain limited. At the same time, traditional medicinal knowledge in the region is increasingly threatened by socio-economic changes, rural depopulation, and market-driven overexploitation of certain plant species. Unsustainable harvesting practices, particularly of root- and bark-harvested taxa, pose a growing risk to local plant populations, while unprofessional use of toxic species may have adverse health consequences.

Ethnobotanical research in such contexts is therefore of particular importance, as it enables the documentation and preservation of traditional knowledge, contributes to biodiversity conservation, and provides a scientific basis for the sustainable management of medicinal plant resources. Furthermore, understanding the timing of collection activities in relation to plant phenology offers valuable insight into traditional ecological knowledge and rational harvesting strategies that are often closely aligned with periods of maximum bioactive compound accumulation.

The aim of this study was to document medicinal plant species used in traditional medicine on Bjelasica Mountain and to synthesize data on their taxonomic diversity, patterns of use, and harvesting practices. Specifically, the objectives were to: (i) Record medicinal plant species and their traditional applications; (ii) Analyze the overlap between traditional use and modern medicine and pharmacy; (iii) Examine the timing of collection activities in relation to phenological stages; and (iv) Assess the socio-economic and conservation implications of medicinal plant harvesting in the study area.

MATERIAL AND METHODS

Research area

The study was conducted on Bjelasica Mountain, located in northern Montenegro (Figure 1).

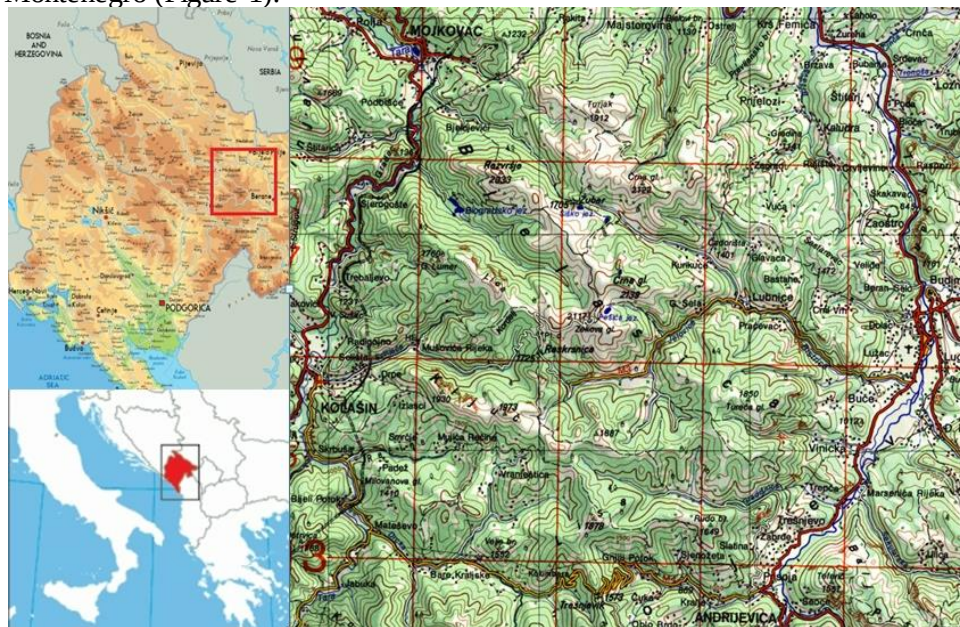


Figure 1. Location of the studied area of Bjelasica Mt. and Montenegro

The mountain massif extends between 42°40' - 42°55' N latitude and 19°30' - 19°50' E longitude and is characterized by pronounced altitudinal variation, ranging from approximately 600 to over 2,100 m a.s.l. Bjelasica is

distinguished by a complex geological structure dominated by silicate substrates, locally interspersed with limestone formations, as well as by abundant surface and groundwater resources (Fušić, 1991).

The area of hills and slopes above river valleys and basins, and even plateaus up to about 1300 m above sea level, is depicted by a transitional variant of the continental climate, which is modified by the influence of the alpine climate. At elevations above 1300 m, there is alpine and subalpine climate with long, severe and snowy winters, and very short and cool summers. The municipalities of Berane and Bijelo Polje, located in the valley of the river Lim, have a continental climate, while the municipality of Kolašin in the valley of the river Tara is a representative of the transitional climate type - from the continental to the alpine climate.

The average annual rainfall of the studied area ranges from about 1750 mm (lowland) to about 2000 mm (high-altitude and eastern regions). During the vegetation season, the amount of precipitation is about 500 mm to about 700 mm.

The largest gradients - the concentration of isotherms (Figure 2) are present in the zones with sudden changes in altitude. In the area of Bjelasica Mountain, there are two centres with temperatures below 2° C, of which one covers a much larger area. Moving to the west or to the east, the concentration increases. The south-north direction of isotherms indicates the fact that the temperature changes very quickly in the west-east direction, and very slowly in the south-north direction.

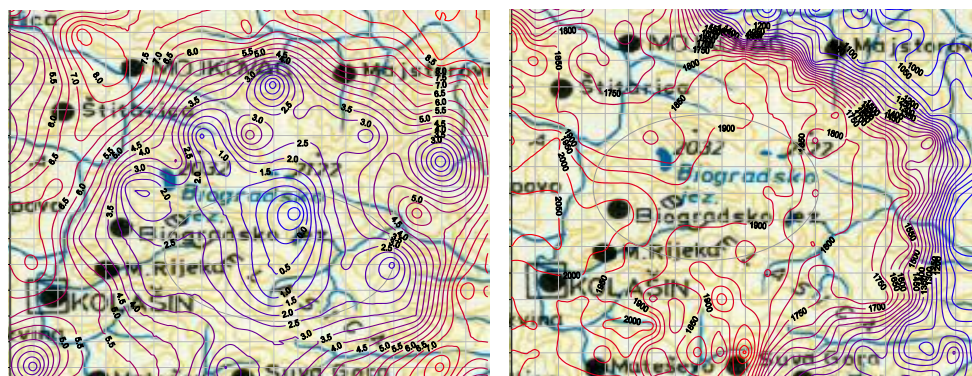


Figure 2. Maps of the study region with isotherms (a) and isohyets (b) (Source: Curović M, 2011)

The isohyets (b) do not change significantly in the whole area of Bjelasica Mountain. Although it is a high mountain zone, there is much less rainfall than in the southern lowlands due to the generation of precipitation in the mountain zones that precede Bjelasica. Since the eastern side of Bjelasica Mt. has significantly less precipitation, there is an intensive concentration of isohyets with extreme gradients. This zone covers the entire eastern side with a longitudinal arc of 180 degrees around Bjelasica itself, where precipitation is less by as much as 1000 mm per year and amounts to about 1850 mm per year.

Fieldwork and material processing

Ethnobotanical fieldwork was carried out during the vegetation seasons from 2017 to 2020. Data were collected through direct interviews with 90 informants residing in villages and rural settlements located on and around Bjelasica Mountain. Informants included local inhabitants, traditional healers, and experienced collectors of medicinal plants. Participation was voluntary, and information was obtained through oral communication.

Semi-structured interviews were conducted using open-ended questions focusing on local plant names, parts used, medicinal applications, preparation methods, and timing of collection. Additional information regarding the mode of administration and perceived effectiveness of remedies was recorded when provided by informants. Particular attention was paid to the seasonal timing of harvesting activities in relation to plant phenological stages. Thus, we recorded a number of plant families and number of species within each family, their use in traditional medicine (T), in modern medicine and pharmaceutical industry (MPh), but we also recorded poisonous plants that can be harmful to humans due to unprofessional use (P).

Plant species were identified using standard floristic literature. The following literature was consulted for different floristic categories: Mountain Plants (Lakušić, 1990); Endemic Plants (Šilić, 1983); Forest Herbaceous Plants (Šilić, 1983), Medicinal Herbs of the Bijelo Polje Region (Baljagić *et al.*, 2010), Medicinal Herbs of Serbia (Sarić M., 1989), as well as key for identification (Josifović *et al.*, 1970-1977; Jančić, 1990). Scientific names and family assignments follow accepted botanical nomenclature. For each recorded species, the local (vernacular) name, plant part used, mode of preparation, and medicinal application were documented. Species were classified according to their use in traditional medicine, modern medicine and pharmacy, and toxicity, based on available literature and informant reports.

Ethnobotanical data were analysed using descriptive statistical methods. The frequency of citation for each species was calculated based on the number of informants who reported its use. Medicinal plant species were grouped according to their therapeutic applications, plant parts used, and harvesting periods. The proportion of species used exclusively in traditional medicine, those shared with modern medicine and pharmacy, and toxic species was determined.

Harvesting periods were analysed in relation to plant phenology, with particular emphasis on flowering and fruiting stages, in order to assess the ecological and empirical basis of traditional collection practices. The results were compared with previously published ethnobotanical and pharmacological studies from Montenegro and neighbouring regions.

RESULTS AND DISCUSSION

The timing of medicinal plant collection on Bjelasica Mountain varies depending on altitude, exposure, and microclimatic conditions. Informants determine the optimal harvesting period primarily according to plant phenological stages observed on their own properties and surrounding habitats. These local indicators serve as practical guidelines for collecting plants even in remote mountain sites. In most cases, the reported harvesting periods coincide with those

described in scientific literature, particularly with the beginning of flowering, when the concentration of bioactive compounds is generally highest. This correspondence confirms the empirical ecological basis of traditional harvesting practices.



Figure 3. Blueberry pickers



Figure 4. Drying of medicinal herbs



Figure 5. Sorting and storage of medicinal herbs



Figure 6. Presentation of dried herbs



Figure 7. Exhibition and sale of tea in Bjelasica



Figure 8. Sales of tea on the market

Processing activities—including drying, sorting, storage, and marketing—are largely organized at the local level (Figures 3–8). Generational differences were evident: younger collectors tend to focus on fewer species with higher commercial value and greater abundance, whereas middle-aged and elderly informants possess broader ethnobotanical knowledge and more frequently collect plants for household and community healthcare purposes. Both men and women participate equally in collection activities.

A total of 102 medicinal plant species belonging to 44 families were recorded in the study area (Table 1). The most represented families are Lamiaceae and Asteraceae, which is consistent with floristic patterns observed in other mountainous regions of the Balkans. Their dominance reflects both ecological adaptability to diverse altitudinal belts and the high concentration of aromatic and bioactive compounds characteristic of these taxa.

When compared with ethnobotanical studies from other regions of Southeast Europe, the results show comparable richness. For example, 195 species were documented in the Ancona district (Central Italy), while 69 species were recorded in the Zlatibor district (Southwestern Serbia) and 136 taxa in Stara Planina (Southeastern Serbia). Although methodological differences and sample sizes vary, these comparisons confirm that Bjelasica Mountain represents an important regional centre of medicinal plant diversity and traditional knowledge. In the Ancona district, in the Marche region the ethnobotanical data concern medicinal (122 species), food (119), veterinary (53), superstitious/religious (61), cosmetic (30), domestic (27), dyeing (17), recreational (17), repellent (15), craft (10), and miscellaneous (29) uses, along with inclusion in local sayings and proverbs (25). The species with the greatest number of categories of use here was *Sambucus nigra* L. (Lucchetti *et al.* 2019). In the Zlatibor district, Southwestern Serbia, 220 informants (mean age 47 years, 79% female, 21% male) provided data for 69 medicinal plants belonging to 36 families (Šavikin *et al.* 2013), while in Southeast Serbia in Stara Planina the study identified 136 vascular medicinal plant taxa belonging to 53 families and 116 genera (Jarić *et al.* 2024).

Species with the highest frequency of citation (Table 2) among informants include:

- *Ononis spinosa* L. (79.01%)
- *Taraxacum officinalis* Weber (79.01%)
- *Achillea millefolium* L. (60.49%)
- *Betula pendula* Roth. (60.49%)
- *Hypericum perforatum* L. (60.49%)
- *Primula acaulis* L. (60.49%)
- *Sambucus nigra* L. (60.49%)
- *Thymus serpyllum* L. (60.49%)
- *Tussilago farfara* L. (60.49%)
- *Viscum album* L. (60.49%)

These taxa exhibit high cultural importance and represent key elements of the local pharmacopoeia. A similar pattern was observed in Stara Planina (Serbia), where *Hypericum perforatum* and *Thymus serpyllum* also showed maximum use values (Jarić *et al.*, 2024), suggesting a broader Balkan ethnomedicinal core group of species.

Table 1. Plant families, species and parts used in traditional medicine

No Family	Plant species and folk names	Part(s) used	Usage
01 <i>Alijaceae</i>	<i>Allium ursinum</i> L. (bear's garlic)	leaf-folium	It eliminates toxins from the body, regulates blood pressure.
02 <i>Anacardiaceae</i>	<i>Cotinus coggygria</i> Scop (smoke bush)	leaf	It helps the thyroid gland function, treats bacteria in the urinary tract in children.
03 <i>Apiaceae</i>	<i>Angelica silvestris</i> L. (wild root, fruit angelica)		It treats urinary tract ailments, biliary disorders, and respiratory diseases.
	<i>Sanicula europaea</i> L. (wood sanicle)	root, herb	It is used for the treatment of wounds, as a remedy for diarrhoea.
	<i>Coriandrum sativum</i> L. (Coriander)	fruit	Abdominal pain
	<i>Foeniculum vulgare</i> Mill. (fennel)	fruit	Infections of the urinary tract.
	<i>Petroselinum crispum</i> (Mill.) Nym. (parsley)	leaf	Kidneys, prostate
04 <i>Aspleniaceae</i>	<i>Asplenium scolopendrium</i> L. (hart's-tongue fern)	herb, leaf, flower	Pancreas, diuretic
05 <i>Asteraceae</i>	<i>Achillea millefolium</i> L. (common yarrow)	leaf-folium	Skin complaints, coronary heart disease, immune system, external treatment of wounds, stomach problems.
	<i>Artemisia absinthium</i> L., (wormwood)	herb	Lungs, cancer, viral diseases
	<i>Calendula officinalis</i> L. (pot marigold)	flower	It treats fungi, diarrhoea, and it is used for making skin creams.
	<i>Cichorium intybus</i> L. (blue daisy)	Radix, rarely herb or flower	It treats diarrhoea, and recovers liver.
	<i>Inula helenium</i> L. (elecampane)	root	Lung, rheumatic disease. It prevents pneumonia and coughing.
	<i>Helianthus tuberosus</i> L. (sunroot)	leaf, root	Diabetes
	<i>Matricaria chamomila</i> L. (chamomile)	flower	It is used in tea blends, for mouthwash, for eye compresses, and as a tranquilizer.
	<i>Petasites hybridus</i> (L.) G.M. Sch. (butterbur)	leaf	It treats rheumatic ailments.
	<i>Silybum marianum</i> (L.) Gaertn. (milk thistle)	fruit	After cancer surgery, for liver cleanse
	<i>Solidago virgaurea</i> L. (true goldenrod)	herb	Inflammatory processes, urinary tract ailments, renal disorders, rheumatic diseases
	<i>Taraxacum officinalis</i> Weber. (dandelion)	root flower	For weakened immune system, lungs, after liver cancer surgery, dissolve gallstones in gallbladder
	<i>Tussilago farfara</i> L. (coltsfoot)	flower	Bronchitis, expectoration, tea blends

06	<i>Betulaceae</i>	<i>Betula pendula</i> Roth. (silver birch)	Betule succus, Folium, gemmae.	It cleanses blood, strengthens immunity, stimulates kidney function, for urinary tract problems, as a shampoo.
07	<i>Boraginaceae</i>	<i>Pulmonaria officinalis</i> L. (lungwort) <i>Symphytum officinale</i> L. (comfrey)	herb root	Lungs, respiratory organs, asthma Bone regeneration, varicose veins, fractures, hard-to-heal wounds, rheumatic diseases, lung diseases
08	<i>Brassicaceae</i>	<i>Armoracia rusticana</i> P.Gaertner, B.Mayer &Schreb (horseradish) <i>Capsella bursa-pastoris</i> L. (shepherd's purse)	root herb	Immunity, stomach, appetite Postpartal problems with bleeding, <i>Escherichia coli</i> bacteria, heart
09	<i>Caryophyllaceae</i>	<i>Silene vulgaris</i> Moench. Garcke. (bladder campion)	herb	<i>Escherichia coli</i> bacteria
10	<i>Corylaceae</i>	<i>Corylus avellana</i> L. (common hazel)	leaf	stomach pain and diarrhea treatment
11	<i>Cupressaceae</i>	<i>Juniperus communis</i> L. (common juniper)	fruit	Problem with the urinary tract, kidneys, glands, as a diuretic
12	<i>Ericaceae</i>	<i>Arctostaphylos uva-ursi</i> (L.) Spreng. (Bearberry) <i>Vaccinium myrtillus</i> L. (blueberry).	leaf, fruit fruit, leaf	Urinary tract infections, kidneys As a vitamin supplement. improves blood count. anti-diabetes effects. improves vision, boosts immunity.
13	<i>Equisetaceae</i>	<i>Equisetum arvense</i> L. (field horsetail)	herb	Urinary tract infection, prostate problems. abdominal pain. lung disease, anti-virus, fractures
14	<i>Euphorbiaceae</i>	<i>Euphorbia cyparissias</i> L. (cypress spurge)	herb	Wart removal
15	<i>Fabaceae</i>	<i>Anthyllis vulneraria</i> L. (common kidney vetch) <i>Phaseolus vulgaris</i> L. (common bean) <i>Robinia pseudacacia</i> L. (black locust) <i>Trifolium pratense</i> L. (red clover) <i>Ononis spinosa</i> L. (spiny retharrow)	flower, herb pods flower flower, herb radix	Helps in eliminating gallstones and kidney stones, for wound healing. It lowers blood sugar level. Lung tea, blended tea Estrogen, help manage menopause symptoms Mild diuretic/prostate, urinary tract infections, mayasil
16	<i>Fagaceae</i>	<i>Quercus cerris</i> L. (Turkey oak)	Cortex - bark	For the stomach, for better digestion and against constipation
17	<i>Gentianaceae</i>	<i>Centaurium umbellatum</i> Gilib. (European centaury) <i>Gentiana asclepiadea</i> L. (willow gentian) <i>Gentiana cruciata</i> L. (cross gentian) <i>Gentiana lutea</i> L. (bitterwort)	herb root herb root	It helps digestion, in helicobacter treatment. Treatment of digestive problems, fever, weight reduction Cirrhosis of the liver, fatty liver, persistent cough Stimulates appetite Dilutes blood, improves digestion; it is used in the treatment of heart disease.
18	<i>Geraniaceae</i>	<i>Geranium macrorrhizum</i> L. (bigroot geranium)	flower, leaf, rhizome	Fertility problems, after radiation therapy

19	<i>Hipericaceae</i>	<i>Hypericum perforatum</i> L. (perforate St John's-wort)	herb	Haemorrhoid's, nervous system problems, wounds, depression, as a balm
20	<i>Juglandaceae</i>	<i>Juqlans regia</i> L. (walnut)	young leaf	For rinsing female reproductive organs, treating the tapeworms, gastrointestinal diseases
21	<i>Lamiaceae</i>	<i>Acinos alpinus</i> (L) Moench (rock thyme)	herb	Reduce high fever
		<i>Ballota nigra</i> L. (black horehound)	herb	Against hysteria, urinary incontinence
		<i>Glechoma hederacea</i> L. (ground-ivy)	herb	Respiratory system
		<i>Calamintha officinalis</i> Moench (pennyroyal)	herb	Anxiety, urinary incontinence
		<i>Melissa officinalis</i> L. (lemon balm)	leaf	As a tranquilizer, for headaches and migraines especially when triggered by stress.
		<i>Menta piperita</i> L. (Peppermint)	folium	For tea blends, upset stomach, as a tranquilizer
		<i>Origanum majorana</i> L. (majoran)	herb	Stimulates appetite
		<i>Oriqanum vulgare</i> L. (oregano)	herb	Inflammatory processes
		<i>Satureja montana</i> L. (winter savory)	herb	Peripheral blood circulation
		<i>Stahys officinalis</i> (L.) Trevis. (common hedgenettle)	herb	Bronchitis, wound healing
		<i>Teucrium chamaedrys</i> L. (wall germander)	herb	Against abdominal pain and diarrhea.
		<i>Teucrium montanum</i> L. (mountain germander)	herb	Strengthening immune svstem. for digestive and respiratory systems
		<i>Thymus serpyllum</i> L. (breckland thyme)	herb	Upper respiratory infection, tea blends
22	<i>Loranthaceae</i>	<i>Viscum album</i> L. (mistletoe)	herb	For hypertension, prostate, milder forms of paresis, cholesterol, liver cirrhosis
23	<i>Lycopodiaceae</i>	<i>Lycopodium clavatum</i> L. (common club moss)	spore	It can treat liver cirrhosis, all prostate diseases, and peeled skin.
24	<i>Malaceae</i>	<i>Crataequs monoqyna</i> L. (common hawthorn)	flower	Strengthens the heart muscle, lowers and regulates blood pressure.
25	<i>Malvaceae</i>	<i>Althaea officinalis</i> L. (marshmallow)	radix, leaf flower	Respiratory problems, stomach problems
		<i>Malva silvestris</i> L. (common mallow)	herb	Respiratorv and bladder problems, for wound dressings
26	<i>Oenotheraceae</i>	<i>Epilobium angustifolium</i> L. (rosebay willowherb)	herb, folium	Bacteria. swellings. and prostate, bladder, and kidneys diseases
27	<i>Papaveraceae</i>	<i>Chelidonium majus</i> L. (greater celandine)	herb, radix	It treats warts, lichen planus, early stage of cataracts, liver diseases. It is used as a narcotic.
28	<i>Parmeliaceae</i>	<i>Cetraria islandica</i> Achar. (Iceland lichen)	dried talus	Lung diseases, viruses

29	<i>Pinaceae</i>	<i>Pinus sylvestris</i> L. (Scots pine)	tips of the saplings	Bronchial tubes
30	<i>Plantaginaceae</i>	<i>Plantago lanceolata</i> L. (ribwort plantain)	seed, folium	Hemorrhoids, prostate, esophagus. Treat acute and chronic bronchitis, but not the allergic one.
		<i>Plantago major</i> L. (broadleaf plantain)	folium	Coughing and bronchitis
		<i>Plantago media</i> L. (Hoary plantain)	folium	Bronchitis, wounds
31	<i>Poaceae</i>	<i>Avena sativa</i> L. (common oat)	tips of the saplings	Bronchial tubes, cholesterol.
		<i>Zea mays</i> L. (corn)	Maydis stigma	Mild diuretic; relieves rheumatic pain.
32	<i>Polygonaceae</i>	<i>Polygonum aviculare</i> L. (common knotgrass)	herb	Prostate, urinary tract infection, kidney stones, lung disease
		<i>Polygonum bistorta</i> L. (bistort, snakeroot)	rhizome	Diarrheal, heart, stinking wounds
33	<i>Primulaceae</i>	<i>Anagallis arvensis</i> L. (scarlet pimpernel)	herb	Eye problems
		<i>Primula acaulis</i> L. (common primrose)	flower	Stomach, lungs, coughing, heart problems
		<i>Primula elatior</i> L. (oxlip)	flower	Lungs
		<i>Primula veris</i> Huds (cowslip)	Radix, flower	Lungs
34	<i>Rhamnaceae</i>	<i>Rhamnus frangula</i> L. (alder buckthorn)	bark	Cleansing the body, treats constipation and other digestive problems
35	<i>Rosaceae</i>	<i>Agrimonia eupatoria</i> L. (agrimony)	herb	Kidneys, liver, gall bladder, break up stones in the kidney
		<i>Alchemilla vulgaris</i> L. (common lady's mantle)	herb	Fertility, female diseases, female reproductive system. Diarrhea, wounds.
		<i>Cydonia oblonga</i> Mill. (quince)	leaf, fruit	Diarrhea
		<i>Fragaria vesca</i> L. (wild strawberry)	leaf	Respiratory system disorders, diarrhea, heartburn, bleeding
		(wood avens) <i>m</i> L. (wood avens)	Radix, herb	Energising preparations
		<i>Rosa canina</i> L. (dog rose)	fruit	Vitamin C, immunity, colds, inflammation
		<i>Rubus caesius</i> L. (dewberry)	leaf, fruit	Boost immunity, inflammatory processes in the throat, Diarrhea
		<i>Rubus idaeus</i> L. (raspberry)	leaf	Antidiarrheal, mouthwash, source of vitamins
		<i>Sorbus aucuparia</i> L. (mountain-ash)	fruit	Source of vitamins, flower tea for liver diseases, cough, hemorrhoids
36	<i>Rubiaceae</i>	<i>Asperula odorata</i> L. (sweet woodruff)	herb	Respiratory system disorders, gall bladder problems, skin diseases, liver, diuretic
		<i>Galium verum</i> L. (lady's bedstraw)	herb	Diuretic, for heart disease, sedative
37	<i>Salicaceae</i>	<i>Salix alba</i> L. (white willow)	bark	Calming, convulsions, insomnia, fever

38	<i>Sambucaceae</i>	<i>Sambucus nigra</i> L. (black elder)	flower, fruit	Colds, antivirals, urinary tract, cause Diarrhea
39	<i>Scrophulariaceae</i>	<i>Euphrasia rostkoviana</i> Hayek. (eyebright)	herb	Eye inflammation, for nasal irrigation
		<i>Scrophularia nodosa</i> L. (figwort)	herb, radix	Hemorrhoid's, tumors, stimulates appetite
		<i>Verbascum phlomoides</i> L. (orange mullein)	flower	Pulmonary diseases, respiratory tract, cough, bronchitis
		<i>Veronica officinalis</i> L. (heath speedwell)	herb	A medicine for strengthening, against nervousness, for treating the liver and gall bladder.
40	<i>Thymelaeaceae</i>	<i>Daphne blaaviana</i> Frey. (Blagay's daphne)	Bark, flower	Externally in tumors, rheumatism
41	<i>Tiliaceae</i>	<i>Tilia platyphyllos</i> Scop. (large-leaved lime)	flower	Relieves cough irritation, feverish colds that require sweating, nervous tension
42	<i>Urticaceae</i>	<i>Urtica dioica</i> L. (common nettle, stinging nettle)	leaf, seed	Anemia, immunity, menstrual complaints, rheumatism
43	<i>Valerianaceae</i>	<i>Valeriana officinalis</i> L. (Valerian, Garden heliotrope)	root	For soothing fever
44	<i>Violaceae</i>	<i>Viola odorata</i> L. (wood violet, sweet violet)	Flower	Lung diseases, coughing, sore throat,
		<i>Viola tricolor</i> L. (wild pansy, Johnny Jump up, heartsease)	herb	Skin diseases, for rinsing with sore throats and colds.
Σ 44 families		102 species		

The aerial parts of plants during flowering are most frequently harvested. Herb (entire aboveground part) is collected from 32 species (31.37%), leaves from 14 species (13.72%), and flowers from 9 species (8.82%). Root and bark harvesting occurs less frequently but is of greater ecological concern due to its destructive impact on plant survival.

Of the 102 recorded species:

- 58 species (59.16%) are used exclusively in traditional medicine
- 34 species (34.68%) are used both in traditional medicine and modern pharmacy
- 4 species are classified as toxic but used traditionally (T/P)
- 6 species are both pharmacologically recognized and toxic (MPH/T/P)

The relatively high proportion of species shared between traditional and modern medicine (34.68%) indicates strong concordance between empirical knowledge and scientifically validated therapeutic properties. This overlap confirms that local ethnomedicinal practices are largely grounded in long-term experiential selection of effective taxa.

Some plants (10) used in traditional medicine contain poisonous substances, which can cause adverse reactions if the plants are not processed professionally. According to Šarić *et al.* (1989) *Anagallis arvensis* L. has a harmful effect as it is a narcotic poison. *Artemisia absinthium* L. has a beneficial effect if used in small doses.

Due to the toxicity of thujone, some countries have forbidden the use of wormwood alcoholic extracts in the production of alcoholic beverages based on wormwood essential oil. *Chelidonium majus* L. is a spicy narcotic poison and in a large dose can be lethal.

Daphne blagayana Frey is very toxic if used too often and unprofessionally. *Euphorbia cyparissias* L. while fresh contains spicy milk that can cause inflammation and blisters. *Equisetum arvense* L. toxic alkaloids can cause bloody diarrhoea and bloody urination in case of irrational use. Hypericin, the constituent of *Hypericum perforatum* L. or St. John's wort, increases the sensitivity of skin to sunlight, so that people with fair skin can develop inflammation similar to that of sunburn. Moreover, St. John's wort berries are poisonous.

Although traditional knowledge includes awareness of dosage limitations, improper preparation or commercialization without professional guidance may pose health risks.

Market demand significantly influences harvesting pressure. A notable example is *Scrophularia nodosa*, which experienced a temporary surge in demand following claims of anti-cancer properties that were not scientifically confirmed. Such trends may lead to overharvesting and resource depletion.

Species harvested for roots or bark, such as *Gentiana lutea* or *Quercus cerris*, require particular attention due to the destructive nature of collection. Sustainable harvesting strategies and potential cultivation initiatives should therefore be prioritized.

The ecological conditions of the locality and the biological properties of the plants themselves determine the representation of individual plant families in the area. In Bjelasica Mt., the altitude difference between the sites is 1,569 m above sea level (2139 m-570=1569 m,) with several types of climates, from temperate continental to alpine climate.

For instance, *Gentiana lutea* is most abundant above 1000 m a.s.l., where environmental conditions favour higher concentrations of bioactive compounds. Similarly, *Vaccinium myrtillus* occupies acidic soils along a broad altitudinal gradient (650–2000 m a.s.l.). These patterns demonstrate the close relationship between mountain ecology and ethnomedicinal practices.

Generational differentiation in plant use suggests gradual erosion of comprehensive traditional knowledge. While younger collectors are economically oriented, older informants retain broader therapeutic applications. Without systematic documentation and knowledge transfer, this intangible heritage may decline.

A special danger for the preservation of natural resources is the sensational attribution of healing power to certain plants, especially in the treatment of serious diseases. About ten years ago, such a power was attributed to a black nettle (*Scrophularia nodosa* L.) in the treatment of malignant diseases. Its value and use have increased sharply in the market. As the science has not confirmed its value, the demand for it in Bjelasica Mt. has declined over time. Many traditional healers (60.49%) harvest *Achillea millefolium* L., *Betula pendula* Roth., *Hypericum perforatum* L., *Primula acaulis* L., *Sambucus nigra* L., *Thymus serpyllum* L., *Tussilago farfara* L., *Viscum album* L.

Table 2. The use of plants in traditional medicine

The collected species	Usage T-traditional medicine MPh- pharmacy P-poisonous	Number of study participant s	Annual	Biennial	Perenn ial	H- herbaceous W-woody plants S-shrub
<i>Achillea millefolium</i> L. (common yarrow)	MPh/T	60.49%			+	h
<i>Acinos alpinus</i> (L) Moench (rock thyme)	T	19.75%			+	h
<i>Agrimonia eupatoria</i> L. (agrimony)	T	11.11%			+	h
<i>Alchemilla vulgaris</i> L. (common lady's mantle)	MPh/T	30.86%			+	h
<i>Allium ursinum</i> L. (bear's garlic)	T	19.75%			+	h
<i>Althaea officinalis</i> L. (marshmallow)	T	11.11%			+	h
<i>Anagallis arvensis</i> L. (scarlet pimpernel)	P/T	1.23%	+			h
<i>Angelica silvestris</i> L. (wild angelica)	T	1.23%		+	+	h
<i>Anthyllis Vulneraria</i> L. (common kidney vetch)	T	1.23%	+	+	+	h
<i>Arctostaphylos uva-ursi</i> (L.) Spreng (bearberry)	MPh/T	19.75%			+	evergreen shrub
<i>Artemisia absinthium</i> L. (wormwood)	MPh/T/P	19.75%			+	R/w Arial parts/h Semi shrub
<i>Armoracia rusticana</i> P.Gaertner, B.Mayer & Schreb (horseradish)	T	4.94%			+	h
<i>Asperula odorata</i> L. (sweet woodruff)	MPh/T	4.94%			+	h
<i>Asplenium scolopendrium</i> L. (hart's-tongue fern)	T	4.94%			+	h
<i>Avena sativa</i> L. (common oat)	T	1.23%	+			h
<i>Ballota nigra</i> L. (black horehound)	T	11.11%			+	h
<i>Betula pendula</i> Roth. silver birch)	MPh/T	60.49			+	w
<i>Calamintha officinalis</i> Moench (pennyroyal)	T	1.23%			+	h
<i>Calendula officinalis</i> L. (pot marigold)	T	11.11%	+	rarely		h
<i>Capsella bursa-pastoris</i> L. (shepherd's purse)	MPh/T	44.44		+		h

<i>Cetraria islandica</i> Achar. (Iceland lichen)	MPh/T	1.23%				lichen
<i>Centaureum umbellatum</i> Gilib. (European centaury)	MPh/T	30.86%	+	+		h
<i>Chelidonium majus</i> L. (greater celandine)	MPh/T/P	19.75%			+	h
<i>Cichorium intybus</i> L. (blue daisy)	MPh/T	19.75%			+	
<i>Coriandrum sativum</i> L. (coriander)	T	1.23%	+			h
<i>Corylus avellana</i> L. (common hazel)	T	1.23%			+	w
<i>Cotinus coggygria</i> Scop (smoke bush)	T	11.11%			+	w
<i>Crataegus monogyna</i> L. (common hawthorn)	MPh/T	44.44			+	tree
<i>Cydonia oblonga</i> Mill. (quince)	T	1.23%			+	w
<i>Daphne blagayana</i> Frey. (Blagay's daphne)	MPh/T/P	11.11%				shrub-w
<i>Equisetum arvense</i> L. (field horsetail)	MPh/T/P	44.44			+	h
<i>Enilobium anaestifolium</i> L. (rosebay willowherb)	T	44.44			+	h
<i>Euphorbia cyparissias</i> L., (cypress spurge)	T/P	1.23%			+	h
<i>Euphrasia rostkoviana</i> Hayek. (eyebright)	T	4.94%	+			h - parasite
<i>Foeniculum vulgare</i> Mill. (fennel)	T	1.23%			+	h
<i>Fragaria vesca</i> L. (wild strawberry)	T	1.23%			+	h
<i>Galium verum</i> L. (lady's bedstraw)	T	1.23%			+	h
<i>Gentiana asclepiadea</i> L. (willow gentian)	T	30.86%			+	h
<i>Gentiana cruciata</i> L. (cross gentian)	MPh/T	1.23%			+	h
<i>Gentiana lutea</i> L. (bitterwort)	MPh/T	11.11%			+	h
<i>Geranium macrorrhizum</i> L. (bigroot geranium)	T	11.11%			+	h
<i>Geum urbanum</i> L. (wood avens)	T	1.23%			+	h
<i>Glechoma hederacea</i> L. (ground- ivy)	T	1.23%			+	h
<i>Helianthus tuberosus</i> L. (sunroot)	T	1.23%			+	h
<i>Hypericum perforatum</i> L. (perforate St John's-wort)	MPh/T/P	60.49%			+	h
<i>Inula helenium</i> L. (elecampane)	MPh/T	19.75%			+	h
<i>Juglans regia</i> L. (walnut)	T	11.11%			+	W
<i>Juniperus communis</i> L. (common juniper)	MPh/T	11.11%			+	w
<i>Lycopodium clavatum</i> L. (common club moss)	MPh/T	11.11%				

<i>Malva silvestris</i> L. (common mallow)	T	4.94%		+	+	Woody at the lower part
<i>Matricaria chamomila</i> L. (chamomile)	MPh/T	19.75%	+			h
<i>Melissa officinalis</i> L. (lemon balm)	MPh/T	44.44			+	h
<i>Menta piperita</i> L. (peppermint)	MPh/T	19.75%			+	h
<i>Ononis spinosa</i> L. (spiny restharrow)	MPh/T	79.01%			+	h
<i>Origanum majorana</i> L. (majoran)	T	1.23%			+	h
<i>Origanum vulgare</i> L. (oregano)	MPh/T	11.11%			+	h
<i>Petasites hybridus</i> (L.) G.M. Sch. (butterbur)	T/P	1.23%			+	h
<i>Petroselinum crispum</i> (Mill.) Nym. (parsley)	T	4.94%		+		h
<i>Phaseolus vulgaris</i> L. (common bean)	<u>I</u>	1.23%	+			h
<i>Pinus sylvestris</i> L. (Scots pine)	T	11.11%			+	w
<i>Plantago lanceolata</i> L. (ribwort plantain)	T	4.94%			+	h
<i>Plantago major</i> L. (broadleaf plantain)	T	30.86%	+	+	+	h
<i>Plantago media</i> L. (Hoary plantain)	T	1.23%			+	h
<i>Polygonum aviculare</i> L. (common knotgrass)	MPh/T	11.11%	+			h
<i>Polygonum bistorta</i> L. (bistort, snakeroot)	T	1.23%			+	h
<i>Primula acaulis</i> L. (common primrose)	T	60.49%			+	h
<i>Primula elatior</i> L. (oxlip)	MPh/T	1.23%			+	h
<i>Primula veris</i> Huds (cowslip)	MPh/T	4.94%			+	h
<i>Pulmonaria officinalis</i> L. (lungwort)	T	30.86%			+	h
<i>Quercus cerris</i> L. (Turkey oak)	MPh/T	4.94%			+	w
<i>Rhamnus frangula</i> L. (alder buckthorn)	T	4.94%			+	w
<i>Robinia pseudacacia</i> L.(black locust)	T	1.23%			+	w
<i>Rosa canina</i> L. (dog rose)	MPh/T	4.94%			+	w-shrub
<i>Rubus caesius</i> L. (dewberry)	T	19.75%			+	w-shrub
<i>Rubus idaeus</i> L. (raspberry)	T	19.75%			+	w-shrub
<i>Salix alba</i> L. (white willow)	MPh/T	19.75%			+	w
<i>Sambucus nigra</i> L. (black elder)	T	60.49%			+	w

<i>Sanicula europaea</i> L. (wood sanicle)	T	1.23%			+	w
<i>Satureja Montana</i> L. (winter savory)	T	19.75%			perennial	semibush
<i>Scrophularia nodosa</i> L. (figwort)	T	4.94%			+	h
<i>Silene vulgaris</i> (Moench) Garcke. (bladder campion)	T	1.23%				h
<i>Silybum marianum</i> (L.) Gaertn. (milk thistle)	T	1.23%	+ or	+ over winter		
<i>Solidago virgaurea</i> L. (true goldenrod)	MPh/T	11.11%			+	
<i>Sorbus aucuparia</i> L. (mountain-ash)	T	1.23%			+	w
<i>Stachys officinalis</i> (L.) Trevis. (common hedgenettle)	T	11.11%			+	h
<i>Symphytum officinale</i> L. (comfrey)	T/P	44.44			+	semishrub
<i>Taraxacum officinalis</i> Weber. (dandelion)	MPh/T	79.01%			+	h
<i>Teucrium chamaedrys</i> L. (wall germander)	T	44.44			+	semishrub
<i>Teucrium montanum</i> L. (mountain germander)	T	44.44			+	h
<i>Thymus serpyllum</i> L. (breckland thyme)	MPh/T	60.49%			+	h
<i>Tilia platyphyllos</i> Scop. (large-leaved lime)	T	1.23%			+	w
<i>Trifolium pratense</i> L. (red clover)	T	19.75%				h
<i>Tussilago farfara</i> L. (coltsfoot)	MPh/T/P	60.49%			+	h
<i>Urtica dioica</i> L. (common nettle, stinging nettle)	MPh/T	44.44			+	h
<i>Vaccinium myrtillus</i> L. (blueberry)	MPh/T	44.44			+	evergreen
<i>Valeriana officinalis</i> L. (Valerian, Garden heliotrope)	MPh/T	30.86%			+	h
<i>Verbascum phlomoides</i> L. (orange mullein)	T	30.86%		+		h
<i>Veronica officinalis</i> L. (heath speedwell)	T	4.94%	+	+	+	h
<i>Viola odorata</i> L. (wood violet, sweet violet)	T	30.86%			+	h
<i>Viola tricolor</i> L. (wild pansy, Johnny Jump up, heartsease)	MPh/T	11.11%	+		+	h
<i>Viscum album</i> L. (mistletoe)	MPh/T 14	60.49%			+	(80) evergreen shrub
<i>Zea mays</i> L. (corn)	T 8	1.23%	+			h

A total of 102 plant species are used in traditional medicine.						
T 102 species	58	59,16%				
MPh/T 34 species	34	34,68				
T/P 4 species						
MPh/T/P 6 species	10	9,80				

A large proportion of informants (79.01%) reported harvesting a core group of species, including *Achillea millefolium*, *Betula pendula*, *Hypericum perforatum*, *Primula acaulis*, *Sambucus nigra*, *Thymus serpyllum*, *Tussilago farfara*, and *Viscum album*, indicating their high cultural and therapeutic importance.

Similar patterns have been documented in other mountainous regions of the Western Balkans, such as Stara Planina (Serbia), where *Gentiana lutea*, *Hypericum perforatum*, and *Thymus serpyllum* were also among the most frequently cited species (Jarić *et al.*, 2024). This recurrence suggests a shared ethnomedicinal framework shaped by comparable ecological and cultural conditions.

The considerable proportion of species used both in traditional medicine and modern pharmacy confirms a strong correspondence between empirical knowledge and scientifically validated properties. However, the presence of toxic taxa and increased market demand for certain species highlight the need for careful management and sustainable harvesting. Ecological factors, particularly altitude and soil conditions, significantly influence species distribution and quality, further emphasizing the importance of site-specific knowledge in traditional plant use.

CONCLUSIONS

This study provides comprehensive ethnobotanical documentation of medicinal plants used in traditional medicine on Bjelasica Mountain, Montenegro. A total of 102 plant species belonging to 44 families were recorded, confirming the exceptional floristic richness of the region and its importance as a reservoir of traditional ecological knowledge. The predominance of species from the families Lamiaceae, Asteraceae, and Rosaceae reflects both the ecological characteristics of the mountain and the long-term empirical selection of therapeutically effective taxa.

The timing of collection activities is closely aligned with plant phenology and altitude-dependent vegetation dynamics. Harvesting periods largely coincide with the stages described in the scientific literature, particularly the beginning of flowering, when the concentration of biologically active compounds is considered highest. This alignment demonstrates that local collectors rely on accumulated empirical knowledge linked to seasonal indicators and developmental stages of vegetation, confirming the ecological grounding of traditional harvesting practices.

The relatively high proportion of species used both in traditional medicine and in modern pharmacy (34.7%) indicates substantial concordance between

local ethnomedicinal knowledge and scientifically validated therapeutic properties. Species such as *Ononis spinosa*, *Taraxacum officinale*, *Achillea millefolium*, *Hypericum perforatum*, *Sambucus nigra*, *Thymus serpyllum*, and *Tussilago farfara* exhibit particularly high cultural importance, as reflected by their frequent citation among informants, and represent priority taxa for further phytochemical and pharmacological investigation.

Socio-economic patterns reveal generational differences in plant use: younger collectors predominantly harvest species of higher commercial value, whereas older informants retain broader knowledge of plants used for household and community healthcare. This differentiation highlights the ongoing risk of erosion of traditional knowledge and underscores the importance of systematic documentation and intergenerational knowledge transfer.

From a conservation perspective, the intensive collection of certain high-demand species, particularly those harvested for roots or bark, raises concerns regarding long-term sustainability. The documented use of toxic species further emphasizes the need for education, professional guidance, and regulatory oversight to prevent adverse health effects and ecological degradation. Sustainable harvesting strategies, cultivation initiatives, and integration of traditional knowledge into conservation planning are essential to ensure the preservation of medicinal plant diversity on Bjelasica Mountain.

Overall, this research establishes a valuable baseline for future interdisciplinary studies linking ethnobotany, mountain ecology, pharmacology, and rural development. By documenting species diversity, phenological harvesting patterns, and socio-economic dynamics, the study contributes to the safeguarding of both biological resources and intangible cultural heritage in mountainous regions of the Western Balkans.

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COMPARISON OF DISTRIBUTION OF PINE PROCESSIONARY THAUMETOPOEA PITYOCAMPA (DENIS & SCHIFFERMÜLLER, 1775) IN THE AREAS OF MOSTAR, LIVNO AND TOMISLAVGRAD MUNICIPALITIES IN BOSNIA AND HERZEGOVINA

SUMMARY

The pine processionary, *Thaumetopoea pityocampa* (Denis and Schifferrmüller, 1775), is described in the scientific literature as a major pest of forest ecosystems in the Mediterranean region (Androić, 1957). It was first recorded in Bosnia and Herzegovina during the 1960s and 1970s in the area of Trebinje and the Neretva River canyon (Konjic and Prozor). Its harmful impact is emphasized by its geographical expansion and high adaptability, influenced by both internal and external factors such as climate change and the expansion of areas under coniferous forests.

The aim of this study was to determine and compare the distribution of this pest at three locations-Mostar, Livno, and Tomislavgrad. Field investigations were conducted during 2023 and 2024 at ten microlocations, including five in Mostar, two in Livno, and three in Tomislavgrad. The study included pest identification, determination of the number of nests, and assessment of their position and exposure within the tree canopy. The highest number of nests was recorded in the upper third of the canopy, on southern exposures, and within the 30-50 cm diameter class. Statistical analysis revealed significant differences among canopy sections only at Location 3 (Privala, Tomislavgrad), while the remaining locations showed a more uniform nest distribution. Urbanized sites in Mostar had a minimal number of nests (five in total), and at the University Campus (Location 9) a decrease in nest numbers was observed compared to

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previous studies. Successful recovery of previously infested trees was recorded, manifested through the emergence of new green shoots and regeneration of damaged branches. The attack of the pine processionary is localized and currently does not threaten forest stands; however, it poses a health risk to visitors and domestic animals. Regular removal of nests remains a key management measure, while climate change and milder winters may increase the intensity of attacks, particularly on weakened and older trees.

Keywords: *Thaumetopoea pityocampa*, distribution, pine trees, defoliator pest-caterpillar, health risk

INTRODUCTION

The pine processionary, *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775) (order Lepidoptera), is one of the most destructive pests causing defoliation of pine forests in the Mediterranean region, while simultaneously representing a serious allergen hazardous to human and animal health. Its harmful impact is largely determined by its geographical expansion and population increase over recent decades (Čolak *et al.*, 2024). The biological characteristics of the pest-including phenotypic plasticity, high adaptability, preference for warmer and sunnier exposures, and considerable reproductive potential-are key drivers of its spread (Mirchev *et al.*, 2021). Favorable factors such as mild winters and the expansion of coniferous forests (particularly pine stands) significantly influence the distribution, abundance, and population dynamics of *T. pityocampa* (Mirchev *et al.*, 2000; Battisti *et al.*, 2005; Hrašovec *et al.*, 2011). From all the above mentioned reasons, larvae of the pine processionary are considered to be the main pest of pine trees in the entire Mediterranean region (Hodar *et al.*, 2003).

The pine processionary is characteristic of the Mediterranean region with mild winters, and its northward expansion and spread to higher altitudes in recent decades have been closely associated with climate change. In Bosnia and Herzegovina, the pest was first recorded in the early 1960s in the Trebinje area and later, in the late 1970s, in the Neretva River canyon (Androić, 1978), while its occurrence in the northern part of the country was first documented in 2007 (Canton 10) (Mirchev *et al.*, 2000). In the sub-Mediterranean zone of Bosnia and Herzegovina, the pine processionary represents a significant pest of black pine (*Pinus nigra*), and it has also been recorded on *P. sylvestris*, *P. halepensis*, *P. mugo*, and *P. heldreichii* (Dautbašić *et al.*, 2018).

The aim of this study was to determine and compare the distribution of the pest at three locations-Mostar (Canton 7), Livno, and Tomislavgrad (Canton 10)-in order to assess the level of infestation in two climatically distinct areas within a limited range of pine forests. Particular emphasis was placed on identifying the canopy sections where infestation was most pronounced, considering both attack intensity and the risk posed by the pest to humans and animals. Contact with pine processionary larvae may cause severe allergic reactions affecting the skin and mucous membranes of humans and animals (Kaszak *et al.*, 2015).

MATERIAL AND METHODS

The aim of this study was to determine and compare the distribution of the pine processionary at three different locations-the municipalities of Mostar, Livno, and Tomislavgrad in Bosnia and Herzegovina-which are situated within two distinct climatic zones. Field research was conducted during 2023 and 2024. A total of ten microlocations were selected: five in Mostar, two in Livno, and three in Tomislavgrad. Microlocations were chosen based on the presence of pine forests, their representativeness of the forest ecosystems of the respective municipalities, terrain accessibility, and differences in altitude. Transects with a width of 10 m (5 m to the left and 5 m to the right of the transect centerline) were established at the selected microlocations. The total number of trees assessed per transect varied depending on stand density at each microlocation. For each tree, the number of nests, the position of nests within the canopy (upper, middle, and lower third), and tree exposure (north, south, east, west) were recorded. The “degree of infestation” was determined through a visual assessment based on the number of nests per tree and the intensity of defoliation, with each canopy third evaluated separately. All pine species present at the microlocations were treated equally in the study, including *Pinus nigra*, *P. sylvestris*, *P. halepensis*, *P. mugo*, and *P. heldreichii*.

Overall, data were collected on pest identification, the number of nests, and their position and exposure. For statistical analysis, the chi-square test was applied to determine differences in nest numbers among canopy sections and diameter classes of trees.

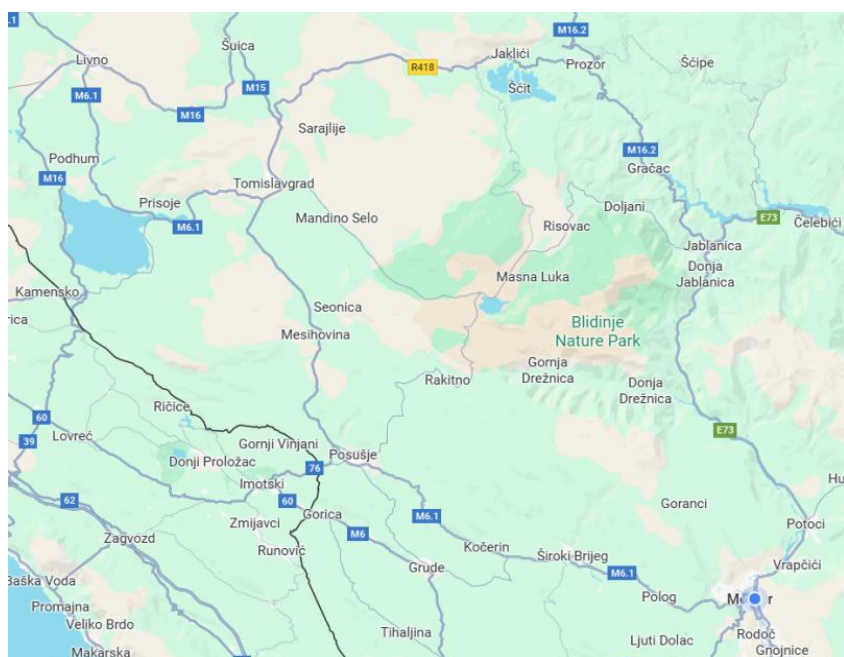


Fig 1. Area of the investigated locations (Google maps)

Microlocations were selected at different altitudes as follows:

Livno

Location 1 43.72669719037238, 17.022689132245436 altitude of 880 m

Location 2 43.725534207884365, 17.022517470868483 altitude of 870 m

Tomislavgrad

Location 3 43.70344876282711, 17.127548736534667 altitude 950 m

Location 4 43.70261109279604, 17.12274221797998 altitude 970 m

Location 5 43.700470327318165, 17.11694864650781 altitude 980 m

Mostar

Location 6 43.45865094194867, 17.956777826766483 altitude 980 m

Location 7 43.356114728635234, 17.809698350611146 altitude 65 m

Location 8 43.340972778007824, 17.794024054164765 altitude 74 m

Location 9 43.300990094022644, 17.843153539724884 altitude 120 m

Location 10 43.34966770010976, 17.830673755524916 altitude 435 m

RESULTS AND DISCUSSION

The Table 1. is presenting overview of all the locations and number of pine processionary nests spotted on pine trees in the locations and altitudu of the sites.

Table 1. Number of nests per location and altitude

Microlocation	Number of nests	Altitude
Location 1 – Livno Batinića brdo area	39	880m
Location 2 - Livno Batinića brdo area	60	870m
Location 3 – Tomislavgrad Privala area	101	950m
Location 4 - Tomislavgrad Privala area	34	970m
Location 5 - Tomislavgrad Privala area	42	980m
Location 6 – Mostar Rujište (Humi)	20	980m
Location 7 - Mostar Trimuša	5	65m
Location 8 – Mostar Carski vinogradi	5	74m
Location 9 – MostarUniversity campus	29	120m
Location 10 – Mostar Fortica	45	435m
TOTAL:	381	-

Location 3, situated above the reservoir (Buško Lake), recorded the highest number of nests (101 nests) compared to the other two locations in the same region. This can be explained by highly favorable microclimatic conditions, including a combination of sunny exposure, warm air currents originating from the water surface, and protection from strong winds, all of which promote the development of larvae and the pest on edge and individual trees. At this location, damage to older branches resulting from previous pine processionary attacks was

also observed, along with clear signs of recovery of affected pine branches, manifested by the emergence of new green needles. The lowest number of nests was recorded in the Mostar area (Locations 7 and 8-University Campus and Carski Vinogradi), which are urban environments with individual trees at lower altitudes.

In a regional comparison, Livno and Tomislavgrad (characterized by higher altitudes, cooler conditions, and less sunny exposures) exhibited a lower number of nests per tree than Mostar, indicating that microclimatic factors-such as temperature, solar radiation, and air humidity-play a significant role in determining attack intensity. At higher-altitude locations, nests were predominantly concentrated in the upper third of the canopy, whereas at lower elevations the infestation was more dispersed, which may be attributed to differences in solar exposure and temperature fluctuations.

Table 2. Analyses of statistical significance of number of nests in the upper, middle and bottom part of the tree by diameter class

Tree diameter (cm)						Total
0-10	10-20	20-30	30-50	>50		
Number of nests						
Upper third of the canopy	8	22	49	75	21	175
Middle third of the canopy	1	6	32	45	28	112
Botom third of the canopy	1	17	38	18	20	94
Total	10	45	119	138	69	381
Tree diameter (cm)						Total
0-10	10-20	20-30	30-50	>50		
Number of nests						
Upper third of the canopy	4.60	20.66	54.7	63.4	31.7	175
Middle third of the canopy	2.94	13.22	35.0	40.6	20.3	112
Botom third of the canopy	2.47	11.10	29.4	34.0	17.0	94
Total	10	45	119.0	138.0	69.0	381
DATA			RESULTS			
Level of significance		0.05		Critical value		15.51
Degree of freedom		8		Chi square		32.47

It can be seen in the Table 2., in the upper third of the tree, the largest number of nests was in the thickness class 30-50 cm (75 nests), followed by the thickness class 20-30 cm (49 nests). Regarding the middle third of the tree canopy, the largest number of nests was found in the thickness class 30-50 cm (45 nests), followed by the thickness class 20-30 cm (32 nests), similar results were reported by Dautbasic *et al.* 2018. In the lower third of the tree canopy, the largest number of nests was in the thickness class 20-30 cm (38 nests), followed by the thickness class over 50 cm (20 nests). Observing the number of nests by

tree parts, the largest number of nests was located in the upper third of the tree (175 nests or 45%), followed by the middle third (112 nests or 30%), while the lowest number of nests was located in the lower third (94 nests or 25%).

Statistical analysis (Chi-square test) has shown that there are statistically significant differences between the number of pine conifer nests in the upper, middle and bottom parts of the tree by thickness classes.

Table 3. Analyses of statistical significance of number of nests in the upper, middle and bottom part of the tree by diameter classe Location 3

	<i>Tree diameter (cm)</i>					<i>Total</i>
	0-10	10-20	20-30	30-50	>50	
	Number of nests					
<i>Upper third of the canopy</i>	0	0.39	5.50	16.89	10.21	33
<i>Middle third of the canopy</i>	0	0.46	6.50	19.96	12.07	39
<i>Botom third of the canopy</i>	0	5.32	4.35	5.32	14.02	29
Total	0	6	16	43	36	101
DATA	RESULTS					
<i>Level of significance</i>	0.05		<i>Critical value</i>		15.51	
<i>Degree of freedom</i>	8		<i>Chi-square</i>		19.37	

Statistical analysis for all the locations showed no statistically significant differences for all examined locations, except the Location 3, where there was statistically significant differences between the number of nests in the upper, middle and bottom parts of the tree by thickness classes (Table 3). Statistical analyses for Mostar locations 7 and 8, at Trimusa and Carski vinogradi were not performed due to the lack of valuable data, since only 5 nests were spotted in total. Location 9 – University campus, which was previously subject of research by Čolak *et al.* 2024 showed a decrease in a number of spotted nests, from 42 to 29.

Table 4. Number of nests of the pine processionary by tree part and exposure

	<i>Diameter class (cm)</i>				<i>Total</i>
	north	east	west	south	
	Number of nests				
<i>Upper third of the canopy</i>	0	0	31	154	185
<i>Middle third of the canopy</i>	0	14	23	82	119
<i>Botom third of the canopy</i>	0	3	9	65	77
Total	0	17	63	301	381

Microclimatic factors significantly influence the distribution of nests. At higher altitudes (Livno and Tomislavgrad), nests were predominantly concentrated in the upper third of the canopy, whereas at lower altitudes (Mostar) nests were more dispersed (Table 4). Sunny exposure, temperature, air humidity,

and the local influence of water bodies or urban surfaces act as key drivers of attack intensity. Comparison with similar studies conducted in the Mediterranean region shows that the observed patterns of nest distribution are consistent with the findings of Dautbašić *et al.* (2018) and Battisti *et al.* (2006), who reported a dominant concentration of nests in the upper third of the canopy and a preference for warmer exposures. Localized but numerous infestations in urban and peripheral areas represent an increased risk of contact between humans, domestic animals, and larvae, while in high-altitude forests the intensity of infestation may affect the future structure of forest stands. These findings enable more targeted control measures and timely nest removal, taking into account microclimatic conditions and regional differences, thereby contributing to risk reduction and improved forest management planning.

In Canton 10, the pine processionary currently does not pose a serious threat to forest stands; infestations are localized and do not occur within the interior of the canopy. Compared with lower-altitude and more urbanized areas (Mostar, Canton 7), the number of nests per tree is lower and infestations are more dispersed, further indicating the influence of microclimatic factors. Nevertheless, regular monitoring and control of this pest are necessary, as climate change is becoming increasingly pronounced, creating more favorable conditions for its spread.

The current intensity of infestation has not caused significant damage, as needle-feeding caterpillars-typically forming one to two nests per tree-do not yet interfere with normal tree development. At sites where nests were present in the past, the emergence of young green needles indicates regeneration of previously damaged canopy sections.

CONCLUSIONS

The highest number of pine processionary nests was recorded on warmer exposures, particularly southern aspects, in the upper part of tree canopies and within the 30–50 cm diameter class.

Successful recovery of previously infested trees was also observed, indicating that the current intensity of infestation is not at a critical level. Given the overall vitality of the trees, the pine processionary represents a greater risk to nature visitors and domestic animals within the study area. It is necessary to continue the timely removal of nests, as this method is environmentally friendly; the removal and destruction of nests contribute to environmental protection and enable safe use of natural areas.

Based on evaluation, identification, and analysis of the numerical status of the pine processionary population, it is evident that infestation intensity has increased in peripheral zones of pine forests and on individual trees, particularly on southern aspects, which receive greater solar radiation and thus favor pest development. Mild winter conditions have further contributed to increased attack intensity, although they have not resulted in a significant expansion of the pest's range.

Ongoing climate change further facilitates the spread of the pine processionary and causes negative impacts on pine forest ecosystems. Consequently, an increase in infestation intensity is expected in the future, particularly on weakened and older trees with reduced physiological activity and, therefore, lower resistance.

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TOTAL PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF CORNUS MAS L. FRUIT EXTRACTS

SUMMARY

In this study, the contents of phenolic compounds (phenols, hydrolysable tannins, flavonoids, and monomeric anthocyanins) and the antioxidant capacity of extracts obtained from *Cornus mas* L. fruits were determined. The plant material was collected in September 2023 at Duklja, near Podgorica, Montenegro. Destoned fruits were used to obtain the juice, the extraction of which was performed by the Kutscher-Steudel liquid-liquid extraction method. Soxhlet solid-liquid extractions were performed on the remaining by-products: pomace (consisting of exocarp and mesocarp) and stones/pyrenes (endocarp and seed). Different solvents were applied depending on the fruit part and the extraction method used. The highest phenolic and flavonoid contents were detected in the pyrene extracts: 85.85-105.86 mg/g gallic acid equivalents (GAE) and 60.37-71.77 mg/g quercetin equivalents (QE), respectively. The highest tannin content was found in the extracts of pomace (6.61-25.19 mg/g tannic acid equivalents, TAE) and juice (17.59-19.75 mg TAE/g). The strongest antioxidant activity, assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, was observed in the acetone extract from pyrenes and the methanol extract from pomace (IC₅₀ of 58.27 and 63.66 µg/mL, respectively). The ferric reducing antioxidant power (FRAP) assay showed the highest activity in the acetone extract from pyrene (40.01 mmol Fe²⁺/g). For comparison, all analyses were also performed using freshly squeezed juice; the contents of phenols, tannins, flavonoids, and anthocyanins were 1383.56 mg GAE/L, 1529.68 mg TAE/L, <5 mg QE/L and 79.49 mg/L cyanidin-3-glucoside equivalents, respectively. The antioxidant capacities of the juice were IC₅₀ 20.97 µg/mL (DPPH) and 39.07 mmol Fe²⁺/L (FRAP).

Keywords: cornelian cherry; extraction; fruit extract; bioactive compounds; DPPH; FRAP

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INTRODUCTION

Cornus mas L. (cornelian cherry, cornel, dogwood; CM) is a shrub species belonging to the family Cornaceae and is native to Southern and Central Europe and parts of Asia (Kazimierski *et al.*, 2019). It can reach up to 9 m in height and grows well in various soil types, including clay and sandy soils with low pH values, as well as in areas with low temperatures. However, it does not tolerate coastal regions with high air and soil salinity (Dokoupil and Řezníček 2012). The plant is entomophilous, heliophilous, and thermoxerophytic. Its fruit is a drupe, approximately 12 mm long and 5 mm wide, with an elliptic, smooth endocarp containing a seed (Šilić, 1990). The fruit ripens in late summer, and its color ranges from yellow to red depending on the stage of maturity (Da Ronch *et al.*, 2016). Fruit weight varies from 2 to 9 g and is influenced by genotype, cultivation practices, and environmental conditions (Szczepaniak *et al.*, 2019).

CM is valued for its nutritional and therapeutic properties and is classified as a medicinal plant (Kaya and Koca, 2021). Traditionally, it has been used to treat inflammation, kidney and intestinal disorders, maintain blood glucose levels, prevent liver fat accumulation, and potentially support cardiovascular health and cancer prevention (Hosseinpour-Jaghdani *et al.*, 2017). Due to the durability and hardness of its wood, it has historically been used in weapon making and construction (Da Ronch *et al.*, 2016). In Balkan folklore, CM is considered a magical plant and was often planted near homes to promote health and longevity (Radaković, 2012). The fruit can be consumed dried or used to prepare compotes and teas (D' Antuono *et al.*, 2014). Among the most important bioactive compounds in CM are phenolic compounds, which are stable secondary metabolites with protective roles in plants. They contribute to defense against herbivores and pathogens and may influence plant growth and interactions with the environment (Pratyusha, 2022). In human nutrition, phenolic compounds contribute to reducing oxidative stress and may offer benefits in the prevention or management of cardiovascular diseases, diabetes, and cancer (Lin *et al.*, 2016). Although phenolic composition and antioxidant activity of CM fruits have been previously studied, limited attention has been given to the chemical composition and bioactive potential of pyrenes. This represents a significant research gap, considering that pyrenes are often treated as by-products despite their possible functional value. Moreover, in this study, juice samples were extracted using a Kutscher-Steudel apparatus, a liquid-liquid extraction technique that has been applied in only a few previous studies. This approach provides a more efficient separation of bioactive compounds and represents a methodological innovation in the analysis of CM fruit constituents.

The aim of the study was to investigate the phenolic composition and antioxidant activity of different extracts of CM fruit, with particular emphasis on pyrenes and pomace, in order to evaluate their potential as valuable bioactive sources.

MATERIAL AND METHODS

Sampling of Cornelian Cherry

Plant material was collected in September 2023 at the Duklja site (42.467638, 19.263933) near Podgorica. The harvested fruits were mostly dark red, indicating full ripeness. On the same day, the fruits were processed by removing the endocarp with seed (pyrene), and juice was manually squeezed from the remaining pulp using a pestle and mortar. The juice was strained through cheesecloth (a simple traditional method for juice preparation) and stored in glass bottles at low temperature. The remaining dry residue (pomace) and pyrenes were dried for 20 days in a well-ventilated, dark area. Additionally, freshly squeezed juice and concentrated juice (obtained by water evaporation under heating) were included as samples.

Liquid-liquid Extraction

Juice samples were extracted using a Kutscher-Steudel apparatus according to a previously reported methodology (Božović *et al.*, 2025). This liquid-liquid extraction apparatus is suitable for the analysis of volatile solvents, as it requires temperatures typically near the boiling point of the selected solvent (Rausch, 2008). The device is intended for the extraction of aqueous samples using solvents that are less dense than water; in this study, ethyl acetate and diethyl ether were used. An 80 mL aliquot of the prepared juice sample was subjected to extraction for 24 hours. The obtained extracts were evaporated and stored in dark glass vials at 4°C until further analysis.

Solid-liquid Extraction

Soxhlet extraction was performed according to a previously published method (Božović *et al.*, 2026). This technique is based on the cyclic percolation of the plant material, which is continuously wetted by a solvent that extracts the bioactive compounds and collects them in a round-bottom flask. The apparatus is used for solid-liquid extraction, in which the selected plant material (in this study, the endocarp with seeds and dry residue of pulp) is first pulverized to increase the contact surface, thereby enhancing extraction efficiency. A total of 20 g of material was weighed, pulverized in an electric blender, and packed in filter paper. For the pyrenes, diethyl ether, hexane, and acetone were used as solvents, while for the pomace, methanol, hexane, and ethyl acetate were employed. The volume of solvent used for each extraction was 300 mL. The extraction cycles lasted on average 6-7 hours, with a total of 20 cycles, each lasting approximately 20 minutes.

Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). From each 1 mg/mL stock solution, 1 mL was taken, mixed with 0.5 mL of Folin-Ciocalteu reagent and 2.5 mL of 7.5% sodium carbonate. The mixture was kept in the dark for 120 minutes before absorbance

was measured at 740 nm. Results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract, or per L in the case of juice. All samples were tested in triplicate, and values are expressed as mean $\pm$ standard deviation (SD).

Total Flavonoid Content (TFC)

The aluminum chloride (AlCl_3) colorimetric method was used to determine total flavonoids (Ardekani *et al.*, 2011). Each reaction mixture consisted of 1.2 mL of extract and 1.2 mL of 2% AlCl_3 . After 60 minutes at room temperature, absorbance was measured at 420 nm. Results were calculated using a quercetin calibration curve and expressed as mg of quercetin equivalents (QE) per g of dry extract, or per L in the case of juice. All samples were analyzed in triplicate, and values are expressed as mean $\pm$ standard deviation (SD).

Total Anthocyanin Content (TAC)

TAC was measured using the pH differential method (Lee *et al.*, 2005), following the procedure described by Giusti and Wrolstad (2003), which is based on the structural transformation of anthocyanins as a function of pH in two buffer solutions: 0.025 M potassium chloride at pH 1.0 and 0.4 M sodium acetate at pH 4.5. Each test included 2 mL of extract (stock solution) and 8 mL of buffer. Absorbance was measured at 520 and 700 nm after 50 minutes. Values were expressed as mg of cyanidin-3-glucoside equivalents (C3GE) per g of extract, or per L in the case of juice. Assays were performed in triplicate, and values are expressed as mean $\pm$ standard deviation (SD).

Total Tannin Content (TTC)

Tannins were determined using the modified Prussian blue method described by Price and Butler (1977). The stock solutions were diluted 10-fold, and 500 μL from each diluted solution was mixed with 8 mL of distilled water, 0.5 mL of 0.1 M FeCl_3 , and 0.5 mL of 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$. After 10 minutes at room temperature, absorbance was measured at 720 nm. Tannic acid was used as the standard and the results were expressed as mg tannic acid equivalents (TAE) per g of dry extract, or per L in the case of juice. Assays were performed in triplicate, and values are expressed as mean $\pm$ standard deviation (SD).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The method is based on the reduction of the purple-colored DPPH radical by antioxidants (Gülçin and Alwasel, 2023; Baliyan *et al.*, 2022). From 0.1 g of extract dissolved in 10 mL methanol, 2 mL was taken and diluted to prepare 11 concentrations ranging from 1 to 0.0009375 mg/mL. Each concentration was tested in triplicate. A volume of 3 mL of each diluted extract was mixed with 1 mL of 0.1 mM DPPH solution and kept in the dark for 30 minutes. Absorbance was measured at 517 nm. Vitamin C was used as the standard. The experiment was performed in triplicate. The inhibition percentage of antioxidant activity (%AA) was calculated by the equation:

$$\%AA = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of the extract or the standard solution. The IC_{50} (the concentration required to inhibit 50% of the DPPH free radical; $\mu\text{g/mL}$) value of the extracts or ascorbic acid was calculated using log dose inhibition curve; the lower IC_{50} value means a higher antioxidant activity.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay measures the ability of the extract to reduce Fe^{3+} to Fe^{2+} , forming a blue Fe^{2+} -TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) complex (Benzie and Devaki, 2017). Each test used 3 ml of TPTZ reagent, 1 mL of distilled water, and 100 μL of extract. After incubation at 37°C for 15 minutes and cooling for 5 minutes, absorbance was measured at 595 nm. Results were expressed as $\mu\text{mol Fe}^{2+}$ equivalents per mL of sample. The standard was prepared using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Statistical Analysis

All statistical analyses were performed using Statistica 10.0 software package. Pearson correlation coefficients ($p < 0.05$) were used to examine relationships between antioxidant activity (DPPH and FRAP values) and total content of phenols, flavonoids and tannins. Correlation analyses were initially conducted for all extract types. However, as no statistically significant correlations were observed for most of the extracts, only the results for pomace are presented. A total of 18 correlations were calculated for pomace, including three phytochemical parameters (TPC, TFC, TTC) and two antioxidant assays (DPPH and FRAP) across all solvent extracts.

RESULTS AND DISCUSSION

Table 1. Yields of extracts (E1–E10) obtained from different fruit parts of CM and corresponding solvents

Extract	Fruit part	Solvent	Yield%
E1	juice	Ethyl acetate	3.03
E2	juice	Diethyl ether	0.75
E3	pomace	Ethyl acetate	6.05
E4	pomace	Methanol	45.3
E5	pomace	Hexane	1.4
E6	pyrene	Diethyl ether	8.45
E7	pyrene	Acetone	11.2
E8	pyrene	Hexane	5.13
E9 ^a	concentrated juice	–	15.2
E10 ^a	freshly squeezed juice	–	n.a. ^b

^a E9 and E10 represent concentrated and freshly squeezed juice respectively. They are labeled as “E” for consistency with the extract samples (E1–E8), although they are not obtained by extraction.

^b n.a. = not applicable (the yield was not determined). “–” indicates that no solvent was used.

The yields (%) of all extracts obtained and analyzed in this study are summarized in Table 1, with the corresponding fruit part and solvent indicated for each extract.

The results of the analyses of TPC, TFC, TAC, and TTC are presented in Table 2. Analyses of freshly squeezed juice and juice concentrated by evaporation were performed directly, without any additional extraction steps. Results for extracts are expressed per gram of dry extract, whereas juice samples are expressed per liter due to differences in sample matrices and preparation methods. This approach ensures that concentrations are presented appropriately for each sample type and allows for meaningful comparison of antioxidant activity and phytochemical content within each matrix.

Table 2. Analytical results of phenolic compounds of CM fruit extracts

Extract ^a	TPC (mg GAE/g)	TFC (mg QuE/g)	TTC (mg TAE/g)	TAC (mg C3GE/g)
E1	48.39 ± 3.38	< 5 ^b	17.59 ± 0.73	n.d. ^c
E2	51.18 ± 2.67	< 5	19.75 ± 0.81	n.d.
E3	52.38 ± 2.83	< 5	24.76 ± 0.85	n.d.
E4	52.54 ± 3.26	8.31 ± 0.33	25.19 ± 0.98	n.d.
E5	34.38 ± 1.48	< 5	6.61 ± 0.38	n.d.
E6	85.85 ± 4.46	63.53 ± 2.30	10.86 ± 0.61	n.d.
E7	105.86 ± 4.34	60.37 ± 2.17	19.81 ± 0.70	n.d.
E8	86.35 ± 3.62	71.77 ± 2.25	10.03 ± 0.53	n.d.
E9	36.47 ± 1.58	< 5	9.96 ± 0.60	n.d.
E10 ^d	1383.56 ± 95.63	< 5	1529.68 ± 101.26	79.49 ± 1.73

a The extract names (E1–E10) are consistent with those used in Table 1. E9 and E10 correspond to concentrated and freshly squeezed juice, respectively, and are labeled as “E” for uniformity, although they were not obtained by extraction. *b* Below the detection limit of 5 mg/g). *c* n.d. = not detected; the compound was below the detection limit of the applied method and standard concentration range. *d* The contents of phenols, flavonoids, tannins, and anthocyanins for E10 are expressed per liter (mg/L)

TPC ranged from 34.38 to 105.86 mg GAE/g extract (Table 2); the lowest value was observed in the hexane extract of the dry pomace (E5), whereas the highest phenolic content was recorded in the acetone extract of the pyrenes (E7). Among the solvents used, methanol was the most effective for extracting phenolics from the pomace, whereas acetone was the most effective for the pyrenes, whose extracts exhibited the highest values among all samples. The TPC of freshly squeezed juice was 1383.56 mg GAE/L.

For comparison, the present results are evaluated alongside previously reported values. In the study by Cosmulescu *et al.* (2018), phenolic values ranged from 163.69 to 359.28 mg GAE/100 g FW, depending on genotype and processing method, while Gąstoł *et al.* (2013) reported values of approximately 45.6 mg GAE/g, which are similar to those obtained in the present study. In a

study conducted in Serbia (Stanković *et al.*, 2014), the mean phenolic contents were 31.36 mg GAE/g for methanol extracts, 179.05 mg GAE/g for ethyl acetate extracts, and 55.38 mg GAE/g for acetone extracts. Among these solvents, methanol showed values most similar to those obtained in the present study, whereas ethyl acetate and acetone differed considerably; the value for ethyl acetate was approximately three times higher, while that for acetone was about half of the value reported in this paper. In a recent study conducted in Ukraine (Lidiková *et al.*, 2024), TPC ranged from 0.583 to 1.877 mg GAE/g FW. These differences can be attributed to analyses performed on fresh fruits, as opposed to extracts analyzed in the present study.

Flavonoid contents ranged from 8.31 to 71.77 mg QE/g extract, the lowest being observed in the methanol extract of the pomace (E4) and the highest recorded in the hexane extract of the pyrenes (E8). Moreover, extracts of the pyrenes obtained with all three solvents exhibited high flavonoid contents. For the pomace, flavonoids in the ethyl acetate and hexane extracts were not detected (below the detection limit of 5 mg/g), and the same was observed for the juice extracts, and both concentrated and freshly squeezed juice. Therefore, the results indicate that flavonoids are predominantly concentrated in the pyrenes.

In the study by Aurori *et al.* (2023), flavonoid contents averaged 0.139 mg/g, with ethanol used as the extraction solvent. Considering that most extracts in the present study were below measurable levels, these low values support the conclusion that flavonoids are weakly present in this plant's fruit. Gelo (2022) reported TFC values in fruits from Croatia without the endocarp, using methanol as the solvent, ranging from 0.18 to 1.07 mg QE/g. The authors also noted that the aluminum chloride method may be inadequate for determining flavonoid content in this plant, due to the presence of glycosylated hydroxyl carbon groups, which interfere with chelation by the reagent (Gelo, 2022; Mammen and Daniel, 2012).

Anthocyanins were not detected in the extracts of juice, pomace, or pyrenes, nor in concentrated juice, but were detected in freshly squeezed juice at 79.49 mg C3GE/L (E10). This suggests that anthocyanins are likely rapidly degraded during storage and upon exposure to high or low temperatures. Moldovan and David (2020) reported a TAC value of 93.43 mg C3GE/L in juice, measured using the pH differential method, which is comparable to the value obtained in the present study.

Tannin contents ranged from 6.61 to 25.19 mg TAE/g extract; the lowest value was found in the hexane extract of the pomace (E5), whereas the highest was recorded in the methanol extract of the pomace (E4). When comparing the same solvents across different parts of the fruit, ethyl acetate yielded higher tannin contents in the pomace than in the juice extract, hexane was more effective for the pyrenes than for the pomace, and diethyl ether resulted in higher values in the juice extract compared to the one obtained from pyrenes. Freshly squeezed juice contained 1529.68 mg TAE/L. In the study by Blagojević *et al.* (2023), using the percentage method prescribed by the European Pharmacopoeia (Ph.

Eur. 9.0), tannin content was higher in the pomace (0.81%) than in juice (0.65%), a result that aligns with the observations of the present study.

Given that only ripe fruits were used in the present study, this may explain the relatively low tannin content, as tannin levels are known to decrease during fruit ripening (Gunduz *et al.*, 2013). Bayram and Ozturkcan (2020) noted that only few studies have investigated tannin content in CM, highlighting the limited available data. Tannins in pomegranate (Fernandes *et al.*, 2015), which was selected for comparison with the present results, showed varying values depending on the part of the fruit analyzed. Notably, tannins were not detected in pomegranate juice, whereas in CM juice, they were present at significant level. Tannin contents in pomegranate seeds ranged from 2.4 to 12 mg/g, which is markedly lower than that observed in the pyrene extracts analyzed in the present study.

The results of antioxidant properties are presented in Table 3. Antioxidant activity measured by the DPPH assay showed IC₅₀ values ranging from 58.27 to 4560.89 µg/mL; the lowest value, indicating the highest antioxidant activity, was observed in the acetone extract of the pyrenes (E7), whereas the highest value was recorded for the hexane extract of the pyrenes (E8).

Table 3. DPPH (IC₅₀) and FRAP values of extracts (E1–E10) from CM fruits

Extract ^a	DPPH (IC ₅₀ µg/mL)	FRAP (mmol Fe ²⁺ /g)
E1	134.54 ± 4.57	13.69 ± 0.45
E2	362.09 ± 9.25	15.66 ± 0.55
E3	108.96 ± 4.36	15.96 ± 0.55
E4	63.66 ± 2.34	13.69 ± 0.56
E5	1273.07 ± 60.38	6.75 ± 0.35
E6	534.85 ± 25.10	7.51 ± 0.40
E7	58.27 ± 2.60	40.01 ± 1.52
E8	4560.89 ± 260.72	7.21 ± 0.40
E9	447.19 ± 17.87	7.09 ± 0.41
E10	20.97 ± 1.15	39.07 ± 1.37 ^b

^a The extract names (E1–E10) are consistent with those used in Table 1. E9 and E10 correspond to concentrated and freshly squeezed juice, respectively, and are labeled as “E” for uniformity, although they were not obtained by extraction. ^b The FRAP value for juice is expressed per liter (mmol Fe²⁺/L)

Large differences in IC₅₀ values were observed in the pomace extracts, highlighting the significant influence of solvent choice in this assay; methanol was the most effective solvent (E4), while hexane was the least effective (E5). The juice extract obtained with ethyl acetate (E1) showed higher activity than that obtained using diethyl ether (E2). A comparison of the same solvents across sample types revealed that ethyl acetate was slightly more effective in the pomace

extracts than in the juice extracts. The hexane extracts displayed generally low antioxidant activity, although those obtained from pomace performed better than those from pyrenes. Diethyl ether, in contrast, was more effective in the juice extracts than in those obtained from pyrenes. These results indicate that acetone and methanol are the most suitable solvents for maximizing antioxidant activity, while hexane and diethyl ether are less effective. The low antioxidant activity observed in concentrated juice is likely attributable to thermal degradation occurring during the juice concentration process. Freshly squeezed juice exhibited an IC_{50} value of 20.97 $\mu\text{g/mL}$.

IC_{50} values reported by Stanković *et al.* (2014) for fruit extracts prepared with methanol, ethyl acetate, and acetone, were 251.87, 11.06, and 107.99 $\mu\text{g/mL}$, respectively. In their study, ethyl acetate was the most effective solvent, whereas in the present research, acetone showed the highest activity and ethyl acetate was the least effective. These differences are likely due to the fact that Stanković *et al.* (2014) used whole CM fruits. In the study by Milenković-Andelković *et al.* (2015), DPPH activity expressed as IC_{50} was evaluated in fruits and leaves. Fruit extracts had IC_{50} values of 1090 $\mu\text{g/mL}$ in the first year and 940 $\mu\text{g/mL}$ in the following year. These values indicate considerably weaker antioxidant activity compared to the present results, which may be attributed to differences in extract preparation and solvent choice.

FRAP activity measured in juice by Gaštoł *et al.* (2013) was 23.5 mmol Fe^{2+}/L , which is comparable to the values obtained for freshly squeezed juice in the present study. In Turkey, Tural and Koca (2008) reported FRAP values ranging from 16.21 to 94.43 mmol Fe^{2+}/g , which partially correspond to the samples tested here; however, among 24 native plants covered in their work, some showed substantially higher FRAP values than those measured here. FRAP activity measured by Blagojević *et al.* (2023) in juice and pomace of CM from Montenegro was exceptionally low, ranging from 0.000094 to 0.000112 mmol Fe^{2+}/g and 0.000107 to 0.000120 mmol Fe^{2+}/g , respectively. In comparison, the present study showed considerably higher values for juice than for pomace across all solvents, and in general, higher FRAP values were detected. These differences may reflect variation in sample composition, extraction methods, and experimental conditions, rather than solely the analytical procedure.

Correlation analysis of phenolic content and antioxidant activity

In this study, the correlation between phenol, flavonoid and tannin contents and antioxidant activity was calculated using the Pearson correlation coefficient, with significance set at $p < 0.05$ (Table 4).

The content of phenolic compounds in ethyl acetate, methanol, and hexane extracts showed a negative correlation with antioxidant activity as determined by the DPPH assay. In contrast, the FRAP assay revealed significant positive correlations between phenolic compound content and antioxidant activity. A strong positive correlation was observed for phenols extracted with ethyl acetate ($r=0.85$, $p < 0.05$), while a moderate positive correlation was found for methanol

extracts ($r=0.53$, $p<0.05$). Regarding flavonoid content, a high significant positive correlation was found between ethyl acetate extracts and FRAP activity ($r=0.94$, $p<0.05$). For tannins, a very strong significant correlation was observed between ethyl acetate extracts and FRAP activity ($r=0.99$, $p<0.05$).

These results indicate that ethyl acetate is an effective solvent for extracting phenols, flavonoids, and especially tannins, which contribute significantly to antioxidant activity measured by the FRAP assay. Methanol also showed a positive correlation between flavonoid content and FRAP activity, suggesting it can influence the antioxidant potential of flavonoids.

Table 4. Correlation of phenol, flavonoid, anthocyanin, and tannin contents with antioxidant activity in extracts of CM

	TPC EA	TPC M	TPC H	TFC EA	TFC M	TFC H	TTC EA	TTC M	TTC H
DPPH EA	-0,99			-0,99			-0,92		
DPPH M		-0,69			-0,83			-0,85	
DPPH H			-0,88			-0,58			-0,98
FRAP EA	0,85*			0,94*			0,99*		
FRAP M		0,53			-0,73			0,29	
FRAP H			-0,46			-0,02			-0,69

*Bold values represent strong correlations ($p < 0.05$); TPC=Total Phenolic Content; TFC=Total Flavonoid Content; TTC=Total Tannin Content; EA=Ethyl Acetate; M=Methanol; H=Hexane; DPPH=antioxidant activity measured by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (IC_{50} , $\mu g/mL$); FRAP=antioxidant activity measured by the Ferric Reducing Antioxidant Power assay ($mmol Fe^{2+}/g$)

These findings are generally in agreement with previous studies. For instance, Guzel (2021) reported strong positive correlations between antioxidant activity and the content of phenolic compounds, including total phenols ($r=0.965$), flavonoids ($r=0.890$), and hydrolysable tannins ($r=0.882$). Similarly, Blagojević *et al.* (2023) demonstrated a high correlation between flavonoid content and antioxidant capacity measured by the FRAP assay ($r=0.96$), whereas the association with DPPH activity was minimal, which is consistent with the trends observed in the present study. In contrast to Blagojević *et al.* (2023), who reported very low correlations for tannin content with both DPPH and FRAP assays ($r=-0.04$ and $r=0.14$, respectively), the results in the study presented herein indicate a strong positive correlation between tannins and FRAP activity, underscoring their substantial contribution to the overall antioxidant potential. These discrepancies may reflect differences in extraction solvents, plant material, or methodological approaches, emphasizing the influence of experimental conditions on the observed antioxidant activities.

CONCLUSIONS

It can be concluded that acetone and methanol were the most effective solvents for extracting antioxidant compounds, while hexane was less successful. Nevertheless, the highest flavonoid content in the pyrenes extracts was obtained using hexane, and in pomace samples, methanol successfully extracted flavonoids

despite lower overall values. Both tests showed similar trends, indicating that the highest antioxidant activity was found in the acetone extract of pyrenes, whereas the lowest activities were recorded in hexane extracts. Among fruit parts, the extracts obtained from pyrenes exhibited the strongest antioxidant activity.

These findings provide valuable insights into the composition and bioactivity of CM, particularly the pyrenes, which has been the least investigated fruit part to date. Future research could focus on the mineral composition of fresh juice, the potential uses of the pyrenes, and other under-examined organs such as flowers, bark, and leaves. Environmental factors influencing antioxidant activity, including altitude and proximity to the sea (salinity being a known limiting factor), should also be considered. Additionally, exploring different extraction methods could further enhance understanding of the plant's chemical composition and medicinal properties, supporting its potential applications in pharmaceutical, cosmetic, and food industries.

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GROWTH PERFORMANCE AND BLOOD SERUM PARAMETERS OF PIGLETS USING DIETS WITH DIFFERENT RATIOS OF STANDARDIZED ILEAL DIGESTIBLE ISOLEUCINE TO LYSINE

SUMMARY

Isoleucine is a branched-chain essential amino acid in pig nutrition that regulates protein metabolism, fat deposition, and glucose utilisation, and serves as an energy substrate for immune protein synthesis, thereby enhancing immune function. It also improves growth performance, feed intake, and nutrient utilisation. However, the optimal standardized ileal digestible (SID) isoleucine:lysine ratio for young pigs remains controversial. This study aimed to determine the optimal SID isoleucine:lysine ratio for pigs weighing 7–30 kg. Four groups of piglets were fed diets with SID isoleucine:lysine ratios of <0.5, 0.5, 0.55, and 0.6. The results demonstrated that a ratio of 0.55 was optimal, increasing live weight at 70 days of age by 8.8% ($p<0.05$) without adverse physiological effects. This ratio reduced serum glucose, creatinine, urea, total cholesterol, low-density lipoprotein cholesterol, and triglycerides, while increasing total protein and high-density lipoprotein cholesterol and decreasing ALT and AST activity. A ratio of 0.5 was less effective but still increased live weight by 6.2% ($p<0.05$) and decreased serum glucose, creatinine, urea, and triglycerides, with a concomitant rise in total protein. In contrast, increasing the ratio to 0.6 did not improve body weight and altered protein and lipid metabolism, as evidenced by increased total protein and globulins, decreased albumins and albumin-to-globulin ratio, elevated cholesterol due to higher low-density lipoproteins, and reduced ALT and AST activity.

Keywords: SID isoleucine:lysine, pigs, live weight, protein, triglycerides, glucose

INTRODUCTION

Excessive nitrogen emissions from pigs raised on modern farms have detrimental effects through their contribution to acidification and eutrophication

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of sensitive ecosystems, as well as odour emissions (Almeida *et al.*, 2024). Reducing crude protein in pig diets has been shown to reduce these negative effects by improving nitrogen use efficiency, positively affecting both performance and animal health, and reducing environmental impacts (Chae *et al.*, 2024). However, this feeding strategy should be combined with an adequate supply of amino acids in quantity and quality to meet the genetic potential of the animals for protein deposition (Adhikari *et al.*, 2025). Until now, NRC (2012) has been the guideline for ensuring adequate amino acid requirements in pig diets. However, recent studies on various animals (Sychoy *et al.*, 2022; Plyska *et al.*, 2021; Shkarban and Sychoy 2025; Sychoy *et al.*, 2025) have shown that the standardized ileal digestibility (SID) ratio of amino acids to lysine has changed significantly over the past two decades, while some publications on pigs have reported contradictory results (Kwon *et al.*, 2022; Goodarzi *et al.*, 2022; Habibi *et al.*, 2022a; Zong *et al.*, 2022). Therefore, a more precise study is necessary, considering each factor that affects the amino acid requirements (especially the limited amino acids) in pig feeding.

Among the essential amino acids, tryptophan, valine, isoleucine, and methionine play important roles in several biological functions of porcine metabolism. In particular, isoleucine belongs to the group of essential branched-chain amino acids (BCAA), which are interdependent amino acids that affect the incorporation rate of other limiting amino acids. Isoleucine is a branched-chain amino acid that is essential for pigs and can regulate protein metabolism, fat deposition and glucose utilisation in the body (Nie *et al.*, 2018; Le Couteur *et al.*, 2020; Zhao *et al.*, 2024). In addition, isoleucine serves as an important energy substrate and is required for the synthesis of immune proteins (Gu *et al.*, 2019). Previous studies have shown that isoleucine can improve growth parameters, feed intake and utilisation, and relative intestinal length and morphology in pigs (Wang *et al.*, 2024). In addition, it can regulate intestinal immune function by increasing the secretion of β -defensins through the activation of the Sirt1/ERK/90RSK signalling pathway (Ren *et al.*, 2016; Zhang *et al.*, 2024).

Given the numerous functions that isoleucine performs in the body of pigs, over the past decades, many scientists have been devoted to establishing the optimal SID ratio of isoleucine:lysine in their diet. In particular, Goodarzi P. *et al.* (2022) recommend an optimal SID Ile:Lys ratio of 0.30–0.55 for pigs with a live weight of up to 25 kg. However, in subsequent studies, the authors increased the recommended SID Ile:Lys ratio to 0.29–0.60 (Goodarzi *et al.* 2023).

According to Habibi M. *et al.* (2022a), to maintain the normal physiological state of the pigs' body, it is necessary to maintain the SID Ile:Lys ratio at the level of 0.31–0.55. While Soumeh E. A. *et al.* (2014) and Nørgaard J. V. *et al.* (2013) recommend a SID Ile:Lys of 0.42–0.62, and Nørgaard J. V. and Fernández J. A. (2009) recommend 0.53–0.62. At the same time, Clark A. B. *et al.* (2016) suggest an even higher SID Ile:Lys ratio of 0.40–0.63 to maintain optimal growth parameters in pigs. According to Soumeh E.A. *et al.* (2014), for piglets with a body weight ranging from 11 to 25 kg, the optimal SID Ile:Lys

ratio in diets is between 0.42 and 0.62, whereas Barea R. *et al.* (2009) recommend a ratio of 0.46 to 0.65. For piglets with a body weight of 15–30 kg, Lazzeri D. B. *et al.* (2017) consider the optimal SID Ile:Lys ratio to be 0.45–0.73, and according to Waguespack, A. M. *et al.* (2012) SID Ile:Lys for young pigs 20–45 kg should be 0.52–0.61. For pigs with a live weight of 25 to 50 kg, Clizer D. A. *et al.* (2022) established an optimal SID Ile:Lys ratio of 0.55–0.75. However, Kerkaert H.R. and colleagues (2021) suggest a lower SID Ile:Lys ratio for pigs of this body weight, ranging from 0.61 to 0.66.

Summarising and generalising the data described in the literature, the optimal SID Ile:Lys ratio in pigs up to 11 kg live weight is between 0.58 and 0.65 (Chae *et al.*, 2024). These higher requirements compared to the NRC recommendation (2012) and several articles in the literature (Htoo *et al.*, 2014; Soumeh *et al.*, 2014; Clark *et al.*, 2017) can be explained by the previous assessment related to genetic improvement and excessive inclusion of leucine or valine in the piglet diet. For piglets with a live weight of 11 to 25 kg, the optimal SID Ile:Lys ratio ranged from 0.55 to 0.61, and for young pigs with a live weight of 25 to 50 kg, it ranged from 0.55 to 0.58 (Chae *et al.*, 2024). These results are also higher than those reported in the literature (Waguespack *et al.*, 2012; Gloaguen *et al.*, 2013; Nørgaard *et al.*, 2013; Lazzeri *et al.*, 2017) regarding the needs of the SID Ile:Lys ratio for pigs. Thus, despite a fairly large number of studies on determining the optimal SID Ile:Lys ratio in the diet of pigs with different body weights, i.e. for different periods of growth, the established values are contradictory and fluctuate in a fairly wide range. Therefore, this study aimed to establish the optimal SID Ile:Lys ratio in the diet of pigs aged 24 to 70 days with a body weight of 7–30 kg, which would provide the necessary growth parameters and maintain the normal physiological state of their body.

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 organised. The local Commission approved the experimental protocol on Bioethics of the National University of Life and Environmental Sciences of Ukraine.

Experimental Treatment Groups. From young pigs of the industrial herd, Danish selection (♀ Landrace/Large White X ♂ Dyurek) aged 24 days, 4 groups of piglets were formed (all animals were either half-brothers or half-sisters), which were kept in 10 m² pens on a slatted floor. Each pen was equipped with a 2.5 m² cover with a heating lamp, which was used in the first week of life after weaning. The experiment was conducted in December 2024 and January 2025.

The housing conditions (microclimate parameters, functioning of automatic drinkers) and the health status of the experimental animals were monitored daily. Before the start of the experiment, the premises were disinfected, and the

experimental pigs were subjected to preventive deworming. The average daily air temperature in the pigsty where the experimental pigs were kept was at 30-23 °C, depending on the period of detention, the relative humidity fluctuated within 70–75%.

The experimental design is presented in Table 1.

Table 1. Experimental design

Groups	Characteristics of feed	SID isoleucine:lysine
1 (control)	Basic feed (BF)	<0.5
Experimental 2	Basic feed (BF)	0.5
Experimental 3	Basic feed (BF)	0.55
Experimental 4	Basic feed (BF)	0.6

Piglets were fed manually using a TR-4 ROTECNA feeding device. 1 feeding device per group (hopper volume – 120 l, intended for piglets 6–40 kg, feeding type – dry, number of animals served by 1 hopper – 30 animals, feeding place width 18.7 cm. Feeding principle: every morning from 9.00 to 10.00 – the rest of the feed from the previous day was taken out, weighed and a new amount of feed was poured in.

The differences between the groups concerned only the composition of the diet, namely the ratio of SID isoleucine to lysine. Thus, in piglets of the Con group, the SID isoleucine:lysine ratio in the diet was unlimited and less than 0.5, in piglets of the Ile: Lys 0.5 group the SID isoleucine:lysine ratio was 0.5, in the Ile: Lys 0.55 group – 0.55, respectively, and in the Ile: Lys 0.6 group – 0.6.

Zootechnical records. Throughout the rearing period, the number of piglets removed from the experiment (due to mortality and culling) was recorded. Also, every seven days, the live weight of pigs was determined on the AXIS BDU300C-0608X scales (Ukraine). Electronic scales AXIS BDU300C-0608X (manufactured in Ukraine) are intended for weighing animals (in particular piglets) and general cargo, with a maximum capacity of 300 kg and a minimum load of 1 kg, a readability and verification scale interval of 50 g, a medium accuracy class, and a tare compensation range up to 100% of the maximum capacity.

Blood sampling. Blood sampling was performed from 8 pigs (♀4 & ♂4) of each group at the end of the experiment. The sampling was performed from the axillary vein, and 1.0–1.5 ml of blood was taken into a K2 EDTA tube. Blood sampling, as well as the selection of randomised individuals, was performed according to generally accepted methods.

Haematological methods. Haematological parameters of pig blood were determined on the IDEXX ProCyte Dx haematological analyser (IDEXX Europe B.V.) at the LLC "Veterinary Diagnostics Centre". The following parameters were determined: erythrocyte, leukocyte, and platelet counts - by impedance change (conductometric method); haemoglobin concentration - by the spectrophotometric method.

Biochemical parameters and enzyme activity of piglet blood serum were determined on an automatic Beckman Coulter biochemical analyser (USA) in the laboratory of LLC "Centre for Veterinary Diagnostics" using the manufacturer's original test systems and commercial diagnostic kits High Technology Inc. (USA). Determination of the main serum metabolites (High Technology Inc., USA). The following reagent sets were used to determine the concentrations of metabolites: glucose – set HT-G242 (method: glucose oxidase, endpoint; $\lambda=500$ nm); total protein – HT-T251 (biuret reaction, endpoint; $\lambda=540$ nm); albumin – HT-A203 (reaction with bromocresol green; $\lambda=630$ nm); creatinine – HT-C225 (Jaffe method, kinetic; $\lambda=510$ nm); urea – HT-U254 (Trinder/uricase method, endpoint; $\lambda=520$ nm); inorganic phosphorus – HT-P244 (ammonium molybdate method; $\lambda=340$ nm). calcium – HT-C216 (orthocresolphthalein method, endpoint; $\lambda=570$ nm); ALT (alanine aminotransferase) – HT-A206 (IFCC method, kinetic; $\lambda=340$ nm). The methods were performed according to the kit manufacturer's instructions.

Statistical analysis of biometric data was carried out on a PC using Statistica 10. The significance of differences between groups was assessed using one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparison test as a post hoc procedure. The normality of the sample data distribution was tested using the Shapiro-Wilk, Kolmogorov-Smirnov, and Box-Plot tests. If the data distribution was significantly different from normal, the nonparametric Mann-Whitney U-test was used. Differences between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The experimental young pigs were fed complete mixed feeds, balanced in all nutrients, in which the main nutritional indicators, including the levels of the first essential amino acids (lysine, methionine + cystine, tryptophan, valine) covered the needs of the recommendations of the CVB (2023). Before compiling the diets, the main ingredients (wheat, barley, soybean meal, soybean concentrate and dry aerosol-dried haemoglobin) were analysed for the content of dry matter, crude protein, crude fibre, starch, fat and crude ash. Based on the indicators obtained, the nutritional value of the main ingredients in the feed was calculated according to the formulas and nutritional matrices of the CVB database (2018). The nutritional value of the mixed feed used for feeding piglets is given in Tables 2–4.

Table 2. Nutritional value of the diet for young pigs from 24 to 38 days of age

Indicator	Group of piglets			
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6
1	2	3	4	5
Dry matter, g.	904.1	904.2	904.2	904.3
Humidity, g.	95.9	95.8	95.8	95.7

Crude protein, g.	179.5	179.9	180.2	180.6
Crude fibre, g.	43.1	43.1	43.1	43.0
Raw ash, g.	47.9	47.9	47.9	47.9
Crude fat, g.	70.1	70.0	70.0	70.0
Starch according to Evers	390.7	390.2	389.8	389.4
Net energy, kcal	2605.1	2605.0	2605.0	2605.0
Net energy, MJ.	10.9	10.9	10.9	10.9
Lysine, g.	13.2	13.2	13.2	13.2
Methionine, g.	5.0	5.0	5.0	5.0
Methionine + Cystine, g.	8.1	8.1	8.1	8.1
Threonine, g.	9.8	9.8	9.8	9.8
Tryptophan, g.	3.0	3.0	3.0	3.0
Valine, g.	9.5	9.5	9.5	9.5
SID Lysine, g.	12.1	12.1	12.1	12.1
SID Methionine, g.	4.7	4.7	4.7	4.7
SID Methionine + Cystine, g.	7.3	7.3	7.3	7.3
SID Threonine, g.	8.5	8.5	8.5	8.5
SID Tryptophan, g.	2.7	2.7	2.7	2.7
SID Valine, g.	8.3	8.3	8.3	8.3
SID Isoleucine, g.	5.3	6.1	6.7	7.3
SID Leucine, g.	12.1	12.1	12.1	12.1
SID Isoleucine/Lysine	0.439	0.5	0.55	0.6
SID Leucine/ Lysine	1.0	1.0	1.0	1.0
Calcium, g.	6.0	6.0	6.0	6.0
Total phosphorus, g.	5.0	5.0	5.0	5.0
Absorbable phosphorus, g.	3.8	3.8	3.8	3.8
Sodium, g.	3.0	3.0	3.0	3.0
Iron, mg.	110.0	110.0	110.0	110.0
Zinc, mg.	150.0	150.0	150.0	150.0
Manganese, mg.	72.0	72.0	72.0	72.0
Copper, mg.	150.0	150.0	150.0	150.0
Iodine, mg.	0.8	0.8	0.8	0.8
Selenium, mg.	0.4	0.4	0.4	0.4
Vit. A, IU	15000	15000	15000	15000
Vit. D3, IU	2500	2500	2500	2500
Vit. E, mg.	200.0	200.0	200.0	200.0
Vit. K3, mg.	2.5	2.5	2.5	2.5
Vit. B1 (Thiamine), mg.	1.875	1.875	1.875	1.875

Vit. B2 (Riboflavin), mg.	6.25	6.25	6.25	6.25
Vit. B3 (Niacin), mg.	25.0	25.0	25.0	25.0
Choline, mg.	300.0	300.0	300.0	300.0
Vit. B5 (Pantothenic acid), mg.	12.5	12.5	12.5	12.5
Vit. B6 (Pyridoxine), mg.	3.75	3.75	3.75	3.75
Vit. H (Biotin), mcg.	125.0	125.0	125.0	125.0
Vit. B9 (Folate), mg.	0.625	0.625	0.625	0.625
Vit. B12 (Cyanocobalamin), mcg.	31.25	31.25	31.25	31.25

Table 3. Nutritional value of the diet for young pigs from 39 to 55 days of age

Indicator	Group of piglets			
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6
1	2	3	4	5
Dry matter, g.	895.6	895.6	895.7	895.7
Humidity, g.	104.4	104.4	104.3	104.3
Crude protein, g.	174.9	175.3	175.6	176.0
Crude fibre, g.	42.4	42.4	42.4	42.4
Raw ash, g.	46.7	46.7	46.7	46.7
Crude fat, g.	57.4	57.2	57.0	56.7
Starch according to Evers	426.7	426.5	426.3	426.1
Net energy, kcal	2509.0	2509.0	2509.0	2509.0
Net energy, MJ.	10.5	10.5	10.5	10.5
Lysine, g.	12.7	12.7	12.7	12.7
Methionine, g.	4.9	4.9	4.9	4.9
Methionine + Cystine, g.	7.7	7.7	7.7	7.7
Threonine, g.	8.7	8.7	8.7	8.7
Tryptophan, g.	2.8	2.8	2.8	2.8
Valine, g.	9.0	9.0	9.0	9.0
SID Lysine, g.	11.5	11.5	11.5	11.5
SID Methionine, g.	4.5	4.5	4.5	4.5
SID Methionine + Cystine, g.	6.9	6.9	6.9	6.9
SID Threonine, g.	7.5	7.5	7.5	7.5
SID Tryptophan, g.	2.5	2.5	2.5	2.5
SID Valine, g.	7.8	7.8	7.8	7.8
SID Isoleucine, g.	5.2	5.8	6.3	6.9
SID Leucine, g.	11.5	11.5	11.5	11.5
SID Isoleucine/Lysine	0.452	0.5	0.55	0.6
SID Leucine/Lysine	1.0	1.0	1.0	1.0
Calcium, g.	5.5	5.5	5.5	5.5

Total phosphorus, g.	5.0	5.0	5.0	5.0
Absorbable phosphorus, g.	3.5	3.5	3.5	3.5
Sodium, g.	2.5	2.5	2.5	2.5
Iron, mg.	110.0	110.0	110.0	110.0
Zinc, mg.	150.0	150.0	150.0	150.0
Manganese, mg.	72.0	72.0	72.0	72.0
Copper, mg.	150.0	150.0	150.0	150.0
Iodine, mg.	0.8	0.8	0.8	0.8
Selenium, mg.	0.4	0.4	0.4	0.4
Vit. A, IU	15000	15000	15000	15000
Vit. D3, IU	2500	2500	2500	2500
Vit. E, mg.	200.0	200.0	200.0	200.0
Vit. K3, mg.	2.5	2.5	2.5	2.5
Vitamin B1 (Thiamine), mg.	1.875	1.875	1.875	1.875
Vit. B2 (Riboflavin), mg.	6.25	6.25	6.25	6.25
Ex. B3 (Niacin), mg.	25.0	25.0	25.0	25.0
Choline, mg.	400.0	400.0	400.0	400.0
Vit. B5 (Pantothenic acid), mg.	12.5	12.5	12.5	12.5
Vit. B6 (Pyridoxine), mg.	3.75	3.75	3.75	3.75
Vit. H (Biotin), mcg.	125.0	125.0	125.0	125.0
Vit. B9 (Folate), mg.	0.625	0.625	0.625	0.625
Vit. B12 (Cyanocobalamin), mcg.	31.25	31.25	31.25	31.25

Table 4. Nutritional value of the diet for young pigs from 56 to 70 days of age

Indicator	Group of piglets			
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6
1	2	3	4	5
Dry matter, g.	895.0	895.0	895.0	895.1
Humidity, g.	105.0	105.0	105.0	104.9
Crude protein, g.	173.8	174.0	174.4	174.7
Crude fibre, g.	39.2	39.2	39.1	39.1
Raw ash, g.	51.2	51.2	51.2	51.2
Crude fat, g.	46.9	46.7	46.5	46.3
Starch according to Evers	412.5	412.4	412.2	412.1
Net energy, kcal	2348.0	2348.0	2348.0	2348.0
Net energy, MJ.	10.2	10.2	10.2	10.2
Lysine, g.	12.2	12.2	12.2	12.2
Methionine, g.	4.5	4.5	4.5	4.5

Methionine + Cystine, g.	7.5	7.5	7.5	7.5
Threonine, g.	8.3	8.3	8.3	8.3
Tryptophan, g.	2.7	2.7	2.7	2.7
Valine, g.	8.4	8.4	8.4	8.4
SID Lysine, g.	11.0	11.0	11.0	11.0
SID Methionine, g.	4.2	4.2	4.2	4.2
SID Methionine + Cystine, g.	6.6	6.6	6.6	6.6
SID Threonine, g.	7.2	7.2	7.2	7.2
SID Tryptophan, g.	2.4	2.4	2.4	2.4
SID Valine, g.	7.2	7.2	7.2	7.2
SID Isoleucine, g.	5.2	5.5	6.1	6.6
SID Leucine, g.	11.0	11.0	11.0	11.0
SID Isoleucine/Lysine	0.473	0.5	0.55	0.6
SID Leucine/Lysine	1.0	1.0	1.0	1.0
Calcium, g.	5.5	5.5	5.5	5.5
Total phosphorus, g.	5.4	5.4	5.4	5.4
Absorbable phosphorus, g.	3.4	3.4	3.4	3.4
Sodium, g.	2.5	2.5	2.5	2.5
Iron, mg.	110.0	110.0	110.0	110.0
Zinc, mg.	150.0	150.0	150.0	150.0
Manganese, mg.	72.0	72.0	72.0	72.0
Copper, mg.	100.0	100.0	100.0	100.0
Iodine, mg.	0.8	0.8	0.8	0.8
Selenium, mg.	0.4	0.4	0.4	0.4
Vit. A, IU	15000	15000	15000	15000
Vit. D3, IU	2500	2500	2500	2500
Vit. E, mg.	200.0	200.0	200.0	200.0
Vit. K3, mg.	2.5	2.5	2.5	2.5
Vitamin B1 (Thiamine), mg.	1.875	1.875	1.875	1.875
Vit. B2 (Riboflavin), mg.	6.25	6.25	6.25	6.25
Ex. B3 (Niacin), mg.	25.0	25.0	25.0	25.0
Choline, mg.	300.0	300.0	300.0	300.0
Vit. B5 (Pantothenic acid), mg.	12.5	12.5	12.5	12.5
Vit. B6 (Pyridoxine), mg.	3.75	3.75	3.75	3.75
Vit. H (Biotin), mcg.	125.0	125.0	125.0	125.0
Vit. B9 (Folate), mg.	0.625	0.625	0.625	0.625
Vit. B12 (Cyanocobalamin), mcg.	31.25	31.25	31.25	31.25

The effect of using different SID isoleucine to lysine ratios in diets for young pigs on their body weight

Isoleucine has traditionally been considered an essential amino acid for pigs, as it cannot be synthesised in their body (Wang *et al.*, 2024). Optimal isoleucine intake positively affects growth performance and the physiological status of pigs (van Milgen *et al.*, 2012). According to the ideal protein concept, the requirement for isoleucine is determined by the SID isoleucine:lysine ratio, with lysine typically being the first limiting amino acid, and isoleucine thus considered the second limiting amino acid when assessing its requirement (Soumeh *et al.*, 2014; van Milgen *et al.*, 2012).

In this study, four different SID isoleucine:lysine ratios, ranging from less than 0.5 to 0.6, were used for young pigs with a live weight of 7–30 kg. At the beginning of the experiment, at 24 days of age, the body weights of young pigs were 7.40–7.43 kg, with no significant difference between the groups (Table 5). Thus, piglets were selected for the control and experimental groups, which were similar in age and body weight.

Table 5. Body weight of piglets depending on the ratio of SID isoleucine to lysine in the diet, kg, M $\pm$ SEM, n=21/group

Age, days	Group of piglets			
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6
24	7.43 $\pm$ 0.048 ^a	7.42 $\pm$ 0.045 ^a	7.40 $\pm$ 0.043 ^a	7.42 $\pm$ 0.045 ^a
38	11.08 $\pm$ 0.149 ^a	11.49 $\pm$ 0.138 ^b	11.67 $\pm$ 0.193 ^{bc}	11.13 $\pm$ 0.147 ^{ab}
55	18.42 $\pm$ 0.230 ^a	19.35 $\pm$ 0.23 ^b	19.79 $\pm$ 0.289 ^{bc}	18.70 $\pm$ 0.179 ^{ab}
70	27.57 $\pm$ 0.332 ^a	29.27 $\pm$ 0.357 ^b	30.00 $\pm$ 0.434 ^{bc}	28.27 $\pm$ 0.262 ^{ab}

Note: Values are presented as mean $\pm$ SEM (n=21 per group). Means within a row with different superscript letters differ significantly ($P<0.05$, Tukey–Kramer test)

However, during the growing period, the effect of different SID isoleucine:lysine ratios due to the addition of synthetic isoleucine on the body weight of young pigs was revealed. In particular, at 38 days of age, the body weight of piglets in the Ile:Lys0.5 group was 3.7% higher ($P<0.05$), at 55 days it was 5.0% higher ($P<0.05$), and at 70 days it was 6.2% higher ($P<0.05$), compared to the Con group. Young pigs in the Ile:Lys0.55 group were characterized by a higher body weight at 38 days of age by 5.3% ($P<0.05$), at 55 days of age by 7.4% ($P<0.05$), and at 70 days of age by 8.8% ($P<0.05$), compared to the Con group.

At the same time, no statistically significant differences were observed between the Ile:Lys0.55 and Ile:Lys0.5 groups at any stage of the experiment. Furthermore, piglets in the Ile:Lys0.6 group did not differ significantly in body weight from the Con, Ile:Lys0.5, or Ile:Lys0.55 groups throughout the experimental period.

The obtained data are consistent with the meta-analysis published by van Milgen J. *et al.* (2012), in which the authors concluded that the SID Ile:Lys level for maximising pig growth parameters should be 0.50–0.55. In general, studies on

the effect of the dose-dependent SID isoleucine:lysine ratio in diets without the addition of blood products on growth parameters of pigs with a live weight of 7–30 kg are few (Wiltafsky *et al.*, 2009). However, it is known that excessive isoleucine intake can increase BCAA metabolism and thus negatively affect growth parameters in pigs (Soumeh *et al.*, 2014). Thus, according to Nørgaard J. V. *et al.* (2013), pigs fed a diet containing excessive levels of isoleucine are characterised by reduced feed intake and reduced body weight gains. The negative consequences of excessive isoleucine intake may be due to interactions among BCAAs. Wiltafsky M. K. *et al.* (2009) also found that too high a SID isoleucine:lysine ratio can lead to reduced performance in pigs, and suggested that the synthesis of α -keto- β -methylvalerate by excessive isoleucine may activate the branched-chain ketoacid dehydrogenase complex, which can catalyse all irreversible steps of BCAA catabolism, and thus limit leucine metabolism.

Biochemical profile of piglets depending on the ratio of SID isoleucine to lysine in diets

Serum biochemical parameters can reflect the metabolic function in the pig body (Oliveira *et al.*, 2025). This study showed that when using all the studied SID isoleucine:lysine ratios in the diet for young pigs, namely from < 0.5 to 0.6, the clinical biochemistry parameters of their serum were within the physiological norm (Table 5). However, certain differences were still observed within the reference intervals depending on the ratios mentioned above. In particular, a slight increase in the concentration of total protein in the serum of piglets was found in proportion to the increase in the SID isoleucine:lysine ratio in the diet. Thus, in the young pigs of the Ile:Lys0.5 group, the concentration of total protein in the serum was lower by 1.9 % ($p < 0.05$), and in the group Ile: Lys 0.55 - by 4.8% ($p < 0.05$), while in piglets of the Ile:Lys0.6 group - by 5.1% ($p < 0.05$), compared to the Con group.

Table 6. Biochemical parameters of piglet blood serum depending on the ratio of SID isoleucine to lysine in the diet, $M \pm SEM$, $n=8/\text{group}$

Indicator	Group of piglets				PI ¹
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6	
Total protein, g/L	56.7 $\pm$ 0.31 ^a	57.8 $\pm$ 0.45 ^b	59.4 $\pm$ 0.55 ^c	59.6 $\pm$ 0.46 ^c	33–63
Albumin, g/L	31.5 $\pm$ 0.87 ^a	29.5 $\pm$ 0.76 ^a	31.5 $\pm$ 0.69 ^a	29.5 $\pm$ 0.95 ^a	20–39
Glucose, mmol/L	7.5 $\pm$ 0.21 ^a	6.2 $\pm$ 0.24 ^b	5.4 $\pm$ 0.25 ^c	7.7 $\pm$ 0.36 ^a	5.1–8.3
Creatinine, $\mu\text{mol/L}$	98.8 $\pm$ 2.78 ^a	89.8 $\pm$ 2.73 ^b	88.9 $\pm$ 2.32 ^c	101.9 $\pm$ 2.46 ^a	73–111
Urea, mmol/L	3.8 $\pm$ 0.48 ^a	1.9 $\pm$ 0.49 ^b	1.5 $\pm$ 0.42 ^b	2.6 $\pm$ 0.32 ^c	0.5–3.8
Uric acid, $\mu\text{mol/L}$	3.6 $\pm$ 0.34 ^a	3.5 $\pm$ 0.27 ^a	3.7 $\pm$ 0.22 ^a	3.2 $\pm$ 0.31 ^a	–
Calcium, mmol/L:					
- general	2.8 $\pm$ 0.07 ^a	2.8 $\pm$ 0.06 ^a	2.9 $\pm$ 0.08 ^a	2.6 $\pm$ 0.09 ^a	2.4–3.3
- ionised	1.3 $\pm$ 0.02 ^a	1.4 $\pm$ 0.01 ^a	1.4 $\pm$ 0.01 ^a	1.3 $\pm$ 0.02 ^a	–
Phosphorus, mmol/L	4.3 $\pm$ 0.07 ^a	4.4 $\pm$ 0.03 ^a	4.3 $\pm$ 0.05 ^a	4.2 $\pm$ 0.03 ^a	2.6–4.0

Note: Values are presented as mean $\pm$ SEM ($n=21$ per group). Means within a row with different superscript letters differ significantly ($P < 0.05$, Tukey–Kramer test). 1 RI - reference intervals according to Egeli A. K. *et al.* (1998)

Glucose is the primary source of energy and carbon for most eukaryotic cells, as it can be oxidised to provide energy and has a wide range of effects on cell function (Gaster *et al.*, 2000). Amino acids can be involved in the regulation of glucose levels. BCAAs, and especially isoleucine, improve glucose uptake and utilisation. Isoleucine can suppress increased blood glucose concentration (Doi *et al.*, 2003). According to Zhang, S. *et al.* (2016), the intake of isoleucine alone can increase glucose utilisation in pigs. In this study, a decrease in serum glucose concentration was observed in young pigs with an increase in the SID isoleucine:lysine ratio in the diet. Thus, in the young pigs of the Ile:Lys0.5 group, the concentration of glucose in the blood serum was lower by 17.3% ($p<0.05$), and in the young pigs of the Ile:Lys0.55 group - by 2.1 mmol/l or 28.0% ($p<0.05$), compared to the Con group. However, a further increase in the SID isoleucine:lysine ratio in the diet caused an increase in the concentration of glucose in the blood serum of piglets to the level of the Con group. The hypothesis of the mechanism by which isoleucine and leucine regulate the level of glucose in the blood serum may be associated with an increase in glucose uptake by muscles, glucose oxidation in the body and a decrease in gluconeogenesis in the liver of pigs (Doi *et al.*, 2007; Zhang *et al.*, 2016). Glycogen, which is synthesised and stored in the liver and skeletal muscle, plays an important role in the control of blood glucose levels. BCAAs promote glucose uptake by the liver and muscle and enhance glycogen synthesis. Isoleucine lowers plasma glucose levels by increasing muscle glucose uptake by activating the glucose transporter. This involves phosphatidylinositol-3-kinase and protein kinase C, which are insulin-independent (Doi *et al.*, 2005). BCAAs, in addition to mediating muscle glucose uptake and upregulating glycogen synthesis through activation, are also involved in fat and energy metabolism. The above statement is generally consistent with evidence indicating that isoleucine can reduce glycemia by enhancing skeletal muscle glucose uptake and activating glucose transport, including through PI3K/PKC signaling pathways, partially in an insulin-independent manner, thereby potentially supporting glycogen synthesis and storage in muscle. These effects were demonstrated in classic studies by Doi *et al.*, where isoleucine administration in rats reduced plasma glucose levels and increased muscle glucose uptake; subsequent work further clarified the involvement of PI3K-dependent pathways and effects on glucose oxidation and hepatic gluconeogenesis (Doi *et al.*, 2005; 2007).

Also, within the physiological norm, a decrease in the serum concentration of creatinine in young pigs was observed when diets with a SID isoleucine:lysine ratio of 0.5 and 0.55 were used for their feeding. In the piglets of the Ile:Lys0.5 group, the creatinine concentration in the serum was 9.1% lower ($p<0.05$), while in the other group, it was 10.0% lower ($p<0.05$), compared to the Con group. However, further increasing the SID isoleucine:lysine ratio in the diet to 0.6 resulted in an opposite effect, with the creatinine level in the serum of piglets in the Ile:Lys0.6 group rising to levels comparable to those of the Con group. Your observation that feeding young pigs diets with SID isoleucine:lysine ratios of 0.5

and 0.55 lowered serum creatinine within the physiological range, but increasing the ratio to 0.6 restored creatinine to control levels, aligns with emerging evidence that amino acid balance affects nitrogen metabolism and renal biomarkers indirectly rather than directly altering creatinine metabolism. Recent work indicates that functional amino acids such as isoleucine can influence overall nitrogen utilization, protein turnover, and metabolic health in growing pigs, which may in turn modulate serum creatinine concentration as part of broader metabolic shifts rather than a direct renal effect. For example, a 2024 study on dietary functional amino acid supplementation reported improvements in growth and health indicators in pigs, suggesting that balanced dietary amino acids can optimize protein metabolism and reduce metabolic stress markers (e.g., metabolites related to protein catabolism) without necessarily altering renal function directly (França *et al.*, 2024). In another recent analysis of SID branched-chain amino acid (BCAA) requirements, meta-analysis models confirmed that appropriate Ile:Lys ratios support optimal growth performance and efficient amino acid utilization in growing pigs, which likely reflects more efficient muscle protein synthesis and reduced amino acid catabolism — processes that could decrease creatinine release from muscle turnover. Additionally, a 2023 study on optimal Ile:Lys ratios in piglets found similar recommendations around the mid-range of ratios (close to ~0.5–0.55) for performance and physiological balance, supporting your empirical finding that moderate proportions are beneficial (Kerkaert *et al.*, 2021). From a mechanistic standpoint, serum creatinine in pigs tends to reflect lean tissue mass and protein turnover more than acute dietary changes. Thus, the reduction in creatinine at SID Ile:Lys ratios of 0.5 and 0.55 may indicate more efficient amino acid utilization and reduced muscle protein breakdown, whereas the return to control levels at 0.6 suggests that exceeding the optimal balance does not further improve nitrogen metabolism and may instead increase amino acid catabolism back to baseline. This interpretation is consistent with broader literature showing that maintaining amino acid balance near predicted requirements minimizes unnecessary catabolism and supports growth efficiency (França *et al.*, 2024; Sarri *et al.*, 2024; Bertolinir *et al.*, 2026).

Enriching the pig diet with isoleucine reduces blood urea and affects lipid metabolism (Spring *et al.*, 2020; Habibi *et al.*, 2022b). The mechanisms by which isoleucine regulates nitrogen utilisation and lipid metabolism in pigs are still poorly understood. However, in support of this, this study showed that increasing the SID isoleucine:lysine ratio in the diet for young pigs was associated with a decrease in serum urea concentrations within the physiological norm. Urea is a serum by-product of protein catabolism and a key indicator of the efficiency of amino acid utilisation in pigs (Marín-García *et al.*, 2022). In young pigs of the Ile:Lys0.5 group, the concentration of urea in the blood serum was lower by 50.0% ($p < 0.05$), and in the Ile:Lys0.55 group - by 60.5 % ($p < 0.05$), compared to the Con group. However, a further increase in the SID isoleucine:lysine ratio caused, on the contrary, an increase in the serum urea level of piglets of the Ile:Lys0.6 group to the values of the Con group, which indicates a negative effect of

excessive isoleucine consumption on the efficiency of protein utilisation in the body of pigs. This effect can be explained by the fact that pigs fed a balanced diet demonstrate a low level of urea in the blood, which indicates a decrease in protein catabolism, a more efficient use of total nitrogen and, consequently, a decrease in urea synthesis (Waguespack *et al.*, 2012; Toledo *et al.*, 2014). Increased blood urea concentration is associated with decreased protein synthesis and increased protein catabolism, and its level is negatively correlated with amino acids and protein utilisation efficiency (Heo *et al.*, 2008). Isoleucine deficiency leads to decreased protein synthesis, which may promote deamination of other amino acids and subsequently to increased blood urea levels (Kwon *et al.*, 2019).

The reduction in serum urea seen at moderate SID isoleucine:lysine ratios followed by a return to control levels at the higher ratio aligns with contemporary evidence that blood urea nitrogen (BUN) reflects the efficiency of nitrogen and amino acid utilization in pigs, rather than acute changes in crude protein alone. Recent studies have confirmed that BUN concentration is a reliable marker of protein degradation and nitrogen utilization, with lower values generally indicating improved matching of dietary amino acid supply to metabolic requirements and reduced excess amino acid catabolism. Such decreases in serum urea are interpreted as evidence of enhanced nitrogen retention and more efficient use of dietary amino acids for protein synthesis, as pigs on diets with better balanced amino acid profiles exhibit reduced hepatic ureagenesis and nitrogen excretion. Furthermore, current pig nutrition research underscores that oversupply or imbalance of indispensable amino acids, including branched-chain amino acids, can diminish the efficiency of nitrogen utilization, often resulting in higher urinary nitrogen outputs and less favorable metabolic profiles. The observed pattern in your data, where moderate optimization of Ile:Lys improved nitrogen metabolism but further increases had no additional benefit, is consistent with meta-analytical findings pointing to evolving and refined standardized ileal digestible amino acid requirements that support nitrogen efficiency only within an optimal range (Wang *et al.*, 2024; Chae *et al.*, 2024).

The activity of serum enzymes in piglets was within the physiological norm (Table 6). However, certain differences were still observed within the reference interval. In particular, in young pigs of the group Ile: Lys 0.55 showed a decrease in ALT activity by 20.5% ($p<0.05$) and AST by 11.7% ($p<0.05$), and in piglets of the group Ile: Lys 0.6 – ALT by 24.2% ($p<0.05$) and AST – by 13.1% ($p<0.05$), compared to the Con group.

Table 7. Activity of serum enzymes in piglets depending on the ratio of SID isoleucine to lysine in the diet, IU/L, M $\pm$ SEM, n=8/group

Enzyme	Group of piglets				PI ¹
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6	
ALT	82.5 $\pm$ 4.33 ^a	73.5 $\pm$ 3.23 ^a	65.6 $\pm$ 3.28 ^b	62.5 $\pm$ 4.11 ^b	15–108
AST	83.0 $\pm$ 2.74 ^a	81.8 $\pm$ 3.01 ^a	73.3 $\pm$ 3.11 ^b	72.1 $\pm$ 2.98 ^b	8–84

Note: Values are presented as mean $\pm$ SEM (n=21 per group). Means within a row with different superscript letters differ significantly ($P<0.05$, Tukey–Kramer test). 1 RI - reference intervals according to Egeli A. K. *et al.* (1998)

BCAA, especially isoleucine, is involved in the regulation of amino acid and fatty acid profiles of the pig body (Doudou *et al.*, 2022). This study confirmed that changes in the SID isoleucine:lysine ratio in the diet of young pigs affect the protein profile of their blood serum (Table 7). In particular, no differences were found in the concentration of serum albumins and globulins, as well as in the individual globulin fractions and the albumin-globulin ratio, in the piglets of the Con, Ile:Lys0.5, and Ile:Lys0.55 groups. However, in the piglets of the Ile:Lys0.6 group, a 12.7% decrease in albumins ($p<0.05$), a 13.2% increase in globulins ($p<0.05$), and a 0.45% reduction in the albumin-globulin ratio ($p<0.05$) were observed, compared to the Con group. The increase in globulins in the serum of piglets in the Ile:Lys0.6 group was due to an increase in all fractions, except for β -globulins. Specifically, there was a 2.5% increase in $\alpha 1$ -globulins ($p<0.05$), a 6.3% increase in $\alpha 2$ -globulins ($p<0.05$), and a 3.2% increase in γ -globulins ($p<0.05$), compared to the Con group.

Branched-chain amino acids, particularly isoleucine, not only influence lipid and nitrogen metabolism but also affect systemic protein profiles in growing pigs, with serum albumin and globulin fractions serving as sensitive indicators of metabolic balance and immune modulation. In the present study, no differences were detected in albumins, total globulins, individual globulin fractions, or the albumin-globulin ratio among piglets fed control or moderate SID Ile:Lys diets, underscoring metabolic stability when amino acids are balanced within recommended ranges. However, at a higher SID Ile:Lys ratio (0.6), a marked decrease in albumin and concomitant increases in total globulins –driven by elevated $\alpha 1$ -, $\alpha 2$ -, and γ -globulin fractions –suggest a shift in hepatic protein synthesis and possibly mild activation of immune-related protein production. This interpretation is supported by recent research showing that alterations in dietary protein and amino acid supply can significantly affect serum protein fractions in pigs, with both albumin and globulin responding in nonlinear ways to nutritional imbalances (Hlathini *et al.*, 2024). Moreover, contemporary studies on amino acid-balanced diets demonstrate that dietary protein and amino acid composition shape nitrogen metabolism and blood biochemical profiles, including serum proteins, and that deviations from ideal patterns can lead to compensatory responses in immune-related protein fractions.

Table 8. Protein profile of piglet blood serum depending on the ratio of SID isoleucine to lysine in the diet, %, $M\pm SEM$, $n=8/\text{group}$

Indicator	Group of piglets			
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6
Albumins	52.2 $\pm$ 1.13 ^a	53.5 $\pm$ 1.35 ^a	50.5 $\pm$ 1.67 ^a	39.5 $\pm$ 1.42 ^b
Globulins	47.3 $\pm$ 1.12 ^a	46.5 $\pm$ 1.36 ^a	49.5 $\pm$ 1.31 ^a	60.5 $\pm$ 1.21 ^b
$\alpha 1$ -globulins	2.5 $\pm$ 0.27 ^a	3.2 $\pm$ 0.29 ^a	2.5 $\pm$ 0.22 ^a	5.0 $\pm$ 0.26 ^b
$\alpha 2$ -globulins	4.5 $\pm$ 0.57 ^a	5.0 $\pm$ 0.21 ^a	5.8 $\pm$ 0.49 ^a	10.8 $\pm$ 0.22 ^b
β -globulins	15.0 $\pm$ 0.85 ^a	14.3 $\pm$ 0.77 ^a	15.5 $\pm$ 0.57 ^a	16.2 $\pm$ 0.73 ^a
γ -globulins	25.3 $\pm$ 0.56 ^a	24.0 $\pm$ 0.97 ^a	25.7 $\pm$ 0.73 ^a	28.5 $\pm$ 0.67 ^b
Albumin-globulin ratio	1.12 $\pm$ 0.048 ^a	1.25 $\pm$ 0.196 ^a	1.03 $\pm$ 0.074 ^a	0.67 $\pm$ 0.061 ^b

Note: Values are presented as mean $\pm$ SEM ($n = 21$ per group). Means within a row with different superscript letters differ significantly ($P < 0.05$, Tukey–Kramer test)

BCAAs are known to be involved in fatty acid metabolism (Cheng *et al.*, 2010). Insufficient or excessive levels of BCAA in the diet can negatively affect lipid metabolism in pigs (Zhang *et al.*, 2017). Serum triglyceride and total cholesterol levels are related to lipid absorption, and low-density lipoprotein and high-density lipoprotein levels are related to lipid breakdown and transport in pigs (Heng *et al.*, 2020). Therefore, at the final stage of this study, the serum lipid profile of young pigs was analysed depending on the SID isoleucine:lysine ratio in their diet (Table 8). It was found that the content of total cholesterol in the serum of young pigs using all the studied SID isoleucine:lysine ratios in the diet was within the physiological norm. However, certain differences were still observed within the reference intervals. In particular, in young pigs of the Con and Ile:Lys0.5 groups, the concentration of total cholesterol was at the same level. In the piglets of the Ile:Lys0.55 group, the total cholesterol content was 8.7% lower ($p<0.05$) compared to the Con group. However, in the piglets of the Ile:Lys0.6 group, a 10.6% increase in serum cholesterol levels ($p<0.05$) was observed, compared to the Con group.

Table 9. Lipid profile of piglet blood serum depending on the ratio of SID isoleucine to lysine in the diet, mmol/L, $M\pm SEM$, $n=8/\text{group}$

Indicator	Group of piglets				PI
	Con	Ile:Lys0.5	Ile:Lys0.55	Ile:Lys0.6	
Total cholesterol	2.65 ± 0.063^a	2.68 ± 0.031^a	2.42 ± 0.094^b	2.93 ± 0.063^c	$1.6-4.3^1$
Triglycerides	0.58 ± 0.049^a	0.37 ± 0.019^b	0.30 ± 0.038^b	0.46 ± 0.053^b	$0.2-2.25^1$
High-density lipoproteins	0.88 ± 0.028^a	0.95 ± 0.033^a	0.99 ± 0.026^b	0.93 ± 0.02^{ab}	$0.59-2.10^2$
Low-density lipoproteins	1.62 ± 0.059^a	1.61 ± 0.019^a	1.28 ± 0.062^b	1.81 ± 0.042^c	$0.42-3.72^2$
Very low-density lipoproteins	0.15 ± 0.021^a	0.12 ± 0.024^a	0.15 ± 0.019^a	0.19 ± 0.024^a	—

Note: Values are presented as mean $\pm$ SEM ($n = 21$ per group). Means within a row with different superscript letters differ significantly ($P < 0.05$, Tukey–Kramer test). 1 RI - reference intervals according to Egeli A. K. *et al.* (1998); 2 RI - reference intervals according to Sucheong S. *et al.* (2012).

High-density lipoproteins, known as “good cholesterol,” are a type of lipoprotein that transports excess cholesterol from the body tissues of pigs to the liver for further removal (Kiss *et al.*, 2025). Its amount in young pigs of the Con and Ile:Lys0.5 groups was at the same level within the physiological norm. In young pigs of the Ile: Lys 0.55 group, the level of high-density lipoproteins was 12.5% higher ($p<0.05$) compared to the Con group. With a further increase in the SID isoleucine:lysine ratio in the diet of young pigs, no differences in the serum content of high-density lipoproteins were observed in pigs of the Ile: Lys 0.6 group. As for low-density lipoproteins, their concentration in the blood serum of young pigs of the Con and Ile:Lys0.5 groups was at the same level within the physiological norm. While in young pigs of the Ile:Lys0.55 group there was a

decrease in the content of low-density lipoproteins by 21.0% ($p < 0.05$) compared to the Con group. Further increase in the SID isoleucine:lysine ratio in the diet of young pigs of the Ile: Lys 0.6 group was accompanied by an increase in the content of low-density lipoproteins in their blood serum by 11.7% ($p < 0.05$) compared to the Con group.

Serum triglyceride content is related to lipid absorption (Wang *et al.*, 2024). Isoleucine deficiency is known to dramatically increase fat mobilisation in white adipose tissue and reduce fat quality (Du *et al.*, 2012). Excess isoleucine in the diet of pigs can enhance the synthesis of monounsaturated fatty acids and fat accumulation in skeletal muscle by regulating lipid metabolism (Luo *et al.*, 2019). Therefore, isoleucine may play a potential role in the regulation of lipid metabolism. Oxidative metabolism by isoleucine can reduce lipid accumulation in cells (Solon-Biet *et al.*, 2019). Furthermore, excessive BCAA intake may lead to increased catabolism of all BCAAs, and studies have shown that excessive isoleucine may have an antagonistic effect and negatively affect adipose tissue function and lipid metabolism (Morales *et al.*, 2016; Zhang *et al.*, 2021). This study revealed changes, within the physiological norm, in serum triglyceride concentrations in young pigs depending on the SID isoleucine:lysine ratio in their diet. Thus, in piglets of the Ile:Lys0.5 group, serum triglyceride concentrations were 36.2% lower ($p < 0.05$) compared to the Con group. Young pigs of the Ile:Lys0.55 group were characterised by an even lower serum triglyceride concentration, namely 48.3% ($p < 0.05$) compared to the Con group. However, with a further increase in the SID isoleucine:lysine ratio in the diet of piglets in the Ile: Lys 0.6 group, a decrease in serum triglyceride concentration was found by only 25.9% ($p < 0.05$) compared to the Con group. The increase in triglyceride content is associated with increased serum BCAA levels, insulin resistance in muscles and impaired glucose utilisation, which clearly links BCAA oxidation with glucose and fat metabolism (She *et al.*, 2007). Isoleucine reduces muscle triglyceride levels and increases free fatty acid oxidation in skeletal muscle and liver by promoting the expression of peroxisome proliferator-activated receptor (PPAR- α) and uncoupling protein. These results indicate that isoleucine may prevent the development of visceral obesity and high insulin levels by affecting white adipose tissue, liver, and muscle adiposity (Nishimura *et al.*, 2010). Inhibition of fat synthesis in adipose tissue has also been reported by many other studies, using animal models or cell lines under conditions of BCAA deficiency (Du *et al.*, 2012; Freudenberg *et al.*, 2013) or excess (Bong *et al.*, 2010; Vianna *et al.*, 2012).

CONCLUSIONS

Based on the obtained findings, for pigs (7–30 kg), the optimal SID Ile:Lys was 0.55, which increased live weight at 70 days by 8.8% ($p < 0.05$) without negative physiological effects. At this ratio, serum glucose decreased by 28.0% ($p < 0.05$), creatinine by 10.0% ($p < 0.05$), urea by 60.5% ($p < 0.05$), total cholesterol by 8.7% ($p < 0.05$), low-density lipoproteins by 21.0% ($p < 0.05$), triglycerides by

48.3% ($p < 0.05$), ALT activity by 20.5% ($p < 0.05$), and AST by 11.7% ($p < 0.05$), whereas total protein increased by 4.8% ($p < 0.05$) and high-density lipoproteins by 12.5% ($p < 0.05$). The 0.5 ratio was less effective, increasing body weight by 6.2% ($p < 0.05$) and reducing glucose by 17.3% ($p < 0.05$), creatinine by 9.1% ($p < 0.05$), urea by 50.0% ($p < 0.05$), and triglycerides by 36.2% ($p < 0.05$), while total protein increased by 1.9% ($p < 0.05$). Increasing the ratio to 0.6 did not affect growth but led to a 5.1% rise in total protein ($p < 0.05$), a 12.7% decrease in albumins ($p < 0.05$), a 13.2% increase in globulins ($p < 0.05$), and a 10.6% elevation in total cholesterol ($p < 0.05$) due to higher low-density lipoproteins, which increased by 11.7% ($p < 0.05$). ALT activity decreased by 24.2% ($p < 0.05$) and AST by 13.1% ($p < 0.05$).

The results of this study demonstrate that the ratio of standardized ileal digestible (SID) isoleucine to lysine in pig diets significantly influences growth performance and metabolic indicators in young pigs.

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BIOACTIVE COMPOUNDS CONTENT AND ANTIOXIDANT POTENTIAL OF BEETROOT (*Beta vulgaris* L.) PEEL, POMACE, AND JUICE

SUMMARY

Scientific research in the 21st century has increasingly focused on bioactive compounds from medicinal plants and their antioxidant properties due to their potential benefits for human health. The aim of this study was to evaluate the antioxidant activity and determine the total content of bioactive compounds (phenols, flavonoids, tannins, and betalains) in peel and pomace extracts, as well as in freshly squeezed juice of *Beta vulgaris* L. Plant material was collected from two locations in Montenegro (Nikšić and Mojkovac) in September 2023. To compare extraction efficiency, two extraction methods (Soxhlet extraction and conventional maceration) and three solvents (water, methanol, and ethyl acetate) were used. Antioxidant activity was assessed using DPPH and FRAP assays. The highest total phenolic content (70.01 mg GAE/g extract) and total tannin content (49.27 mg TAE/g extract) were observed in the ethyl acetate peel extract obtained by the Soxhlet method from the Nikšić location. The highest total flavonoid content (88.31 mg QuE/g extract) was recorded in the ethyl acetate peel extract from Mojkovac. Higher concentrations of betacyanins and betaxanthins in peel and pomace were detected in samples from Nikšić. Freshly squeezed beetroot juice from this location exhibited the highest levels of all analyzed bioactive compounds and showed pronounced antioxidant activity (DPPH IC₅₀=24.99 µg/mL; FRAP=49.93 mmol Fe²⁺/L). Among the extracts, the strongest DPPH scavenging activity was observed in the ethyl acetate peel extract from Mojkovac, while the highest FRAP value was recorded for the methanolic peel extract from Nikšić. These results indicate that *Beta vulgaris* L., particularly its peel and fresh juice, is a valuable source of bioactive compounds with strong antioxidant potential, influenced by extraction method, solvent type, and geographical origin.

Keywords: *Beta vulgaris* L., antioxidant activity, phenolics, flavonoids, tannins, betalains

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INTRODUCTION

For centuries, medicinal and aromatic plants have played an essential role in human life and culture worldwide. They have traditionally been used in pharmacy, cosmetics, and nutrition, and in recent years their application has expanded into food technology, particularly as natural sources of antioxidants (Stefanou *et al.*, 2014). Herbal-based medical systems represent some of the oldest forms of health care, dating back to the earliest stages of human civilization (Thirumalai *et al.*, 2019). A thorough understanding of plant biochemical composition is fundamental for elucidating their potential medicinal properties, which are primarily attributed to secondary metabolites. These compounds have historically been used to alleviate various ailments in traditional and folk medicine and are currently exploited in the development of pharmaceutical agents targeting a wide range of diseases, from migraines to cancer (Hussein *et al.*, 2019).

Plants of the genus *Beta* originate from North Africa, from where their distribution expanded across the Mediterranean region and subsequently to Europe, Asia, and the Americas (Ceclu and Nistor, 2020). Beetroot has been cultivated for several centuries in temperate coastal regions, initially for its leaves, which were consumed as salad greens, while the thin and fibrous roots were occasionally used for medicinal purposes. Systematic cultivation for root consumption began in the 16th century in Germany and Italy, leading to the development of beet varieties that are still cultivated today (Sentkowska and Pyrzyńska, 2023). Currently, the largest production of beetroot occurs in northern and central Europe, although it is also widely grown in North Africa and Asia, extending as far as India (Yashwant, 2015). Beetroot thrives in moderately warm climates and grows optimally in clayey, slightly acidic soils with a pH ranging from 5.5 to 6.2. Sowing is generally performed between March and June to avoid low temperatures, while optimal growth occurs at temperatures between 15 and 19 °C; lower temperatures are known to enhance the development of the characteristic dark red pigmentation (Giampaoli *et al.*, 2021, Chhikara *et al.*, 2019). In recent decades, *Beta vulgaris* L. has attracted increasing scientific interest as a functional food with significant health-promoting properties. Although its medicinal use was documented as early as Roman times, systematic scientific investigations into its bioactive potential have intensified only recently. Beetroot is recognized as a rich source of bioactive compounds, many of which are associated with beneficial effects related to chronic inflammatory conditions (Clifford *et al.*, 2015).

Beetroot contains dietary fiber, essential minerals, and vitamins, including vitamins A, C, and those of the B complex, as well as betalains and phenolic compounds. Its high nutritional value is largely attributed to its considerable sucrose content. These bioactive constituents exert their effects through diverse mechanisms, including free radical scavenging, interaction with enzymatic active sites, and modulation of gene expression involved in intracellular defense systems against oxidative damage (Baião *et al.*, 2017). Betalains derived from beetroot

have been reported to reduce the risk of certain cancers, cardiovascular and cerebrovascular diseases, as well as liver and kidney damage (Chen *et al.*, 2021). Moreover, beetroot extracts exhibit a wide range of biological activities, such as antioxidant, anti-inflammatory, antihyperglycemic, and anticancer effects. However, the extraction techniques and solvents employed significantly influence the qualitative and quantitative composition of bioactive compounds and, consequently, their biological potential (Borjan *et al.*, 2022). Additionally, substantial variability in phytochemical content has been observed among different beetroot varieties, as well as a result of processing and storage conditions, including temperature and light exposure (Wruss *et al.*, 2015, Pavlović *et al.*, 2022).

This study aimed to evaluate the bioactive profile of *Beta vulgaris* L., focusing on phenols, flavonoids, tannins, and betalains in various extracts and freshly squeezed juices. In addition to qualitative and quantitative characterization, the antioxidant activity of these samples was assessed. Although beetroot has been widely recognized as a rich source of bioactive compounds, comparative data on different extraction methods and freshly squeezed juices remain limited. Therefore, this research seeks to clarify how extraction procedures influence the recovery of key phytochemicals and their antioxidant potential. Furthermore, the associations between different polyphenol classes and antioxidant capacity were explored using Pearson's correlation coefficient to better understand their relative contribution to oxidative stress mitigation. The findings aim to provide a clearer insight into the functional potential of beetroot-derived products and support their application in food and nutraceutical development.

MATERIAL AND METHODS

Chemicals and Instruments

All phenolic standards and other chemicals/reagents were obtained from Cayman Chemical (Michigan, USA), Acros Organics (Antwerp, Belgium), Sigma-Aldrich Chem (Steinheim, Germany), VWR International (Pennsylvania, USA), MP Biomedicals (California, USA), Alfa Aesar (Massachusetts, USA), Carl Roth (Karlsruhe, Germany), and Centrohem (Stara Pazova, Serbia). All reagents used in this study were of analytical grade. The instruments employed included a UV-Vis spectrophotometer (Cecil Aurius Series CE 2021, UK), a digital pH meter (Jenway 3505, UK), an incubator (LSW-33, Vims Elektrik, Serbia), and an analytical balance (Kern ABS 220-4, Philippines).

Plant Samples

The sample was collected at two locations in the municipality of Nikšić (Vidrovan, 42° 50' 21.3" N 18° 55' 36.3" E) and Mojkovac (Polja, 42° 57' 35.3" N 19° 32' 57.8" E) in September 2023. The beetroot sample was collected from arable fields/gardens from local households that grow beetroot only for household needs.

Preparing samples for extraction

The collected beets were washed, the petioles and leaves were separated from the roots. The beet root was peeled, cut into pieces and left to dry at room temperature. The juice was separated from the pomace with a juicer and immediately subjected to analysis. The pomace, which was additionally squeezed for faster and more efficient drying, was placed in the drying room together with the peel. Before the actual extraction, the peel (root periderm) and the pomace were additionally ground with an electric blender. The same procedure was carried out for both localities. For the analysis of the total content of phenols, flavonoids, and tannins, as well as for the determination of antioxidant activity through the FRAP test, the extracted juice was diluted 10 times. All analyses were performed in triplicate.

Soxhlet solid-liquid extraction

Peel (root periderm) and pomace from two localities were extracted using the Soxhlet solid-liquid extraction method. Two solvents were used for this type of extraction: methanol and ethyl acetate. 15 g of mechanically ground sample was packed in filter paper and then sealed. 300 ml of solvent was added to the distillation flask in contact with the heat source. After the boiling of the solvent, the extraction cycle is started, which was continuously repeated for the next 6 hours that the extraction lasted. Solvents were evaporated by air-drying at room temperature until a constant weight was achieved. The procedure was repeated for both solvents, for peel and pomace from two different sites, yielding 8 different samples. A total of eight final extracts were stored at 4°C in an airtight container until further use. Stock samples were made by dissolving 0.01g of each of the six obtained extracts in 10 ml of methanol. Their concentration was 1 mg/ml.

Maceration solid-liquid extraction

Three solvents were used for this type of extraction: distilled water, methanol and ethyl acetate. 10 g of mechanically ground sample was extracted with 100 ml of solvent, then left to stand for 48 h at room temperature. After every 24 h, the samples were macerated. When the maceration procedure was finished, the macerate was filtered with filter paper, and the sample was steamed in a water bath. The procedure yielded 12 samples: peel and pomace from two localities extracted with three different solvents. A total of twelve final extracts were stored at 4°C in an airtight container until further use. Stock samples were made by dissolving 0.01 g of each of the six obtained extracts in 10 ml of methanol. Their concentration was 1 mg/ml.

Determination of total phenolics

The colometric Folin-Ciocalteu method (Singleton and Rossi, 1965). was used to determine the content of total phenols in different extracts and extracted juice. Reaction mixture were obtained with 1 ml of sample (1 mg/ml), 0.5 ml of

Folin-Ciocalteu solution and 2.5 ml of 7.5% Na₂CO₃. Three repetitions were performed for each sample. A blank sample was also measured, which differed in the preparation in the addition of 1 ml of distilled water instead of the sample. The prepared samples were stored in a dark room for 120 minutes, then their absorbance was measured at 740 nm. The gallic acid calibration curve was used to calculate the content of total phenols. TPC (Total phenolic content) was expressed in mg/g equivalent of gallic acid (mg/g GAE).

Determination of flavonoids

The colorimetric aluminum-chloride method was used to determine the content of total flavonoids in different extracts and extracted juice (Ardekani *et al.*, 2011). 1.2 ml of 2% AlCl₃ solution and 1.2 ml of sample (1 mg/ml) are added to the test tube in order to obtain a reaction mixture. Three repetitions were performed for each sample. Incubation of the prepared mixture took place at room temperature for an hour, then the absorbance was read at 420 nm. The absorbance of the blank (extraction solvent - methanol) was also measured. The flavonoid content was calculated using the calibration curve for quercetin. TFC (Total flavonoid content) was expressed in mg/g quercetin equivalent (mg/g QuE).

Determination of tannins

The total content of tannins in different extracts and the squeezed juice was determined according to the modified method according to Price and Butler, 1977. 500 µl of sample (1mg/ml), 0.5 ml of 0.1 M FeCl₃, 0.5 ml of 0.008 M K₃Fe(CN)₆ and 8 ml of distilled water were used to obtain a reaction mixture. Three repetitions were performed for each sample. A blank sample was measured, which differed in the preparation only in the addition of 500 µl of distilled water instead of the sample. The samples were left for 10 minutes in room temperature, after which the absorbance was measured at a wavelength of 720 nm. The tannin content was calculated according to the calibration curve of the tannic acid standard. TTC (Total tannin content) was expressed in mg/g tannic acid equivalent (mg/g TAE).

Determination of betalains

The method is based on spectrophotometric measurement of absorbance at different wavelengths for betacyanins and betaxanthins (Shakir and Simone, 2024). 0.1 g of mechanically ground sample was dissolved in 10 ml of distilled water. The mixture was stirred for one minute, and then the contents were centrifuged for 10 minutes at 15,000 g. All four samples (peel and pomace from two locations) were made in three repetitions. After centrifugation, the supernatant was separated and subjected to spectrometric analyses. The absorbance was measured at two different wavelengths: 480nm for betaxanthines and 538nm for betacyanins. The content of total betalains was calculated using a mathematical equation.

The betalain content in the squeezed beetroot juice was determined by a spectrophotometric method, measuring content betanin and vulgaxanthin. 15 ml of phosphate buffer pH 6.5, 0.15 ml of squeezed juice sample were used to obtain a reaction mixture. Absorbance of the solution was determined at three different wavelengths: 600, 476, 538. Both juice samples were made in three repetitions. Phosphate buffer was measured on a spectrophotometer as a blank. The content of total betalains was calculated using a mathematical equation

DPPH radical-scavenging activity

The DPPH test was used to evaluate the free radical scavenging activity of various extracts as well as beetroot juice (Gülçin and Alwasel, 2023; Baliyan *et al.*, 2022). For the analysis of the antioxidant activity, serial dilutions of the extracts were made at concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015, 0.0075 mg/ml. Three repetitions were made for each dilution, 24 test tubes for each sample. In each test tube, 0.1mM DPPH solution and 3 ml of juice/extract of a certain concentration were prepared. All test tubes were incubated in a darkened room for a period of thirty minutes, and then measured at a wavelength of 517 nm. A blank sample consisting of 3 ml of methanol, a control prepared with 3 ml of methanol, and 1 ml of DPPH were also measured.

The percent DPPH inhibition (percentage of antioxidant activity - %AA) was calculated by the equation: $\%AA = (A_0 - A_1) / A_0 \times 100$, where A_0 was the absorbance of the control, and A_1 is the absorbance of the sample. Antioxidant activity was expressed as the IC_{50} value, which represents the concentration of the extract or reference compound required to inhibit 50% of DPPH radicals. IC_{50} values were determined by plotting the percentage of DPPH inhibition against the sample concentration, followed by interpolation using nonlinear regression.

FRAP assay

The FRAP test was used to determine the total antioxidant activity of various extracts of the bark and roots, as well as of the squeezed beetroot juice (Benzie and Devaki, 2017). 100µl of sample, 3 ml of TPTZ reagent and 1 ml of distilled water were added to the test tube to make the reaction mixture. Each one of the samples had three replicates. The reaction mixture was incubated in a thermostat (37°C) for 15 minutes. After the test tubes were removed from the thermostat, they stood for five minutes (room temperature), after which the absorbance value was read at a wavelength of 595 nm. A blank sample was also measured, with the addition of 100µl of distilled water instead of the sample. The value of the antioxidant potential is expressed as the equivalent of mmol Fe^{2+} in 1 ml of the sample.

Statistical analysis

All experimental data were processed using the statistical software package Statistica 10.0. The relationships between the contents of total phenolics, flavonoids, and tannins in the plant extracts and their antioxidant activities,

determined by the DPPH and FRAP assays, were evaluated using Pearson's correlation analysis ($p < 0.05$). The strength of the correlation coefficient (r) was interpreted as follows: $|r| < 0.3$, negligible or no correlation; $0.3 \leq |r| < 0.5$, weak correlation; $0.5 \leq |r| < 0.7$, moderate correlation; $0.7 \leq |r| < 0.9$, strong correlation; and $0.9 \leq |r| \leq 1.0$, very strong correlation.

Differences among experimental groups were analyzed using one-way analysis of variance (ANOVA). When statistically significant differences were detected, Tukey's honestly significant difference (HSD) post hoc test was applied to identify differences between individual mean values. A p -value < 0.05 was considered indicative of statistical significance. Furthermore, principal component analysis (PCA) was conducted to characterize the distribution of bioactive compounds in the tested *Beta vulgaris* L. extracts in relation to the extraction solvent used.

RESULTS AND DISCUSSION

Extraction Yields

Comparing the two extraction methods, a significantly higher yield (34.2%) was obtained by the Soxhlet method using methanol as a solvent, while the ethyl acetate extract had the lowest yield in both extraction methods (0.17%). Overall, the yield of the pomace extracts was significantly higher compared to the peel.

Bioactive Compounds Content of Beetroot Peel, Pomace, and Juice

The content of bioactive compounds in different peel and pomace extracts and in squeezed juice of *Beta vulgaris* L. is presented in Tables 1, 2, and 3.

Table 1. Total phenolic (TPC), flavonoid (TFC), tannin (TTC) and in peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid Soxhlet method, as well as squeezed juice (SJ); values represent mean + SD of three measurements ($n=3$)

Sample/solvent	Method/location	TPC mgGAE ¹ /g dw	TFC mg QE ² /g dw	TTC mg TAE ³ /g dw
PL/ethyl acetate	S/Nikšić	70.01 ± 0.78 ^c	88.318 ± 1.33 ^c	49.27 ± 1.77 ^c
PL/ethyl acetate	S/Mojkovac	64.34 ± 1.27 ^c	73.20 ± 0.45 ^c	28.60 ± 1.32 ^d
PL/methanol	S/Nikšić	63.91 ± 0.89 ^c	n.d ⁴	38.46 ± 0.78 ^{cd}
PL/methanol	S/Mojkovac	65.35 ± 1.23 ^c	n.d ⁴	26.58 ± 0.24 ^d
PC/ethylacetate	S/Nikšić	50.43 ± 0.32 ^d	32.61 ± 1.76 ^d	16.26 ± 0.68 ^e
PC/ethylacetate	S/Mojkovac	41.191 ± 2.21 ^e	n.d ⁴	8.634 ± 0.56 ^{ef}
PC/methanol	S/Nikšić	46.451 ± 0.67 ^d	n.d ⁴	24.09 ± 0.75 ^d
PC/methanol	S/Mojkovac	40.53 ± 1.44 ^e	2.03 ± 1.11 ^e	12.914 ± 1.28 ^e
Squeezed juice (SJ)	Nikšić	722.6 ± 1.12 ^a	698.44 ± 0.97 ^a	601.6 ± 0.56 ^a
Squeezed juice (SJ)	Mojkovac	554.37 ± 0.76 ^b	293.27 ± 0.86 ^b	336.84 ± 1.16 ^b

*GAE–gallic acid equivalent; 2QE–quercetin equivalent; 3TAE–tannic acid equivalent; 4n.d.–not detected with the standard concentration range. Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, $p < 0.05$). The contents of phenols, flavonoids and tannins for the juice samples are expressed per liter (mg/L)

Table 2. Total phenolic (TPC), flavonoid (TFC), tannin (TTC) and in peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid maceration method; values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	TPC mgGAE ¹ /g dw	TFC mg QE ² /g dw	TTC mg TAE ³ /g dw
PL/ethyl acetate	M/Nikšić	69.70 ± 2.41 ^a	85.71±5.34 ^a	28.71± 1.76
PL/ethyl acetate	M/Mojkovac	60.70 ±1.56 ^a	60.09± 3.21 ^b	16.56±2.34
PL/methanol	M/Nikšić	59.42 ±1.12 ^a	n.d ⁴	24.53± 3.44
PL/methanol	M/Mojkovac	61.82 ± 2.78 ^a	n.d ⁴	21.57±1.56
PL/water	M/Nikšić	52.12 ±3.56 ^b	n.d ⁴	25.72±0.95
PL/water	M/Mojkovac	48.08 ±0.78 ^b	n.d ⁴	24.29±0.78
PC/ethylacetate	M/Nikšić	50.02 ± 4.56 ^b	26.20± 0.98 ^c	7.34±1.67
PC/ethylacetate	M/Mojkovac	36.378 ± 1.23 ^c	29.04±1.32 ^c	8.53±1.66
PC/methanol	M/Nikšić	40.04±0.78 ^b	n.d ⁴	9.03±2.34
PC/methanol	M/Mojkovac	35.893 ±2.15 ^c	n.d ⁴	9.96±0.68
PC/water	M/Nikšić	34.846 ±2.23 ^c	n.d ⁴	13.01±0.88
PC/water	M/Mojkovac	27.974 ±1.65 ^c	n.d ⁴	8.53± 1.45

*GAE–gallic acid equivalent; 2QE–quercetin equivalent; 3TAE–tannic acid equivalent; n.d.–not detected with the standard concentration range. Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, $p < 0.05$)

Table 3. Betalain content of peel (PL), pomace (PC) and squeezed juice (SJ) of *Beta vulgaris* L. at two different locations; values represent mean + SD of three measurements (n=3)

Sample/location	Betacyanins (mg/l) for extracts (mg/ml) for SJ	Betaxanthin (mg/l) for extracts (mg/ml) for SJ
PL/Nikšić	206.25 ±6.3 ^a	132.82± 0.98 ^a
PL/Mojkovac	181.5 ± 4.22 ^b	111.65± 1.24 ^b
PC/Nikšić	143 ± 2.39 ^c	90.47±2.22 ^c
PC/ Mojkovac	132± 2.67 ^c	90.47± 1.76 ^c
SJ/Nikšić	185.62±1.98 ^b	94.5± 1.89 ^c
SJ/Mojkovac	71.69±3.87 ^d	30.8±3.14 ^d

*Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, $p < 0.05$)

The total phenolic content (TPC) in the extracts ranged from 27.97 to 70.01 mg GAE/g dw. The highest TPC was found in the ethyl acetate peel extract from the Nikšić locality, while the lowest value was recorded in the aqueous pomace extract from the Mojkovac site. Regardless of the extraction method, the most pronounced differences in TPC were observed between aqueous and ethyl acetate extracts of both peel and pomace at both localities. Analysis of the results indicates that ethyl acetate and methanol were more efficient solvents for the extraction of total phenols compared to water, which proved to be the least

effective solvent. Slight variations in TPC were observed depending on the extraction method, with Soxhlet extraction yielding marginally higher values than maceration. In general, except for peel extracts, TPC values were slightly higher in samples from the Nikšić locality. At both localities, the TPC was significantly higher in peel extracts compared to pomace, regardless of the solvent and extraction method used. The total phenolic content in juice samples ranged from 554.37 to 722.60 mg GAE/L. Beetroot juice from the Nikšić locality exhibited higher TPC values than juice samples from Mojkovac.

The total flavonoid content (TFC) in the extracts ranged from 26.20 to 88.32 mg QuE/g dw. Detectable flavonoid levels were observed exclusively in ethyl acetate extracts, regardless of the extraction method or locality. The highest TFC values were obtained in peel extracts prepared by Soxhlet extraction, while the lowest values were recorded in pomace extracts obtained by maceration. As observed for total phenols, flavonoid content was significantly higher in peel than in pomace samples, irrespective of the extraction method and locality. Overall, only minor variations in TFC were noted between localities and extraction methods. In juice samples, TFC values varied considerably, ranging from 293.27 mg QuE/L in Mojkovac to 698.44 mg QuE/L in Nikšić.

The total tannin content (TTC) in beetroot extracts ranged from 7.34 mg TAE/g dw, recorded in pomace extracts obtained by maceration with ethyl acetate, to 49.28 mg TAE/g dw in ethyl acetate peel extracts prepared by Soxhlet extraction. The efficiency of solvent extraction depended on both the extraction method and the plant part. Under Soxhlet extraction conditions, ethyl acetate showed the highest efficiency for tannin extraction from peel, whereas methanol was more effective for pomace samples. In contrast, no consistent solvent efficiency pattern was observed for maceration. Regardless of the extraction method, solvent, or locality, TTC values were significantly higher in peel than in pomace. Soxhlet extraction generally resulted in higher tannin yields compared to maceration, and samples from the Nikšić locality exhibited higher tannin contents, particularly in ethyl acetate extracts. The tannin content in juice samples was 336.84 mg TAE/L for Mojkovac and 601.60 mg TAE/L for Nikšić.

Betalain content ranged from 30.80 to 132.83 mg/L for betaxanthins and from 71.70 to 206.25 mg/L for betacyanins. In all analyzed samples (peel, pomace, and juice), betacyanin content was higher than betaxanthin content. The peel samples from both localities contained higher levels of betalains compared to pomace samples. Only minor differences in betalain content were observed between the two localities.

Antioxidant Potential of Beetroot (*Beta vulgaris* L.) Peel, Pomace, and Juice

The antioxidant potential of peel and pomace extracts and squeezed juice of *Beta vulgaris* L., determined by the DPPH assay and expressed as IC₅₀ values, is presented in Tables 4 and 5.

Table 4. Antioxidant activity of peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid Soxhlet method, as well as squeezed juice (SJ); values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	DPPH IC50 ($\mu\text{g/ml}$)	FRAP ($\text{mmol Fe}^{2+}/\text{g dw}$)
PL/ethyl acetate	S/Nikšić	84.64 $\pm$ 1.55 ^d	19.91 $\pm$ 0.88 ^c
PL/ethyl acetate	S/Mojkovac	52.98 $\pm$ 0.67 ^e	17.04 $\pm$ 0.76 ^d
PL/methanol	S/Nikšić	99.01 $\pm$ 1.73 ^d	21.37 $\pm$ 1.22 ^c
PL/methanol	S/Mojkovac	81.08 $\pm$ 2.79 ^d	21.03 $\pm$ 3.98 ^c
PC/ethylacetate	S/Nikšić	373.89 $\pm$ 3.45 ^b	12.87 $\pm$ 4.56 ^e
PC/ethylacetate	S/Mojkovac	923.46 $\pm$ 2.28 ^a	4.22 $\pm$ 1.57 ^f
PC/methanol	S/Nikšić	317.15 $\pm$ 1.75 ^b	9.66 $\pm$ 1.29 ^{ef}
PC/methanol	S/Mojkovac	332.32 $\pm$ 1.66 ^b	10.26 $\pm$ 2.33 ^e
Squeezed juice (SJ)	Nikšić	24.99 $\pm$ 2.99 ^f	398.6 $\pm$ 6.78 ^a
Squeezed juice (SJ)	Mojkovac	127.27 $\pm$ 1.43 ^c	227.8 $\pm$ 7.89 ^b

*Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, $p < 0.05$). For DPPH IC50, lower values indicate stronger antioxidant activity; for FRAP, higher values indicate stronger antioxidant activity. The FRAP value for juice is expressed per liter ($\text{mmol Fe}^{2+}/\text{L}$)

Table 5. Antioxidant activity of peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid maceration method; values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	DPPH IC50 ($\mu\text{g/ml}$)	FRAP ($\text{mmol Fe}^{2+}/\text{g dw}$)
PL/ethyl acetate	M/Nikšić	136.04 $\pm$ 5.7 ^e	12.31 $\pm$ 2.35 ^b
PL/ethyl acetate	M/Mojkovac	331.7 $\pm$ 8.97 ^{cd}	6.91 $\pm$ 1.22 ^c
PL/methanol	M/Nikšić	114.95 $\pm$ 3.87 ^e	13.18 $\pm$ 2.09 ^a
PL/methanol	M/Mojkovac	131.98 $\pm$ 3.56 ^e	14.4 $\pm$ 1.89 ^a
PL/water	M/Nikšić	85.10 $\pm$ 2.38 ^f	14.14 $\pm$ 1.56 ^a
PL/water	M/Mojkovac	113.17 $\pm$ 1.77 ^e	13.28 $\pm$ 2.34 ^a
PC/ethylacetate	M/Nikšić	5298.82 $\pm$ 7.67 ^a	4.11 $\pm$ 1.46 ^d
PC/ethylacetate	M/Mojkovac	2198.63 $\pm$ 8.84 ^b	3.55 $\pm$ 2.35 ^{ed}
PC/methanol	M/Nikšić	635.07 $\pm$ 4.67 ^c	4.15 $\pm$ 0.78 ^d
PC/methanol	M/Mojkovac	512.76 $\pm$ 4.45 ^c	4.74 $\pm$ 0.56 ^d
PC/water	M/Nikšić	563.8 $\pm$ 3.45 ^c	7.24 $\pm$ 2.78 ^c
PC/water	M/Mojkovac	1813.34 $\pm$ 3.34 ^b	5.12 $\pm$ 0.97 ^{cd}

*Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, $p < 0.05$). For DPPH IC50, lower values indicate stronger antioxidant activity; for FRAP, higher values indicate stronger antioxidant activity

The IC₅₀ values ranged from 81.08 $\mu\text{g/mL}$ for the methanolic peel extract obtained by Soxhlet extraction from the Mojkovac locality to 5298.82 $\mu\text{g/mL}$ for the ethyl acetate pomace extract obtained by maceration from Nikšić. In general, peel and pomace extracts obtained by the Soxhlet method exhibited higher antioxidant potential compared to those obtained by maceration, particularly ethyl

acetate and methanolic peel extracts, with only minor variations between localities. An exception was the aqueous peel extract from the Nikšić locality, which also demonstrated high antioxidant activity. Differences in antioxidant potential between the two localities were generally insignificant. Notably, peel extracts exhibited significantly higher antioxidant potential than pomace extracts. Analysis of the IC_{50} values of juice samples revealed a clear difference between the two localities, with juice from Nikšić showing stronger antioxidant activity ($IC_{50} = 24.99 \mu\text{g/mL}$) compared to juice from Mojkovac ($IC_{50} = 127.27 \mu\text{g/mL}$).

Antioxidant potential measured by the FRAP assay showed the lowest value in the ethyl acetate pomace extract obtained by maceration from Mojkovac ($3.55 \text{ mmol Fe}^{2+}/\text{g}$) and the highest value in the methanolic peel extract obtained by Soxhlet extraction from the same locality ($21.37 \text{ mmol Fe}^{2+}/\text{g}$). Extracts obtained by the Soxhlet method generally exhibited higher FRAP values regardless of locality. Juice samples from both localities showed particularly high FRAP values, with $398.6 \text{ mmol Fe}^{2+}/\text{L}$ for Nikšić and $227.8 \text{ mmol Fe}^{2+}/\text{L}$ for Mojkovac.

DISCUSSION

Total Phenolic content

The total phenolic content (TPC) of beetroot extracts varied considerably depending on the plant part, solvent, and extraction method. In this study, TPC values ranged from 27.97 to 70.01 mg GA/g dry weight, with ethyl acetate extracts of the peel yielding the highest concentrations. These results are lower than previously reported ranges (136–1026 mg GA/g, Guamán-Balcáza *et al.* 2024), but are consistent with the trend observed in other studies showing that solvent polarity and extraction methodology significantly influence phenolic recovery (Čanadanović-Brunet, 2011, Arjeh *et al.*, 2022, Vaitkevičienė *et al.* 2022., Abadi *et al.* 2024). Specifically, water was generally less efficient for phenolic extraction, whereas ethyl acetate and methanol proved more effective. Soxhlet extraction also yielded slightly higher TPC values compared to maceration, corroborating earlier findings (Borjan *et al.*, 2022). Across studies, peel extracts consistently contain higher phenolic levels than pomace extracts. Interestingly, the TPC of freshly squeezed juice in this study (up to 722.6 mg GA/L) exceeded values reported in earlier studies (11.58–98.08 mg GA/L, Abdo *et al.*, 2020, Olumese and Oboh. 2016), highlighting the efficiency of the extraction procedure and the influence of geographical origin.

The elevated total phenolic content (TPC) in beetroot peel can be attributed to its protective role against environmental stress. Phenolic compounds accumulate in the peel to provide defense against UV radiation, pathogens, and oxidative stress, which are more intense at the surface tissues (Duthie *et al.*, 2000). Additionally, secondary metabolites such as phenols are biosynthesized at higher levels in outer tissues to enhance antioxidant capacity and structural defense, whereas inner tissues, including the pomace, have lower phenolic

concentrations (Leong and Shui, 2002; Pérez-Gregorio *et al.*, 2010; De Vit *et al.*, 2020; Bhat *et al.*, 2025).

Total Flavonoid content

The flavonoid content in whole beetroot extracts has been reported as 38.5 mg QuE/g dry matter, (Nugraha *et al.* 2022) which is comparable to the pomace extracts obtained in the present study. Peel extracts, however, exhibited approximately twice this flavonoid content. Significantly lower flavonoid levels (1.54–8.35 mg QuE/g dry matter) have been reported (Hikmawanti *et al.*, 2021, El-Beltagi *et al.*, 2018) in various whole beetroot extracts, indicating that the flavonoid content observed in this study is considerably higher. Comparison of the two extraction methods, Soxhlet and maceration, revealed no significant differences in flavonoid yield (Dianursanti *et al.*, 2020), although other studies suggest that Soxhlet extraction may provide higher flavonoid recovery. Factors such as an increased number of extraction cycles and optimal extraction temperature can further enhance flavonoid recovery using this method (Wardhani *et al.*, 2023). Furthermore, the flavonoid content in the juice samples (83.34 ± 4.47 mg QuE/L) was substantially higher than values previously reported in the literature (Olumese and Oboh, 2016), indicating the high efficiency of the applied extraction procedure.

Total Tanin content

The tannin content in various beetroot extracts obtained in this study ranged from 7.34 mg TAE/g of dry matter, recorded in the pomace extract prepared with ethyl acetate by maceration, to 49.278 mg TAE/g of dry matter in the ethyl acetate peel extract obtained by Soxhlet extraction. The efficiency of tannin extraction was clearly dependent on both the method and the plant part analyzed. Soxhlet extraction with ethyl acetate showed the highest tannin recovery from peel at both collection sites, whereas methanol was more efficient for pomace extracts. In contrast, maceration did not show a consistent pattern in solvent efficiency; at the Mojkovac site, water provided the highest tannin content in peel extracts, while methanol was more effective for pomace. Overall, tannin content was consistently higher in peel than in pomace, independent of extraction method, solvent, or site, highlighting the differential accumulation of tannins in protective plant tissues.

Comparing these results with literature data, tannin levels in our pomace extracts were generally higher than those reported in whole beetroot extracts (6.055 mg TAE/g and 5.13 mg TAE/g; Odoh *et al.*, 2012; El-Beltagi *et al.*, 2018), while water extracts in previous studies often did not yield detectable tannin levels (Omogbai and Omoregie, 2016), in contrast to our measurable values. Methanolic extracts obtained by ultrasonic extraction also confirmed higher tannin content in peel 4.3 mg GA/g compared to pomace 3.7 mg GA/g (Arjeh *et al.*, 2022). While Manea (2013) reported extremely high tannin concentrations in juice (3059 g/L), the maximum tannin content observed in our study reached 601.5 mg TAE/g dw, suggesting notable differences that may be attributed to variations in plant material, extraction procedures, or analytical methods.

The relatively low tannin content observed in our extracts can be explained by several factors. Beetroot (*Beta vulgaris* L.) naturally contains comparatively low amounts of tannins relative to other phenolic compounds, such as flavonoids and betalains, which dominate its phytochemical profile, with betalains constituting the major class of phenolic-related constituents in red beetroot. (Mirmiran *et al.*, 2019).

Additionally, the distribution of specific phenolic classes in beetroot is influenced by biosynthetic pathways that prioritize betalain production a tyrosine derived pigment class that dominates the phenolic profile in *Beta vulgaris* L. over other phenolic secondary metabolites such as tannins, leading to comparatively limited tannin accumulation in this species. The betalain biosynthetic pathway has been extensively characterized as a tyrosine derived metabolic process responsible for high betalain content in beetroot tissues, which may reduce flux toward other phenolic pathways including tannin formation. (Khan and Giridhar, 2015).

Betalain Content

The obtained betalain content ranged from 30.8 to 132.825 mg/L for betaxanthins and from 71.696 to 206.25 mg/L for betacyanins, depending on the drying method and solvent used. These results are consistent with the study by Shakir and Simone (2024), highlighting that the method of drying plant material significantly affects betalain stability and content. Zin *et al.*, (2021) reported lower betacyanin (165.24 mg/L) and betaxanthin (97.56 mg/L) levels in peel extracts, whereas Ayk n-Din er *et al.*, (2021) and Jahan *et al.*, (2021) observed higher betalain concentrations in pomace (300.82 and 364.93mg/L for betacyanins; 261.27 and 249.40 mg/L for betaxanthins) compared to our results.

Freshly squeezed beetroot juice exhibited maximum values of betacyanin (119.53 mg betanin/100 mL) and betaxanthin (48.27 mg vulgasanthin I/100 mL) in the study by Fern ndez-L pez *et al.* (2023), which is consistent with the juice obtained from the Mojkovac locality in the present study. In all samples, betalain content was consistently higher in the peel than in the pomace, as reported by Sokolova (2022), in agreement with our findings. This pattern aligns with previous studies showing that betalains are predominantly localized in protective tissues such as the peel, where they contribute to antioxidant defense and color pigmentation (Kujala *et al.*, 2000; Mirmiran *et al.*, 2019).

Environmental factors, particularly temperature, influence betalain biosynthesis, with higher temperatures generally increasing betalain content (Sokolova *et al.*, 2024). This effect may partly explain the observed differences between the Mojkovac and Nik i  localities. Moreover, betacyanins are typically present at higher concentrations than betaxanthins in beetroot, reflecting their dominance in the plant's phenolic profile and metabolic prioritization over other phenolic compounds (Stintzing and Carle, 2004; Clifford *et al.*, 2017).

Antioxidant activity

The antioxidant potential of beetroot extracts was evaluated using DPPH and FRAP assays, revealing notable differences depending on solvent, plant part, and extraction method. In previous studies, IC₅₀ values for methanol, water, and

ethyl acetate extracts of whole beetroot ranged from 34.9 to 88.6 µg/mL (Omer *et al.*, 2022), with ethyl acetate generally showing the lowest antioxidant activity. In contrast, in the present study, the ethyl acetate peel extract obtained by Soxhlet extraction exhibited the lowest IC₅₀ value (52.98 µg/mL), indicating the highest antioxidant potential. These results emphasize the significant influence of extraction methodology on the recovery of bioactive compounds. Methanolic extracts consistently showed higher antioxidant activity than other solvents, in agreement with Saani and Lawrence (2017) and John *et al.* (2017). Similarly, Arjeh *et al.*, (2022) observed higher IC₅₀ values in pomace compared to peel, consistent with our findings, where peel extracts exhibited stronger antioxidant activity than pomace. Overall, Soxhlet extraction proved more effective than maceration for antioxidant recovery.

In freshly squeezed juice samples, antioxidant activity measured by DPPH and FRAP was higher than previously reported values (IC₅₀=16.25 µg/mL, Khider *et al.*, 2019; FRAP=8.354 mmol/L, Wootton-Beard and Ryan, 2011), with juices from the Nikšić locality showing 51.54% inhibition. This activity also exceeded that reported for carrot juice in comparative studies (Arora *et al.*, 2019). The enhanced activity corresponds to the higher betacyanin content in juices from both Nikšić and Mojkovac, supporting the role of betacyanins as key contributors to antioxidant potential (Czapski *et al.*, 2009). Overall, trends observed in DPPH and FRAP assays were consistent across most samples, although minor variations between localities reflected differences in secondary metabolite composition.

Correlation Between Bioactive Compounds and Antioxidant Activity

A very high positive correlation was observed between total phenolic content (TPC) and antioxidant potential (FRAP assay) in the methanolic peel extract ($r=0.97$, $p<0.05$) (Table 6).

Table 6. Pearson's correlation coefficients ($p<0.05$) between total phenols, flavonoids, tannins, and antioxidant capacity in methanol and ethyl acetate peel and pomace extracts of *Beta vulgaris* L. from Mojkovac, prepared by maceration

PEEL (PL)	TPC-EA	TPC-M	TPC-W	TFC-EA	TFC-M	TFC-W	TTC-EA	TTC-M	TTC-W
DPPH-EA	-0.29			0.53			0.26		
DPPH-M		-0.41			-0.21			-0.62	
DPPH-W			-0.07			-0.18			-0.11
FRAP-EA	-0.20			-0.34					
FRAP-M		0.97*			-0.56			0.96*	
FRAP-W			-0.61			-0.45			0.52
POMACE (PC)	TPC-EA	TPC-M	TPC-W	TFC-EA	TFC-M	TFC-W	TTC-EA	TTC-M	TTC-W
DPPH-EA	-0.31			-0.78			-0.63		
DPPH-M		-0.29			-0.98			0.73	
DPPH-W			0.97			-0.79			-0.79
FRAP-EA	0.45			0.96*			0.99*		
FRAP-M		0.14			0.65			0.62	
FRAP-W			0.34			-0.88			0.76

*Bold values represent strong correlations ($p < 0.05$)

It is noteworthy that most extracts showed positive correlations when antioxidant activity was assessed using the FRAP method, whereas the DPPH assay predominantly revealed negative correlations. This observation is consistent with a previous study by Olumese and Oboh (2016), which reported a significant positive correlation ($r=0.98$) between flavonoid content and FRAP-measured antioxidant activity in various extracts of peeled beetroot and juice. However, in the same study, a relatively low correlation between total phenolic content and FRAP activity ($r=0.36$) was observed.

Furthermore, a strong positive correlation between betacyanin content and DPPH-determined antioxidant activity of whole beetroot ($r=0.80$) was reported by Sokolova *et al.*, (2024). This trend aligns with the present study, where a significant positive correlation was observed between betacyanin content and FRAP-measured antioxidant activity in pomace extracts ($r=0.81$, $p<0.05$). Additionally, beetroot juice showed significant correlations between FRAP-measured antioxidant potential and the contents of both total phenols and betacyanins, as reported by Wruss *et al.*, (2015). It is interesting to note that at the Nikšić locality, no statistically significant correlations were observed between the contents of the analyzed components and the values of antioxidant potential, regardless of the type of sample or the method used. The absence of statistically significant correlations at the Nikšić site may be due to the complex interplay of multiple bioactive metabolites beyond the measured components, environmental influences on phytochemical biosynthesis, and variability in how different antioxidant assays reflect the activities of specific compounds. Previous studies have noted that the overall antioxidant response of plant extracts can be affected by synergistic or antagonistic interactions among constituents, as well as by external ecological and methodological factors, which can obscure simple linear correlations between individual compounds and antioxidant outcomes. (Jacobo Velázquez and Cisneros Zevallos, 2009., Villegas-Aguilar *et al.*, 2024).

Principal component analysis (PCA)

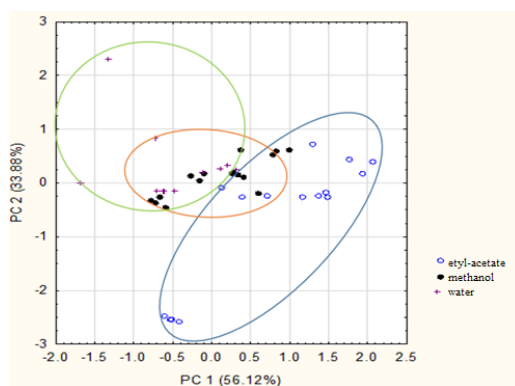


Figure 1. Principal Component Analysis (PCA) of total phenolic, flavonoid and tannin content in relation to type of solvent in peel and pomace samples of *Beta vulgaris* L. Projection of the samples on the first two principal components (PC1: 56.12%, PC2: 33.88%)

By analyzing the obtained data, it can be observed that the first two principal components account for approximately 90% of the variability in the content of phenols, flavonoids, and tannins (PC1: 56.12%, PC2: 33.28%) in relation to the type of solvent. The PCA results indicate that ethyl acetate extracts form a relatively distinct group, as evidenced by the ellipse in Figure 1, which slightly overlaps with the other solvent groups, highlighting differences in the composition of bioactive compounds compared to the remaining solvents.

CONCLUSIONS

The results of this study indicate that the content of secondary metabolites and the antioxidant potential of *Beta vulgaris* L. are significantly influenced by the extraction method, the solvent used, and the ecological conditions of the collection site. Soxhlet extraction, particularly with ethyl acetate, proved to be more effective in isolating phenolic compounds, flavonoids, tannins, and betalains compared to maceration. The peel generally contained higher amounts of bioactive components than the pomace, while freshly squeezed juices, especially from the Nikšić site, exhibited the highest antioxidant potential, which can be attributed to the absence of thermal degradation.

Comparative analysis of the DPPH and FRAP assays suggests that the FRAP method may provide a more reliable assessment of antioxidant activity. Although the collection site did not show a consistent effect on peel and pomace extracts, environmental factors clearly contribute to the variability of chemical composition. These findings contribute to a better understanding of the biochemical profile of *Beta vulgaris* L. and highlight the need for further research involving different varieties, additional extraction techniques, and other plant parts with potential bioactive properties.

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SHORT FOOD SUPPLY CHAINS AND LOCAL VALUE IN DAIRY PRODUCTION

SUMMARY

This paper examines the potential for enhancing direct sales in small family farms, with a specific focus on the case study farm in Bosnia and Herzegovina. Short food supply chains and direct sales represent an increasingly relevant marketing model in modern agribusiness, offering farmers opportunities to increase profitability, build closer relationships with consumers, and preserve rural traditions. The survey was conducted on a sample of 245 respondents who consumed the farm dairy products. The respondents preferred the intrinsic over the extrinsic factors of the product when purchasing. The results also showed that trust and quality influence purchasing a product. The growing trend of a healthy diet will increase the short food supply chains for milk and dairy products, which opens the possibility to increase the assortment and supply, and the obtained results could have a positive impact on the development of a strategy for improving direct sales on the farm. However, challenges such as limited financial resources and competition from imported goods remain significant barriers. Findings suggest that strengthening direct sales can contribute not only to farm income stability but also to broader rural economic development.

Keywords: direct sales, consumer behavior, local dairy products, family farm

INTRODUCTION

An increasing number of consumers are orienting themselves toward local food (Kneafsey *et al.*, 2013; Feldmann and Hamm, 2015) and purchasing food through short food supply chains (SFSC) directly from farmers (Aguiar *et al.*, 2018; González-Azcárate *et al.*, 2021; Renkema and Hilletoft, 2022; Evola *et al.*, 2022). From this perspective, research interest focused on food distribution through various forms of direct sales has become increasingly relevant (Belletti

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and Marescotti, 2020; Paciarotti and Torregiani, 2021). At the same time, as a consequence of food market development and new market trends, new forms of direct sales channels (Winarno *et al.*, 2020; Lankauskienė *et al.*, 2022) are emerging and developing, to connect consumers directly with the producers of primary and processed agricultural products, predominantly from small family farms. Therefore, it is essential to assess the current state of this segment of the distribution channels by examining consumer attitudes toward food purchased via direct sales, as well as by conducting analyses of consumer satisfaction with the provided services and the quality of the offered products. This also involves identifying the potential advantages and disadvantages of the existing direct sales channels, along with opportunities to overcome the observed shortcomings.

Kneafsey (2013) defines short food supply chains as those in which the number of intermediaries between farmers and consumers is minimal or equal to zero. Renting (2003) argues that shortening food supply chains creates new market relationships, with direct sales representing one of the most important forms of short food supply chains. Compared to indirect sales, direct sales allow producers to achieve significantly higher selling prices (Stojanović *et al.*, 2016). Direct sales are conducted at the farm gate, at farmers' markets, and in some cases via the internet or direct home delivery—door-to-door (Vlahović, 2016), which corresponds to the case examined here.

Kovačić (2005) notes that engaging in direct sales entails a wide range of additional responsibilities. Beyond agricultural production, farms must also take on the role of storage, transportation, processing, and marketing of their products. The most common motives for direct consumer purchases include: traditional production, transparency of the production process, personal contact with the producer, mutual trust, and similar factors. The same author emphasizes that market proximity, the type of product, and the level of farm equipment are key prerequisites for successful direct sales. Farms located near consumers can offer multiple forms of direct sales and market all their products. A key advantage of short supply chains is that they provide family farms with an excellent means of product diversification, allow them to retain a larger share of added value, and secure more stable income streams (Gajdić, 2019).

According to Feher (2007), the essence of direct sales lies in the notion of a “shared advantage.” Both producers and consumers are beneficiaries: producers secure work and income, while consumers gain access to healthy food. For consumers, the benefits include access to locally grown, fresh, healthy, and often organic products. By purchasing locally produced food, consumers support local farmers and contribute to the revitalization of rural areas (La Trobe, 2008).

Aguglia *et al.* (2009) highlight that direct sales represent a form of marketing that enables farmers to retain a larger share of the final value of their products. Online purchasing of organic food has emerged as a growing trend within online marketing (Hanpermchai and Dejtanasoontorn, 2011). This sales method is employed by the analyzed farm, which serves as a case study. Rocchi *et al.* (2019) found that for farmers to sell their products directly to consumers,

they must possess additional IT skills, which are not determined by education level but rather by age. For small agricultural holdings producing dairy products, the most effective sales channels are short supply chains based on customer orders placed via phone or online (Horská *et al.*, 2020). O'Hara and Low (2020) argue that using the internet as a form of direct sales reduces transportation costs from producers to end consumers. Furthermore, the use of social media in agricultural marketing improves efficiency, reduces costs, and increases farmers' revenues through higher demand for agricultural products (Inegbedion *et al.*, 2021).

The primary aim of this study is to analyze the opportunities, challenges, and prospects of SFSC of milk and dairy products on farms of the analyzed type, as well as to identify possibilities for improving the functioning of short food supply chains. Short supply chains with dairy products have already been the subject of scientific considerations in other countries (Sellitto *et al.*, 2018; Prišenk *et al.*, 2018; Mancini *et al.*, 2019; Horská *et al.*, 2020; Brumă *et al.*, 2021), while in BiH, this topic has almost not been considered from a scientific point of view so far. In addition to the primary objective, the secondary aim of the research is to determine the influence of specific factors such as the use of social media, income levels, number of children, and educational attainment on consumer attitudes toward purchasing through SFSCs.

MATERIAL AND METHODS

The family farm "Mijatović", which is the subject of this research, has been a registered farm in the Farm Registry of the Republic of Srpska since 2013, but several generations of the family have worked and produced on the farm. Today, the farm is managed by a young couple and is primarily engaged in milk production and processing as its core activity. Both spouses have also graduated from the Faculty of Agriculture and possess formal education and expertise in the field in which they work. The family consists of three adult members, excluding children, who are actively working on the farm. The farm cultivates 10 hectares of agricultural land, producing sufficient quantities of cereals and forage to meet the feed requirements of its 8–10 dairy cows and other livestock. The entire volume of milk and dairy products is marketed through direct sales channels. The most common distribution methods are home delivery and on-farm sales. Promotion is carried out primarily through social media and word-of-mouth recommendations from satisfied customers. Milk and dairy products are marketed exclusively through a short food supply chain, namely by door-to-door delivery or sales at the farm gate, using social media for the promotion of the farm's products. This farm was used as a case study to research the functioning of the short dairy supply chain in Bosnia and Herzegovina.

An analysis of attitudes, motivations, facts, opinions and consumer satisfaction with the products and sales methods of the family farm "Mijatović" was conducted using primary data. The aim of the analysis was to identify

opportunities for improving direct sales. The following research hypothesis was formulated and tested based on the results of the conducted research:

H1: For dairy product consumers, intrinsic attributes of milk and dairy products are more important than extrinsic attributes.

Primary data were collected through an online survey using a structured questionnaire that included sociodemographic characteristics of respondents, as well as questions on purchasing frequency, the farm's product assortment, product quality, delivery services, product promotion, online purchasing, and related topics. The survey was conducted in the cities of Banja Luka and Laktaši, where the consumers of this farm's products are located (within a radius of about 30 km from the farm). In total, 245 survey responses were collected from consumers of the farm's products. The first phase of data collection involved testing the clarity and applicability of the questions on a sample of 20 pilot respondents. The primary data were sorted and coded in Microsoft Excel and subsequently transferred into IBM SPSS Statistics (version 22) for further processing and analysis. The analysis of the collected data initially employed descriptive statistics (frequencies and data distribution). Thereafter, appropriate comparative methods were applied to test the significance of differences between groups of responses, in accordance with the data type (ANOVA and general linear models). The significance level for observed differences was set at $p < 0.05$. The research findings are presented in tabular and graphical form, based on the survey responses, accompanied by the authors' interpretation.

RESULTS AND DISCUSSION

Among the customers of the analyzed farm's products, women are predominant, which is also reflected in the survey sample, where female respondents accounted for 67.35%, while 32.65% were male. In terms of age structure, the largest group of respondents was between 30 and 40 years old (47.35%), followed by those between 40 and 50 years old (32.65%). The third-largest group consisted of respondents aged 50 to 60 years (10.61%). Thus, 80% of the farm's customers and respondents are between 30 and 50 years of age. Based on this case study, the most common participants in of direct buying of food are middle-aged consumers.

Regarding educational background, the majority of respondents hold a university degree (58.78%), followed by those with secondary education (22.45%), while 13.47% hold a master's degree. This indicates that supporters of direct food purchases are more educated consumers.

The most frequent household size among respondents was four members (37.96%), followed by three-member households (28.98%), two-member households (12.56%), and five-member households (12.24%). Households with six members accounted for 6.67%, while those with one member represented 2.86%, and seven-member households 1.63%. Overall, three and four-member households predominate. The largest share of respondents (40%) reported having two children, followed by those with one child (24.49%). Respondents without

children accounted for 19.18%, while 12.65% had three children, 2.86% had four children, and only 0.82% had five children.

As for income levels, 40.82% of respondents reported monthly incomes between 2,000 and 4,000 BAM, while 39.59% had incomes ranging from 1,000 to 2,000 BAM. A smaller portion (10.20%) reported incomes below 1,000 BAM, whereas 9.39% of respondents had monthly incomes exceeding 4,000 BAM.

Milk consumption depends on the population structure, purchasing power, and dietary habits (as found by Deže *et al.*, 2021). The results from Chart 1 indicate that respondents most frequently (daily) purchase yogurt, followed by cream and milk. On a weekly basis, milk is purchased most often, followed by cream and yogurt. The least frequently purchased products are flavored sliced cheese, cheese in olive oil, smoked cheese, and semi-hard cheese. Products with a shorter shelf life are purchased more frequently, while longer shelf-life products, such as various categories of cheese, are purchased less often, as they are also the most expensive products.

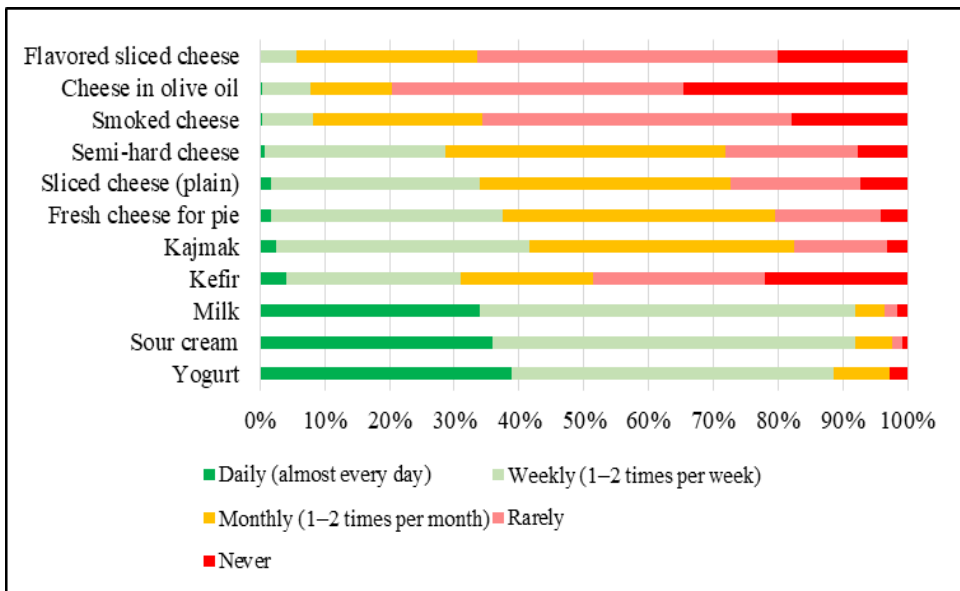


Chart 1. Frequency of purchase of milk and dairy products

*Note: 1€ = 1,95573 BAM

Heitmann *et al.* (2007) state that a larger assortment should benefit consumers and consequently lead to increased sales. The questions related to the product assortment (Chart 2), consumer satisfaction with the variety of products offered by the analyzed farm, and the possibilities of alternative purchasing options, such as direct on-farm purchase (Chart 3), were analyzed are discussed in this section.

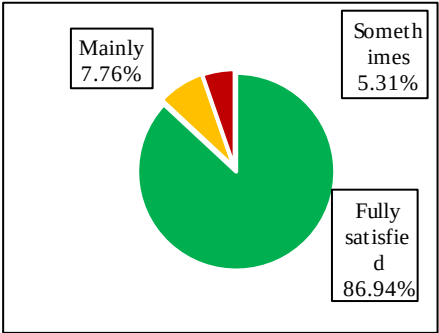


Chart 2. Satisfaction with the product assortment

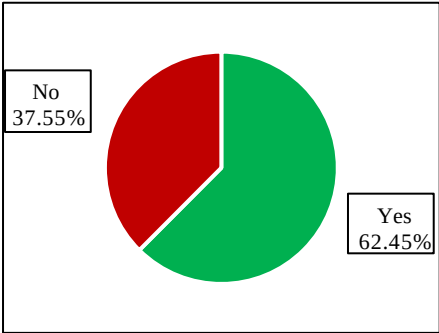


Chart 3. The willingness to purchase products at the farm gate

According to the survey results, 86.94% of respondents are satisfied with the broad assortment of milk products offered by the farm (Chart 2). Additionally, one of the questions concerned the days and times of delivery for milk and dairy products, to which 93.88% of respondents replied that the delivery days were suitable for them, and 96.33% stated that the delivery time was convenient. Thus, in addition to offering high-quality products, it is important to deliver them to customers in a way that is acceptable to them.

The next question concerned the possibility of consumers visiting the farm and buying products directly at the farm, if the delivery to their door were interrupted (Chart 3). If the farm stopped delivering products to the doorstep, 62.45% of respondents indicated that they would be willing to visit the farm to purchase products, while 37.55% stated that they would not, with the most common reason cited being lack of time. The reason for such a large share of consumers willing to purchase domestically produced products, even in a more demanding and expensive way, is supported by the study of Jalić *et al.* (2024), who concluded that homemade products are significantly more desirable compared to industrially produced ones.

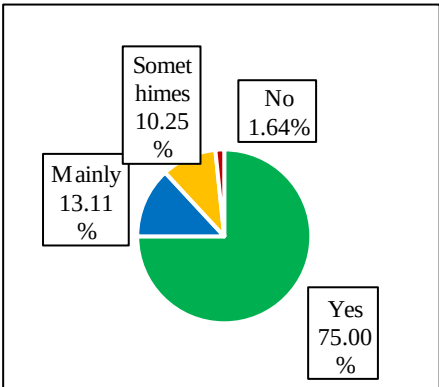


Chart 4. Level of satisfaction with the current packaging

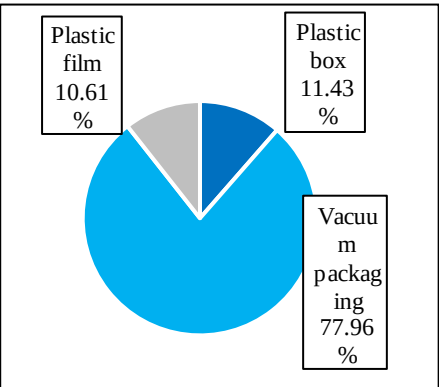


Chart 5. Most preferred types of cheese packaging

Modern packaging is much more than just the product wrapper or a cardboard box for transport (Paraušić *et al.*, 2006). There are two main functions of packaging: protecting the product from spoilage and providing consumers with information about the product (Muhamedbegović *et al.*, 2015). The milk from the household is packaged in 1-liter glass bottles, as are curd, kefir, and yogurt. The results showed that 75% of respondents are satisfied with the farm's products packaging (Chart 4). In total, 77.96% indicated that they prefer cheese vacuum packaging for cheese, 11.43% preferred a single-use plastic boxes, and 10.61% preferred plastic film (Chart 5). Regarding cheese packaging size, 63% of respondents prefer 0.5 kg packages, 33% prefer 1 kg packages, and the remaining 4% prefer other sizes.

Many studies have identified concern for health as the main motivation for food purchase (e.g., Vehapi and Dolićanin, 2016). For this reason, the following analysis highlights the importance of various elements and examines the factors that influence the purchase of products directly from the producer.

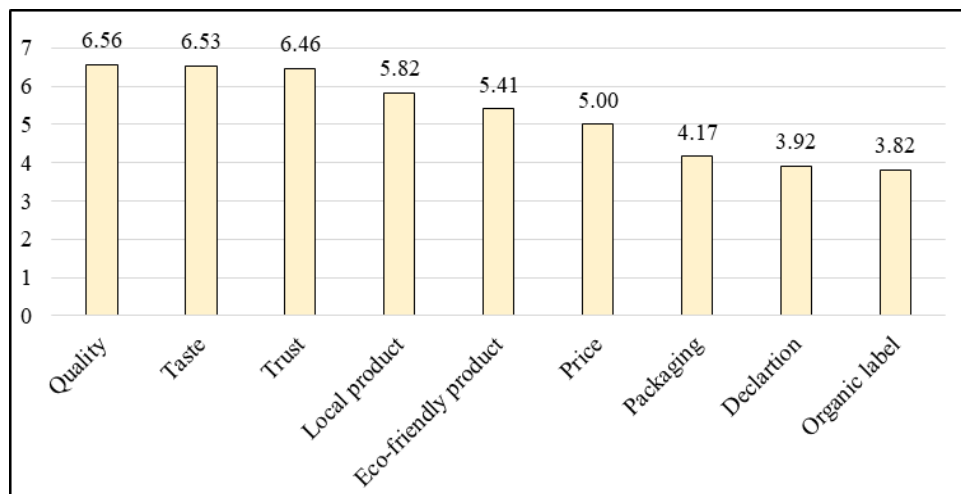


Chart 6. The importance of different elements and factors influencing the purchase of products through SFSC

According to Jalić *et al.* (2022), transitioning from milk production to cheese processing and selling through SFSCs in the market of the Republic of Srpska, as part of BH, is economically justified, as small-scale milk production is financially unsustainable due to the limited production volume and the high share of fixed costs in overall production. Matić (2008) states that when purchasing products, consumers place the greatest emphasis on quality and freshness, while brand and price are less important to them. The results of this study determine that the most significant factors influencing customers to purchase dairy products directly from these farms are product quality, taste, and trust in the producer (Chart 6). Similar to the results of this study, Ostojić *et al.* (2023) found that freshness, quality, aroma and taste, as well as the origin of a product are the most

important factors when purchasing vegetables. The second group of factors, in order of importance, includes the preference for local products and the perception that these products are produced in an eco-friendly manner, followed by the acceptability of the price. The third, least important group of factors for purchasing dairy products through short supply chains from small farms includes packaging, the content of the label, and having an organic certification. This indicates that consumers pay more attention to personal acquaintance with the manufacturer than to specific certificates.

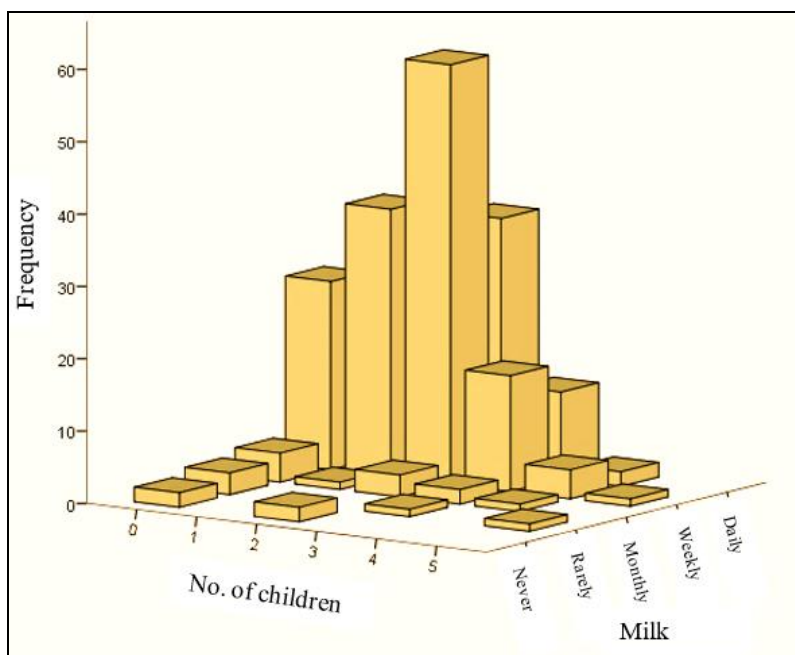


Chart 7. The impact of the number of children on the frequency of milk purchases

According to the survey results it was observed that respondents with two or three children purchase milk most often, both on a weekly and daily basis (Chart 7). Chiciudean *et al.* (2016) note that extrinsic attributes are applied to all types of products, while intrinsic attributes are specific to certain products. Intrinsic attributes define the physical aspects of a product, whereas extrinsic attributes are related to the product itself but are not part of it. Consumers often make purchase decisions based on extrinsic attributes (origin, price, production method, etc.) or intrinsic attributes (taste, texture, color, etc.). According to Veale *et al.* (2006), consumers are often unable to evaluate both intrinsic and extrinsic attributes before making a purchase decision. Grgurić (1996) states that milk is an essential food in a child's diet. The most important properties of milk are its role as a source of calcium and minerals necessary for bone growth. Colić-Barić *et al.* (2000) further note that for children, foods from the group of milk and dairy

products are of personal importance. By cross-tabulating the results on the frequency of milk purchases with the number of children in the household, it was found that respondents with two or three children buy milk daily and weekly much more often than those with fewer children, and for this reason, the hypothesis can be considered confirmed.

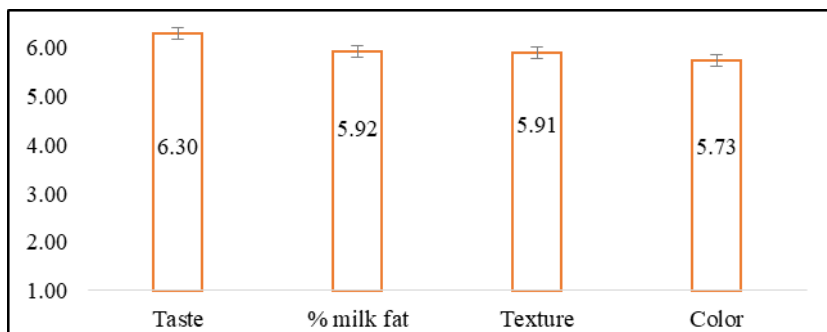


Chart 8. Intrinsic characteristics of milk

When purchasing fresh milk, traditional attributes of milk are highly valued, such as local origin, high nutritional value, and good taste of the product (Merlino *et al.*, 2022). The most important intrinsic attribute of milk, according to the research results, is considered to be taste (mean value 6.30), followed by milk fat content (mean value 5.92), then texture (mean value 5.91), and color (mean value 5.73) (Chart 8).

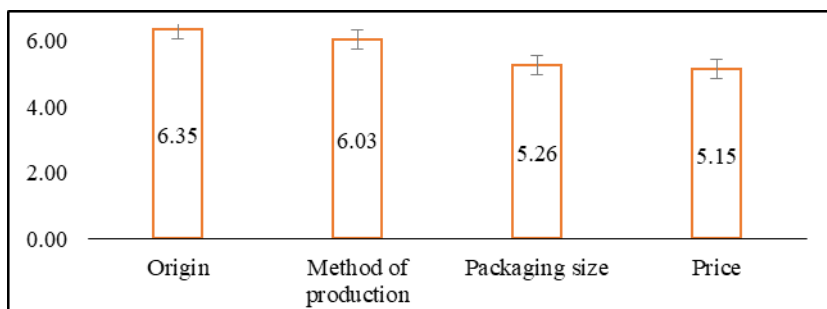


Chart 9. Extrinsic characteristics of milk

Regarding extrinsic characteristics, the origin of the milk was identified as the most important attribute, with a mean value of 6.35, followed by the production method with a mean value of 5.26 (Chart 9). The package size was rated with a mean value of 5.26, and the price with 5.15. Kristić *et al.* (2015) state that extrinsic product attributes increasingly represent an important reference point in the consumer decision-making process. According to Mesić *et al.* (2017), consumers pay particular attention to the origin and price of products when considering extrinsic attributes.

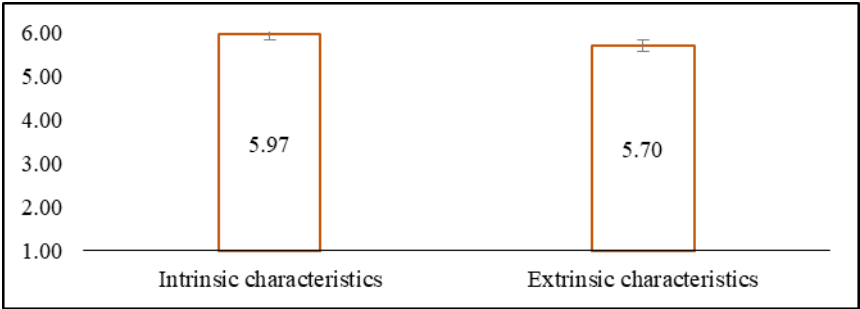


Chart 10. Evaluation of the intrinsic and extrinsic attributes of milk from the farm

Respondents rated the importance of intrinsic attributes with an overall average score of 5.96 ± 1.1 , while the importance of extrinsic attributes was rated with an average score of 5.7 ± 1.09 (Chart 10). The difference is small and of little practical significance, but nevertheless statistically highly significant ($F=13.44$, $p<0.001$). This indicates that intrinsic attributes of milk are significantly more important to respondents.

According to Chiciudean *et al.* (2016) and Mesias *et al.*, (2023), taste is the most important intrinsic attribute when purchasing cheese, which was also confirmed by this research (mean value 6.19). Taste is followed by aroma (mean value 5.89), texture (mean value 5.85), and the presence of spices (mean value 5.74). All scores (Chart 11) are generally high, which indicates that respondents are satisfied with the products they purchased from the farm.

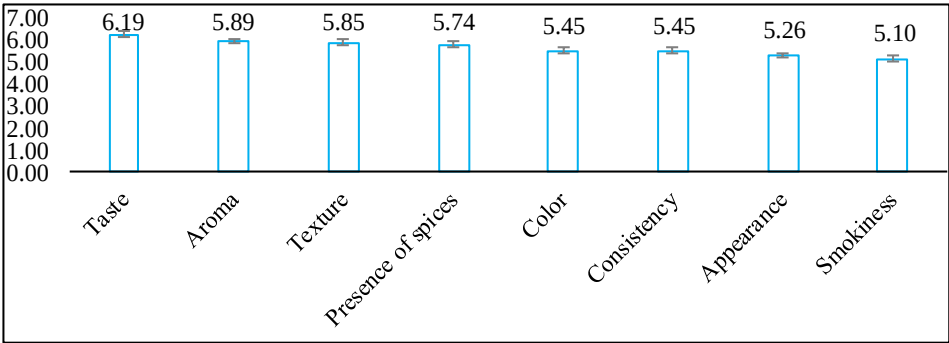


Chart 11. Intrinsic characteristics of cheese

Regarding the extrinsic attributes of cheese, the highest ratings were given to the same attributes as for milk: origin (mean value 6.24), production method (mean value 5.99), packaging method (mean value 5.28), and package size (mean value 5.20) (Chart 12). Some consumers make purchasing decisions based on the extrinsic attributes of the product, while for others, intrinsic attributes are more important. Chiciudean (2016) states that consumers are more interested in intrinsic attributes such as taste and the fact that the cheese is made from natural ingredients.

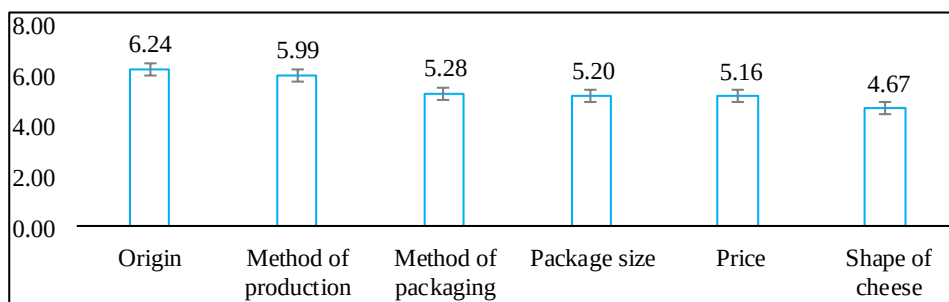


Chart 12. Extrinsic characteristics of cheese of the Farm “Mijatović”

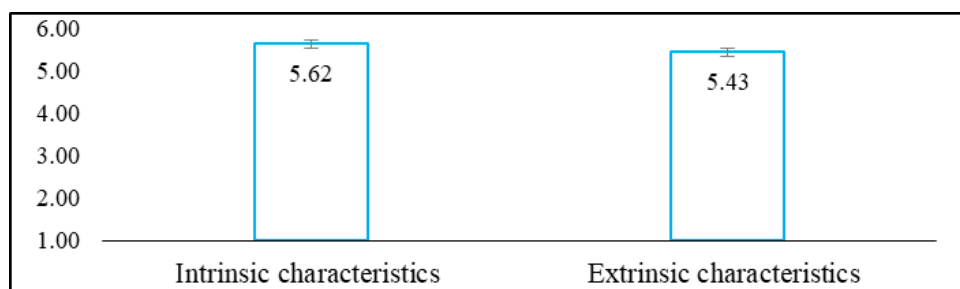


Chart 13. Evaluation of the intrinsic and extrinsic attributes of the farm “Mijatović”

Respondents rated the importance of intrinsic attributes with an overall average score of 5.62 ± 1.21 , while the importance of extrinsic attributes was rated with an average score of 5.42 ± 1.12 (Chart 13).

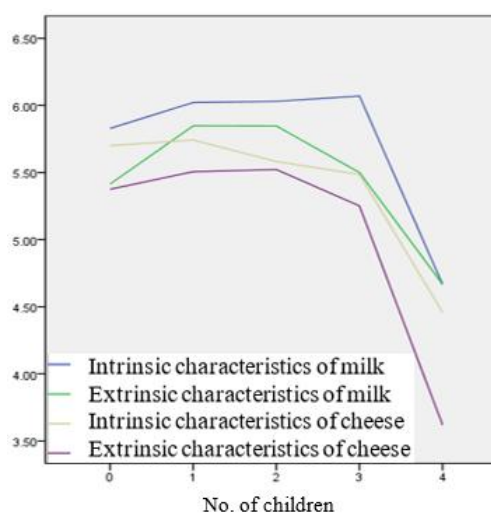


Chart 14. The relationship between the importance of intrinsic and extrinsic characteristics of milk and cheese, with the number of children

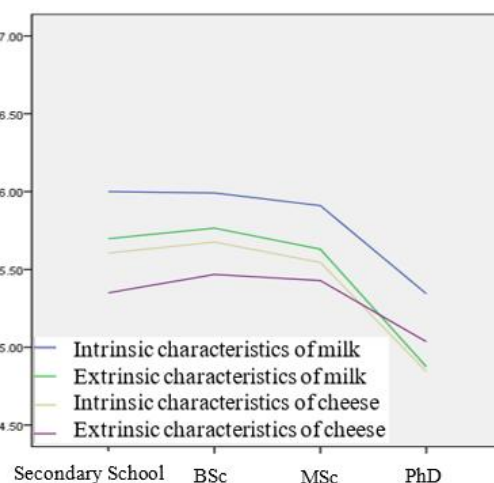


Chart 15. The relationship between the importance of intrinsic and extrinsic characteristics of milk and cheese, with the level of education

The difference is small and of little practical significance, but nevertheless statistically highly significant ($F=9.67$, $p=0.002$). For cheese as well, it is indicative that respondents consider intrinsic attributes to be significantly more important.

The analysis of the impact of the number of children on the evaluation of intrinsic and extrinsic attributes of milk and cheese shows that, as the number of children per household increases, these evaluated characteristics are considered less important (Chart 14). The only sustained increase was observed in the importance of intrinsic attributes of milk among families with three children. Respondents with four children rated all these attributes lower, meaning they considered them less important compared to respondents with fewer children.

When analyzing the impact of education (Chart 15), intrinsic attributes were rated as more important than extrinsic ones across all categories, except among the most highly educated respondents. In this case, a paradox emerged: they rated the intrinsic attributes of milk higher than extrinsic ones, while the extrinsic attributes of cheese were the only category they rated as more important than intrinsic attributes.

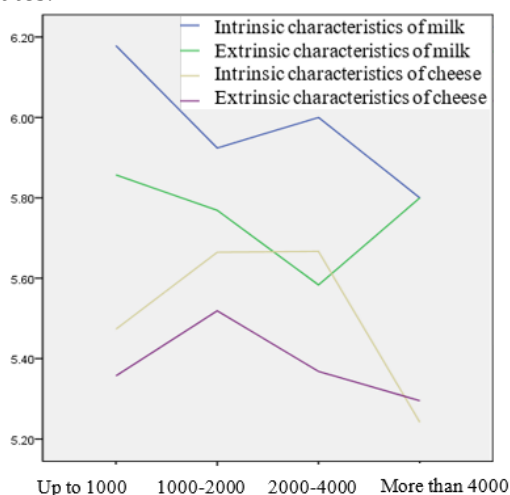


Chart 16. The relationship between the importance of intrinsic and extrinsic characteristics of milk and cheese and the monthly income in BAM

According to Užar (2023), respondents' monthly income has a significant impact on the intention to purchase cheese directly from the farm. An analysis of the ratings given by respondents regarding the importance of specific attributes reveals an interesting finding: only respondents with middle-high incomes gave the highest ratings to intrinsic attributes for cheese (Chart 16). Regarding milk, intrinsic factors are rated best by those with the lowest income. When it comes to the extrinsic properties of milk, they are rated best by respondents with the lowest income, while the extrinsic properties of cheese are rated best by those with a middle-income level. It is also noteworthy that respondents with high incomes

above 4,000 BAM gave equal ratings to both intrinsic and extrinsic characteristics, whether for cheese or milk.

The findings confirm that intrinsic product attributes, particularly taste and freshness, play a more significant role than extrinsic attributes in shaping consumer preferences for dairy products purchased directly from farms. Furthermore, the results highlight the importance of trust in producers, product quality, and local origin as key drivers of consumer participation in SFSCs. These findings are consistent with previous studies conducted in other European contexts, but they also provide new insights into consumer behavior in Southeast Europe, where small-scale farms and direct marketing channels are becoming increasingly important for rural economic sustainability.

As part of the research, certain statements were defined concerning the factors influencing purchases through short supply chains and the impact of online posts on product purchases. Respondents rated these statements on a scale from 1 to 7, and they are presented in the following chart. Higher statement values indicate a greater level of agreement with the given assertion.

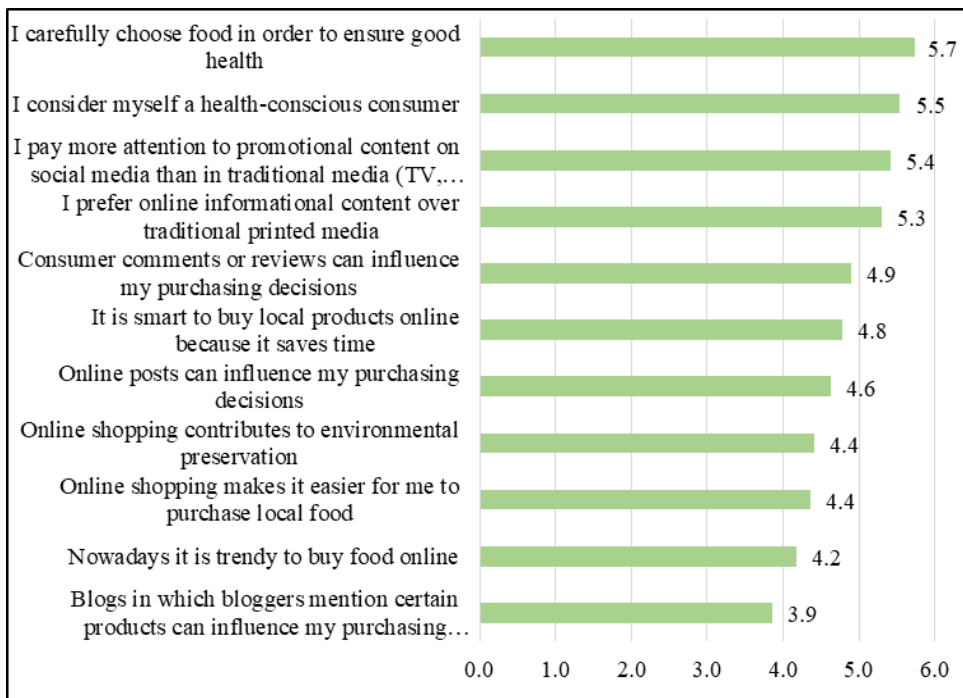


Chart 17. Degree of respondents' agreement with statements on food and the impact of online posts on purchasing behavior

According to Mesić and Šaban (2020), online sales have become the most popular channel for selling food products over the past decade. Ćendić and Zarić (2019) note that there are various motives for purchasing organic food, the most important being concern for one's own health and care for the environment.

Consumers of dairy products from the farm expressed the highest level of agreement with the statement that they choose food to ensure their health, followed by another health-related statement with which they also strongly agreed (Chart 17). After the statements focused on health factors, the third highest-rated statement was that social networks are more important for promotion than traditional media. Moderately high ratings were given to the statements that consumers’ attitudes and opinions influence purchasing decisions, that it is wise to buy local products online as this saves time, and that online posts can affect purchase decisions. Somewhat lower ratings were given to the claims that online shopping contributes to environmental protection, facilitates the purchase of domestic products, and that current trends lean toward buying local food. The least accurate statement, according to respondents, was that bloggers can influence purchasing decisions for certain products. The results suggest that consumer loyalty and purchasing decisions are strongly influenced by personal relationships with producers and perceived product quality, which represents an important characteristic of SFSC systems in transitional agricultural markets such as Bosnia and Herzegovina.

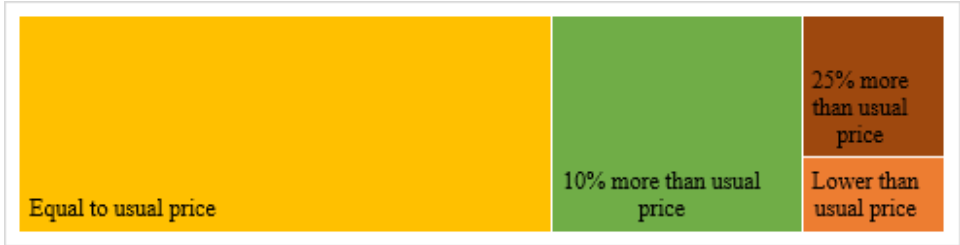


Chart 18. Willing to pay higher milk prices

Out of the total number of respondents, the majority (58%) stated that the prices of milk from the farm under study were identical to those in nearby supermarkets (Chart 18). The second-largest group, 27%, believed that the prices were about 10% higher than in retail shops. In addition, 10% of respondents considered the prices to be significantly higher, even by a quarter higher than the usual price. A very small number of respondents thought the prices were actually lower than usual. Based on the above, it is evident that price is not such a primary factor. A similar conclusion regarding wine as an agricultural product was reached by Jalić *et al.* (2022). Their study demonstrated that price does not have a particularly significant influence on the purchase of agricultural products when compared to other factors such as taste, aroma, color, freshness, and similar attributes of tangible product quality.

Čehić (2020) stated that the intrinsic attributes of products refer to their composition and represent an objective measure of product quality, while the technological production process is a key factor in defining these intrinsic attributes. The research results showed that taste is the most important intrinsic attribute when purchasing milk and cheese, while product origin was the highest-

rated extrinsic attribute. Kristić (2015), in his research, found that more than half of respondents, 52.46%, answered that taste is the most important characteristic when purchasing milk. Based on the research, the set hypothesis „Intrinsic attributes of milk and dairy products are more important than extrinsic attributes“ was confirmed. Respondents rated the importance of intrinsic attributes of milk with an overall average score of 5.96 ± 1.1 , while extrinsic attributes were rated with an average score of 5.7 ± 1.09 . The difference is small, but statistically significant ($F=13.44$, $p<0.001$), indicating that intrinsic attributes of milk are significantly more important to respondents. The intrinsic attributes of cheese were rated with an overall average score of 5.62 ± 1.21 , while extrinsic attributes were rated with an average score of 5.42 ± 1.12 . The same was observed for cheese as with milk. The difference is small, but statistically highly significant ($F=9.67$, $p=0.002$). The presented results clearly show that intrinsic attributes are more important to respondents than extrinsic ones. These data confirm the stated hypothesis.

CONCLUSIONS

Direct sales for small farmers and processors of agricultural products provide new opportunities for marketing and distributing food products. Direct sales via a short supply chain represent an effective strategy for small farmers. The research results, based on the case study of Farm “Mijatović” show that consumers who use SFSCs are mostly female, belong to the middle age group, are predominantly highly educated, have a medium level of monthly income, and mostly live in families with two or three children. This suggests that women are primarily responsible for purchasing food, including dairy products, for their families, including children. Education plays an important role in purchasing decisions. Highly educated consumers prefer local products produced using traditional methods on small family farms, focusing on quality over price, which aligns with the principles of short food supply chains. The number of children in the household also significantly affects the frequency of milk and dairy product purchases, as families having two or three children buy these products more frequently. When purchasing milk and dairy products from the “Mijatović” farm, consumers rated taste as the most important intrinsic attribute, while origin was rated as the most important extrinsic attribute. Across both milk and cheese, intrinsic attributes were consistently rated higher than extrinsic attributes, indicating that consumers prioritize product quality and health benefits in their purchasing decisions. Consumers rely more on online information than on traditional media content and carefully select food to ensure their health and the health of their children. More than half of the respondents consider the prices of locally produced products comparable to the prices of products of industrial origin in supermarkets. Overall, consumers prefer local products from small family farms, emphasizing product quality and trust in the producer. Trust was rated higher than any other purchase or consumption motive, highlighting the importance of direct contact between consumers and producers in short food

supply chains. These results confirm that consumers value healthy, locally produced food and prioritize intrinsic product attributes when purchasing milk and cheese.

Direct sales through short food supply chains (SFSC) of agricultural products, and in the examined case specifically dairy products, represent a significant opportunity for small farms to strengthen their competitive and market position based on the trust they build with consumers. The results of this research show that consumers prefer locally produced dairy products from small farms, emphasizing trust in product quality, good taste, and the importance of product origin, particularly when it refers to local producers, while paying less attention to the price of the product. These findings suggest that farmers should focus on maintaining high product quality, even at the cost of higher prices, promoting the local origin of their products, and using direct communication channels with consumers.

The limitation of this research is reflected in the sampling procedure itself, as the sample represents a convenience sample based on consumers of a single dairy producer. Furthermore, future research should focus on consumers from a larger number of farms that sell their products through short supply chains.

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Alam, S., Ginting, S., Suleman, D., Kilowasid, L.M.H., Muhidin, M., Kandari, A.M. (2026). Exploring the relationship between vegetation diversity and soil fertility in a tropical forest ecosystem of southeast Sulawesi. *Agriculture and Forestry*, 72 (1): 373-385. <https://doi:10.17707/AgricultForest.72.1.22>

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EXPLORING THE RELATIONSHIP BETWEEN VEGETATION DIVERSITY AND SOIL FERTILITY IN A TROPICAL FOREST ECOSYSTEM OF SOUTHEAST SULAWESI

SUMMARY

Many studies have investigated the relationship between vegetation density and soil quality. However, the influence of vegetation diversity on soil fertility is still not documented. This study evaluated the relationship between vegetation diversity and soil fertility in a tropical forest ecosystem located in Southeast Celebes. An ecological survey was conducted using the quadrat method at three forest sites in different toposequences, including upper, middle, and lower. Three indicators were selected to describe the vegetation diversity, namely richness, evenness, and heterogeneity. Soil fertility was evaluated by six parameters, including soil acidity, organic carbon, total nitrogen, available phosphorus, available potassium, and cation exchange capacity. We used correlation analysis to examine the relationship between the two variables. Nineteen species were recorded from the field observation. The highest species abundance was noted in the lower forest. This site was also superior in richness, heterogeneity, and evenness. Soil fertility among sites was relatively similar, with low values for all parameters. There was no significant correlation between vegetation diversity and soil fertility. Further investigation is still required to verify this finding in other ecosystems.

Keywords: soil quality, plant biodiversity, soil organic matter, toposequence

INTRODUCTION

Understanding the interaction between soil and plant is necessary to support sustainable ecosystem management. Soil naturally supplies water and nutrients for plant growth and development (FAO, 2022; Huntley, 2023). Inversely, the plant has the potential to improve soil fertility by providing organic matter through litter fall (Giweta, 2020; Alam *et al.*, 2022; Xu *et al.*, 2022). When

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the litter is decomposed, nutrients will be released into the soil (Krishna and Mohan 2017; Liu *et al.*, 2022). This process is generally known as the close nutrient cycle and becomes one of the specific attributes in tropical forest ecosystems.

Several studies have investigated the soil and plant interactions, wherein higher plant density will improve soil fertility (Ndabamenye *et al.*, 2013; Brauner-Otto, 2014; Duan *et al.*, 2019). It can happen because there is a high accumulation of organic matter from litter. Besides containing several nutrients, the dense litter can also maintain soil moisture (Deutsch *et al.*, 2010). It indirectly influences soil organism activity that makes meaningful contributions to the biogeochemical cycle (Chen *et al.*, 2020). However, the interaction between soil and plant in the tropics still has many interesting questions that require further investigation. One of them is the significant effect of vegetation diversity on soil fertility.

Vegetation diversity is an important indicator of environmental stability in tropical forest ecosystems (Majeed *et al.*, 2022; Suyanto *et al.*, 2022). This indicator is also used to assess the resilience of ecosystems to natural disturbances, such as pests and diseases, wind damage, wildfire, etc (Cantarello *et al.*, 2017). Previous studies also reported that higher diversity described better environmental capability for facilitating life cycle (Isbell *et al.*, 2011; Souza *et al.*, 2014; Asselin *et al.*, 2019; Lindner *et al.*, 2019; Sanyé-Mengual *et al.*, 2023). In other words, vegetation diversity should also affect soil fertility, as soil is the major component of the ecosystem.

However, most empirical findings discussing the relationship between vegetation diversity and soil fertility are still dominated by studies in temperate ecosystems, while similar studies on tropical forests are relatively limited and have not been comprehensively integrated (Prober and Wiehl 2012; Li *et al.*, 2018; Chen *et al.*, 2020; Xie *et al.*, 2021). Tropical ecosystems have distinctive biogeochemical characteristics, such as pronounced phosphorus limitation, high nutrient recycling rates, and highly intense weathering. There is no adequate information to support this claim in the tropical forest ecosystem. Meanwhile, previous studies in tropical ecosystems have only focused on the relationship between vegetation density and soil fertility (Song *et al.*, 2013; Dibala *et al.*, 2021; Tokozwayo *et al.*, 2021).

To date, empirical evidence specifically examining soil fertility variations along the vegetation diversity gradient in tropical forest ecosystems remains limited. This limitation hinders a deeper understanding of the extent to which biodiversity itself plays a role in shaping soil nutrient dynamics, independent of the influence of vegetation structure alone. Therefore, this study aims to analyze patterns of soil fertility change along the vegetation diversity gradient in tropical forest ecosystems. By directly testing the relationship between vegetation diversity and various indicators of soil fertility in a tropical context, this study contributes to enriching the existing ecological framework by providing region-based evidence on the feedback mechanisms between biodiversity and soil. The

result is expected to provide a deeper understanding of the ecological relationship between the diversity of vegetation and soil fertility dynamics as the primary component of a forest ecosystem.

MATERIAL AND METHODS

This study was undertaken in a tropical forest ecosystem located at Moramo Education Estate (Figure 1). It is a special-purpose area managed by Universitas Halu Oleo in Southeast Sulawesi. The site has a geographic position in E4°6'30"-4°7'30" and S122°35'0"-122°35'30". Moramo Education Estate is a natural ecosystem consisting of three land cover types, including forest (70%), shrub (20%), and savanna (10%) (Alam *et al.*, 2022). Altitude varies from 25 to 137 m above sea level—topography dominated by a hilly area with a slope level of 8–15%. The mean daily temperature reaches 27.6°C, with a minimum of 23.1°C and a maximum of 32.2°C. Annual rainfall is 3,179.70 mm year⁻¹ with air humidity of 81%. Dry periods occur from September to October.

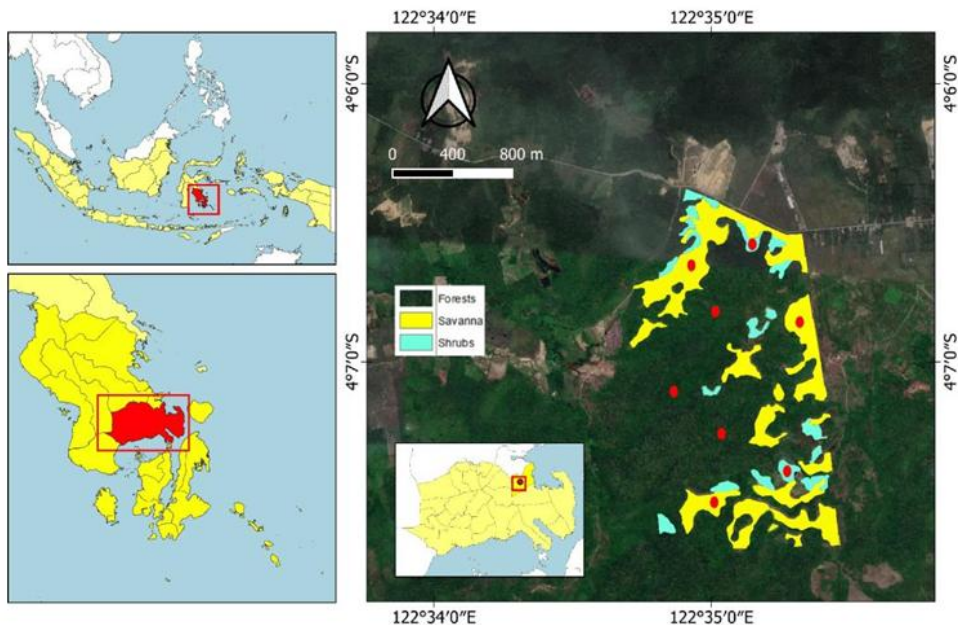


Figure 1. Study site of Moramo Education Estate in Southeast Sulawesi. Red circles indicate sampling locations for vegetation and soil survey (Source: Alam *et al.*, 2022).

An ecological survey was carried out using the quadrat method at three forest sites in different toposequences, including upper, middle, and lower (Alam *et al.*, 2020). We used a sampling plot of 20×20 m to facilitate the measurement process (Grussu *et al.*, 2016). The number of plots in each toposequence was five units. These plots were placed systematically with a distance between plots of 50 m (Sadono *et al.*, 2020). Data collection in each plot was divided into two stages, namely vegetation inventory and soil sampling.

We conducted a vegetation inventory using a nested method, wherein each plot was divided into four subplots with a specific size and objective: 1×1 m (understorey), 2×2 m (seedlings), 5×5 m (saplings), 10×10 m (poles), and 20×20 m (trees) (Rambey *et al.*, 2021). However, the life stage of vegetation in this site was only in poles and trees; thus, there were only two subplots in every sampling plot. Several parameters were recorded from the vegetation inventory, including species name, density, and diameter (Nugroho *et al.*, 2022a). The diameter was measured at 1.3 m above ground using a diameter tape.

In parallel, we took soil samples from three different positions in every plot at the depths of 0–10 cm, 11–20 cm, and 21–30 cm (Nugroho *et al.*, 2022b). Afterward, these samples were composited and brought to the laboratory for quality analysis. We used six parameters to quantify the soil fertility, namely soil acidity (pH), organic carbon (SOC), total nitrogen (TN), available phosphorus (AvP), exchangeable potassium (ExK), and cation exchange capacity (CEC) (Alam *et al.*, 2022). Soil pH was determined using a pH meter, while SOC was quantified using the Walkley–Black method. TN was analyzed using the Kjeldahl method. The ammonium acetate (NH₄OAc) 1 M, pH 7.0 extraction was applied to determine ExK, while AvP was enumerated using the Bray method. We used the ammonium acetate method to quantify the CEC. The soil analysis protocol was conducted following the guidance of soil analysis published by Estefan *et al.*, (2013).

We conducted descriptive analysis to identify the vegetation structure in each toposequence. First, data from the vegetation inventory were analyzed to calculate species density, species dominance, and frequency distribution. The generated values were then utilized to compute relative abundance, dominance, and frequency from every species (Matatula *et al.*, 2021). Then, the importance value index was calculated by summing up these three indicators. It is an essential parameter to recognize the strategic position of species in each toposequence (Yuliana *et al.*, 2019).

The diversity of vegetation from every toposequence was evaluated using three indicators, including richness, heterogeneity, and evenness (Wirabuana *et al.*, 2021). We used the Margalef Index to determine vegetation richness (Singh, 2020), while the Shannon-Wiener Index was applied to quantify the vegetation heterogeneity (Li *et al.*, 2018). The evenness level of vegetation was assessed using the Pielou-Evenness Index (Nugroho *et al.*, 2022a). Furthermore, the analysis of Spearman correlation was applied to examine the relationship between vegetation diversity and soil fertility for each parameter (Alam *et al.*, 2020).

RESULTS AND DISCUSSION

Vegetation diversity patterns along the toposequence

Nineteen species were recorded from the forest inventory (Table 1). Most species had a life stage in the pole, although some species also existed at the tree level. The highest species abundance was found in LF (15), followed by UF (7) and MF (5). Interestingly, the number of species in every life stage was relatively

different (Figure 2), although LF showed the highest species abundance at each phase. Among the observed species, *Castanopsis buruana* was the only one that appeared at every site (Table 1).

Table 1. Importance value index of every species in different forest sites

No.	Species	Upper Forest (UF)		Middle Forest (MF)		Lower Forest (LF)	
		Poles	Trees	Poles	Trees	Poles	Trees
1	<i>Castanopsis buruana</i>	58.51	190.92	126.48	300	43.84	117.51
2	<i>Xanthophyllum sp.</i>	-	109.08	-	-	-	-
3	<i>Lasia hildebrandi</i>	47.52	-	55.23	-	-	-
4	<i>Palmaceae sp.</i>	21.41	-	56.61	-	-	-
5	<i>Barringtonia reticulate</i>	24.07	-	-	-	13.8	-
6	<i>Syzygium subglauc</i>	52.41	-	32.26	-	-	-
7	<i>Cleistanthus sumatranus</i>	96.08	-	-	-	20.94	-
8	<i>Syzygium sp.</i>	-	-	29.41	-	23.06	-
9	<i>Santiria laevigata</i>	-	-	-	-	-	83.97
10	<i>Dillenia serrata</i>	-	-	-	-	23.49	98.53
11	<i>Vitex cofassus</i>	-	-	-	-	18.53	-
12	<i>Lagerstroemia speciosa</i>	-	-	-	-	19.09	-
13	<i>Gardenia sp.</i>	-	-	-	-	13.8	-
14	<i>Guensia cinnamomea</i>	-	-	-	-	22.98	-
15	<i>Fagrarea fragrans</i>	-	-	-	-	13.8	-
16	<i>Nauclea orientalis</i>	-	-	-	-	34.59	-
17	<i>Hibiscus tiliaceus</i>	-	-	-	-	13.8	-
18	<i>Colona scabra</i>	-	-	-	-	25.36	-
19	<i>Ficus fistulosa</i>	-	-	-	-	12.9	-
	Important value index	300	300	300	300	300	300

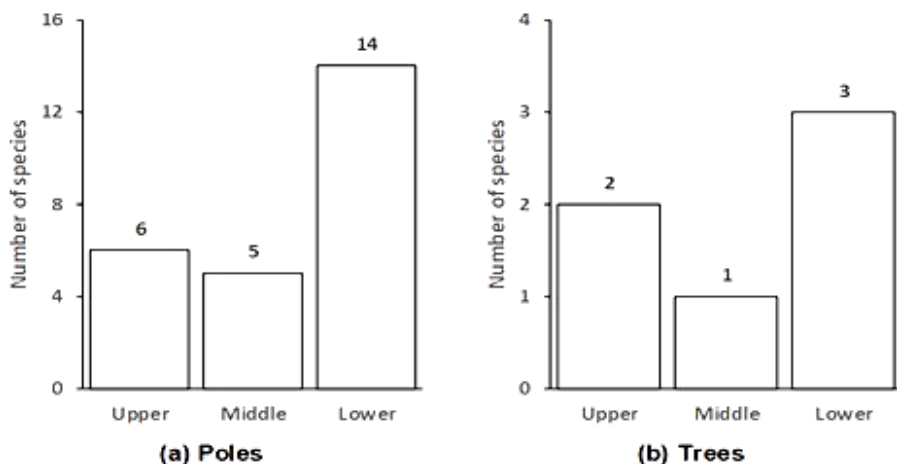


Figure 2. Number of species in every life stage at different forest sites

Our study reported that vegetation diversity among toposequences varied relatively, with the lower forest demonstrating the highest richness (4.08), heterogeneity (2.52), and evenness (0.93) (Figure 3). This could be attributed to the lower forest having the highest species abundance among sites (Table 1). In addition, the species distribution in this site exhibited a better proportion of important value index than the upper and middle forests, particularly at the pole stage.

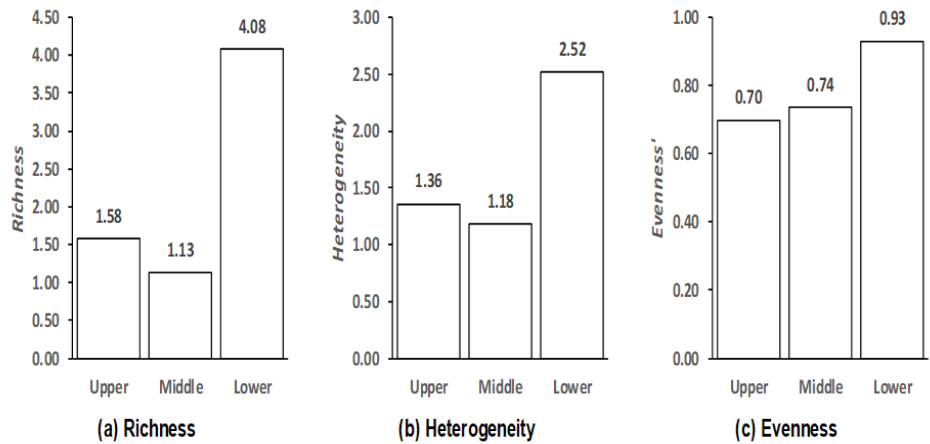


Figure 3. Plant diversity among forest sites based on richness, heterogeneity, and evenness

Soil fertility characteristics

Soil properties among sites generally had high acidity with a pH of lower than 5.0 (Table 2). Most sites also had low organic carbon content (<2.0%), except for the upper forest, which had moderate levels. Meanwhile, other parameters such as total nitrogen, available phosphorus, exchangeable potassium, and cation exchange capacity were classified as low concentrations. This trend was consistently found in all sites.

Table 2. Soil fertility in different forest sites

Soil attributes	Symbol	Unit	Upper	Middle	Lower
pH H ₂ O	pH	-	4.4	4.5	4.4
Organic carbon	SOC	%	2.19	1.38	1.43
Total nitrogen	TN	%	0.18	0.14	0.14
Available phosphorus	AvP	ppm	6.72	3.66	4.94
Exchangeable potassium	ExK	cmol(+) kg ⁻¹	0.32	0.29	0.3
Cation exchange capacity	CEC	cmol(+) kg ⁻¹	15.54	11.46	12.63

Vegetation diversity–soil fertility correlation

The correlation analysis between vegetation diversity indicators and soil fertility parameters shows that there is no statistically significant relationship between the two variables (Table 3). All correlation values have a p-value greater than 0.05, indicating that vegetation diversity is not significantly related to the observed soil fertility parameters. However, the relationship between vegetation diversity indicators and soil pH showed a moderate negative correlation.

Table 3. Correlation results between vegetation diversity and soil fertility

	Richness	Heterogeneity	Evenness
pH	-0.655	-0.618	-0.601
	(0.546)	(0.576)	(0.590)
SOC	-0.275	-0.320	-0.341
	(0.823)	(0.792)	(0.778)
TN	-0.327	-0.372	-0.392
	(0.788)	(0.757)	(0.743)
AvP	0.096	0.048	0.026
	(0.939)	(0.969)	(0.983)
ExK	0.000	-0.048	-0.070
	(1.000)	(0.970)	(0.956)
CEC	-0.051	-0.099	-0.121
	(0.967)	(0.937)	(0.923)

Note: The number inside the breakers indicates

DISCUSSION

The dominance of *Castanopsis buruana* in all locations indicates that this species has a good ability to adapt to environmental conditions in the study area. This is reflected in the high importance value index (IVI) values, especially at the tree level in all observation locations. These high IVI values indicate that *C. buruana* plays a significant role in shaping the structure of the vegetation community and contributes significantly to the ecological dynamics of the forest in the study area.

The interaction between biotic and abiotic factors, such as soil, climate, vegetation, and topography, naturally influences vegetation dynamics in a tropical forest ecosystem. This process generates a high variation of vegetation at the microsite level, like toposequences. Hussain *et al.*, (2012) explained that the vegetation condition in forest ecosystems was principally assessed using three biodiversity indicators, such as richness, heterogeneity, and evenness. According to the richness value, the vegetation dynamics of tropical forests could be classified into three levels, including disturbed (<2.0), semi-disturbed (2.0–5.0), and integrated (>5.0). Our results indicated that the vegetation of the upper and middle forest had a disturbed condition, while the lower forest was semi-disturbed.

Hussain *et al.*, (2012) also classified the heterogeneity of vegetation into three statuses, namely poor (<2.0), moderate ($2.0-3.0$), and good (>3.0). Meanwhile, the species evenness was stratified into three stages, i.e., unbalanced (<0.5), semi-balanced ($0.5-0.8$), and balanced (>0.8). Referring to our results, the upper and middle forests had relatively poor heterogeneity with semi-balanced species distribution. In contrast, the lower forest showed moderate heterogeneity and balanced species evenness. Our findings were also similar to Suyanto *et al.*, (2022), who reported that tropical forest ecosystems in lowland areas had relatively low-moderate diversity with semi-balanced species distribution. It could happen since most species in tropical forests naturally live in groups and have a habitat preference (Purwaningsih, 2004; Hadi *et al.*, 2019; Sari *et al.*, 2019).

The soil fertility characteristics observed in this study showed generally low nutrient levels across the study sites. The soils were strongly acidic, with pH values below 5.0, and most nutrient parameters such as soil organic carbon, total nitrogen, available phosphorus, exchangeable potassium, and cation exchange capacity were classified as low. These conditions are typical for highly weathered tropical soils where intensive rainfall and leaching processes reduce nutrient availability (Purwanto and Alam 2020; Basak *et al.*, 2022; Liu *et al.*, 2024).

Interestingly, we reported there was no significant correlation between the vegetation diversity and soil fertility in the study site for all parameters, although there was a moderate correlation between diversity indicators and soil acidity (Table 3). This outcome was in contrast to other previous studies in temperate forests, which reported a significant influence of plant diversity and soil fertility (Prober and Wiehl 2012; Li *et al.*, 2018; Chen *et al.*, 2020; Xie *et al.*, 2021). Several factors may influence this different finding. One of them is related to the species composition between the two forest ecosystems. In general, tropical forests had substantially more abundance of species than temperate forests since they had humid conditions with full sun intensity. This condition is suitable for plant species to establish and grow. In contrast, the temperate forest had more extreme conditions, particularly related to temperature and sunlight. The number of species in a temperate forest is relatively lower than in a tropical forest. Thereby, the increase in plant diversity in temperate forests may provide a faster impact on soil fertility than tropical forests.

Forest fires also occurred frequently in this site, which decreased the litter accumulation as input for improving soil fertility. This observation is consistent with AlKhayri and Khan (2024), who reported that forest fires can influence soil fertility by altering organic matter inputs and nutrient cycling in the soil. It typically happened in the dry season, wherein the monthly precipitation was substantially lower than in the wet season. Meanwhile, during the rainy season, the root systems of vegetation can minimize runoff and maintain soil fertility in forest sites. It also possibly means there is no significant correlation between vegetation diversity and soil fertility across the toposequence.

Furthermore, there are also other factors that affect soil fertility in the tropics, such as topography, climate, and natural disturbance. Topography is highly related to the risk of runoff and erosion that can leach topsoil (Zhao *et al.*, 2022; Supandi *et al.*, 2023; Zhou *et al.*, 2023). Meanwhile, climate condition determines the rate of decomposition since they correlate with soil temperature and moisture (Wang *et al.*, 2016; Krishna and Mohan 2017; Petraglia *et al.*, 2019). Natural disturbance can also affect soil fertility since it can change vegetation dynamics aboveground (Maynard *et al.*, 2014; Guo *et al.*, 2019; Cornejo *et al.*, 2023). Our study also found that vegetation diversity in the majority of sites had a disturbance condition based on the range of species richness (Table 1).

CONCLUSIONS

This study concluded that there was no significant relationship between vegetation diversity and soil fertility in the tropical forest ecosystem in the study area. Although vegetation diversity varied among sites, soil fertility indicators remained relatively similar, suggesting that soil nutrient status may be influenced more strongly by inherent edaphic and topographic factors than by vegetation diversity alone. From a forest management perspective, these findings indicate that increasing vegetation diversity does not necessarily lead to immediate improvements in soil fertility. Therefore, sustainable forest management in tropical ecosystems should integrate biodiversity conservation with careful consideration of soil physicochemical properties and landscape characteristics. While vegetation diversity remains essential for maintaining ecosystem resilience and other ecological functions, it may not directly predict soil fertility conditions.

This study is limited by the relatively small sample size and the use of correlation analysis, which may not fully capture complex multivariate interactions among ecological variables. Future research should include broader spatial gradients, larger datasets, and more comprehensive analytical approaches, such as multivariate or long-term studies to better understand biodiversity–soil feedback mechanisms in tropical forest ecosystems.

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THE EFFECT OF VARIETIES AND FERTILIZATION ON YIELD AND SOIL MICROBIOLOGICAL DIVERSITY UNDER LUCERNE

SUMMARY

Lucerne represents one of the most important perennial forage legumes in Bosnia and Herzegovina, recognized for its high yield and nutritional value. It is characterized by a high content of crude protein, almost all essential vitamins, and is a rich source of mineral elements. The yield and quality of lucerne are largely determined by agroecological conditions, the genetic potential of the cultivar, soil fertility, as well as the phenological stage of the plant at the time of mowing. The aim of this study was to determine the effects of cultivar and fertilization on dry matter yield, crude protein content, and microbial diversity under alfalfa in the agroecological conditions of the Butmir locality, near Sarajevo. The study included two lucerne cultivars (Banat VS and Soča), two fertilization treatments (N0 - control, application of 50 kg N/ha, and incorporation of straw into the soil at 5.0 t/ha), as well as the abundance of systematic and physiological groups of microorganisms. Mowing was carried out at the flowering stage of lucerne. The results showed that the application of nitrogen fertilizer had a positive effect on almost all analyzed parameters. The highest dry matter yield, crude protein content, and total number of bacteria were recorded in treatments with applied fertilizer at a rate of 50 kg N/ha, regardless of cultivar. In contrast, the total number of ammonifiers and actinomycetes was highest in the control treatment. The Banat VS cultivar exhibited a more diverse soil microbial community, with higher numbers of bacteria, fungi, and actinomycetes compared to the other cultivar.

Keywords: Lucerne, cultivars, fertilization, dry matter, crude protein, microbial diversity

INTRODUCTION

Lucerne (*Medicago sativa* L.) represents the most important perennial legume in modern livestock and crop production, primarily due to its high yield

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and exceptional forage quality. The nutrients of lucerne are characterized by high digestibility, which decreases as the plant advances in its developmental stage at the time of mowing. Owing to its high nutritional value, particularly its high content of proteins, vitamins, and mineral elements, lucerne is rightly regarded as a leading forage crop (Lukić, 2000; Katić, 2002; Stjepanović, 2009). Under optimal agroecological conditions, lucerne exhibits strong regenerative capacity, enabling four to six cuts per year, and up to seven under irrigated conditions. Green mass yields may reach 60–80 t/ha, while dry matter yields exceed 20 t/ha (Katić *et al.*, 2002). However, in Bosnia and Herzegovina, achieved yields are significantly lower, averaging around 4 t/ha of dry matter, indicating suboptimal agronomic practices and considerable potential for improving production. Lucerne requires carefully planned agronomic management, including appropriate placement in crop rotation, high-quality soil tillage, proper fertilization, and optimal utilization of cuts. Earlier perceptions of its modest requirements are no longer sustainable in modern production systems, as high yields can only be achieved through the application of a complete technological package. In addition to its direct production importance, lucerne also has a positive effect on soil fertility. After cultivation, it leaves substantial amounts of organic matter in the soil, equivalent to 50–80 t of high-quality manure, as well as 200–300 kg/ha of biologically fixed nitrogen (Halagić, 2005), thereby contributing to improved crop performance in subsequent seasons. As a perennial crop with multiple cuts during the growing season, lucerne meets a large proportion of its nitrogen requirements through symbiotic fixation of atmospheric nitrogen by root nodule bacteria, making it an important component of environmentally sustainable and economically efficient forage production. Monitoring agronomic practices, including cultivar selection and fertilization strategies, is essential for achieving high yields and stable forage quality, as well as for preserving and enhancing soil microbial diversity (Vučković, 2019; Yaoquan *et al.*, 2025). The types and amounts of mineral fertilizers that satisfy crop requirements also positively influence the abundance and enzymatic activity of soil microorganisms (Govedarica and Jarak 1995). The aim of this study was to determine the effects of cultivar and fertilization on dry matter yield, crude protein content, and microbial diversity under alfalfa in the agroecological conditions of the Butmir locality, near Sarajevo.

MATERIAL AND METHODS

The study was conducted at the Butmir experimental field (520 m a.s.l.), where the experiment was arranged in a randomized complete block design with four replications. The size of the basic plot was 6 m² (3×2 m). The experiment included two lucerne cultivars (Banat VS and Soča) and two plant nutrition treatments: N0 (control) and fertilization with 50 kg N/ha combined with the incorporation of 5 t/ha of straw into the soil. Sowing was performed in early June 2021 using a seed drill at an inter-row spacing of 12 to 15 cm. Total nitrogen was determined using the Kjeldahl method. Ground cover was assessed based on the percentage of plot surface covered by plants. Dry matter yield was calculated based on green mass yield and its dry matter percentage. In the samples, dry

matter content was determined by drying to 13–15% moisture. Total nitrogen was determined by the Kjeldahl method, and crude protein content was calculated using a conversion factor of 6.25. Crude protein yield was obtained based on dry matter yield and crude protein content. The main systematic groups of microorganisms were analyzed, including the total number of microorganisms, fungi, and actinomycetes, using appropriate culture media. Isolated fungi were identified to the genus level. The results were statistically processed using analysis of variance (ANOVA), and differences between means were tested using Tukey's test. Soil chemical properties were analyzed at the beginning and at the end of the experiment.

Table 1. Chemical properties of the soil at the Butmir experimental field

Soil layer	Depth (cm)	pH value		Humus (%)	P ₂ O ₅ /100g tla	K ₂ O/100 g tla
		H ₂ O	KCl			
Layer I	0-30	5.98	4.91	2.24	17.28	22.59
Layer II	30-60	6.08	4.97	2.20	15.26	19.62

Soil pH was determined electrometrically, humus content colorimetrically, and available phosphorus and potassium using the AL method. Chemical analysis showed that the soil of the Butmir experimental field (Table 1) had an acidic reaction, with pH values ranging from 4.91 to 4.97. The humus content amounted to 2.24% in the surface layer and 2.20% in the deeper layer, indicating a low to moderately humic soil. Available phosphorus was present at levels of 17.28 and 15.26 mg/100 g of soil, suggesting low to moderate availability. Potassium was present at levels of 22.59 and 19.62 mg/100 g of soil, corresponding to good potassium availability.

RESULTS AND DISCUSSION

Weather conditions during the study. Weather conditions during the study year were unfavorable. They were characterized by high air temperatures and a lack of precipitation during the vegetation period.

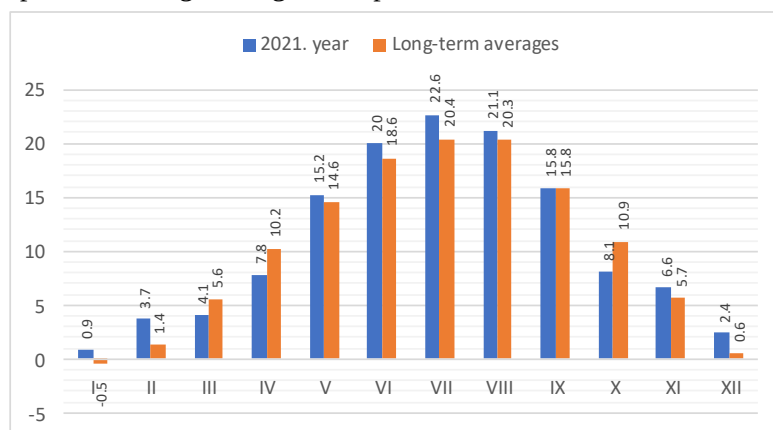


Figure 1. Mean monthly air temperatures for 2021 and long-term averages for the Butmir area

In 2021, the average annual air temperature (Figure 1) was 10.7 °C, which is 0.4 °C higher than the long-term average (10.3 °C). The lowest mean monthly temperature was recorded in January (0.9 °C), while the highest was recorded in July (22.6 °C). The total annual precipitation amounted to 501.8 mm, which is 309.4 mm lower than the long-term average, clearly indicating a pronounced moisture deficit (Figure 2). In June, only 11.7 mm of precipitation was recorded, which is 60.9 mm below the average, while in July precipitation increased to 25.8 mm but remained significantly below typical values.

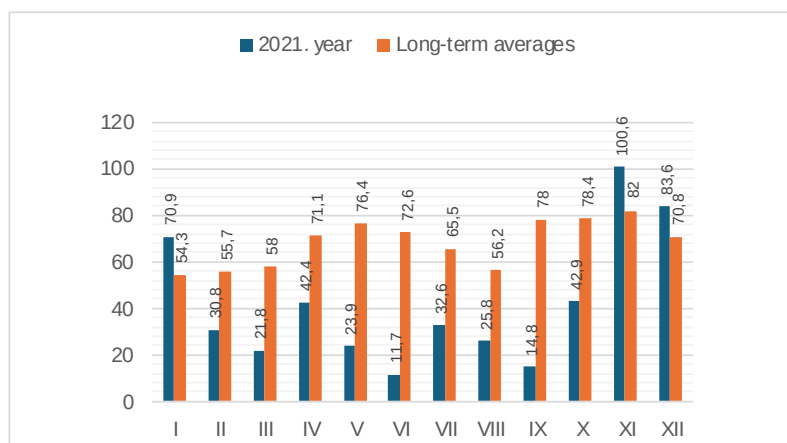


Figure 2. Average precipitation amounts (mm) for the Butmir locality

Effect of weather conditions on lucerne emergence and ground cover

The results of the study showed that unfavorable weather conditions in the year of sowing, particularly moisture deficit and elevated temperatures, had a pronounced effect on the germination, emergence, and early development of alfalfa. These findings are consistent with the reports of Hayat *et al.*, (2010), who emphasize that optimal soil moisture and moderate temperatures are key factors for successful emergence and stand establishment of alfalfa, whereas drought and high temperatures significantly reduce ground cover and biomass accumulation. During the research year, a pronounced lack of precipitation was recorded in the post-sowing period, accompanied by unfavorable temperature conditions. Such agroecological conditions negatively affected germination energy, uniformity of emergence, and final crop ground cover in both tested cultivars. For the cultivar Banat VS, the highest ground cover (Table 2) was recorded in the control treatment (36.2%), while treatments with the application of 50 kg N/ha and straw incorporation achieved 33.7% and 29.2%, respectively. Analysis of variance (ANOVA) showed that differences among treatments were not statistically significant ($p=0.673$; $p>0.05$). The obtained results indicate that neither nitrogen fertilization nor straw incorporation had a statistically significant effect on the ground cover of the Banat VS cultivar. The observed differences were numerical in nature and can be attributed to variability caused by unfavorable weather

factors. For the Soča cultivar, the highest ground cover was recorded in the nitrogen fertilization treatment (53.7%), followed by the straw incorporation treatment (51.2%), while the lowest value was observed in the control treatment (45.0%). Analysis of variance showed that, for this cultivar as well, differences among treatments were not statistically significant ($p=0.714$; $p>0.05$).

Table 2. Alfalfa plant cover (%) depending on treatment and variety

Variants	Cultivars	
	Banat VS	Soča
Control	36.2 ^b	45.0 ^a
Straw (5,0 t/ha)	29.2 ^b	51.2 ^a
50 kg N/ha	33.7 ^b	53.7 ^a
Average	33,0 ^b	49,9 ^a

^{ab}Values followed by different letters differ significantly ($p<0.05$). Letters within columns indicate comparisons among treatments within the same cultivar, while letters next to the mean indicate significant differences between cultivars

He obtained results indicate that, for both the Banat VS and Soča cultivars, the application of nitrogen fertilizer and straw incorporation did not have a statistically significant effect on ground cover. However, in the Soča cultivar, a slight increasing trend in ground cover was observed in treatments with nitrogen application and straw incorporation. When compared by cultivars, the average ground cover of the Soča cultivar was 49.9%, which was 16.9% higher than that of the Banat VS cultivar. Comparison of cultivars within individual treatments revealed statistically significant differences in all variants (control $p=0.035$; straw $p=0.041$; nitrogen fertilizer $p=0.016$; $p<0.05$), with the Soča cultivar achieving significantly higher ground cover than the Banat VS cultivar. These results indicate pronounced genotypic differences in the ability of early growth and stand establishment. The obtained data are consistent with the findings of Miletić and Radulović (2005), who emphasize that genotypic differences among alfalfa cultivars may result in faster and more uniform early plant growth under the same agroecological conditions, which is directly reflected in the degree of crop ground cover.

Dry matter yield and crude protein content

Results of the study showed that dry matter yield and crude protein yield were numerically higher in treatments with mineral fertilization (50 kg N/ha) and straw incorporation (5 t/ha); however, within cultivars, these differences were not statistically significant (Table 3). Analysis of variance for dry matter yield revealed no statistically significant differences among treatments ($p=0.913$; $p>0.05$), indicating that the applied treatments did not have a significant effect on this parameter in the research year. Additionally, no statistically significant difference was found between the Banat VS and Soča cultivars in average dry matter yield ($p=0.459$; $p>0.05$). At the cultivar level, the average dry matter yield of the Soča cultivar was 0.50 t/ha, which was approximately 0.01 t/ha higher than that of the Banat VS cultivar. Although this difference suggests a slight genotypic

advantage of the Soča cultivar, it was not statistically confirmed. A similar pattern was observed for crude protein yield. No statistically significant differences were detected among treatments ($p=0.890$; $p>0.05$), and no significant differences were found between the average values of the Banat VS and Soča cultivars ($p=0.765$; $p>0.05$).

Table 3. Dry matter yield (t/ha) and crude protein yield (kg/ha)

Variants	Dry matter yield		Crude protein yield	
	Banat VS	Soča	Banat VS	Soča
Control	0.47 ^{ns}	0.49 ^{ns}	95.5 ^{ns}	96.1 ^{ns}
Straw 5,0 t/ha	0.50 ^{ns}	0.51 ^{ns}	97.2 ^{ns}	97.3 ^{ns}
50 kg N/ha	0.51 ^{ns}	0.52 ^{ns}	97.8 ^{ns}	98.8 ^{ns}
Average	0.49 ^{ns}	0.50 ^{ns}	96.8 ^{ns}	97.4 ^{ns}

Values marked with “ns” indicate no significant differences ($p > 0.05$). Letters within columns compare treatments within the same cultivar, while letters next to the mean compare treatments between cultivars

The average crude protein yield of the Soča cultivar was slightly higher than that of the Banat VS cultivar; however, the difference was minimal and not statistically significant. The obtained results are partially consistent with the findings of Aon and Colaneri (2001), who reported that nitrogen addition or the incorporation of organic matter may increase alfalfa yield and quality, but that under stress conditions, such as moisture deficit and high temperatures, the effects of fertilization may be limited. In the research year, unfavorable climatic conditions represented the dominant limiting factor, reducing the potential effects of the applied treatments. Furthermore, although the Soča cultivar showed a tendency toward slightly higher dry matter and crude protein yields, statistical analysis did not confirm a significant genotypic advantage under the given conditions. This indicates that climatic stress had a stronger influence on yield and quality than genetic differences between cultivars, which is consistent with the findings of Stamenov (2013) regarding the variable efficiency of cultivars in protein accumulation depending on environmental conditions.

Soil microbial community under Lucerne

The results of the study indicate that different treatments (control, fertilization with 50 kg N/ha, and straw incorporation at 5 t/ha) and lucerne cultivars (Banat VS and Soča) significantly influenced the soil microbial community, including bacteria, ammonifiers, fungi, and actinomycetes. Bacteria were the most abundant group of microorganisms, which is consistent with general knowledge that their abundance reflects overall soil biological activity and ecosystem quality (Miletić and Radulović, 2005; Hayat *et al.*, 2010). In our study (Table 4), the highest number of bacteria was recorded in the treatment with mineral nitrogen, while the lowest was observed in the straw incorporation treatment. These results confirm the findings of Li *et al.*, (2020), who demonstrated that the addition of mineral nitrogen can stimulate bacterial

populations in the lucerne rhizosphere, potentially enhancing biological processes that support plant growth. The number of ammonifiers was highest in the control treatment, whereas fertilization with 50 kg N/ha resulted in a reduction in their abundance. This trend can be explained by the fact that the availability of mineral nitrogen reduces the soil's need for the decomposition of organic nitrogen, which is consistent with the basic principles of the soil nitrogen cycle (Stamenov, 2013; Aon and Colaneri, 2001). Fungi, on the other hand, showed the highest abundance in the straw incorporation treatment and the lowest in the mineral nitrogen treatment. This confirms their role in the decomposition of complex organic matter and soil humification, in accordance with previous studies by Yudiarti *et al.*, (2010).

Table 4. Total number of bacteria and ammonifiers, fungi and actinomycetes

Cultivar	Variants	Total number of bacteria CFU x 10 ⁴ /g	Total number of ammonifiers CFU x 10 ³ /g	Total number of fungi CFU x 10 ² /g	Total number of actinomycetes CFU x 10 ² /g
Banat VS	Straw 5,0 t/ha	33.7 x 10 ⁴	25.1 x 10 ³	12.5 x 10 ²	10.2 x 10 ²
	50 kg N/ha	59.5 x 10 ⁴	22.5 x 10 ³	7.1 x 10 ²	18.4 x 10 ²
	Control	45.3 x 10 ⁴	34.5 x 10 ³	9.3 x 10 ²	24.8 x 10 ²
Soča	Straw 5,0 t/ha	39.4 x 10 ⁴	21.2 x 10 ³	11.2 x 10 ²	8.5 x 10 ²
	50 kg N/ha	47.5 x 10 ⁴	13.5 x 10 ³	6.7 x 10 ²	15.5 x 10 ²
	Control	40.8 x 10 ⁴	25.5 x 10 ³	8.2 x 10 ²	22.3 x 10 ²

CFU/g stands for colony-forming units per gram of sample.

Organic amendments such as straw stimulate the development of fungal populations, thereby contributing to long-term soil fertility. Actinomycetes exhibited the highest abundance in the control treatment, slightly lower abundance in the mineral nitrogen treatment, and the lowest abundance in the straw incorporation treatment. This indicates their sensitivity to changes in the availability of organic and mineral substrates and highlights their role in the decomposition of complex organic compounds and soil humification (Miletić and Radulović 2005). Overall, the results confirm that agronomic practices, such as cultivar selection and fertilization strategy, significantly influence the soil microbial community. The Banat VS and Soča cultivars exhibited similar trends, although Banat VS generally showed slightly higher numbers of bacteria and actinomycetes, while Soča had higher fungal abundance in certain treatments. These findings are consistent with the literature emphasizing the interaction between cultivar and soil microbial activity (Jarak *et al.*, 2005). The application of mineral nitrogen stimulates bacterial populations, whereas organic amendments (straw) increase fungal abundance, indicating the potential for targeted manipulation of the soil microbial community to improve soil fertility. These results contribute to the understanding of the long-term effects of different

treatments on soil and support the need for an integrated approach to soil management in lucerne production.

CONCLUSIONS

The results of the study on the effects of cultivar and fertilization on yield and soil microbial diversity under lucerne showed the following. Late sowing and unfavorable weather conditions, particularly moisture deficit and high temperatures, negatively affected plant growth, ground cover, and the yields of dry matter and crude protein in both the Banat VS and Soča cultivars, and also contributed to a reduction in microbial activity. Cultivars differed significantly in ground cover, with Soča achieving statistically significantly higher values compared to the cultivar Banat VS. Mineral fertilization increased dry matter yield, while straw incorporation partially enhanced soil microbial activity, particularly fungal abundance. Overall microbial activity was higher in the Banat VS cultivar, indicating different interactions between cultivars and the soil microbial community. Since lucerne sowing was carried out later than the optimal period, this factor had a decisive negative impact on all examined parameters. Delayed sowing, in combination with unfavorable weather conditions, limited plant development and reduced the effectiveness of applied agronomic measures. This highlights the importance of timely sowing as a fundamental prerequisite for achieving stable yields and maintaining soil microbial activity. Further research should focus on optimizing sowing dates and combinations of organic and mineral fertilizers in order to improve productivity and biological soil activity.

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CHANGES IN FATTY ACID COMPOSITION DURING THE PRODUCTION OF TRADITIONAL BEEF PROSCIUTTO RELATED TO TECHNOLOGICAL PROCESSING

SUMMARY

This study investigates changes in the fatty acid composition of beef prosciutto during traditional production, with particular attention to seasonal variations in raw material quality. Three production batches were prepared under traditional conditions in a certified slaughterhouse facility, differing according to slaughter season (spring, summer, and winter). Samples were collected at key technological stages—before salting, after 21 days of dry salting, and after thermal processing—and analyzed to monitor changes in fatty acid composition.

Seasonal factors significantly influence nutritional characteristics, as cattle finished in spring and summer show higher levels of polyunsaturated and omega-3 fatty acids due to improved feeding conditions. Saturated fatty acids (SFA) reached their highest proportion after brining (50.276%) and lowest after thermal treatment (46.326%). Monounsaturated fatty acids (MUFA) were highest immediately after thermal processing (46.523%) and lowest after brining (42.477%). Polyunsaturated fatty acids (PUFA) were most abundant before brining (5.408%), decreasing during processing and reaching their lowest values after thermal treatment. Trans fatty acids were highest before brining (3.908%) and lowest after thermal processing (2.947%).

Observed variations result from chemical transformations occurring during processing, affecting the physical and chemical properties of fats and consequently the product's nutritional value and quality. Understanding seasonal and technological influences is essential for optimizing production parameters to preserve beneficial fatty acids, maintain desirable sensory characteristics, and ensure product safety and consistency. The study contributes to a better

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understanding of mechanisms governing quality changes in traditional meat products and supports improvements in processing practices.

Keywords: Fatty acid composition, lipid oxidation, beef prosciutto, quality, traditional processing

INTRODUCTION

The production of beef prosciutto is traditionally associated with Mediterranean countries such as Italy, Spain, France, Portugal, and Croatia, where numerous types of prosciutto with distinctive quality and protected geographical origin have been developed (Krvavica and Đugum, 2006). The characteristics of these products depend on several factors, including animal genetics and rearing conditions, feeding regime, climatic conditions, raw material quality, and applied processing technology. Standardization of production processes contributes to the preservation of traditional manufacturing methods and ensures high product quality. In the Western Balkan region, particularly in Bosnia and Herzegovina and Kosovo, beef prosciutto represents an important traditional dry-cured meat product. Although beef contains less intramuscular and extramuscular fat compared to other types of meat, properly applied processing technology can produce a product with appropriate texture, flavor, and nutritional characteristics (Alfaia *et al.*, 2010a).

Beef prosciutto production involves several technological stages: raw material preparation and cutting, salting or brining, drying, smoking, and aging (Robović *et al.*, 2003; Kurćubić and Vesković-Moračanin, 2017; Ganić *et al.*, 2019). For traditional beef prosciutto produced in Kosovo, this product may include thermal processing as part of the technological procedure, making it regionally unique.

During these processes, significant physicochemical and biochemical changes occur, directly influencing product quality and stability, particularly the lipid fraction and fatty acid composition. Technological and seasonal factors cause variations in the ratio of saturated (SFA) to unsaturated fatty acids (UFA), which are crucial for nutritional value, organoleptic properties (flavor, aroma, texture), and shelf life (Rodríguez *et al.*, 2019; Sampaio *et al.*, 2024; Lorenzo *et al.*, 2014; Garrido *et al.*, 2017; Lorenzo *et al.*, 2012).

However, despite extensive research on Mediterranean dry-cured meats, a key gap persists: the dynamics of fatty acid composition (SFA/UFA ratios and oxidation levels) during the specific stages of traditional beef prosciutto production in Kosovo remain underexplored. Existing studies focus primarily on pork prosciutto from Mediterranean regions or general beef processing, overlooking local rearing conditions, seasonal variations, and Kosovo's unique practices like optional smoking and thermal processing (Alfaia *et al.*, 2010a; Lorenzo *et al.*, 2014).

This study addresses this gap by examining how fatty acid profiles evolve across processing stages (brining → drying → smoking → aging) in Kosovo beef prosciutto. We hypothesize that these stages will drive significant shifts, with

increasing UFA levels and decreasing lipid oxidation in later phases, thereby enhancing nutritional quality and stability. These region-specific insights will optimize production, preserve Kosovo's gastronomic heritage, and boost market competitiveness.

MATERIAL AND METHODS

Three different production batches of traditional beef prosciutto were produced in a traditional manner at the private slaughterhouse "Xhoshkun" in Prizren, Kosovo in accordance with all requirements for the implementation and application of the HACCP and HALAL systems for production. The difference in the production batches is based on the slaughter season. In the first batch, the meat comes from spring slaughter of young cattle (May-June). The second production batch is made from meat of young cattle slaughtered in summer (June-August). The meat used for the production of beef prosciutto in the third batch is obtained from winter slaughter of young cattle (November-February).

Beef prosciutto production and sampling

Traditional Beef Prosciutto from Prizren: Production Process

The traditional beef prosciutto from Prizren stands out as one of the highest quality meat products due to the use of premium cuts from the back-specifically the loin or ribeye muscle (*Musculus longissimus dorsi*). This selection provides optimal texture, juiciness, and rich flavor.

Slaughtering and Initial Preparation

During slaughtering, carcasses from young cattle (typically 18-24 months old, with live weight of 400-500 kg) are halved along the midline, thoroughly exsanguinated to minimize blood residues, and immediately transferred to a cooling chamber. The chamber maintains a temperature of 0-4°C and relative humidity of 85-90% for 24-48 hours. This rapid cooling prevents microbial growth and facilitates further processing. Once the internal temperature drops below 4°C, the halves are quartered, and the target sections for prosciutto production are separated. Shaping involves removing excess fat (to 3-5 mm) and forming into a cylindrical shape for uniform roasting.

Brining - The meat is submerged in cold brine (20-25% salt, 4-6°C) for 21 days with daily agitation. This ensures preservation, flavor, and moisture retention. It is then desalinated in running water for 1-3 hours to reach 2-3% salinity.

Pre-Roasting Preparation - The pieces are tied with string and dried at room temperature for 24 hours to form a surface pellicle.

Thermal processing is conducted in a traditional wood-fired chamber using air-dried beech wood as fuel. The chamber's initial temperature ramps up to 140-160°C with relative humidity of 40-60%; the meat surface reaches 120-130°C, continuing until the internal temperature achieves 58-62°C for medium-rare doneness. The thermal process duration is 270-300 minutes (4.5-5 hours), with continuous rotation of the pieces every 30 minutes until a shiny dark brown crust is obtained.

Cooling and Stabilization - After roasting, they are rapidly cooled to 4°C and rested for 12 hours to set the structure and flavor.



Figure 1. Traditional beef prosciutto in different stages of technological processing -a -brining, -b-during thermal processing, -c- after thermal processing

During the process, exactly 20 samples per group ($n=20$, ~500g each) are taken at three key stages: before brining (7 samples), after 21 days of brining (7 samples for salt uptake), and after thermal processing (6 samples) to monitor fatty acid composition changes and ensure quality control, (Figure 1).

Determination of fatty acid composition

The method of Folch *et al.*, (1957) was used for the extraction of fatty acids from sausages. Obtained fatty acids, were transferred to methyl esters according AOAC Official Method 996.06 (2005) and subsequently subjected to GC-FID analysis. GC-FID analysis was performed on Hewlett Packard 5890 series II gas chromatograph with a flame ionization detector, an auto sampler, and a capillary column HP 88 (60m x 0.250mm x 0.20 μ m). The operation parameters of column were: initial temperature of 70°C (1 minute) ramped at 5°C/minute to 100°C and maintained for 2 minutes. The temperature was then increased at 10°C/minute to 175°C and maintained for 2 minutes, which was followed by last increase at 3°C/minute to 220°C and hold for 5 minutes. Injector and detector temperatures were kept at 250°C and 300°C, respectively. Helium was used as a carrier gas at a flow rate of 1.4 mL/min with split ratio 100:1. 1 μ L volume of each sample was injected two times into GC-FID for separation and identification of the FAMES. Total run was 38.50 minutes. FAMES from samples were identified by comparison of their retention times to those of FAMES mix standard (Supelco 37 component FAME mix, Sigma-Aldrich, Germany). Using conversion factors (AOAC 996.06, 2005) FAMES were converted to corresponding fatty acids. Fatty acids were quantified as percentage of total fatty acids in sample.

Statistical analysis

Prior to statistical analysis, the assumptions of normality and homogeneity of variance were evaluated using SPSS Statistics v.29.0. One-way ANOVA was performed with a confidence interval of 95% ($p<0.05$). Differences between group means were further compared using Student's t-test to determine whether there were statistically significant differences in the total fatty acid profile between the groups.

RESULTS AND DISCUSSION

Beef prosciutto is considered one of the highest quality cured meat products made from beef. The fatty acid composition varies significantly not only between different animal species but also within the same species. These variations depend on the specific cut of beef used for production and the season during which the cattle are slaughtered. The results obtained from our analysis of the fatty acid composition of beef prosciutto before brining is presented in (Table 1) and (Figure 2).

Table 1. Fatty acid composition of beef prosciutto before brining (%)

Fatty acid (%)	Batches		
	I	II	III
C10:0	0,073	0,171	0,071
C11:0	-	0,214	-
C12:0	0,094	0,230	0,104
C14:0	3,013	3,372	3,754
C14:1	0,982	0,874	0,883
C15:0	0,357	0,503	0,770
C15:1	0,095	0,221	0,291
C16:0	25,811	25,461	25,713
C16:1	3,442	3,286	3,272
C17:0	1,390	1,354	1,631
C17:1	0,469	0,603	0,890
C18:0	15,788	15,260	15,542
C18:1n9t	3,637	3,831	1,905
C18:1n9c	36,879	36,495	39,221
C18:1n6t	0,818	1,555	0,978
C18:2n6c	6,331	6,620	3,723
C18:3n6	0,075	0,191	0,092
C20:1	0,151	0,243	0,342
C18:3n3	0,541	0,514	0,733
C22:1n9	-	-	0,080
Total fatty acid profile (%)			
Saturated fatty acids (SFA)	46,532 ^a	46,566 ^a	47,586 ^b
Monounsaturated fatty acids (MUFA)	43,872 ^a	43,820 ^a	43,850 ^a
Polyunsaturated fatty acids (PUFA)	5,139 ^a	5,227 ^b	5,679 ^c
Trans fatty acids (trans FA)	4,455 ^a	4,386 ^b	2,884 ^c

^{a,b,c} statistical differences $p \leq 0.05$ between batches

As shown in Table 1, saturated fatty acids (SFA) represent the dominant fraction in the fat tissue of beef prosciutto before brining. Their relatively stable distribution across the examined batches indicates a consistent lipid composition of the raw material, with only minor variations that may be attributed to natural differences in animal origin or feeding conditions. This aligns with the findings of Wood *et al.*, (2008), who pointed out that saturated fatty acids have a stable presence in cattle fat tissue and significantly influence the texture and organoleptic properties of meat.

Similar data on the dominance of SFA as the main component in the fat tissue is reported by Dinh *et al.*, (2021) and Enser *et al.*, (1996), who emphasize that their proportion varies according to feeding and rearing conditions.

Monounsaturated fatty acids (MUFA) represent the second most abundant group and show relatively consistent distribution across batches, despite statistically significant differences ($p < 0.05$). Their presence plays an important role in shaping both the sensory properties and the nutritional profile of the product. These findings are supported by Bošković *et al.*, (2015), who note that MUFAs, especially oleic acid, play a significant role in enhancing the organoleptic characteristics and beneficial nutritional aspects of meat products. Additionally, Fowler *et al.*, (2023) emphasize that the stability of MUFA in beef products is a key indicator of product quality.

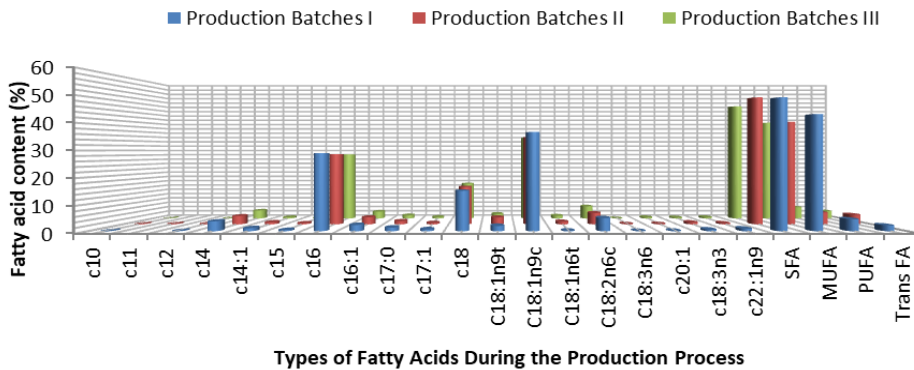


Figure 2. Dynamics of the fatty acid composition in beef prosciutto before brining

Polyunsaturated fatty acids (PUFA) show statistically significant variability ($p < 0.01$), with a tendency to increase in certain batches. This variation may be associated with differences in feeding regimes or seasonal factors influencing the fatty acid composition of the raw material. This variation corresponds with observations by Scollan *et al.*, (2006, 2014) and Dannenberger *et al.*, (2011), which highlight that PUFAs are most influenced by diet and the season of the year. They point out that winter feeding, often consisting of concentrated feed, increases PUFA levels in meat compared to spring and summer periods.

Trans fatty acids, present in the lowest proportions, show statistically significant variability between batches ($p < 0.01$). This variation likely reflects the influence of raw material characteristics and early processing conditions on lipid composition. These changes are consistent with studies by Lorenzo and Franco (2012), Estévez (2011), Estévez (2015), and Wood *et al.*, (2008), which indicate that trans fatty acids are sensitive to biological and seasonal factors, as well as processing and metabolic transformations in the meat. The differences can be linked to rearing conditions, nutrition, and physiological changes occurring in animals across different seasons.

Overall, the results indicate that the fatty acid composition of beef prosciutto is influenced by a complex interaction of biological factors, seasonal

conditions, diet, and processing parameters. This variability reflects both raw material characteristics and technological conditions during production. This is in line with a wide range of scientific literature emphasizing that these factors collectively shape the final fatty acid profile, which is critical for the quality, nutritional value, and organoleptic characteristics of beef prosciutto (Enser *et al.*, 1996; Bošković *et al.*, 2015; Dannenberger *et al.*, 2011; Scollan *et al.*, 2006; Wood *et al.*, 2008; Lorenzo and Franco, 2012; Estévez, 2011).

During the brining process of beef prosciutto, significant changes occur in the fatty acid composition. These changes are driven by lipolytic enzyme activity naturally present in the meat, as well as by structural modifications of lipids induced by brine components, particularly salt and nitrites. As a result, the fatty acid profile is altered during processing.

From a technological perspective, these transformations highlight the importance of controlling brining conditions, such as salt concentration, duration, and temperature. Proper optimization of these parameters can help maintain desirable fatty acid profiles, improve product quality, and enhance the nutritional value of the final product.

The fatty acid content in the fats of the beef prosciutto after brining is shown in (Table 2 and Figure 3).

Table 2. Fatty acid composition of beef prosciutto after brining (%)

Fatty acid (%)	Batches		
	I	II	III
C10:0	0,092	0,172	0,065
C11:0	-	0,211	-
C12:0	0,146	0,234	0,102
C14:0	3,686	3,371	3,504
C14:1	1,139	0,872	0,551
C15:0	0,446	0,502	0,363
C15:1	0,135	0,223	0,194
C16:0	28,969	28,465	28,235
C16:1	2,348	2,891	3,014
C17:0	1,367	1,353	1,393
C17:1	0,687	0,604	0,654
C18:0	15,051	15,261	15,834
C18:1n9t	2,004	2,837	2,050
C18:1n9c	36,881	35,486	35,674
C18:2n6t	0,192	1,102	1,247
C18:2n6c	4,950	4,622	5,390
C18:3n6	0,101	0,193	0,080
C18:3n3	0,623	0,842	0,480
C20:1	0,193	0,244	0,554
C22:1n9	0,984	0,513	0,612
Total fatty acid profile (%)			
Saturated fatty acids (SFA)	49,760 ^a	51,569 ^b	49,498 ^c
Monounsaturated fatty acids (MUFA)	43,540 ^a	41,656 ^b	42,236 ^c
Polyunsaturated fatty acids (PUFA)	4,503 ^a	4,834 ^b	4,967 ^c
Trans fatty acids (trans FA)	2,195 ^a	3,939 ^b	3,298 ^c

^{a,b,c} statistical differences $p \leq 0.05$ between batches

As shown in Table 2 and Figure 3, the fatty acid profile of beef prosciutto after brining differs significantly among the three production batches. Saturated fatty acids (SFA) represent the dominant fraction, with statistically significant differences ($p < 0.05$), indicating that processing conditions and/or seasonal variations in the raw material affect lipid stability and composition. Monounsaturated fatty acids (MUFA) also show significant differences between batches ($p < 0.05$). Polyunsaturated fatty acids (PUFA), although present in lower amounts, show a slight increasing trend across batches, which may be associated with improved nutritional quality under certain production conditions. Trans fatty acids show significant differences ($p < 0.01$), highlighting the influence of technological processes such as brining and drying on lipid transformations. Their variability suggests that processing parameters may affect the formation of these less desirable compounds.

From a technological perspective, these results indicate that controlling key processing factors: such as brining duration, temperature, and drying conditions - can significantly influence lipid composition. Optimization of these parameters may help reduce the formation of trans fatty acids while preserving or enhancing beneficial unsaturated fatty acids, thereby improving the nutritional value and overall quality of the final product.

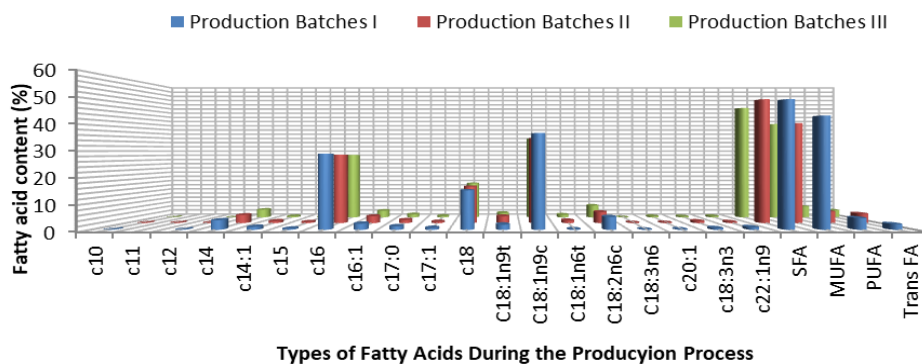


Figure 3. Dynamics of the fatty acid composition in beef prosciutto after brining

The differences in the percentages of MUFA, PUFA, and Trans FA acids between the batches can be linked to different biological and nutritional conditions under which the livestock was raised, as well as seasonal variations in their diet (Enser *et al.*, 1996; Bošković *et al.*, 2015; Dinh *et al.*, 2021; S. M. Fowler *et al.*, 2023).

Changes in fatty acid composition during brining result from the interaction of brine components, lipolytic enzyme activity, and redox processes (Álvarez Sara *et al.*, 2023; Derek and Vale, 1998; Beermann *et al.*, 2003; Kazala *et al.*, 2006). Additionally, microbiological and enzymatic reactions in the salty and acidic environment further influence fatty acid stability and transformation, ultimately affecting the quality, flavor, and nutritional value of beef prosciutto.

During the thermal processing of beef prosciutto, significant changes in fatty acid composition occur due to exposure to high temperatures, leading to lipid denaturation, oxidation, and polymerization. These transformations alter the fatty acid profile and directly affect product quality and stability. From a technological perspective, controlling processing temperature and duration is crucial to limit oxidative degradation and preserve desirable fatty acids. Optimization of thermal conditions can improve lipid stability, enhance sensory properties, and maintain the nutritional value of the final product, (Table 3) and (Figure 4).

Table 3. Fatty acid composition of beef prosciutto after thermal processing (%)

Fatty acid (%)	Batches		
	I	II	III
C10:0	0,072	0,062	0,060
C12:0	0,091	0,088	0,101
C14:0	2,711	2,414	2,500
C14:1	0,432	0,793	0,551
C15:0	0,313	0,386	0,365
C15:1	0,214	0,190	0,194
C16:0	24,151	23,870	23,235
C16:1	2,292	2,865	3,012
C17:0	1,191	1,144	1,395
C17:1	0,584	0,494	0,654
C18:0	18,456	17,565	18,835
C18:1n9t	1,553	2,512	2,324
C18:1n9c	42,962	42,652	39,671
C18:2n9t	1,207	0,264	0,982
C18:2n6c	3,462	3,724	4,390
C18:3n6	0,108	0,094	0,082
C20:1	0,306	0,496	0,552
C18:3n3	0,893	0,750	0,484
C22:1n9	-	0,181	0,6117
Total fatty acid profile (%)			
Saturated fatty acids (SFA)	47,985	45,531	46,491
Monounsaturated fatty acids (MUFA)	44,425	46,853	45,239
Polyunsaturated fatty acids (PUFA)	3,828	4,838	4,962
Trans fatty acids (trans FA)	2,760	2,775	3,306

^{a,b,c} statistical differences $p \leq 0.05$ between batches

In the fats of beef prosciutto after thermal processing, 19 fatty acids were identified. The most abundant fatty acid in this group is palmitic acid (C16:0), with an average content of 23.752%, followed by stearic acid (C18:0) at 18.285%, while others such as myristic acid (C14:0) and heptadecanoic acid (C17:0) occur in smaller amounts. Statistically significant differences ($p < 0.05$) were observed between the first and third batches regarding MUFA content.

Polyunsaturated fatty acids (PUFA), which are the third most abundant group, have an average content of 4.542%, with variations ranging from 3.828% to 4.838% among the batches.

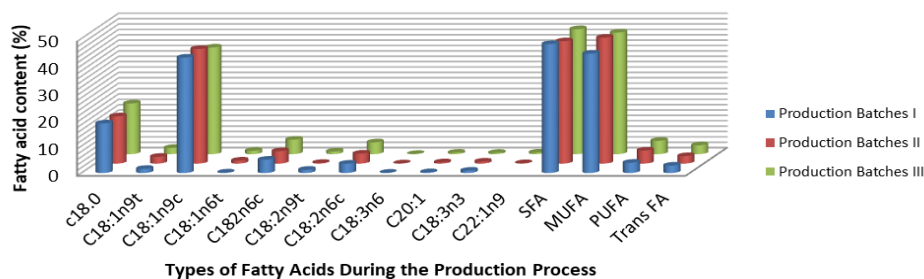


Figure 4. Dynamics of the fatty acid composition in beef prosciutto after thermal processing (%)

These results indicate relative stability in the levels of SFA and MUFA after thermal processing, while variations in PUFA and Trans fatty acids may be attributed to influences related to thermal treatment and the origin of the raw material. Thermal processing causes denaturation, oxidation, and polymerization of fats, which affect their structure and fatty acid profile. Saturated fatty acids (SFA) exhibit greater heat stability, while polyunsaturated fatty acids (PUFA) are more sensitive and prone to oxidative changes, which can influence the flavor and nutritional properties of the product (Alfaia *et al.*, 2010b; Echegaray *et al.*, 2018; Min, 2005).

Additionally, seasonal variations in the diet and rearing conditions of animals significantly affect the fatty acid composition. Winter conditions, when concentrated feeds are used, generally lead to a decrease in PUFA and an increase in Trans fatty acids, whereas in spring and summer periods, when animals graze on fresh grass rich in polyunsaturated fatty acids, higher concentrations of these fatty acids are observed (Scollan *et al.*, 2006; Dannenberger *et al.*, 2011; Wood *et al.*, 2008).

These biological and technical factors together shape the nutritional profile and organoleptic qualities of beef prosciutto, making the control of the production process and understanding of seasonal variations crucial for maintaining the quality and health value of the final product (Zarrdeto *et al.*, 2017; Blanco *et al.*, 2015; Králová, 2015).

CONCLUSIONS

The results demonstrate that both technological processes and seasonal variations significantly influence the fatty acid composition of beef prosciutto. Processing stages, particularly brining and thermal treatment, play a key role in modifying lipid profiles through biochemical and oxidative changes. From a technological perspective, controlling parameters such as salting duration, temperature, and heat treatment intensity is essential to preserve beneficial fatty acids, limit undesirable transformations, and ensure product quality and stability.

Seasonal variation in raw material quality has a notable impact, with spring and summer production associated with more favorable fatty acid profiles, particularly higher levels of polyunsaturated fatty acids due to improved feeding conditions.

These findings have practical relevance for traditional beef prosciutto production, highlighting the importance of adapting processing conditions to both raw material characteristics and seasonal factors in order to achieve consistent quality, improved nutritional value, and desirable sensory properties.

This is particularly important for Kosovo, as it supports the improvement of traditional meat production, enhances product quality, and increases competitiveness in the market

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DOI: 10.17707/AgricForest.72.1.25

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COMPARATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY IN SOME FRUIT SPECIES

SUMMARY

The aim of this work is determination of antioxidant activity of fruits from several species: apple, cherry, pomegranate, strawberry, black currant, white mulberry, chokeberry, blackberry, depending on the content of vitamin C, total anthocyanins and phenols. The highest antioxidant activity, about 86%, is shown by the pomegranate and black currant fruits. Lower values compared to the average antioxidant activity are shown by apple, cherry and strawberry fruits. The highest content of vitamin C (216.0 mg/100g) and the relative contribution of vitamin C on calculated antioxidant activity (250.6) is found in black currant. The highest content of anthocyanins are found in chokeberry (610 mg/100g) and black currant (580.0 mg/100g). Apple fruits had the lowest content of vitamin C (9.4 mg/100g) and anthocyanins (0.7 mg/100g). The highest content of total phenols is found in chokeberry (2390.0 mg/100g) and black currant (1860.0 mg/100g) and the lowest in white mulberry (10.0 mg/100g). A large contribution of vitamin C in the antioxidant activity is found in black currant, strawberry and white mulberry. Anthocyanins contribute most to the antioxidant activity of chokeberry, blackcurrant, blackberry and cherry fruits, while total phenols in chokeberry, blackcurrant, blackberry, pomegranate and apple fruits. In terms of the parameters examined, statistically significant differences were determined between the examined species. A weak positive correlation was determined for total antioxidant activity with vitamin C (0.399) and moderate positive correlation with total anthocyanins (0.534) and phenols (0.556), which indicates that in the examined species, antioxidant activity depends more on the content of total anthocyanins and phenols.

Keywords: fruit species, vitamin C, anthocyanins, total phenols, antioxidant activity

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INTRODUCTION

Fruits are referred to as “functional foods” and have an important place in the human diet. Fresh fruits are irreplaceable, health-safe food rich in bioactive compounds such as vitamins, carotenoids, flavonoids, tannins, proteins, carbohydrates, vitamins, minerals, plant fibers and other phenolic constituents that have a structural, energetic and protective function in the human body. These compounds play a synergistic and cumulative role in promoting human health, they have potent antioxidant activity and exhibit several health-promoting properties, have anti-inflammatory, anti-hyperglycemic, hypo-cholesterolemic activities (Tulipani *et al.*, 2009; Vauzour *et al.*, 2010; Giampieri *et al.*, 2012; Miller *et al.*, 2019). The diversity of secondary metabolites, including flavonoids, carotenoids and terpenoids, contributes to the nutritional richness of fruits and their potential health-promoting effects (Manach *et al.*, 2004; Khoo *et al.*, 2017). Antioxidants, encompassing a diverse array of compounds such as vitamins C and E, polyphenols and carotenoids, have been widely acknowledged for their ability to counteract oxidative stress, a process implicated in the pathogenesis of chronic diseases including cardiovascular disorders and certain cancers (Halliwell and Gutteridge, 2015).

Polyphenols are among the most abundant groups of substances present in fruits, with about 8000 phenolic structures. Some of them contribute to the formation of the taste and color of fruits and vegetables, others play a protective role against predators and pests, still others perform specific physiological functions during plant development and for the most part their functions have not yet been clarified. Phenols are divided into two main groups, flavonoids and non-flavonoids. Flavonoids are the most abundant group of phyto-chemicals, they include flavones (luteolin and apigenin), flavonols (quercetin, kaempferol, and myricetin), flavonones (narigenin), isoflavonoids (daidzein and genistein), anthocyanidins (malvidin and cyanidin) and flavanols (epicatechin, catechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate) (Danyo and Ivantsova, 2025). Anthocyanins are water-soluble pigments and are stored in plant cell vacuoles. Most vascular plant species contain these compounds in various quantities in most of the organs, but mainly fruits, flowers, and leaves (Horbowicz *et al.*, 2008; Viskelis *et al.*, 2010).

Phenolic compounds are a common ingredient in various nutraceutical supplements due to their positive effects on human body. The presence of flavonoids reduces the degree of oxidation of LDL cholesterol and removes free radicals. Flavonoids have been shown to inhibit lipid peroxidation within the phospholipid bilayer by localization within the polar and nonpolar phases (Movileanu *et al.*, 2000). Anthocyanins have various biological functions, provide protection from UV radiation, have antioxidant activity, and protect against various pathogens. They neutralize enzymes that destroy connective tissue, their antioxidant capacity prevents oxidants from damaging connective tissue and they repair damaged proteins in the blood-vessel walls (Horbowicz *et al.*, 2008). Proanthocyanidins have antihistamine, antiviral, anti-inflammatory

and anti-caries effects. They protect the body from cardiovascular diseases, have anticancer effects and protect against UV radiation (Fartzov *et al.*, 1994). Ascorbic acid plays an important role in many metabolic functions, it is an antioxidant that protects the body from the harmful effects of free radicals and is used as a therapeutic agent in many diseases and disorders, protects the immune system, reduces the intensity of allergic reactions and helps fight infections. The recommended daily amount of vitamin C can be achieved by consuming a handful of strawberries (Carr and Frei, 1999). According to Bailey and Gregory (1999) 200-250 grams of strawberries may provide sixty-two percent of the daily folic acid. Thanks to their high-water content and low-calorie content, fruits are recommended in weight loss diets and to combat obesity. The fiber and fructose content can help regulate blood sugar levels (Samia *et al.*, 2019).

The antioxidant power of fruit is closely correlated to the presence of efficient oxygen radical scavengers, such as vitamin C and phenolic compounds. Through comparative analyses of the antioxidant activity of fruits from several fruit species, recommendations can be made for individual species in the prevention or treatment of certain diseases.

MATERIAL AND METHODS

As a result of many years of work in examining the polyphenolic composition of fruits from some fruit species, in this paper are provided comparative values for the antioxidant activity of fruits from different fruit species: pomegranate (*Punica granatum* L.), apple (*Malus domestica* (Suckow) Borkh), cherry (*Prunus avium* L.) and some berry fruits as strawberry (*Fragaria x anannasa* Duch.), black currant (*Ribes nigrum* L.), white mulberry (*Morus alba* L.), chokeberry (*Aronia melanocarpa* (Michx.) Elliott) and blackberry (*Rubus fruticosus* L.). 30 fruits were taken in three replicates from each fruit species. The fruits were grown under natural conditions and harvested at botanical maturity. They were stored in refrigerators under appropriate conditions, for a short time until the samples are analyzed. Part of the data was obtained within the framework of the project titled "Antioxidant activity of fruits from indigenous varieties and populations of fruits, vegetables and grapes". Additionally, chokeberry, blackberry, blackcurrant and mulberry fruits were taken from local markets. There is no information about the method and conditions of cultivation for the commercially available fruits. They are taken from local markets and kept in cold storage.

The laboratory analyses were performed in the oenological laboratory at the Institute of Agriculture in Skopje. The fruits were taken at botanical maturity and the analyses were performed for each species individually according to the time of ripening.

Determination of vitamin C

The content of vitamin C (mg/100 g) was examined by volumetric method which involved titration with 2,6 dihalorophenol indofenol. The filtrate is

titrated to a pale pink color. Samples with a higher degree of red staining were treated with 50 mg of activated carbon before titration, until complete decolourization was achieved (Sadasivam and Balasubramanian, 1987).

Methods for determination of total phenols and anthocyanins

Sample preparation: Take approximately 5 g mass of the sample (the fruit) and let it macerate in a closed container with a solution of ethanol, water and hydrochloric acid in the ratio 70:30:0.1 for 24 h.

Determination of total phenols

Total phenols in the samples are determined according to the Folin-Ciocalteu method. 1 mL of the prepared sample is placed in a 10 mL flask. 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent are added (Slinkard and Singleton, 1977). After 3 minutes, 1.5 mL of 20% solution of Na₂CO₃ is added and the flask is filled up to the mark with distilled water. Then the flask is placed in a water bath heated to 50 °C for 16 minutes. Then they are cooled and total phenols are measured on a spectrophotometer at 765 nm wavelength. In parallel with the samples, a blank sample is prepared, where distilled water is used instead of the tested sample and the rest of the reagents remain the same.

Determination of total anthocyanins

The content of total anthocyanins was performed according to the acid-ethanol method (Somers and Evans, 1977) In a 10 mL flask, add 0.1 mL of the macerated sample and fill up to the mark with the solution of ethanol, water and hydrochloric acid. A solution of ethyl chloride is used as a blank sample. Determination of anthocyanins was carried out by spectrophotometer, at a wavelength of 550 nm.

The content of total phenols and anthocyanins was performed by Agilent 8453 UV-VIS spectrophotometer. These methods are significantly suitable for routine analyses. They are rapid and can be applied to monitor changes in phenols composition during fruit ripening.

Determining of the antioxidant activity was performed as an anti-radical activity against the stable product DPPH (2,2-diphenyl-1-picrylhydrazil). The determination was performed spectrophotometrically, at a wavelength of 517 nm. The antioxidant activity, especially for vitamin C, as well as the inhibitory antioxidant activity of all concentrations are expressed in percent (%).

Statistical analysis

To determine a significant difference from the mean values of the four parameters (vitamin C, antioxidant activity, total phenols and total anthocyanins), ANOVA and the Least Significant Difference (LSD) test at the $p < 0.05$ level were used for all fruit species samples. The correlation between the examined fruit species, as well as for all examined concentrations, was determined through correlation coefficient (r) description (LaMorte, 2021) (Table 1).

Table 1. Correlation coefficient (r.) description (LaMorte, 2021)

<i>Correlation coefficient</i>	<i>Description</i>
+1.0	Perfect positive
+0.8 to 1.0	Very strong positive
+0.6 to 0.8	Strong positive
+0.4 to 0.6	Moderate positive
+0.2 to 0.4	Weak positive
0.0 to +0.2	Very weak positive or no association
0.0 to -0.2	Very weak negative or no association
-0.2 to -0.4	Weak negative
-0.4 to -0.6	Moderate negative
-0.6 to -0.8	Strong negative
-0.8 to -1.0	Very strong negative
-1.0	Perfect negative

RESULTS AND DISCUSSION

The values for antioxidant activity and the content of the most important antioxidants in the fruits of some species are presented in Table 2.

The relative contribution of vitamin C or anthocyanins (expressed in mg/100g) contained in the obtained percentage of antioxidant activity inhibition was calculated as the contribution of vitamin C and anthocyanins in 100%inhibition antioxidant activity. Regarding the examined parameters, it was determined statistically significant differences between the fruit species (Table 3). The very high relative contribution of vitamin C on calculated antioxidant activity in blackcurrant (250.6), in contrast to other examined fruit species (24.4-54.3), is primarily due to the high content of vitamin C (216.0 mg/100g), which is also confirmed by literature data (Moyer *et al.*, 2002). The apple has the lowest vitamin C content (9.4 mg/100g) and the lowest contribution of vitamin C on calculated antioxidant activity (15.6) (Table 2).

The results correspond with available literature data. Several fruits accumulate high amounts of vitamin C at their optimal ripening, especially citrus fruits like grapefruits 40 mg/100 g, orange 41.3-62.7 mg/100 g (Dhuique-Mayer *et al.*, 2005), lemon 26.69-29.91 mg/100 g (Di Matteo *et al.*, 2021), strawberries 56.8-108.1 mg/100 g (Kim *et al.*, 2015; Boonyakiat *et al.*, 2016), kiwi 22.2-93.5 mg/100g (Nishiyama *et al.*, 2004), but other fruits accumulate significantly less ascorbic acid as for example in apple 2–10 mg/100 g, cherry 8 mg/100 g (Davey *et al.*, 2000), pear 4.4-10.2 mg/100g (Abaci *et al.*, 2016), apple (*M.pumila*) 0.05-0.96 mg% (Li *et al.*, 2021).

Compared to other berry fruits (Koca *et al.*, 2008; Selamovska *et al.*, 2024), pomegranate varieties (Selamovska *et al.*, 2022a), cherry varieties (Selamovska *et al.*, 2022b) and apple varieties (Selamovska *et al.*, 2022c), strawberry fruits have a higher content of ascorbic acid. Black currant has a higher content of ascorbic acid than strawberries (Hagg *et al.*, 1995; Paunović *et al.*, 2017).

Table 2. Content of vitamin C (mg/100g), total anthocyanins (mg/100g FW), total phenols (mg/100g FW) and antioxidant activity (%inhibition) in some fruit species

Fruit species	Antioxidant activity (% inhibition)	Vitamin C (mg/100g)	Relative contribution of vit. C in calculated antioxidant activity	Anthocyanins (mg/100g)	Relative contribution of anthocyanins in calculated antioxidant activity	Total phenols (mg/100g)
Apple	60.4	9.4	15.6	0.7	1.2	438.3
Cherry	43.4	12.8	29.6	39.4	90.8	138.6
Pomegranate	86.3	25.0	28.9	50.0	57.9	590.0
Strawberry	50.4	54.3	107.7	3.6	7.1	256.6
Black currant	86.2	216.0*	250.6*	580.0	672.8	1860.0
White mulberry	71.6	29.9	41.7	10.0	14.0	10.0
Chokeberry	76.4	24.2	31.7	610.0*	798.4*	2390.0*
Blackberry	78.6	19.2	24.4	170.0	216.3	760.0
Average	69.2	48.8	66.3	182.9	232.3	805.4

*The highest value for the parameter

The very high content of anthocyanins in chokeberry (610.0 mg/100g) and black currant (580 mg/100g) indicates that anthocyanins have the largest contribution in the total antioxidant activity of the fruits in these species. The lowest content of anthocyanins (0.7 mg/100g) and the lowest relative contribution of anthocyanins on calculated antioxidant activity (1.2) have apple fruits. According to the statistical analysis, it was determined that there is a large variation and statistically significant differences in the content of anthocyanins between fruit species (Table 3). The results of our analyses correspond to the data from the available literature references.

The main dietary sources of anthocyanins are red fruits. Anthocyanins in fruits and vegetables are present in glycosylated forms. They are accumulated in cell vacuoles and they are largely responsible for diverse pigmentation from orange to red, purple and blue in flowers, fruits, such as: blackberry, red and black raspberries, blueberries, bilberries, cherries, currants, blood orange, elderberries, grapes and some vegetables (Horbowicz *et al.*, 2008). Total anthocyanin content in selected common fruits according to literature sources (Horbowicz *et al.*, 2008) is: apple (peel) 100 - 2160 mg/kg, bilberry 4600 mg/kg, blackberry 820 - 1800 mg/kg, blueberry 825 - 5300 mg/kg, cherry 3500 - 4500 mg/kg, chokeberry 5060 - 10000 mg/kg, cranberry 460 - 2000 mg/kg, orange 2000 mg/kg, plum 19 - 250 mg/kg, red raspberry 100 - 600 mg/kg, strawberry

127 - 360 mg/kg, black currant 1300 - 4000 mg/kg, red currant 119 - 186 mg/kg. *Ribes nigrum* and *Vaccinium corymbosum* accumulated slightly fewer anthocyanins up to 380 and 430 mg/100 g FW, respectively (Petruskevicius *et al.*, 2023). Black raspberry had the highest anthocyanin content, over 200 mg/100g of fruit, and red raspberry had the lowest, less than 60 mg/100g. Blackberry was generally intermediate in anthocyanin content between red and black raspberries (Torre and Barritt, 1977). In strawberry, total anthocyanin content ranged between 200 and 600 mg/kg (Lopes-da-Silva *et al.*, 2002). Brazilian blueberry varieties have relatively high concentration of total anthocyanins 140–318 mg/100g (Pertuzatti *et al.*, 2014).

In terms of the content of total phenols, a statistically significant difference was determined between the fruit species. The very high content of total phenols in chokeberry (2390 mg/100 g) and black currant (1860 mg/100 g) indicates that they have a large contribution in the total antioxidant activity of the fruits. The lowest content of total phenols is in white mulberry (10.0 mg/100 g). The low content of polyphenols is not consistent with the obtained results for antioxidant activity, which may be due to the different maturity of the fruits when taking a sample for analysis and performing the chemical analysis on a different day. The results of our analyses correspond to the data from the available literature references. The results of some authors (Proteggente *et al.*, 2002; Pellegrini *et al.*, 2003; Halvorsen *et al.*, 2006) have showed that berries are consistently ranked among the top sources of total phenols and TAC (total antioxidant capacity), with levels up to 4 times greater than other fruits, 10 times greater than vegetables and 40 times greater than cereals. According to Pertuzatti *et al.* (2014) Brazilian blueberry varieties have relatively high concentration of total phenols (1622–3457 mg gallic acid equivalents (GAE) per 100 g dry weight (DW). Wild fruits have the higher TPC value 226.3 ± 1.14 mg GAE/g, than cultivated fruits (135.17 ± 2.02 mg QE/g) (Phuyal *et al.*, 2020). The content of total phenolic in fruit peel of mango (*Mangifera indica*) is 503 mg gallic acid equivalents (GAE)/g and 228 mg gallic acid equivalents (GAE)/g in fruit peel of apple (*Malus domestica*) (Sadef *et al.*, 2022).

Table 3. One-way ANOVA results for differences among fruit species

Source of Variation	Sum of squares (SS)	Degrees of freedom (df)	Mean square (MS)	F- statistic value	P-value	F-critical
Between Groups	3349844	5	669968.9	4.359	0.003	2.438
Within Groups	6455183	42	153694.8			
Total	9805028	47				

The investigated fruit species showed high antioxidative activity, average 69.2%. Blackcurrant and pomegranate have the largest value, over 86%. Lower values compared to the average antioxidant activity are shown by apple, cherry and strawberry fruits. In general, berry fruits show high antioxidant activity, over 50%, compared to other fruit species. According to the statistical analysis, it was

determined that there is a large variation between fruit species in terms of antioxidant activity, which indicates significant differences between species (Table 4).

Table 4. Descriptive statistics of antioxidant parameters across fruit species

Groups	Count	Sum	Average	Variance
Antioxidant activity (%inhibition)	8	553.3	69.1625	260.5541
Vitamin C (mg/100g)	8	390.83	48.85375	4748.177
Relative contribution of vit.C to calculated antioxidant activity	8	530.2	66.275	6364.931
Anthocyanins (mg/100g)	8	1463.7	182.9625	67680.51
Contribution of anthocyanins to calculated antioxidant activity	8	1858.5	232.3125	102425.7
Total phenols (mg/100g)	8	6443.5	805.4375	740689.1

The results from the analyses correspond to the data from the available literature references. According to several authors, the highest antioxidant activity was found in berry fruits. According to Wang *et al.* (1996), Scalzo *et al.* (2005), strawberry have a higher antioxidant capacity than apple, peach, pear, white grape, banana, plum, orange, pink grapefruit, kiwi. On the basis of the dry weight of fruit, strawberries had the highest oxygen radical absorbance capacity (ORAC), followed by black raspberries (cv. Jewel), blackberries and red raspberries (Wang and Lin, 2000). According to Zheng and Wang (2003) chokeberry have higher antioxidant activity compare to blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium macrocarpon*) and lingonberry (*Vaccinium vitis-idaea*). Purple mulberry has lower antioxidant activity than other berry fruits (*Rubus*, *Vaccinium* and *Ribes*) (Koca *et al.*, 2008). In some cherry varieties of the species *Elaeagnus umbellata*, the antioxidant activity is 4.32-9.49 mM TE/100g (Lachowicz *et al.*, 2019), in bird cherry (*Prunus padus*) is 7.26-31.54 mM/kg (Mikulic-Petkovsek *et al.*, 2016), in some apple and strawberry varieties the antioxidant activity is 12- 64 mM FRAP (Kaur and Kapoor, 2005). The percentage of inhibition in apple (*Malus domestica*) and mango (*Mangifera indica*), according to Sadeh *et al.*, (2022) is 67.27% and 73.16%, respectively. Antioxidant activity in orange is 27.12%, in grapefruit is 56.91% (Siddhartha *et al.*, 2025). Prior *et al.*, (1998) were the first authors to notice that blueberries have higher antioxidant activity by the ORAC method, confirming that, compared to other fruits and vegetables, blueberries have a relatively high antioxidant activity.

Given that the antioxidant capacity of fruits is attributed to the presence of phenolic compounds, ascorbic acid, tannins, in this paper we provide comparative values of these three parameters and their influence on antioxidant activity (Table 1). A large contribution of vitamin C in the antioxidant activity is found in black currant, strawberry and white mulberry fruits. Anthocyanins contribute most to the antioxidant activity of chokeberry, blackcurrant, blackberry and cherry fruits, while total phenols in chokeberry, blackcurrant, blackberry, pomegranate and

apple fruits. A weak positive correlation of total antioxidant activity with vitamin C (0.399) and a moderate positive correlation with total anthocyanins (0.534) and total phenols (0.556) was found, indicating that in the studied fruit species, antioxidant activity depends more on the content of total anthocyanins and total phenols. (Table 5).

Table 5. Correlation between vitamin C, total anthocyanins and total phenols with antioxidant activity

	Antioxidant activity (%inhibition)	Vitamin C (mg/100g)	Relative contribution of vit C on calculated antioxidant activity	Anthocyanins (mg/100g)	Relative contribution of anthocyanins on calculated antioxidant activity	Total phenols (mg/100g)
Antioxidant activity (% inhibition)	1					
Vitamin C (mg/100g)	0.399	1				
Relative contribution of vit.C on calculated antioxidant activity	0.267	0.982	1			
Anthocyanins (mg/100g)	0.534	0.590	0.518	1		
Relative contribution of anthocyanins on calculated antioxidant activity	0.493	0.528	0.457	0.996	1	
Total phenols (mg/100g)	0.556	0.468	0.394	0.972	0.973	1

The result from our research aligns well with previous studies. In a study by Tulipani *et al.*, (2008), this individual contribution was investigated in different strawberry cultivars, where vitamin C was found to be one of the most important components, responsible for more than 30 % of the TAC (total antioxidant capacity) of strawberry extracts, followed by anthocyanins contributing 25 - 40 %; and the rest was composed mainly of EA derivatives and flavonols. The antioxidant activity attributed to strawberry is strongly related to the content of extractable polyphenols and vitamin C (Bezerra Almeida *et al.*, 2019). According to Heinonen *et al.*, (1998), Wang and Lin (2000) strawberry fruits (*Fragaria×ananassa* Duch.) have been shown to possess high in vitro antioxidant activity that has been positively correlated with the content of polyphenolic compounds, specifically, anthocyanins.

Citrus fruits have a high content of ascorbic acid. The content of this vitamin is considered a basic indicator of the quality and nutritional value of fruits and fruit products. Although ascorbic acid has lower antioxidant activity than phenolic compounds, including phenolic acids, tannins, catechins, and anthocyanins, its multifaceted antioxidant activity should be stressed (Rafiq *et al.*, 2018).

Several studies (Ovando *et al.*, 2009; Ćujić *et al.*, 2013) indicate a positive correlation between the content of anthocyanins in fruits and high antioxidant activity in some fruit species. About 70% of the total antioxidant capacity comes from anthocyanins (Lopes-da-Silva *et al.*, 2002; Giampieri *et al.*, 2012). Main factors of antioxidant activity in berry fruits are anthocyanins. Blackberries are known to contain a broad range of phenolic compounds and flavonoids, which contribute to their strong antioxidant capacity. Cranberries have big antioxidant punch, thanks to the anthocyanins and polyphenols. Blueberries are among the highest antioxidant-rich fruits, loaded with anthocyanins (Loliger, 1991).

According to Zheng and Wang (2003), Gazdik *et al.*, (2008) the chokeberry had significantly higher antioxidant activity related to the higher content of anthocyanins and phenolics. Prior *et al.*, (1998) investigated the antioxidant capacity of different *Vaccinium* species, emphasizing the role of specific antioxidants, such as anthocyanins, in determining the overall antioxidant potential. Red raspberries antioxidants are primarily from ellagic acid and anthocyanins.

Total phenols participate in the antioxidant activity in walnut (*Juglans regia* L.) (Oliveira *et al.*, 2008), pomegranate, cherry (Selamovska *et al.*, 2022a, b), apple fruits (Eberhardt *et al.*, 2000; Selamovska *et al.*, 2022c) purple mulberry (Koca *et al.*, 2008) and other berry fruits (Wang and Lin, 2000; Kahkonen *et al.*, 2001).

Flavanols (catechin and oligomeric procyanidins) are the major class of apple polyphenols, representing more than 80%, followed by hydroxycinnamic acids (1-31%), flavonols (2-10%), dihydrochalcones (0.5-5%), and in red apples, anthocyanins (1%) (Wojdylo *et al.*, 2008). The results of Pertuzatti *et al.*, (2014) show that Brazilian blueberries have a relatively high concentration of phenolic compounds and total anthocyanins, along with a small concentration of carotenoids. It was also shown that these compounds have a positive correlation with antioxidant activity.

In pecan nuts, ellagic acid, gallic acid, procatechuic, p-hydroxybenzoic acids contribute most to the antioxidant activity (Do Prado *et al.*, 2009). Raspberries (*Rubus idaeus* L.) have high levels of vitamin C and phenolic compounds. Ellagic acid is of particular interest from a dietary view point as it has been reported to have antiviral and accounts for over 30% of the antioxidant activity of the fruit (Mullen *et al.*, 2003). Aaby *et al.*, (2005) determined that strawberry achenes contributed eleven percent of total phenolics and fourteen percent of the antioxidant activity. The authors found that ellagitannins and ellagic acid are the main contributors of antioxidant activity within strawberry achenes.

In addition to the genotype (species and variety) the amounts and types of bioactive compounds and their antioxidant activity are dependent on some factors: plant material (Wang and Lin, 2000; Do Thi and Hwang, 2014; Czech *et al.*, 2021), maturity stage (Kalt and McDonald, 1996; Pineli *et al.*, 2011; Minutti-López Sierra *et al.*, 2019), growing conditions (Wang and Zheng, 2001; Vagiri,

2018), time of harvest, cultivation systems (Wang and Millner, 2009; Melgarejo *et al.*, 2017; Bezerra Almeida *et al.*, 2019), storage period (Kalt *et al.*, 1999; Lee and Kader, 2000), industrial processing (Pellegrini *et al.*, 2003; Wolfe *et al.*, 2003; Aaby *et al.*, 2005).

Fruits and leaves of fruit species have great antioxidant activity. According to Czech *et al.*, (2021) whole lemons, limes, and especially mandarins also had higher antioxidant potentials than their pulps. Compared with fruits, leaves were found to have higher ORAC values. As the leaves become older, the ORAC values and total phenolic contents decreased (Wang and Lin, 2000). According to Do Thi and Hwang (2014) the chokeberry leaves contained a large proportion of the total phenolic and flavonoid contents of the chokeberry fruits. The young leaves contained more phenols and flavonoids than the old leaves, basal leaves and dormant buds have the highest content of total phenols.

Blackberries and strawberries have the highest ORAC values during the green stages, whereas red raspberries had the highest ORAC activity at the ripe stage (Pineli *et al.*, 2011; Minutti-López Sierra *et al.*, 2019). Monomeric anthocyanins, which accounted for greater than 85% of the total anthocyanin content in the samples of lowbush blueberry (*Vaccinium angustifolium* Aiton) were substantially higher in the ripe fruit than in underripe fruit and slightly higher compared to overripe berries (Kalt and McDonald, 1996).

Plants grown in the cool day and cool night temperature (18/12 °C) generally had the lowest phenolic acid, flavonols and anthocyanins. An increase in night temperature from 12 to 22 °C, with the day temperature kept constant at 25 °C, resulted in a significant increase in phenolic acid, flavonols and anthocyanins. These conditions also resulted in a significant increase in antioxidant capacity (Wang and Zheng, 2001). According to Vagiri (2018) blackcurrant cultivars grown in more northern and cooler regions have higher content of flavan-3-ols, phenols, flavonols and antioxidant activity compared to cultivars grown in southern and warmer regions.

The cultivation systems and cultivars influence on the antioxidant activity, physicochemical and sensory properties in fruits. Strawberry cultivated on the conventional system shows higher values for physicochemical variables and antioxidant activity, than strawberry cultivated on hydroponics in gutters and slabs (Bezerra Almeida *et al.*, 2019). Fruit from plants grown in compost socks had significantly higher oxygen radical absorbance capacity (ORAC), flavonoids, anthocyanins, sugars and acids than fruit produced in the black plastic mulch or matted row systems (Wang and Millner, 2009). The strawberries grown under dredged remediated sediment (DRS) and Peat-DRS substrate (mixture 50%) show a high content in vitamin C, total phenols and antioxidant activity, also have the highest sweetness index and a good firmness (Melgarejo *et al.*, 2017).

Kalt *et al.*, (1999) reported changes in phenolic composition, anthocyanin, and antioxidant capacities in Fresh strawberries (*Fragaria x ananassa* Duch.), raspberries (*Rubus idaeus* Michx.), highbush blueberries (*Vaccinium corymbosum* L.) and lowbush blueberries (*Vaccinium angustifolium* Aiton) during postharvest

storage treatments. A study by Lee and Kader (2000) demonstrated that the vitamin C content of strawberries and kiwi fruits declined during storage, with variations attributed to oxygen levels. Thus, the packaging and storage conditions during and after processing emerge as critical factors influencing the nutritional outcomes of processed fruits.

Phenolic content and antioxidant activity of strawberry achenes were reduced by industrial processing (Aaby *et al.*, 2005). Processing methods, such as canning, freezing and drying, subject fruits to various environmental stresses, potentially influencing the stability and bioavailability of antioxidants. Pellegrini *et al.*, (2003) evaluated the impact of different processing methods on the total antioxidant capacity of various plant foods. Their findings highlighted variations in the antioxidant levels of processed fruits compared to their fresh counterparts, emphasizing the need for a nuanced approach in assessing the nutritional consequences of processing. According to Wolfe *et al.*, (2003) conventional heat treatments, such as pasteurization, led to a reduction in the antioxidant capacity of apple peels. The antioxidant activity of fruits is significant from a health perspective. A large contribution of vitamin C in the antioxidant activity is found in black currant, strawberry and white mulberry. Anthocyanins contribute most to the antioxidant activity of chokeberry, blackcurrant, blackberry and cherry fruits, while total phenols in chokeberry, blackcurrant, blackberry, pomegranate and apple fruits.

CONCLUSIONS

The highest value for antioxidant activity is found in pomegranate and black currant fruits. Apple, cherry and strawberry fruits show lower values compared to the average antioxidant activity.

The highest content of vitamin C (216.0 mg/100g) and the highest contribution of vitamin C in antioxidant activity (250.6) has black currant. The highest content of anthocyanins and the highest contribution of anthocyanins in antioxidant activity have chokeberry (610 mg/100g) and black currant (580.0 mg/100g). Apple fruits have the lowest content of vitamin C (9.4 mg/100g) and anthocyanins (0.7 mg/100g). The highest content of total phenols and antioxidant activity have chokeberry (2390.0 mg/100g) and black currant (1860.0 mg/100g).

Vitamin C has a significant impact on the antioxidant activity in black currant, strawberry and white mulberry fruits. Anthocyanins contribute most to the antioxidant activity of chokeberry, blackcurrant, blackberry and cherry fruits, while total phenolics in chokeberry, blackcurrant, blackberry, pomegranate and apple fruits.

In terms of the parameters examined, statistically significant differences were determined between the examined fruit species. A weak positive correlation was determined for total antioxidant activity with vitamin C (0.399) and a moderate positive correlation with total anthocyanins (0.534) and total phenols (0.556), which indicates that in the examined fruit species, antioxidant activity depends more on the content of total anthocyanins and total phenols.

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