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EFFECT OF ZINC OXIDE NANOPARTICLES ON THE MORPHOLOGICAL TRAITS AND NUTRITIONAL QUALITY OF ASPARAGUS (*Asparagus officinalis* L.) IN *in vitro* CULTURE

Anna Krzepiło¹, Roman Prażak², Agata Świącilo³

ABSTRACT

Zinc oxide nanoparticles (ZnONPs) are used to stimulate the growth and development of various plant species, including those grown in tissue cultures. However, there is still a lack of data regarding their effect on the micropropagation of asparagus (*Asparagus officinalis* L.). Asparagus is a valuable vegetable crop due to its taste, easy digestibility, low caloric value, and significant health-promoting properties. In the present study, the effects of different concentrations of ZnONPs on morphological traits and on the content of metals, total phenolic content (TPC), total antioxidant capacity (TAC), and photosynthetic pigments in plantlets of *A. officinalis* derived from tissue cultures were evaluated. Shoot fragments obtained from seedlings grown in *in vitro* culture were used for micropropagation. Explants measuring approximately 0.5 cm were placed on MS medium supplemented with 0.20 mg dm⁻³ BAP, 0.25 mg dm⁻³ Kin, 0.20 mg dm⁻³ NAA, and ZnONPs at concentrations of 0 (control), 10, 20, 30, and 40 mg dm⁻³. The *in vitro* culture was conducted over two subcultures lasting a total of four months. After four months of *in vitro* culture, the developed plantlets were removed from the culture vessels, and their morphological parameters were assessed. A portion of the plantlets was used for further analyses, including the determination of their metal content, photosynthetic pigments, TPC, and TAC using the DPPH assay. ZnONPs at concentrations of 20–40 mg dm⁻³ negatively affected shoot number, root length, and fresh weight of plantlets. At concentrations of 10–20 mg dm⁻³, ZnONPs did not affect shoot length or root number, whereas the 40 mg dm⁻³ treatment negatively affected these parameters. In samples of asparagus plantlets grown under treatment conditions involving 10–40 mg dm⁻³ of ZnONPs, higher levels of K, Zn, and Cu and lower levels of Ca and Mn were found compared with the control group. Samples grown at the 10–20 mg dm⁻³ treatments contained more Mg and Fe than in the control. Strong positive correlations were found between the ZnONPs treatments and the concentrations of Zn and K, and strong negative correlations were found between these treatments and the concentrations of Ca, Mn, and Mg. The total content of phenols and antioxidants measured by the DPPH method, as well as photosynthetic pigments, in plantlets grown at the 10–20 mg dm⁻³ ZnONPs treatments was higher than in the control.

Keywords: *Asparagus officinalis* L., tissue culture, ZnONPs, antioxidants, minerals, photosynthetic pigments, polyphenols

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INTRODUCTION

There are approximately 200 plant species known to belong to the genus *Asparagus* (family *Asparagaceae*). These include edible species such as *A. albus*, *A. acutifolius*, and *A. officinalis*; medicinal species such as *A. adscendens* and *A. racemosus*; and ornamental species such as *A. asparagoides* and *A. densiflorus* [Encina and Regalado 2022]. *Asparagus officinalis* L. grows naturally in the Mediterranean region, adjacent areas, and Central Asia. It is the only edible perennial species of this family cultivated worldwide, particularly in China. Its popularity stems from its ability to provide an early spring supply of fresh, high-quality produce. Unlike many other crops, its underground organs persist after the growing season and overwinter in the soil, enabling the development of new aerial shoots in the following season, which subsequently flower and set fruit [Fuentes-Alventosa 2009, Kobus-Cisowska 2019]. *Asparagus officinalis* L. is valued for its high nutritional quality, low caloric content, delicate taste and health-promoting properties. Young shoots provide approximately 20 kcal per 100 g of fresh weight, and consist mainly of water (approximately 93%), with carbohydrates (2.04%), proteins (1.91%), dietary fiber (1.31%), nitrogen (0.31%), and a low fat content (0.16%) [Olas 2024]. The presence of dietary fiber and inulin contributes to proper digestive function and supports the growth of beneficial intestinal microbiota [Mudannayake et al. 2025]. Asparagus is also a source of minerals, including potassium, phosphorus, magnesium, calcium, iron, zinc, and manganese [Kohli et al. 2023], as well as vitamins A, C, B1, B2, E, and folic acid. Its health-promoting properties are associated with numerous biologically active compounds, including arginine, tyrosine, asparagine, flavonoids (kaempferol, quercetin, and rutin), essential oils, steroidal saponins, and carotenoids [Hamdi et al. 2018, Zhang et al. 2019]. These compounds contribute to antioxidant activity, while chemical analyses have also revealed the presence of organic acids, amino acids, and peptides [Guo et al. 2020, Rolbiecki et al. 2024, Zhang et al. 2024].

Asparagine is a characteristic component of asparagus and is associated with its diuretic properties and high content of non-protein nitrogen [Gupta and Chauhan 2025]. Regular consumption of asparagus may support digestive, cardiovascular, and metabolic health, while its low glycemic index makes it suitable for individuals with carbohydrate metabolism disorders [Hamdi et al. 2018, Guo et al. 2020, Zhang et al. 2024]. Asparagus also contains folic acid, vitamins C and E, carotenoids, potassium, dietary fiber, and glutathione, while its young shoots and roots exhibit diuretic and mild laxative properties [Kulczyński et al. 2016, Javaid et al. 2022]. Furthermore, compounds such as protodioscin, rutin, and quercetin may contribute to the potential cancer-preventive properties of green asparagus [Wang 2022]. The nutritional value and abundance of bioactive compounds, together with the documented health-promoting properties of asparagus, make this vegetable a valuable source of nutrients and biologically active substances [Olas 2024].

For commercial purposes, asparagus is propagated by seeds or vegetatively through division. These traditional propagation methods are time-consuming and may increase the risk of disease transmission. The genetic diversity of cultivated asparagus (*A. officinalis* L.) is relatively limited, which may constrain breeding progress. Therefore, the identification and utilization of diverse genetic resources are important for broadening the genetic base and developing new cultivars with improved agronomic, nutritional, and quality traits [Knaflewski and Kałużewicz 1998].

The cultivation of asparagus in *in vitro* culture is a widely used method for propagation and obtaining plant material of high phytosanitary quality and genetic uniformity [Sharma et al. 2025]. This technique is applied in horticultural practice, particularly for producing pathogen-free plants and rapidly multiplying valuable genotypes. Asparagus is a perennial species with a relatively long production cycle; therefore, micropropagation significantly shortens the time required to obtain planting material [Esmaeili et al. 2016].

Plant growth regulators are widely used in the micropropagation of *Asparagus* species to promote shoot multiplication, rooting, and plant regeneration. In *A. officinalis*, 6-benzylaminopurine (BAP) has been applied at concentrations ranging from 0.1 to 2.0 mg dm⁻³, usually in combination with 1-naphthaleneacetic acid (NAA) at 0.015–0.5 mg dm⁻³ [Štajner et al. 2013, Esmaeili et al. 2016, Minh et al. 2022, Sarabi and Almasi 2010]. Other studies have used kinetin (Kin) in combination with NAA or indole-3-butyric acid (IBA), whereas IBA has been mainly applied for rooting [Pontaroli and Camadro 2005, Esmaeili et al. 2016,

Sarabi and Almasi 2010]. In *A. racemosus*, direct organogenesis has been achieved using BAP in combination with indole-3-acetic acid (IAA) [Singh et al. 2023].

In plant cultures conducted under *in vitro* conditions, nanotechnology is playing an increasingly important role as a tool supporting both propagation and modification of plant material. Nanoparticles are primarily used to reduce microbial contamination and serve as an alternative or complement to traditional disinfectants. They also influence plant morphogenesis, as they have been shown to stimulate somatic embryogenesis, organogenesis, and accelerate tissue growth and differentiation. In many cases, their application leads to increased plant regeneration efficiency and improved quality of the obtained material [Singh et al. 2023]. Additionally, beneficial effects on the biosynthesis of secondary metabolites, such as phenolic compounds, flavonoids, and alkaloids, have been reported, which may enhance the production of plant-derived bioactive substances [Alghamdi et al. 2022, Alharby et al. 2016, Devasia et al. 2020]. In *in vitro* cultures, metal nanoparticles and their oxides are most commonly used, including silver, copper, titanium, zinc, cobalt, nickel, and iron. Carbon-based nanomaterials, such as graphene and carbon nanotubes, as well as nanosilica, are also employed. The diverse physicochemical properties of these materials are contributing to their increasing use in plant biotechnology.

Zinc oxide nanoparticles may stimulate cell division and proliferation, accelerate plant tissue regeneration [Alghamdi et al. 2022, Geremew et al. 2023], and support callus formation, rooting, and somatic embryogenesis [Shafique et al. 2020, Al-Mayahi 2021, Geremew et al. 2023]. Appropriately selected concentrations of zinc oxide nanoparticles (ZnONPs) can have a positive effect on the micropropagation, growth, and *in vitro* development of olive tree explants (*Olea europaea* L. cv. Moraiolo) [Regni et al. 2022]. Beneficial effects of ZnONPs have also been reported for shoot regeneration in blackberry (*Rubus fruticosus* L.) cv. Nawaho [Krzepiłko et al. 2024a], stevia (*Stevia rebaudiana* Bertoni) [Javed et al. 2018, Krzepiłko et al. 2024b], mustard (*Brassica nigra*) [Zafar et al. 2016], *Panicum virgatum* [Nair et al. 2010], *Thymus kotschyianus* and *Zataria multiflora* [Mosavat et al. 2019], and potato (*Solanum tuberosum*) [Alghamdi et al. 2022].

In *in vitro* culture, zinc oxide nanoparticles serve as a source of zinc for plants. This mineral element is essential for chlorophyll synthesis and the production of plant hormones – especially auxins and gibberellins; it also enhances photosynthetic efficiency, and influences nutrient uptake [Hayat et al. 2023]. Zinc also affects cell proliferation and key metabolic processes such as protein biosynthesis, DNA replication, and transcription, and is a component of prosthetic groups of many enzymes [Cabot et al. 2019, Hayat et al. 2023, Shao et al. 2023]. Zinc oxide nanoparticles mitigate stress caused by heavy metals, drought, and salinity [Sun et al. 2021, Jaithon et al. 2022, Liu et al. 2022]. They also exhibit antifungal and antibacterial activity, thereby reducing the incidence of plant diseases [Pena et al. 2022, Zou et al. 2022, Subba et al. 2024].

The aim of this study was to investigate the effects of zinc oxide nanoparticles (ZnONPs), applied at four concentrations (10, 20, 30, and 40 mg dm⁻³), on morphological traits, growth, and the contents of selected metals (potassium, calcium, magnesium, iron, zinc, manganese, and copper), total phenolic content (TPC), total antioxidant capacity (TAC), and photosynthetic pigments (chlorophyll a and b, total chlorophyll: a + b, and carotenoids) in asparagus plants cultured *in vitro*.

MATERIAL AND METHODS

Zinc oxide nanoparticles (ZnONPs) were obtained from Sigma-Aldrich (catalog no. 721077). Particle size was measured using an Aerodynamic Particle Sizer (APS). The average size of individual particles was <35 nm [Wang et al. 2013].

Plant material. The experimental material consisted of garden asparagus (*Asparagus officinalis* L.) cv. Argenteuil (PL-14/32/17672), an early cultivar, characterized by thick, white (or green) shoots with rounded tips and short, pinkish scales. Seeds were obtained from the Polish Breeding and Seed Company (PNOS – Państwowe Przedsiębiorstwo Nasiennictwa Ogrodniczego i Szkółkarstwa) in Ożarów Mazowiecki, Poland. After surface sterilization for 8 min in 0.1% HgCl₂, the seeds were placed in Petri dishes (10 cm in

diameter) containing solid MS medium at pH 5.8 [Murashige and Skoog 1962] without growth regulators. After seedling development, shoots were cut into fragments approximately 0.5 cm in length under sterile conditions and transferred to 200 mL glass jars with heat-resistant Magenta B-caps (Figure 1B). Each jar contained 20 mL of MS medium supplemented with 3% sucrose, 0.8% Difco Bacto agar, 0.20 mg dm⁻³ BAP (6-benzylaminopurine), 0.25 mg dm⁻³ Kin (kinetin), 0.20 mg dm⁻³ NAA (1-naphthaleneacetic acid) and 10, 20, 30, and 40 mg dm⁻³ ZnONPs; a treatment without ZnONPs served as the control. Cultures were maintained in a growth room at 22–24°C, 60% relative humidity, and a 16 h photoperiod with a light intensity of 54 µmol m⁻² s⁻¹ provided by fluorescent lamps (Radium NL 36W/830, Germany). After four months of *in vitro* culture, the regenerated asparagus plantlets were removed from the culture vessels and their shoot and root parameters, as well as their fresh weight, were measured. The plantlets were then transferred into plastic containers filled with sterilized soil (Figure 1). Some of the plant material was used for further analyses, including the determination of metal content (K, Ca, Mg, Fe, Zn, Mn, Cu), chlorophyll a and b, total chlorophyll (a + b), carotenoids, total phenolic content (TPC), and total antioxidant capacity (TAC). Each experiment and analysis was performed in triplicate.

Preparation of plant extracts. The plant material was first homogenized, weighed and mixed with a suitable extraction solvent at a ratio of 1 : 10 (m/v); this was followed by 5 minutes of homogenization during which the samples were kept in an ice bath. After homogenization, samples were centrifuged for 5 min at 5000 rpm. The obtained supernatant was frozen and used for further biochemical analyses. Depending on the parameter analyzed, different extraction solvents were used: 70% methanol in water for phenolic compounds, 80% acetone in water for photosynthetic pigments, and 70% ethanol in water for antioxidant activity (DPPH assay).

Determination of total phenolic content. TPC was determined spectrophotometrically using the Folin–Ciocâlteu reagent. This method is based on the ability of polyphenols to reduce phosphomolybdate–phosphotungstate complexes present in the reagent, resulting in the formation of blue-coloured products. The intensity of the colour is directly proportional to the concentration of phenolic compounds in the sample. The reaction mixture consisted of 0.05 cm³ of methanolic extract from asparagus plantlets, 0.15 cm³ of Folin–Ciocâlteu reagent, 0.3 cm³ of 7.5% sodium carbonate solution, and 2.525 cm³ of distilled water. After incubation for 1 h, absorbance was measured at 765 nm using a Shimadzu spectrophotometer (Japan), model UV-1280. Results were expressed as gallic acid equivalents (GAE) per 1 g of fresh weight (FW), according to the method described by Singleton et al. [1999].

Determination of total antioxidant capacity. TAC was determined using the DPPH method. This method employs the stable radical DPPH• (2,2-diphenyl-1-picrylhydrazyl), which exhibits an intense violet color in ethanol solution. In the presence of antioxidants, the DPPH• radical is reduced, leading to a gradual discoloration of the solution. The decrease in colour intensity, measured as a reduction in absorbance, is proportional to the antioxidant activity of the sample – the stronger the antioxidant properties, the greater the decrease in absorbance. Measurements were taken at 517 nm after 30 min of reaction using the Shimadzu UV-1280 spectrophotometer, according to the method described by Addai et al. [2013]. The reaction mixture consisted of a DPPH• solution prepared in ethanol and 0.05 cm³ of the ethanol extract from asparagus plantlets. TAC was expressed as Trolox equivalents per 1 g fresh weight.

Determination of mineral composition. After harvesting, the plantlets were thoroughly rinsed in distilled water and then dried in a forced-air oven at 70°C for 72 h until constant weight was reached. The dried plant material was then mineralized using a mixture of concentrated nitric acid (V) and perchloric acid (VII) (HNO₃ + HClO₄). After digestion and appropriate dilution, the content of macro- and microelements – potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) – was determined. Mineral content was expressed in mg 100 g⁻¹ DW (dry weight). The Zn content was used as the reference value and set to 1.00. Relative mineral content = mineral content / Zn content.

Statistical analysis. The results were subjected to statistical analysis using analysis of variance (ANOVA). The significance of differences between means was evaluated using Tukey's post hoc test at *p* < 0.05. Additionally, Pearson's correlation coefficients (*r*) were calculated to determine relationships between the analyzed parameters. Statistical analyses were performed using Statistica v.13.1.

RESULTS

The germination rate of garden asparagus seeds was 76% after 14 days and 86% after 21 days of culture on MS medium. The shoots of the seedling were then cut into fragments and transferred to vessels containing solid MS medium, which had been enriched with growth regulators and zinc oxide nanoparticles. The *in vitro* culture was conducted over two subcultures lasting a total of four months. During the first subculture, it was observed that the percentage of explants regenerating organs was slightly lower in the treatments with ZnONPs than in the control. In the control group, 75.6% of the explants developed both shoots and roots. When treated with 10 mg dm⁻³ ZnONPs, 68.0% of the explants developed both shoots and roots; at 20 mg dm⁻³ ZnONPs, 62.2% showed the same response; at 30 mg dm⁻³ ZnONPs, 71.4% of the explants developed both shoots and roots; and at 40 mg dm⁻³ ZnONPs, the corresponding percentage was 70.0%.

Analysis of morphological traits. After the second subculture, plantlets were removed from the culture vessels and morphological traits were analyzed (Figure 1B, 1C).

Table 1. The effect of increasing concentrations of zinc oxide nanoparticles in Murashige and Skoog solid medium on the biometric characteristics of asparagus plantlets (*Asparagus officinalis* L.) after 4 months of *in vitro* culture (mean value of the characteristic per explant)

Biometrical parameter	Concentration of ZnONPs (mg dm ⁻³)					LSDp < 0.05
	0 (control)	10	20	30	40	
Number of shoots	15.93a	14.07ab	11.97b	10.86b	9.83c	2.495
Shoot length (cm)	10.47a	10.02a	9.42ab	7.98b	5.89c	2.150
Number of roots	4.77a	3.73ab	3.57ab	2.63b	1.97c	1.395
Root length (cm)	3.98a	2.71b	2.22c	1.96d	0.71e	1.271
Fresh weight of plant (g)	0.595a	0.515a	0.376b	0.343bc	0.164c	0.132
(Increment, g)	(0.572)a	(0.496)a	(0.355)b	(0.321)bc	(0.145)c	(0.110)

Different letters within a line indicate a significant difference according to the Tukey's test ($p < 0.05$).

Overall, the highest number and length of shoots and roots, as well as the highest fresh weight, were observed in the control and at the lowest ZnONPs concentration (10 mg dm⁻³) (Table 1, Figure 1C). Increasing ZnONPs concentrations generally had a negative effect on plantlet growth and development. At concentrations of 20–40 mg dm⁻³, the number and length of shoots decreased compared with the control, with the most pronounced reductions observed at 40 mg dm⁻³. The number and length of roots were also significantly reduced at the higher ZnONPs concentrations, particularly at 30 and 40 mg dm⁻³. Similarly, fresh weight decreased progressively with increasing ZnONPs concentration, with the lowest value recorded at 40 mg dm⁻³ (Table 1). Negative Pearson correlation coefficients were observed between ZnONPs concentration and all analyzed morphological traits, including the number and length of shoots and roots and plantlet fresh weight.

Mineral content in *Asparagus officinalis* plantlets. ZnONPs influenced the mineral composition of *A. officinalis* (Table 2). The zinc content in plantlets increased as the concentration of ZnONPs in the culture medium increased. At a concentration of 10 mg dm⁻³, the Zn content was four times higher than in the control, while at 40 mg dm⁻³ it was up to fourteen times higher. Plant material from the control treatment most often differed significantly from that obtained from ZnONPs treatments in terms of the content of the analyzed minerals. In plantlets from treatments containing 10–40 mg dm⁻³ ZnONPs, the content of K, Zn, and Cu increased, whereas the content of Ca and Mn decreased compared to the control. The content of Mg and Fe was higher than in the control in plant material from the 10 and 20 mg dm⁻³ ZnONPs treatments; however, in the 30 and 40 mg dm⁻³ treatments, their levels were lower (Table 2).

Figure 1. Effect of different concentrations of ZnONPs on the growth and development of asparagus (*Asparagus officinalis* L.): A) germinating seeds in Petri dishes without ZnONPs, B) developing explants in sterile vessels, C) regenerated plantlets after 4 months (from the left to right: control, 10, 20, 30 and 40 mg dm⁻³), D) regenerated plantlets in plastic cups filled with sterile soil

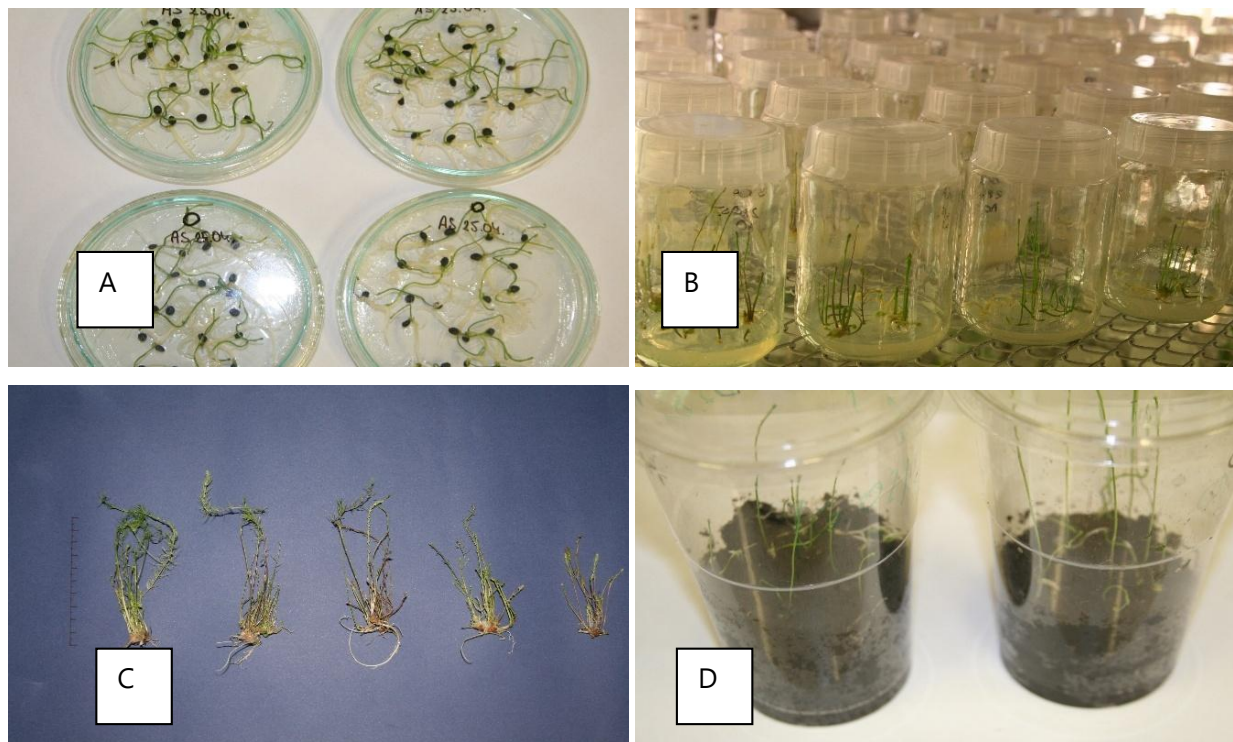


Table 2. The influence of ZnONPs on mineral content in plantlets of asparagus (*Asparagus officinalis* L.) after 4 months of *in vitro* culture

ZnONPs (mg dm ⁻³)	Mineral content (mg 100 g ⁻¹ DW)						
	K	Ca	Mg	Fe	Mn	Zn	Cu
0	2340 ^e	195 ^a	84.0 ^b	17.9 ^b	10.30 ^a	9.29 ^e	0.48 ^d
10	2530 ^d	169 ^b	85.4 ^a	18.4 ^b	9.47 ^b	37.70 ^d	0.57 ^b
20	2740 ^b	158 ^c	85.9 ^a	21.0 ^a	9.31 ^b	82.00 ^c	0.60 ^a
30	2820 ^a	143 ^d	82.4 ^c	17.8 ^b	8.73 ^c	112.00 ^b	0.52 ^c
40	2580 ^c	139 ^e	73.8 ^d	16.2 ^c	8.28 ^c	128.00 ^a	0.49 ^d
LSD _{p < 0.05}	14.50	1.87	1.39	1.30	0.48	1.70	0.03

Mean values assigned the same letter within a column do not differ significantly according to Tukey's test ($p < 0.05$).

Positive Pearson correlation between ZnONPs treatments and the content of elements in *A. officinalis* plant material were observed for potassium ($r = 0.637$) and zinc ($r = 0.989$). In contrast, negative correlations were recorded for calcium ($r = -0.967$), manganese ($r = -0.937$), and magnesium ($r = -0.798$). No correlations were found for copper and iron. In the control treatment without ZnONP, the order of elements based on their content in plantlets was as follows: K > Ca > Mg > Fe > Mn > Zn > Cu. In plantlets treated with 10–40 mg dm⁻³ ZnONPs, zinc content exceeded that of iron and manganese, and in the 30 and 40 mg dm⁻³ treatments, zinc content was higher than that of magnesium (Table 2). The ratio of Cu, Mn, Fe, Mg, Ca, K, and Zn content in the control plantlets was 0.052 : 1.11 : 1.93 : 9.04 : 20.99 : 251.88 : 1.00. The addition of ZnONPs

markedly reduced the relative values of K, Ca, Mg, Fe, Mn, and Cu in relation to Zn content. The most pronounced changes were observed for K and Ca. The decrease in the mineral-to-Zn ratios resulted mainly from the substantial increase in Zn accumulation in plantlets exposed to ZnONPs (Table 3).

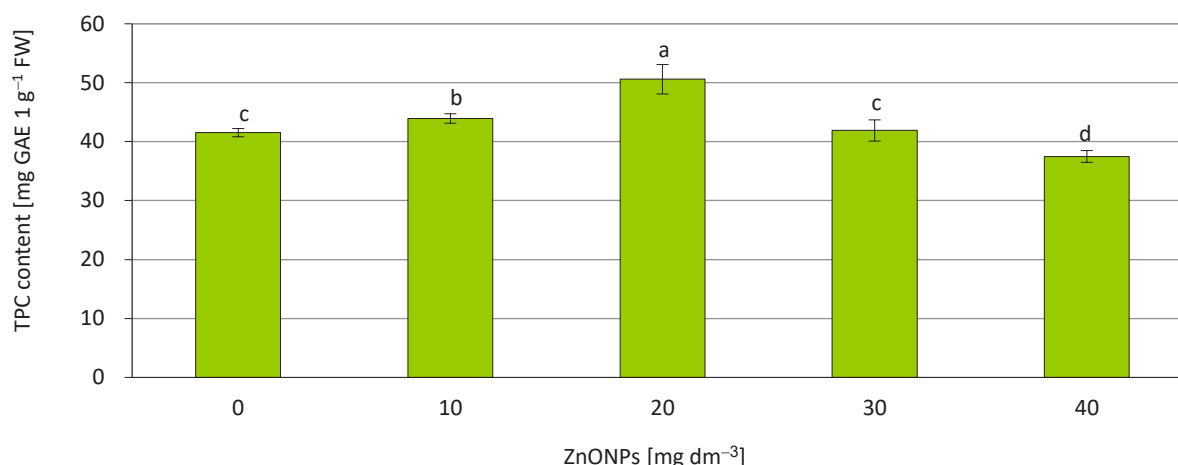
Table 3. The influence of ZnONPs on the relative mineral content in plantlets of asparagus (*Asparagus officinalis* L.) after 4 months *in vitro* culture

ZnONPs (mg dm ⁻³)	The ratio of the mineral content*					
	K	Ca	Mg	Fe	Mn	Cu
0	251.88	20.99	9.04	1.93	1.11	1.00
10	67.11	4.48	2.27	0.49	0.25	1.00
20	33.41	1.93	1.05	0.26	0.11	1.00
30	25.18	1.28	0.74	0.16	0.08	1.00
40	20.16	1.09	0.58	0.13	0.06	1.00

* Zn content was assumed as 1.

Evaluation of TPC in extracts. TPC in methanolic extracts from the fresh weight of asparagus plantlets grown in media supplemented with zinc oxide nanoparticles ranged from 37.49 mg GAE g⁻¹ FW (for 40 mg dm⁻³ ZnONPs) to 50.59 mg GAE g⁻¹ FW (for 20 mg dm⁻³ ZnONPs), as in Figure 2. TPC values of 43.93 and 50.59 mg GAE g⁻¹ FW were higher in the 10 and 20 mg dm⁻³ ZnONPs treatments, respectively, than in the control (41.53 mg GAE g⁻¹ FW). In contrast, samples from the 40 mg dm⁻³ ZnONPs treatment showed significantly lower TPC values compared to the control (Figure 2). No correlation was found between TPC and ZnONPs concentrations 0 (control), 10, 20, 30, and 40 mg dm⁻³.

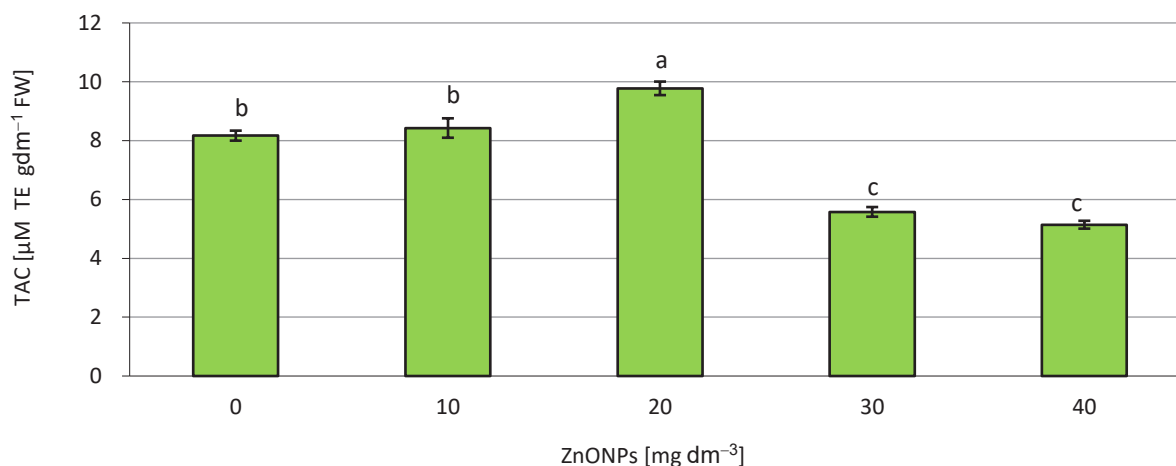
Figure 2. Total phenolic content (TPC) in methanolic extract of fresh asparagus (*Asparagus officinalis* L.) plantlets. Treatments of zinc oxide nanoparticles ZnONPs: 0 (control), 10, 20, 30, 40 mg dm⁻³. Error bars represent $\pm$ SD. Different letters indicate significant differences according to Tukey's test ($p < 0.05$)



Determination of total antioxidant capacity (TAC). TAC was measured in extracts from asparagus plantlets using a method based on the reduction of the DPPH radical and expressed as Trolox equivalents [Addai et al. 2013]. In treatments with ZnONPs at concentrations of 10–40 mg dm⁻³ TAC, ranged from 5.14 μ M TE g⁻¹ FW (40 mg dm⁻³ ZnONPs) to 9.78 μ M TE g⁻¹ FW (20 mg dm⁻³ ZnONPs), as in Figure 3. The Pearson correlation coefficient between ZnONPs concentrations and TAC data was negative ($r = -0.708$), as in Table 4. The antioxidant capacity showed different responses depending on the concentration of ZnONPs. In the

control sample TAC was $8.17 \mu\text{M TE g}^{-1} \text{FW}$ (Figure 3). A concentration of 10 mg dm^{-3} ZnONPs caused a slight increase in antioxidant activity ($8.43 \mu\text{M TE g}^{-1} \text{FW}$). The highest TAC value was recorded at 20 mg dm^{-3} ZnONPs ($9.78 \mu\text{M TE g}^{-1} \text{FW}$), corresponding to 119.71% of the control value. ZnONPs concentrations of 30 and 40 mg dm^{-3} led to a decrease of TAC values by 32% and 37%, respectively (Figure 3).

Figure 3. Total antioxidant capacity (TAC) in extracts of fresh asparagus (*Asparagus officinalis* L.) plantlets determined by DPPH method. Treatments of ZnONPs: 0 (control), 10, 20, 30, 40 mg dm^{-3} . Error bars represent + SD. Different lowercase letters indicate significant differences according to Tukey's test ($p < 0.05$)



Determination of photosynthetic pigment content. The content of photosynthetic pigments in asparagus plantlets was significantly affected by ZnONPs concentration (Figure 4). In the treatments with 10 and 20 mg dm^{-3} ZnONPs, the contents of chl a (12.0% and 28.4% respectively) and chl b (22.4% and 49.5%, respectively) increased compared with the control (Figure 4). The strongest reduction in total chlorophyll was observed in 40 mg dm^{-3} ZnONPs treatment corresponding to a 19.5% decrease compared with the control. The chlorophyll a : b ratio was 2.54 in the control and ranged from 2.13 to 2.33 in the treatments with ZnONPs. Carotenoid content (Figure 4) increased by 2.6% and 23.1% at 10 and 20 mg dm^{-3} ZnONPs, respectively, but decreased by 1.5% and 26.2% at 30 and 40 mg dm^{-3} , respectively, compared to the control ($29.67 \mu\text{g g}^{-1}$).

Figure 4. Content of chlorophylls a, b, a + b and carotenoids in fresh weight of asparagus (*Asparagus officinalis* L.) plantlets. Treatments of ZnONPs: 0 (control), 10, 20, 30, 40 mg dm^{-3} . Error bars represent + SD. The letters refer only to the respective photosynthetic pigment. Different letters indicate significant differences according to Tukey's test ($p < 0.05$)

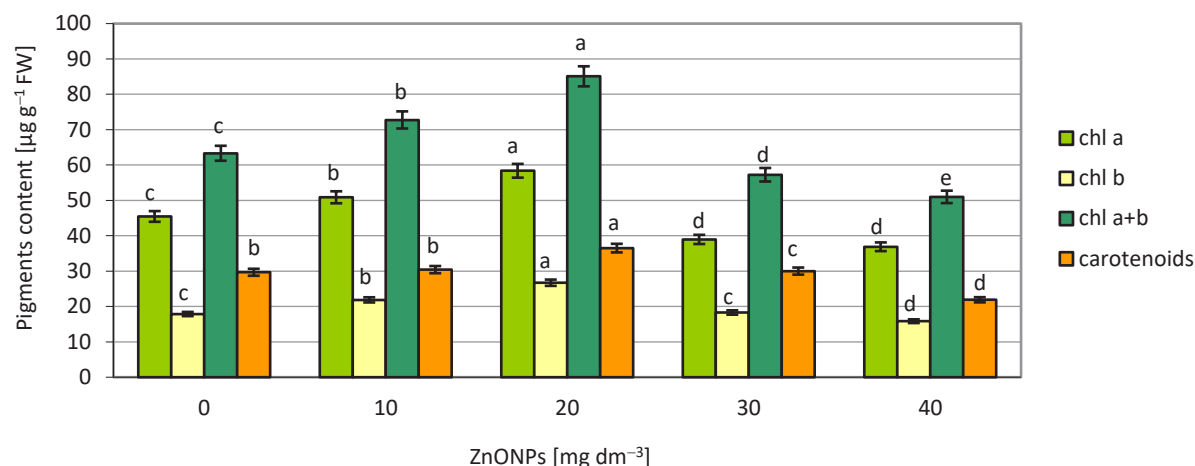


Table 4. Pearson correlation coefficient between the measured parameters

Measurements	ZnONPs treatments	TPC	TAC	chl a	chl b	chl a + b	carotenoids
ZnONPs treatments	1	–	–	–	–	–	–
TPC	–0.331	1	–	–	–	–	–
TAC	–0.708*	0.843*	1	–	–	–	–
chl a	–0.515	0.907	0.952	1	–	–	–
chl b	–0.256	0.852	0.740	0.810	1	–	–
chl a + b	–0.444	0.929	0.918	0.976	0.918	1	–
carotenoids	–0.490	0.940	0.824	0.846	0.770	0.857	1

* correlation coefficients are significant at $p < 0.05$

Pearson correlation analysis revealed negative correlations between ZnONPs concentration and all parameters presented in Table 4, with the strongest negative correlations observed for TAC ($r = -0.708$) and chl a content ($r = -0.515$). The remaining correlation coefficients were positive and high between photosynthetic pigments (ranging from $r = 0.770$ to $r = 0.976$), between photosynthetic pigments and TPC (ranging from $r = 0.852$ to $r = 0.940$), and between photosynthetic pigments and total antioxidant capacity (ranging from $r = 0.740$ to $r = 0.952$) (Table 4). A strong positive correlation was also observed between TPC and antioxidant activity measured by the DPPH method ($r = 0.843$), as in Table 4.

DISCUSSION

To date, asparagus has been propagated *in vitro* using various types of explants (from seedlings, stems, buds, nodes, and internodes) on MS medium supplemented with different growth regulators, employing both direct and indirect organogenesis methods involving callus tissue, for species such as *Asparagus officinalis* [Murashige et al. 1972, Pontaroli and Camadro 2005, Sarabi and Almasi 2010, Štajner 2013, Esmaeili et al. 2016, Rasad et al. 2019, Minh et al. 2022], *Asparagus racemosus* [Singh et al. 2023, Sharma et al. 2025], *Asparagus maritimus* [Štajner et al. 2002], and *Asparagus cochinchinensis* [Wang et al. 2022].

In the present study, seedling explants of the cultivar Argenteuil were used for the direct regeneration of asparagus shoots and roots on MS medium supplemented with 0.20 mg dm^{-3} BAP, 0.20 mg dm^{-3} NAA, and 0.25 mg dm^{-3} Kin, as well as with additional amounts of 10, 20, 30, and 40 mg dm^{-3} ZnONPs. The applied combination of plant growth regulators supported efficient direct regeneration and vigorous growth of asparagus plantlets in the control treatment (Table 1, Figure 1). This response is consistent with findings reported for different asparagus genotypes and species: *A. officinalis* [Štajner 2013], *A. racemosus* [Singh et al. 2023]. Rasad et al. [2019] also used seedling fragments of *A. officinalis* as explants for direct organogenesis. They observed the onset of germination after 2 weeks and obtained fully developed seedlings after 4 weeks. In their study, the highest number of shoots and roots regenerated from seedling fragments on MS medium supplemented with BAP and NAA. Appropriate combinations of plant growth regulators can effectively support direct organogenesis in different asparagus species and cultivars, including *A. officinalis* [Pontaroli and Camadro 2005, Sarabi and Almasi 2010, Esmaeili et al. 2016, Minh et al. 2022], as well as *A. maritimus* [Štajner et al. 2002] and *A. cochinchinensis* [Wang et al. 2022].

A positive effect of ZnONPs has been observed in plants grown *in vitro*. The influence of ZnONPs on plant propagation in tissue culture depends on the species and the applied concentration. In most cases, low and moderate concentrations stimulate growth, regeneration, and the development of callus, shoots, roots, and fresh biomass of plantlets [Singh et al. 2023]. In the present study, ZnONPs at concentrations of 10 and 20 mg dm^{-3} did not stimulate the growth and development of asparagus plantlets compared with the control (Table 1). This finding indicates that the response to ZnONPs may vary considerably among plant species and experimental conditions. However, ZnONPs treatment increased the contents

of several minerals, particularly Zn and K, while low to moderate ZnONPs concentrations also enhanced phenolic content, antioxidant activity, and photosynthetic pigment content compared with the control (Tables 2–3, Figures 2–4). Zinc oxide nanoparticles stimulated the growth and development of *Olea europaea* L. cv. Moraiolo explants *in vitro* [Regni et al. 2022]. Improved shoot regeneration in *Stevia rebaudiana* *in vitro* cultures in the presence of ZnONPs was also reported [Javed et al. 2018]. In *Brassica nigra* *in vitro* culture, ZnONPs stimulated root and shoot regeneration [Zafar et al. 2016]. *Panicum virgatum*, *Thymus kotschyanus*, and *Zataria multiflora* responded to ZnONPs with increased callus induction [Nair et al. 2010, Mosavat et al. 2019]. In blackberry cv. Nawaho and stevia cultivars Morita and Candy, lower concentrations of ZnONPs promoted shoot regeneration [Krzepiłko et al. 2024a, Krzepiłko et al. 2024b].

In the present study, an inhibitory effect of increasing ZnONPs concentrations on shoot number, shoot length, root number, root length, and fresh weight of plantlets was observed. Other authors have also confirmed that zinc oxide nanoparticles may negatively affect plant regeneration processes *in vitro*. Zinc oxide nanoparticles have also been applied in asparagus micropropagation. Desoukey et al. [2019] observed callus formation in all explants of *A. officinalis* in the presence of ZnONPs and 2 mg dm⁻³ BAP. The best results were obtained with 10 mg dm⁻³ ZnONPs, where callus fresh weight increased by 352.5% compared to the control. As ZnONPs concentration increased up to 10 mg dm⁻³, callus fresh weight gradually increased. However, above this concentration, a systematic decrease in callus fresh weight was observed up to 50 mg dm⁻³. The highest concentration (100 mg dm⁻³ ZnONPs) caused a marked reduction in callus fresh weight by 48.8% compared to 50 mg dm⁻³ [Desoukey et al. 2019]. In *Brassica napus* cultures, callus biomass decreased with increasing ZnONPs concentration; however, low concentrations stimulated callus growth and organogenesis [Mousavi Kouhi and Lahouti 2018]. In pot experiments, ZnO nanoparticles at a concentration of 500 ppm provided the best results in terms of morphological traits of *A. officinalis*, including germination capacity, seedling length, vigor index, plant height, number of internodes of the main shoot, number of lateral shoots, fresh and dry weight, and dry-to-fresh weight ratio (DW/FW) compared to the control. One possible reason for growth inhibition is oxidative stress induced by ZnONPs. Biochemical analyses showed increased catalase activity in asparagus plants treated with ZnONPs, indicating activation of antioxidant defense mechanisms and leading to increased production of secondary metabolites [Desoukey et al. 2020].

The use of ZnONPs for zinc biofortification of crops may improve the nutritional value of asparagus. Traditionally grown asparagus plants are rich in trace elements; however, different plant organs accumulate minerals to varying extents. In *in vitro* cultures, the addition of ZnONPs to the medium significantly increased Zn content in plants (Table 2). In the present study, Zn content in plantlets increased with increasing ZnONPs concentration, reaching up to 14 times higher levels than in the control at 40 mg dm⁻³. Moreover, significantly higher levels of K, Cu, Fe, and Mg were recorded in plantlets from ZnONPs treatments compared to the control (Tables 2, 3). Appropriately selected concentrations of ZnONPs may act as environmentally friendly growth stimulators and elicitors of secondary metabolites. In the present study, asparagus plants grown on medium supplemented with 20 mg dm⁻³ ZnONPs showed the highest levels of phenolic compounds, photosynthetic pigments, and antioxidant capacity (Figure 2–4). The phenolic content in asparagus depends on species, cultivation conditions, and extraction methods. In *in vitro* culture, ZnONPs at a concentration of 50 mg dm⁻³ increased total flavonoid content, including rutin [Desoukey et al. 2020]. Increased antioxidant properties and phytochemical content were observed in *A. racemosus* propagated *in vitro* compared to *in vivo* plants [Kumar et al. 2020]. In traditionally cultivated asparagus, TPC varies among green, purple, and white varieties. Purple and green varieties contain the highest levels of phenolics, with rutin being the predominant compound, whereas white varieties contain the lowest levels and lack rutin [Maeda et al. 2005]. The total phenolic content in asparagus extracts also depends on cultivation methods and extraction solvents [Esmaeili et al. 2016]. DPPH radical scavenging is a widely used method for evaluating the antioxidant potential of plant extracts. In the present study, a strong positive correlation between TPC and antioxidant activity measured by the DPPH method was also observed (Table 4). Significant differences in DPPH scavenging capacity have been reported among traditionally cultivated asparagus varieties, with purple varieties showing the highest antioxidant activity, green vari-

eties intermediate activity, and white varieties the lowest. A strong relationship between TPC and DPPH scavenging activity has been demonstrated in edible parts of asparagus from different cultivars [Maeda et al. 2005]. In asparagus plants derived from *in vitro* cultures, *in vivo* plants, and callus tissue, a positive correlation between TPC and DPPH scavenging activity has also been reported [Esmaeili et al. 2016]. Extracts from *in vitro*-grown *A. racemosus* plants showed higher levels of phenolics, flavonoids, and carotenoids compared to *in vivo* plants [Kumar et al. 2020]. In asparagus plants grown *in vitro* with ZnONPs, high positive correlations were also observed between photosynthetic pigment content and TPC and TAC (Table 4). Although chlorophyll content in *in vitro*-grown asparagus plants was lower than in mature plants reported by other authors, ZnONPs at concentrations of 10 and 20 mg dm⁻³ increased chlorophyll content compared to the control (Figure 4). Mature asparagus plants grown under field conditions exhibited total chlorophyll content ranging from 0.20 to 0.38 mg g⁻¹ FW, a chlorophyll a : b ratio of 1.5–2.86, and carotenoid content of 0.059–0.123 mg g⁻¹ FW [López-Moreno et al. 2025]. In greenhouse-grown young shoots of *A. officinalis*, chlorophyll content was reported as 1.27 mg g⁻¹ FW for chlorophyll a, 0.95 mg g⁻¹ FW for chlorophyll b, and 2.23 mg g⁻¹ FW for total chlorophyll (a + b) [Liu et al. 2025]. Overall, ZnONPs may be useful as elicitors and biofortifying agents rather than as growth-promoting additives in asparagus micropropagation. Further studies are needed to optimize their concentrations and assess the persistence of these effects after acclimatization and under *ex vitro* conditions.

CONCLUSION

In vitro culture methods may serve as an effective approach for asparagus propagation, which can be highly beneficial not only for the efficient production of plantlets but also for the acquisition of metabolites. In the present study, successful micropropagation of *A. officinalis* was achieved using seedling explants. Higher concentrations of ZnONPs had a negative effect on the organogenesis of asparagus explants. A negative correlation was observed between ZnONPs concentration and morphological trait values. Additionally, high concentrations of ZnONPs adversely affected the morphological characteristics of *A. officinalis* under *in vitro* conditions. At the same time, it was demonstrated that ZnONPs in *in vitro* cultures may enhance the content of minerals, phenolic compounds, antioxidants, and photosynthetic pigments in asparagus plantlets. Compared with control plantlets, those regenerated on media supplemented with low and moderate ZnONPs exhibited higher levels of metals (K, Mg, Fe, Zn, Cu), phenolic compounds, antioxidants, and photosynthetic pigments. Strong positive correlations were recorded between TPC and TAC, and between these parameters and photosynthetic pigments content. The presented results may be useful for optimizing ZnONPs concentrations in the culture medium in order to improve the growth and development of *A. officinalis* under *in vitro* conditions. Moreover, there is still limited information regarding the content of bioactive compounds and their antioxidant activity in asparagus derived from *in vitro* cultures. The findings presented in this study may be of interest both for expanding scientific knowledge and for potential practical applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF THE USE OF GENERATIVE AI TOOLS IN THE PROCESS OF PREPARING THE PAPER

The authors declare that no generative AI tools were used in the preparation of this manuscript.

AUTHORS CONTRIBUTION

A.K., R.P.: methodology, conceptualization, project administration, data curation, visualization, writing – original draft preparation; A.K., R.P., A.Ś.: formal analysis, investigation, resources, supervision, writing – review and editing. All authors have read and agreed to the published version of the manuscript

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EFFECTS OF NaCl STRESS ON GROWTH, ION HOMEOSTASIS AND SECONDARY METABOLITE ACCUMULATION IN GARDEN CRESS (*Lepidium sativum* L.) AND PURSLANE (*Portulaca oleracea* L.)

Tolga Sarıyer¹, Nusret Özbay²

ABSTRACT

NaCl represents a major source of salinity stress in agricultural lands, particularly in coastal regions where irrigation is practiced. Plant species differ considerably in their tolerance to salinity stress and this variation influences growth performance and plant physiology. In this study, garden cress (*Lepidium sativum* L.), known for its high antioxidant capacity and purslane (*Portulaca oleracea* L.), a halotolerant vegetable species, were cultivated under four irrigation water salinity levels (0, 50, 100 and 200 mM NaCl). Growth parameters (plant fresh weight and plant height), physiological traits (total chlorophyll content, tissue electrical conductivity), biochemical attributes (ascorbic acid and selected flavonoids, namely apigenin and catechin) and mineral nutrient contents (Mg, Na and K) were evaluated. Salinity significantly affected all measured parameters and induced pronounced species-specific responses. The NaCl concentration associated with the highest flavonoid accumulation differed between the two species; for purslane, the maximum flavonoid content was obtained in catechin under 200 mM NaCl treatment (37.806 mg kg⁻¹), whereas for cress, the maximum flavonoid content was obtained in apigenin under 100 mM NaCl treatment (39.074 mg kg⁻¹). Compared with the control treatment, increasing salinity resulted in consistent reductions in plant fresh weight and plant height, whereas tissue electrical conductivity and Na accumulation increased. Changes in K and Mg contents reflected ion imbalance induced by NaCl stress. The results indicate that two species exhibit distinct physiological and biochemical adaptive strategies in response to NaCl stress.

Keywords: *Portulaca oleracea* L., *Lepidium sativum* L., salinity stress, flavonoids, apigenin, catechin

INTRODUCTION

Today, salinity is considered one of the most important abiotic stress factors limiting agricultural production. Particularly in agricultural areas close to coastal regions, irrigation waters used may contain high salt concentrations, or salinity levels may increase over time. An increase in irrigation water salinity reduces soil water potential, thereby limiting water uptake by plants and leading to adverse effects on growth, yield, and quality parameters.

Although sodium chloride is the most abundant salt, it is also present in rainwater at concentrations of 6–50 mg/kg. High salinity can be toxic to plants and also adversely affects water uptake. Cell growth is affected by soil salinity more rapidly than plant functions. In plants, the response to the increase in external osmotic pressure resulting from elevated salt levels around the roots is faster than the response to Na⁺ accumulation in the leaves. Plants can adapt to salinity by tolerating osmotic stress, excluding Na⁺, and exhibiting tolerance to Na⁺, while also differing in their degree of salinity tolerance [Munns and Tester 2008].

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In the study by [Negrão et al. 2017], it was reported that the leaves of some plants were more successful than those of other plants in maintaining their photosynthetic functions as a result of adverse effects, primarily including yellowing, caused by Na accumulation at toxic levels. At the same time, it was recommended that the effects of salinity on plants should be evaluated at the level of components related to salinity tolerance. One of the key strategies to mitigate salinity stress is the characterization of agricultural species that exhibit relatively high salinity tolerance. In addition, understanding how yield, quality traits, and adaptive responses of these species change under different salinity levels is of considerable importance. In the study by Alam et al. [2021], 27 tomato cultivars were grown under high salinity conditions and evaluated based on growth parameters, relative leaf water content, percent yield reduction, membrane stability index, measurement of stress tolerance indices, photosynthetic pigment measurement, mineral analysis, leaf proline content, total phenols and flavonoids, free radical scavenging capacity, and enzyme measurements. The study reported significant findings regarding the salt tolerance levels of certain tomato cultivars and stated that these data could be utilized in tomato improvement and grafting studies.

Some plant species are utilized for human consumption, not only through agricultural production but also by being collected from their natural habitats. The ability of some of these plants to grow without requiring care under conditions in which cultivated plants need maintenance is considered an indication of their tolerance to environmental stress factors such as diseases, pests, drought, and salinity. Polat et al. [2015] reported that 55 wild plants from the Espiye-Giresun region of Türkiye were used for medicinal purposes, including plant parts such as leaves, capitula, flowers, roots and whole plant biomass. It has been stated that some of these plants are used in folk medicine against ailments such as flu, high cholesterol, diabetes disease and the common cold.

Within this framework, purslane (*Portulaca oleracea* L.) emerges as a prominent vegetable due to its resilience. Although it is cultivated to a limited extent in Türkiye, purslane is widely consumed by being collected from the wild, and it has gained significant attention due to its high antioxidant, mineral, and vitamin content. Previous studies have shown that purslane is more tolerant to salinity compared to many other vegetable species [Shannon and Grieve 1999, Alam et al. 2014]. Indeed, studies conducted on different genotypes have reported that purslane exhibits responses ranging from tolerant to sensitive under salinity stress, and that this tolerance varies depending on the genotype [Alam et al. 2014]. Another study has described purslane (*Portulaca oleracea* L.) as a highly salt-tolerant plant, i.e. a halophyte [Yazdani-Biouki et al. 2023].

Garden cress (*Lepidium sativum* L.) is a leafy vegetable with high antioxidant and anti-inflammatory properties, largely due to its high flavonoid content [Vazifeh et al. 2022]. At the same time, in a study [Sat et al. 2013] conducted on two different garden cress cultivars (*Lepidium sativum* cv. Dadas, İzmir), it was reported that cress is a good source of ascorbic acid and mineral elements.

Among the mechanisms of salt stress tolerance in plants are the maintenance of ion homeostasis, regulation of ion transport, osmotic adjustment, and enhancement of antioxidant metabolism. However, information regarding ion transporters involved in salt uptake, transport, and compartmentation remains limited for many agricultural crops [Balasubramaniam et al. 2023].

Flavonoids are important secondary metabolites that are widely present in plants and play a protective role against biotic and abiotic stresses. Apigenin and catechin are flavonoid compounds that stand out for important antioxidant and defensive roles in plants as well as for their health benefits in humans such as antioxidant, anti-inflammatory, and anticancer properties [Patel et al. 2007, Ganeshpurkar and Saluja 2020]. Various stress conditions have been reported to increase flavonoid accumulation, and this effect varies depending on the type and intensity of the stress [Plazas et al. 2019, Zarinkamar et al. 2021].

Ascorbic acid (vitamin C) is also an important antioxidant in plants and exhibits species-dependent responses under salinity stress [Neocleous et al. 2014]. In addition, salinity is known to alter the uptake and distribution of mineral elements such as Na, K, and Mg in plants, and ion imbalance is recognized as one of the primary effects of salt stress [Ghassemi Golezani and Nikpour-Rashidabad 2017, Hannachi and Labeke 2018].

The aim of this study is to comparatively examine the responses of garden cress (*L. sativum* L.) and purslane (*P. oleracea* L.) to different NaCl levels in terms of growth, physiological, biochemical, and mineral nutrition parameters.

MATERIALS AND METHODS

Experimental site and plant material

The experiment was conducted in a greenhouse at the Dardanos Campus of Çanakkale Onsekiz Mart University (ÇOMÜ). Purslane (*P. oleracea* L. cv. Tunç) and garden cress (*L. sativum* L. cv. Bahar) were used as the plant materi-

als. The plants were subjected to four salinity levels using NaCl supplied through irrigation water. Treatments were: 0 (control), 50, 100, and 200 mM NaCl.

Growth conditions and experimental design

Plastic pots (36 cm length × 27 cm width × 7 cm height) with drainage holes and a total surface area of 972 cm² were used in the experiment. Twelve sowing points were established at equal intervals in each pot, providing an allocated surface area of 81 cm² per sowing point and the study consisted of twelve pots for each plant and each pot had 12 sowing points. Ten seeds were sown at the center of each point. Following seedling emergence, thinning was performed to minimize plant competition, leaving four uniform plants per sowing point. The 4 plants located at the center of the 81 cm² area. As a result, each plant had a surface area of 20.25 cm² available for growth.

Growing medium

Field soil sourced from the Dardanos Research and Application Farm of the Faculty of Agriculture at Çanakkale Onsekiz Mart University was used as the growing medium. Prior to sowing, the soil was sieved to remove debris, homogenized, and distributed equally into the pots. The basic physical and chemical properties of the growing medium are presented in Table 1.

Table 1. Physical and chemical properties of the growing medium used in the experiment

Parameter	Unit	Analysis result	Lower limit	Upper limit	Low	Sufficient	High
pH (su)	pH	7.9	6.0	7.2	–	–	*
Organic matter	%	2.5	2.9	6.2	*	–	–
Phosphorus (M3)	mg kg ⁻¹	low	20	40	*	–	–
Total nitrogen	g/kg	sufficient	1	2	–	*	–
Potassium (available)	mmol(+) kg ⁻¹	high	1.5	3	–	–	*
Calcium (available)	mmol(+) kg ⁻¹	high	15	25	–	–	*
Magnesium (available)	mmol(+) kg ⁻¹	high	4.5	10	–	–	*
Organic carbon	g kg ⁻¹	14.2	17	50	*	–	
Potential mineralized nitrogen	mg N kg ⁻¹	high	22	32	–	–	*
Cation exchange capacity	mmol(+) kg ⁻¹	438	75	200	–	–	*
Total aluminum	g kg ⁻¹	low	94	115	*	–	–
Total iron	g kg ⁻¹	high	5	8	–	–	*
Clay	%	40	20	40	–	–	*
Soil moisture	%	6.4	10	30	*	–	–

* Indicates the levels (low, sufficient, or high) of the physical and chemical properties.

Growing conditions and salt stress treatments

Seeds of purslane and garden cress were sown at a depth of approximately 2 mm in the soil. The soil surface was covered with 1 cm of peat, both to prevent seed exposure by surface erosion during irrigation and to provide a more permeable surface for easier seedling emergence compared to field soil. The plants were irrigated every evening with irrigation water used for agricultural purposes in the existing greenhouses (EC: 470 µS cm⁻¹) using a strainer-equipped bucket, until water was observed exiting from the drainage holes at the bottom of the pots.

The newly emerged plants were initially grown under slightly windier external conditions to promote root development and were cultivated in the greenhouse 18 days after sowing (the first week of September). During the experiment, the greenhouse ventilations and doors were kept continuously open. To allow the plants to reach a certain developmental stage before initiating salt stress, they were irrigated only with water in the greenhouse for 14 days. After this period, salt stress treatments were applied exposing the plants to NaCl stress for 17 days. Plants that reached harvest maturity were harvested simultaneously in all treatments. Samples that were not analyzed immediately were stored in a deep freezer (–20 °C) until the time of analysis.

Experimental design

The experiment was established according to a Randomized Complete Block Design, and each treatment was conducted with three replications, with each replication (plot) being consisted of one pot as described above. Accordingly, from the total of 48 plants (12 hills/sowing points × 4 plants) in each replication, 12 morphologically uniform and healthy plants were selected, and all morphological measurements and chemical analyses were carried out on these plants.

Measurements and analyses

Chlorophyll measurements were conducted directly on the leaves, while for all other chemical analyses, the edible parts of purslane and garden cress, including leaves and tender stems, were used.

Plant fresh weight (g)

The harvested plants were cut at the root-shoot junction, and the fresh weights of the plants were determined using a precision balance.

Plant height (cm)

Plant height was measured and recorded from the root collar to the tip of the plant.

Ascorbic acid (vitamin C) content (mg 100 g⁻¹)

Ascorbic acid analysis was performed according to the method of [Pearson and Churchill 1970]. For this purpose, 25 g of fresh sample was weighed and treated with 175 mL of 0.04% oxalic acid. The mixture was filtered through filter paper, and 1 mL of the filtrate was diluted with 9 mL of distilled water. Similarly, 1 mL of the sample was mixed with 9 mL of 2,6-dichlorophenol indophenol. The transmittance values of the reference and sample solutions were measured at a wavelength of 520 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800), and the ascorbic acid contents were calculated.

Apigenin and catechin content (mg kg⁻¹)

For sample extraction, 10 mL of 96% ethanol was added to 2 g of plant material, mixed in a homogenizer for 2 minutes, and incubated overnight in a water bath at 45 °C. The samples were then centrifuged at 4000 rpm for 5 minutes, and the supernatant was completely evaporated using a rotary evaporator. The resulting extracts were dissolved in 1 mL of methanol [Kiselev et al. 2007]. The amounts of apigenin and catechin were determined using high-performance liquid chromatography (HPLC). Analyses were performed with a Shimadzu Prominence HPLC system (DAD detector, SPD-M20A). The mobile phase consisted of A: 3% formic acid and B: methanol, and the method was modified from [Gomes et al. 1999].

Total chlorophyll content (SPAD)

Total chlorophyll content was determined using a portable chlorophyll meter (SPAD). SPAD measurements were taken in the early morning from the upper surface of leaves that were neither too young nor too old.

Tissue membrane permeability

Tissue electrical conductivity was determined according to the method of [Fan and Sokorai 2005]. Five grams of leaf sample were incubated in 50–70 mL of distilled water at 23 °C on a shaker (100 rpm). Tissue electrical conductivity values were measured at the 1st (C₁) and 60th (C₆₀) minutes. The samples were then autoclaved at 121 °C for 25 minutes, cooled, and the total conductivity (C_t) was measured. Membrane permeability was calculated using the following equation:

$$E = (C_{60} - C_1) / C_t \times 100$$

Analysis of Na⁺, K²⁺, and Mg⁺

For elemental analyses, the samples were digested in a microwave oven. For this purpose, 1 g of dry sample was placed in Teflon vessels, and 10 mL of 65% concentrated HNO₃ was added. The dissolution was carried out in a closed system in a CEM MARS 6 microwave oven for a total of 30 minutes, with the temperature raised up to 200 °C. Elemental analyses were performed using an atomic absorption spectrophotometer (A Analyst 800, Perkin

Elmer). Calibration curves were prepared with single-element standards, and the results were calculated in mg L⁻¹. Each sample was read three times to ensure the reliability of the measurements [Jones et al. 1991, Müftüoğlu et al. 2014, Kacar 2016].

Statistical analysis

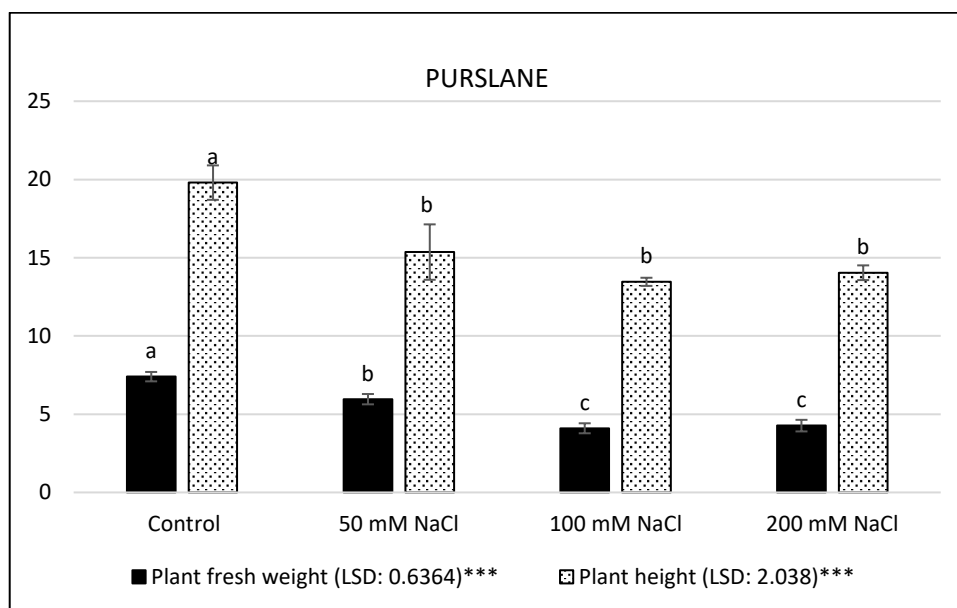
The obtained data were subjected to analysis of variance (ANOVA) using the JMP® ver. 14 statistical packages (SAS Institute, Cary, NC, USA). For factors found to be statistically significant in the ANOVA, the least significant difference (LSD) multiple comparison test was applied to determine differences among treatment means. In all statistical evaluations, the significance level was accepted as $P \leq 0.05$. The data are introduced as mean $\pm$ standard deviation ($n = 3$).

RESULTS

Growth responses of purslane and garden cress to NaCl stress

NaCl treatments significantly affected plant fresh weight and plant height in both purslane and garden cress (Figures 1, 2 and 3). Growth declined progressively with increasing salinity in both species; however, the magnitude of growth inhibition differed markedly.

Figure 1. Effects of salt stress on plant fresh weight (g) and plant height (cm) in purslane. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test



In purslane, fresh weight decreased from 7.40 g in the control to 5.96, 4.10 and 4.27 g under 50, 100 and 200 mM NaCl, corresponding to reductions of 19.5%, 44.5% and 42.3%, respectively. Plant height similarly declined by 22.4–32.0%, although no significant differences were detected between the two highest salinity treatments, indicating that growth inhibition reached a plateau under severe stress.

Garden cress exhibited substantially greater sensitivity to salinity. Fresh weight declined from 5.21 g in the control to 3.42, 2.50 and 1.93 g under 50, 100 and 200 mM NaCl, representing reductions of 34.3%, 52.0% and 62.9%, respectively. Plant height decreased progressively with increasing NaCl concentration, reaching a 58.8% reduction at 200 mM, with all salinity treatments differing significantly from one another.

Figure 2. Effect of salt stress on plant fresh weight (g) and plant height (cm) in garden cress. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. ** and *** indicate significant differences between means at $p < 0.01$ and $p < 0.001$, respectively, according to the least significant difference (LSD) test

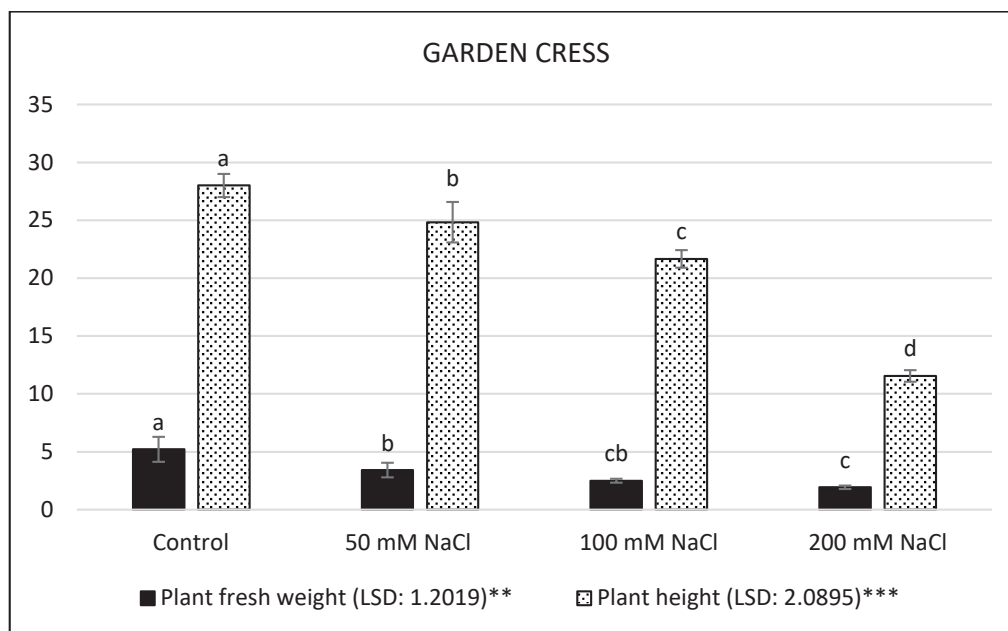
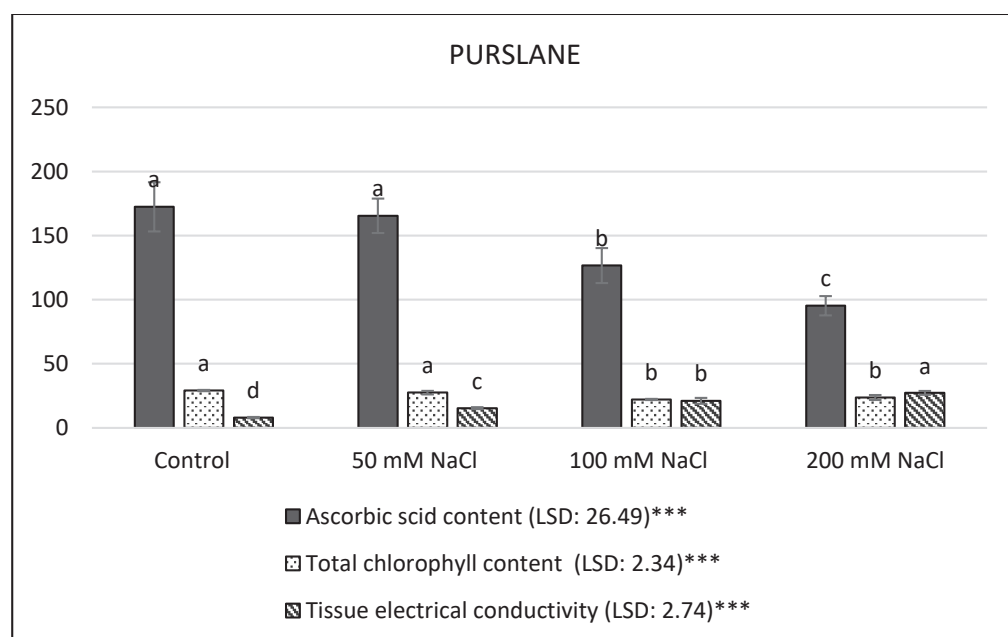


Figure 3. The effect of salt stress on ascorbic acid ($\text{mg } 100 \text{ g}^{-1}$), total chlorophyll (SPAD), and tissue electrical conductivity (%) in purslane. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test



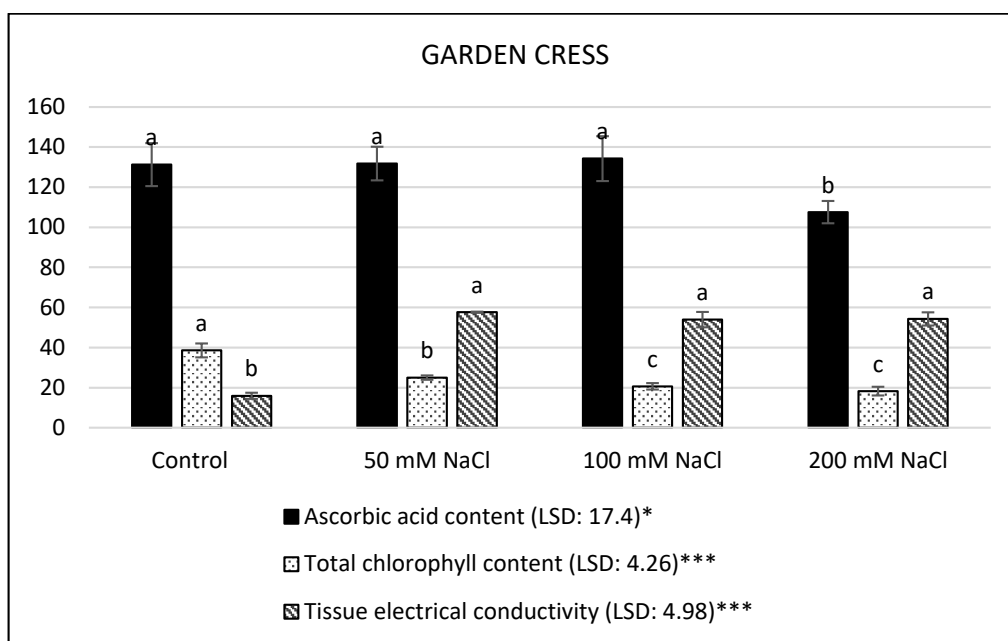
Overall, although salinity reduced vegetative growth in both species, purslane maintained considerably greater biomass and shoot growth than garden cress throughout the salinity gradient, indicating superior morphological tolerance.

Effects of NaCl stress on ascorbic acid, chlorophyll content and tissue electrical conductivity

Salinity significantly influenced ascorbic acid concentration, total chlorophyll content and tissue electrical conductivity in both species (Figures 4 and 5). In purslane, ascorbic acid declined gradually with increasing salinity, with significant reductions becoming evident at 100 and 200 mM NaCl. Total chlorophyll remained relatively stable under mild stress but decreased significantly under moderate and severe salinity. In contrast, tissue electrical conductivity increased continuously with increasing NaCl concentration, indicating progressive membrane damage.

Garden cress displayed a different physiological response. Ascorbic acid remained statistically unchanged up to 100 mM NaCl and declined only under severe salinity. However, chlorophyll content decreased sharply even at 50 mM NaCl and reached a plateau under higher salinity. Tissue electrical conductivity increased dramatically under all NaCl treatments and remained consistently higher than in purslane, suggesting that membrane integrity was disrupted even under relatively mild salt stress.

Figure 4. The effect of salt stress on ascorbic acid (mg 100 g⁻¹), total chlorophyll (SPAD), and tissue electrical conductivity (%) in garden cress. Data are presented as the mean of three replicates ± standard deviation. Means within the same column followed by the same letter are not significantly different. * and *** indicate significant differences between means at $p < 0.05$ and $p < 0.001$, respectively, according to the least significant difference (LSD) test



Collectively, these results indicate that although both species experienced physiological impairment under saline conditions, garden cress exhibited earlier chlorophyll degradation and more severe membrane damage, whereas purslane maintained greater physiological stability.

Effects of NaCl stress on catechin and apigenin accumulation

Salt stress significantly affected catechin and apigenin concentrations in both species (Figures 6 and 7). In purslane, both flavonoids exhibited a progressive increase with increasing salinity. Although slight reductions were observed under 50 mM NaCl, catechin and apigenin increased markedly under moderate and severe salinity, reaching their highest concentrations at 200 mM NaCl.

Figure 5. Effect of salt stress on catechin and apigenin content (mg kg^{-1}) in purslane. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test

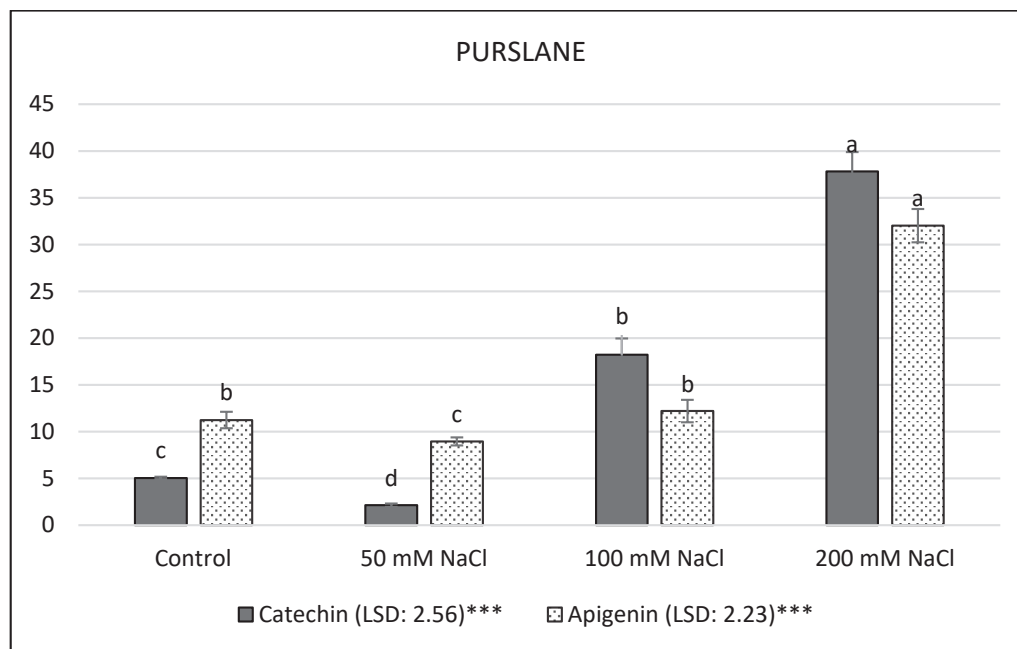


Figure 6. Effects of salt stress on catechin and apigenin contents (mg kg^{-1}) in garden cress. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test

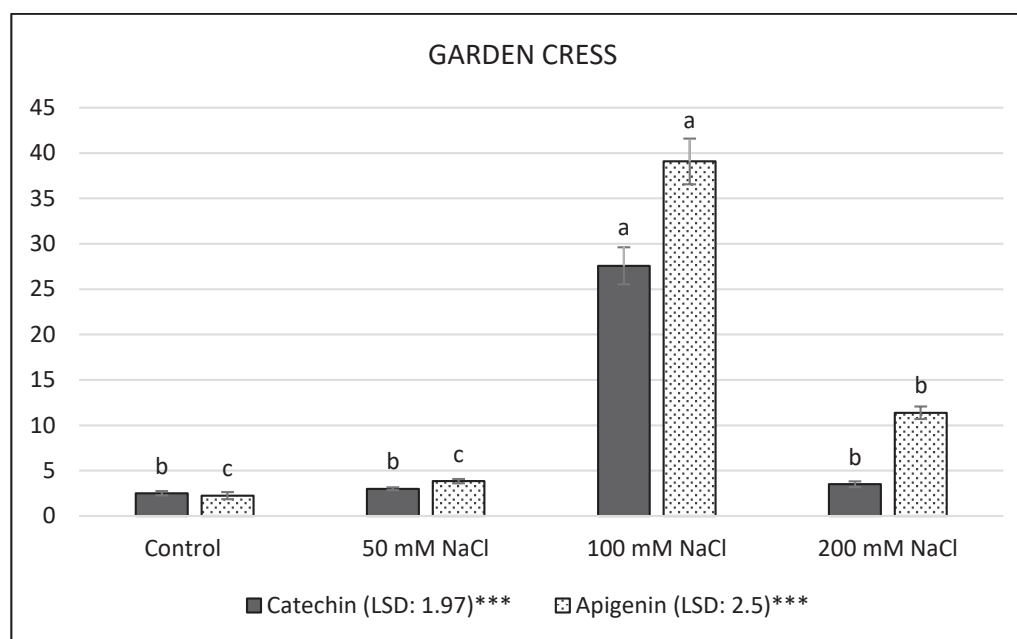
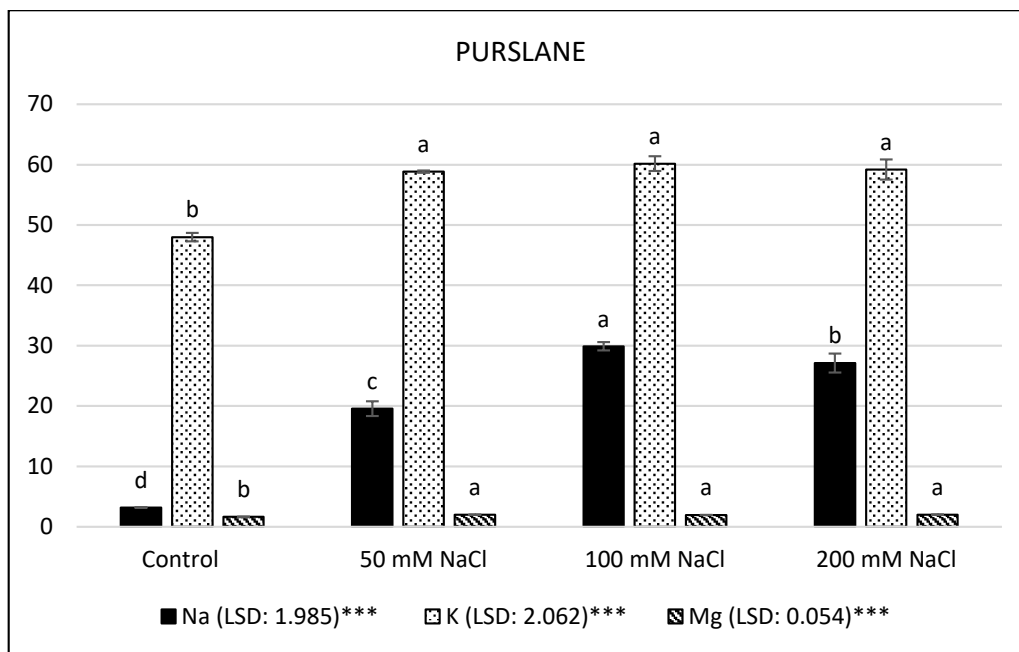


Figure 7. Effects of salt stress on elemental (Na^+ , K^+ , and Mg^{2+}) contents (g kg^{-1}) in purslane. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test



Garden cress exhibited a biphasic response. Catechin and apigenin increased substantially under moderate salinity and reached maximum levels at 100 mM NaCl, but both compounds declined sharply under 200 mM NaCl. Despite remaining above or close to control values, their concentrations were considerably lower than those observed at 100 mM NaCl.

These contrasting patterns indicate that purslane maintained flavonoid biosynthesis under severe salinity, whereas the antioxidant response of garden cress weakened once salt stress exceeded a critical threshold.

Effects of NaCl stress on mineral composition

NaCl treatments significantly affected Na^+ , K^+ and Mg^{2+} concentrations in both species (Figure 8). Sodium accumulation increased markedly with increasing salinity in both species. However, the accumulation pattern differed considerably. Purslane accumulated Na^+ while maintaining relatively stable or even increased K^+ concentrations and only minor changes in Mg^{2+} , indicating effective ionic regulation despite increasing external salinity.

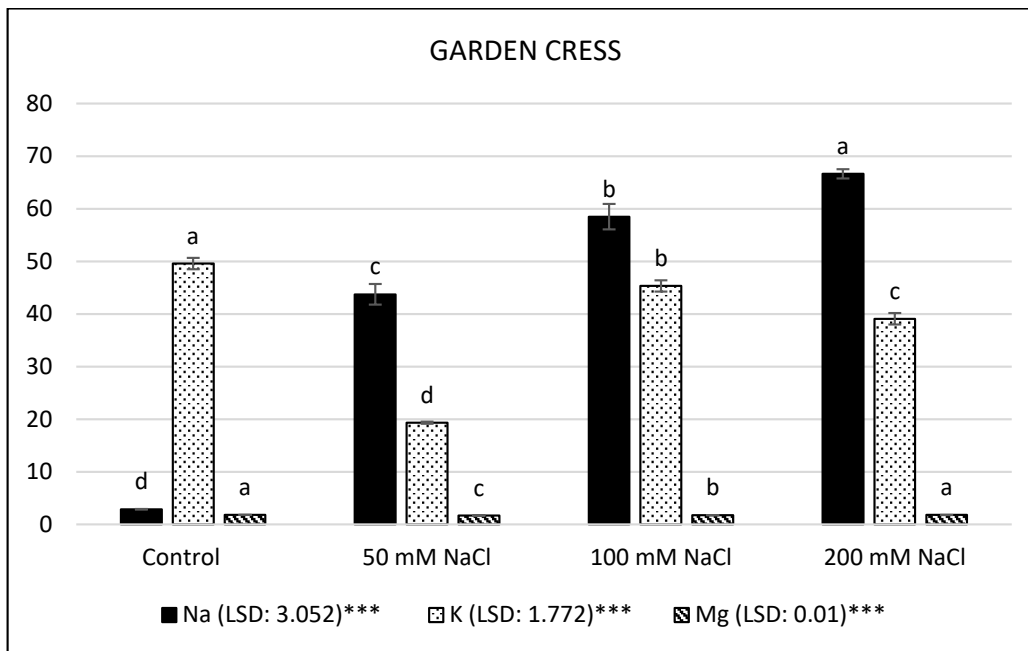
In contrast, garden cress exhibited continuous Na^+ accumulation accompanied by a pronounced reduction in K^+ concentration, particularly under low and moderate salinity. Magnesium content declined under 50 and 100 mM NaCl before partially recovering at 200 mM. Consequently, purslane maintained a substantially more favorable ionic balance than garden cress, particularly through its ability to preserve K^+ homeostasis despite elevated Na^+ accumulation.

Overall comparison between purslane and garden cress

Although salinity adversely affected both species, the severity of the responses differed considerably. Purslane exhibited smaller reductions in growth, greater preservation of chlorophyll content, lower electrolyte leakage, sustained flavonoid accumulation under severe salinity, and a superior capacity to maintain ionic homeostasis. In contrast, garden cress experienced greater growth inhibition, earlier physiological deterioration, reduced antioxidant metabolite accumulation under severe stress, and substantial disruption of mineral balance.

Taken together, the integrated morphological, physiological, biochemical and ionic responses consistently demonstrate that purslane possesses considerably greater tolerance to NaCl stress than garden cress.

Figure 8. Effects of salt stress on elemental (Na^+ , K^+ and Mg^{2+}) contents (g kg^{-1}) in garden cress. Data are presented as the mean of three replicates $\pm$ standard deviation. Means within the same column followed by the same letter are not significantly different. *** indicates significant differences between means at $p < 0.001$, according to the least significant difference (LSD) test



DISCUSSION

Salinity is among the most important abiotic stresses limiting vegetable production worldwide because it simultaneously imposes osmotic stress, ion toxicity, oxidative damage, and nutritional imbalance. Consequently, plant tolerance to salinity depends on the coordinated regulation of multiple physiological and biochemical processes rather than on a single adaptive trait. The present study clearly demonstrates that although both purslane (*Portulaca oleracea* L.) and garden cress (*Lepidium sativum* L.) were adversely affected by increasing NaCl concentrations, the magnitude and nature of their responses differed substantially. The integrated evaluation of growth, physiological characteristics, antioxidant metabolism, flavonoid accumulation, and mineral nutrition consistently identified purslane as considerably more salt tolerant than garden cress.

The reduction in plant fresh weight and plant height observed in both species agrees with the widely documented inhibitory effects of salinity on plant growth. Salt stress initially decreases soil water potential, thereby restricting water uptake and cell expansion, while prolonged exposure results in excessive Na^+ accumulation, nutrient imbalance, and reduced photosynthetic carbon assimilation, ultimately suppressing biomass production. Similar reductions in vegetative growth have been reported for purslane under saline conditions by Xing et al. [2019], and Guevara-Olivar et al. [2024], whereas Kiremit et al. [2023] described comparable responses in garden cress. However, an important distinction emerged between the two species. Growth inhibition in garden cress increased almost proportionally with salinity and became particularly severe at 200 mM NaCl, whereas purslane exhibited moderate reductions and a tendency toward stabilization under severe stress. This response is characteristic of facultative halophytes, which possess efficient mechanisms for osmotic adjustment and ion compartmentation that enable continued growth under saline environments. The contrasting growth patterns therefore indicate that purslane maintains cellular function more effectively once a critical salinity threshold has been reached, whereas the adaptive capacity of garden cress becomes progressively overwhelmed.

The physiological measurements further reinforce this interpretation. Chlorophyll degradation, electrolyte leakage, and changes in ascorbic acid collectively provide insight into the balance between oxidative damage and antioxidant protection during salt stress. Chlorophyll loss is a common consequence of reactive oxygen species (ROS)

accumulation, which accelerates pigment degradation while inhibiting chlorophyll biosynthesis. In the present study, chlorophyll remained relatively stable in purslane under mild salinity and declined only under moderate and severe stress, whereas garden cress exhibited substantial chlorophyll loss even at the lowest NaCl concentration. Similar reductions have been reported in tomato [Krupa-Mańkiewicz et al. 2015], lettuce [Kiran 2019], and garden cress [Kiremit et al. 2023]. The delayed chlorophyll degradation observed in purslane suggests a greater ability to preserve the photosynthetic apparatus and maintain carbon assimilation during salinity.

Membrane stability exhibited an equally contrasting pattern. Electrolyte leakage increased gradually with increasing NaCl concentration in purslane, whereas garden cress showed a sharp increase even under mild salinity, with little additional increase thereafter. Because electrolyte leakage reflects membrane lipid peroxidation caused by ROS, these findings indicate that membrane integrity deteriorated much earlier in garden cress than in purslane. Similar increases in membrane permeability under salt stress have been reported in purslane genotypes by Zaman et al. [2020]. The ability of purslane to delay membrane disruption likely contributes directly to its superior maintenance of growth and photosynthetic performance under saline conditions.

Interestingly, the responses of ascorbic acid differed from those of chlorophyll and membrane stability. Garden cress maintained relatively stable ascorbic acid concentrations up to moderate salinity before declining under severe stress, whereas purslane exhibited a gradual decrease throughout the salinity gradient. At first glance, this pattern could suggest greater antioxidant protection in garden cress. However, the simultaneous occurrence of severe chlorophyll degradation and high membrane leakage indicates that maintenance of ascorbic acid alone was insufficient to prevent oxidative damage. Instead, the lower ascorbic acid concentration in purslane likely reflects its utilization during ROS detoxification, while additional antioxidant mechanisms compensated for its depletion. This observation emphasizes that salt tolerance should not be inferred from a single antioxidant metabolite but rather from the overall effectiveness of the antioxidant network.

The flavonoid responses strongly support this interpretation. Flavonoids such as catechin and apigenin function as important non-enzymatic antioxidants that scavenge ROS, stabilize cellular membranes, contribute to osmotic adjustment, and regulate stress-responsive signaling pathways [Shomali et al. 2022, Wu et al. 2023]. In purslane, both compounds accumulated progressively with increasing salinity and reached their highest concentrations under 200 mM NaCl, indicating sustained activation of secondary metabolism even under severe stress. Similar protective roles of flavonoids have been emphasized by Patil et al. [2024], who identified flavonoid accumulation as an important determinant of salinity tolerance.

Garden cress exhibited a distinctly different pattern. Catechin and apigenin increased markedly under moderate salinity but declined substantially under severe stress. Comparable biphasic responses have been reported for garden cress sprouts exposed to NaCl [Farhat et al. 2023] and for plants subjected to heat stress [Alhaithloul et al. 2021]. Such responses suggest that moderate stress activates flavonoid biosynthesis, whereas prolonged or intense stress exceeds the metabolic capacity required to sustain secondary metabolite production. Consequently, the inability of garden cress to maintain flavonoid accumulation under severe salinity likely contributed to the extensive chlorophyll degradation and membrane damage observed at high NaCl concentrations. In contrast, the sustained induction of catechin and apigenin in purslane appears to compensate for the gradual depletion of ascorbic acid, thereby maintaining antioxidant protection during prolonged salt exposure.

Among all measured parameters, ion homeostasis provides perhaps the clearest mechanistic explanation for the contrasting salinity tolerance of the two species. Excessive Na⁺ accumulation disrupts enzyme activity, osmotic regulation, and nutrient acquisition, particularly through competition with K⁺ for membrane transport systems. Consequently, maintenance of a high cytosolic K⁺/Na⁺ ratio is widely recognized as one of the principal determinants of salt tolerance [Li and Si 2019, Malakar and Chattopadhyay 2021]. Both species accumulated substantial amounts of Na⁺ under saline conditions; however, their ability to regulate accompanying mineral nutrients differed markedly.

Purslane maintained or even increased K⁺ concentrations despite increasing Na⁺ accumulation while also preserving Mg²⁺ levels. Potassium is essential for enzyme activation, stomatal regulation, protein synthesis, and osmotic adjustment, whereas magnesium is indispensable for chlorophyll biosynthesis and numerous enzymatic reactions. The maintenance of these essential cations despite elevated Na⁺ strongly suggests efficient selective ion transport, intracellular Na⁺ sequestration, and effective ionic compartmentation. Similar responses have been reported in purslane by Uddin et al. [2012] and Samet and Çikili [2019], supporting its characterization as a facultative halophyte.

In contrast, garden cress accumulated considerably greater quantities of Na⁺ while simultaneously exhibiting pronounced reductions in K⁺ concentration and transient decreases in Mg²⁺. Such disruption of ionic balance is characteristic of salt-sensitive and has been associated with reduced photosynthesis [Sardar et al. 2023], impaired enzyme

activity [Zhang et al. 2022], and growth inhibition [Breš et al. 2022]. Although Mg^{2+} partially recovered under severe salinity, this adjustment appeared insufficient to compensate for the substantial loss of potassium homeostasis. Therefore, the superior performance of purslane is best explained not by reduced Na^+ uptake itself but by its greater capacity to maintain ionic balance despite elevated sodium accumulation.

Taken together, the present findings demonstrate that salinity tolerance results from the integration of multiple complementary mechanisms rather than from any individual physiological trait. Purslane maintained relatively stable growth, delayed chlorophyll degradation, preserved membrane integrity, sustained flavonoid biosynthesis, and effectively regulated K^+ and Mg^{2+} homeostasis under increasing NaCl concentrations. These coordinated responses collectively limited oxidative damage and maintained cellular function under severe salinity. In contrast, garden cress exhibited progressive growth inhibition, early impairment of photosynthesis, rapid membrane disruption, transient antioxidant activation, and pronounced ionic imbalance, indicating that its protective mechanisms become insufficient as salinity intensifies.

From an agronomic perspective, these contrasting adaptive strategies suggest that purslane is a promising leafy vegetable for cultivation in saline soils or under saline irrigation, where conventional vegetable crops often suffer severe yield reductions. Moreover, the marked accumulation of catechin and apigenin under high salinity indicates that moderate salt stress may further enhance the functional and nutritional value of purslane. Conversely, successful cultivation of garden cress appears to require low-salinity environments or management practices designed to reduce salt accumulation and improve ionic balance, including potassium fertilization, organic amendments, or exogenous biostimulant and antioxidant applications. Future research should investigate the molecular basis of the contrasting responses identified here, particularly genes involved in Na^+ transport, K^+ retention, antioxidant metabolism, and flavonoid biosynthesis, to facilitate the development of leafy vegetable cultivars with improved salinity tolerance.

CONCLUSIONS

The present study demonstrates that salinity significantly affects the growth, physiology, antioxidant metabolism, and mineral nutrition of both purslane (*Portulaca oleracea* L.) and garden cress (*Lepidium sativum* L.), although the magnitude of these responses differs markedly between the two species. The integrated evaluation of morphological, physiological, biochemical, and ionic traits consistently showed that purslane possesses substantially greater tolerance to NaCl stress than garden cress.

Compared with garden cress, purslane maintained higher biomass, delayed chlorophyll degradation, lower membrane damage, sustained accumulation of catechin and apigenin under severe salinity, and a greater capacity to preserve K^+ and Mg^{2+} homeostasis despite increased Na^+ accumulation. In contrast, garden cress exhibited greater growth inhibition, early physiological deterioration, reduced flavonoid accumulation under severe stress, and pronounced disruption of ionic balance, indicating a comparatively limited capacity to adapt to prolonged salinity.

These findings demonstrate that salinity tolerance results from the coordinated regulation of multiple adaptive mechanisms rather than from a single physiological trait. The superior performance of purslane reflects the integration of efficient ion homeostasis, maintenance of photosynthetic integrity, and sustained antioxidant defense, enabling better adaptation to saline conditions.

From an agronomic perspective, purslane appears to be a promising leafy vegetable for cultivation in saline environments while also offering enhanced nutritional value through increased flavonoid accumulation. Conversely, successful cultivation of garden cress is likely to require low-salinity conditions or appropriate stress-mitigation practices. Future studies should focus on the molecular mechanisms underlying these adaptive responses and evaluate the long-term agronomic and nutritional performance of both species under field conditions.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF THE USE OF GENERATIVE AI TOOLS

In the preparation of this manuscript, artificial intelligence (AI) tools were used solely for language editing, translation, and grammatical refinement to improve clarity and consistency. These tools were not used to generate the scientific content of the manuscript. The authors checked the output for errors and take full responsibility for the final text.

AUTHOR CONTRIBUTION

Conceptualization: T.S., N.Ö. Methodology: T.S., N.Ö. Software: T.S., N.Ö. Validation: T.S., N.Ö. Formal analysis: T.S., N.Ö. Investigation: T.S., N.Ö. Resources: T.S., N.Ö. Data curation: T.S., N.Ö. Writing, original draft: T.S., N.Ö. Writing, review and editing: T.S., N.Ö. Visualization: T.S., N.Ö. Supervision: T.S., N.Ö. Project administration: T.S., N.Ö. The authors declare that they have contributed equally to the article. All authors read and approved the final manuscript.

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PLANT GROWTH PROMOTER ENHANCED PHOTOSYNTHESIS AND FRUIT QUALITY IN STRAWBERRY. A CULTIVAR-SPECIFIC MULTIVARIATE ANALYSIS

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ABSTRACT

Spring protected strawberry production in northern China is constrained by elevated temperatures and narrowed diurnal range, yet cultivar-specific management strategies remain undefined. To identify optimized foliar treatments for three cultivars (Benihoppe, Yuexiu, White Snow Princess), plants were sprayed with S-abscisic acid (S-ABA), amino acids, jasmonate, or 5-aminolevulinic acid (5-ALA) at 10, 50, and 100 mg · L⁻¹. Growth, photosynthetic, and fruit quality traits were analyzed using two-way ANOVA, stepwise regression, OPLS-DA, and PLS-SEM. Cultivar was dominant for most traits. Optimal responses were achieved with 50 mg · L⁻¹ 5-ALA for Benihoppe, S-ABA or 10 mg · L⁻¹ 5-ALA for Yuexiu, and 10 mg · L⁻¹ 5-ALA for White Snow Princess. PLS-SEM identified vegetative growth as the central driver of fruit colour (0.922) and fruit quality (0.845), while photosynthesis exerted a significant direct negative effect on growth (−0.709), revealing a resource-based tradeoff under elevated spring temperatures. Stepwise regression confirmed that 5-ALA promoted total soluble solids (TSS) accumulation more strongly than cultivar effects for Benihoppe, whereas high concentration (100 mg · L⁻¹) suppressed fruit weight. These findings support cultivar-specific precision management, emphasizing growth promotion over photosynthetic enhancement to sustain fruit quality under spring stress.

Keywords: *Fragaria × ananassa*, protected cultivation, elevated temperature, foliar plant promoter, 5-aminolevulinic acid, multivariate analysis

INTRODUCTION

In the major strawberry-producing regions of northern China, a recurring production issue characterised by diminished fruit flavour, soft texture, and a sharp decline in marketable yield, typically occurs from late March to early April. This phenomenon is primarily driven by spring heatwaves, triggered by abrupt temperature increases from late March onwards, and by a concurrent narrowing of the diurnal temperature range. As climate change intensifies, high-temperature conditions and reduced diurnal temperature variation have emerged as key climatic constraints on protected strawberry quality [Park et al. 2023]. Strawberries possess shallow, temperature-sensitive root systems, making them highly vulnerable to temperatures outside the optimal range [Thokchom et al. 2022, Akhtar et al. 2025]. Exposure to 35 °C induces significant physiological damage, while 40 °C causes severe injury, including disrupted photosynthesis, hormonal imbalance, and oxidative stress [Manafi et al. 2021, Akhtar et al. 2025]. Moreover, rising nocturnal

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temperatures in spring consistently reduce the protected diurnal temperature range to below 8 °C. This condition, wherein high daytime temperatures suppress photosynthesis and elevated nighttime temperatures increase respiratory consumption, creates a compounded stress that ultimately reduces sugar content, increases acidity, impairs colour development, and decreases fruit size [Menzel 2022, Ullah et al. 2024]. Effective mitigation strategies are therefore essential for the sustainability of protected strawberry production.

Foliar application of bioactive compounds offers a rapid and targeted approach for enhancing plant stress tolerance. Beyond nutritional supplementation, biostimulants and plant growth regulators, such as melatonin, nitric oxide, and calcium, have shown promise in alleviating heat-induced damage in strawberries by activating antioxidant defences or inducing heat shock protein expression [Manafi et al. 2021, Wang et al. 2025]. Nevertheless, critical knowledge gaps persist. First, most studies have evaluated single compounds under controlled environments [Bieniasz et al. 2022], with limited systematic comparison of different signalling molecules under realistic stress conditions. Second, it remains unclear whether compounds such as S-abscisic acid (S-ABA), jasmonic acid, and 5-aminolevulinic acid (5-ALA) primarily enhance source capacity and protect photosynthetic systems, or whether they compensate for reduced diurnal temperature range by suppressing nocturnal respiration and reducing metabolic consumption. More broadly, their dominant physiological targets whether “source” (photosynthetic assimilation and partitioning), “sink” (fruit metabolism and growth), or cellular protection (antioxidant and osmotic regulation) have yet to be resolved [Garrido-Bigotes et al. 2017, Li et al. 2022, Zhong et al. 2025], impeding the development of physiologybased precision management. Third, although strawberry cultivars differ genetically in heat tolerance, the interaction between genotype and treatment remains poorly understood, limiting the transferability of management strategies across cultivars.

To address these gaps, a protected experiment was conducted from 13 March to 10 April 2024, a period encompassing the complete sequence of three thermal phases: an optimal window, a critical discomfort phase, and a fully unsuitable phase. Three representative cultivars Benihoppe, Yuexiu, and White Snow Princess were selected to compare the regulatory effects of foliar sprays of S-ABA, amino acid fertiliser, jasmonic acid, and 5-ALA. Using multivariate statistical approaches [Al-Madhagi et al. 2018], including Two-way analysis of variance, stepwise regression, orthogonal partial least squares discriminant analysis (OPLS-DA), and partial least squares structural equation modelling (PLS-SEM) we aimed to identify the primary physiological targets through which these signalling molecules compensate for combined lowdiurnalrange and hightemperature conditions, and to determine cultivar-specific optimal foliar management strategies. A PLS-SEM approach was employed to elucidate the causal relationships among photosynthesis, vegetative growth, fruit colour, and fruit quality. The findings are expected to provide a theoretical basis for improving stress resilience in spring protected strawberry production.

MATERIALS AND METHODS

Plant materials and experimental site. The experiment was conducted in March–April 2024 in strawberry production facilities under protected cultivation at the Henan Modern Agricultural Research and Development Base (35 °00'19" N, 113 °42'16"E). The trial period, spanning 13 March to 10 April (four weeks), coincided with the natural spring warming phase. According to data from the Yuanyang Meteorological Station, outdoor temperatures ranged from 0–24 °C in mid-March to 8–26 °C in early April, with 18 sunny days recorded during this interval. Internal protected temperature logs indicated that daily maximum temperatures reached or exceeded 35 °C on 11 days, while nocturnal temperatures consistently remained above 12 °C. The diurnal temperature amplitude progressively narrowed from over 10 °C to less than 8 °C over the course of the study. This period comprehensively covered three distinct thermal phases: a suitable window, a critical transition period, and a fully unsuitable phase. Three commercially important strawberry cultivars: Benihoppe, Yuexiu, and White Snow Princess were selected for this study. All cultivars were grown under the same protected cultivation conditions with identical mulching and uniform agronomic management throughout the experimental period.

Experimental design and plant promoter. A completely randomized block design with three replicates was employed. Seven foliar fertilizers were applied, including a water-sprayed control (CK) (Table 1).

Foliar sprays were applied to runoff on four dates (March 13, 20, 27, and April 3, 2024). Each application was performed at a volume sufficient to thoroughly wet both leaf surfaces to the point of imminent run-off. All other agronomic practices were kept uniform across treatments. Growth parameters were recorded on 10 April (after five weeks from first application). Fruits from the retained cohort, those that had developed to marketable maturity, were harvested on the same date for quality assessment.

Table 1. Description of foliar plant promoter applied in the experiment

Order number	Plant promoter description	Commercial source
T1	S-abscisic acid (S-ABA; 10% active ingredient), 1000× dilution	Jiangxi New Reward Biochemical Co., Ltd., China
T2	amino acid water-soluble fertilizer (amino acids $\geq 100 \text{ g} \cdot \text{L}^{-1}$; Mn + Zn $\geq 20 \text{ g} \cdot \text{L}^{-1}$), 500× dilution	Hebei Zhongbao Lvnong Crops Technology Co., Ltd., China
T3	propyl jasmonate-based foliar fertilizer (jasmonic acid propyl ester $\geq 100 \text{ mg} \cdot \text{L}^{-1}$), 500× dilution	Hebei Zhongbao Lvnong Crops Technology Co., Ltd., China
T4	5-aminolevulinic acid (5-ALA), $10 \text{ mg} \cdot \text{L}^{-1}$	Shanghai Macklin Biochemical Co., Ltd., China (purity $\geq 99\%$)
T5	5-aminolevulinic acid (5-ALA), $50 \text{ mg} \cdot \text{L}^{-1}$	
T6	5-aminolevulinic acid (5-ALA), $100 \text{ mg} \cdot \text{L}^{-1}$	
CK	water spray (control)	

Growth measurements. For each experimental unit, ten plants were selected. Prior to the initial application, uniform seedlings with consistent growth status and similar size were selected and labelled to minimize initial plant differences among treatments [Al-Madhangi et al. 2019]. All developed fruits were removed, and only uniform flower buds and young fruitlets (within 7 days after petal fall) were retained.

On April 10 (five weeks after the first application), leaf length, leaf width, petiole length, and petiole diameter were recorded on five randomly chosen plants per experimental unit. All leaf-related morphological parameters were measured on fully expanded functional leaves located in the middle layer of each plant, following a unified sampling standard across all treatments. Considering the short experimental period (28 days), it was difficult to accurately distinguish whether the fully expanded functional leaves emerged before or after foliar spraying; therefore, the uniform middle-layer functional leaf sampling method was adopted to ensure data comparability among different treatments. Given that all plants were selected for uniformity in size and growth status prior to treatment initiation, differences in leaf morphology among treatments after 28 days can be reasonably interpreted as treatment effects on leaf expansion. A digital calliper (Model 500-196-30, Mitutoyo, Japan) was used for petiole diameter measurements.

Leaf photosynthetic characteristics. On the same day (April 10, 09:00–12:00), net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO₂ concentration (Ci), and transpiration rate (Tr) were measured on one functional leaf per plant (three randomly chosen plants per experimental unit) using an LI-6400XT portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA). The leaf chlorophyll index (SPAD value) was determined on all ten plants per experimental unit using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Inc., Tokyo, Japan).

Fruit colour indices. On the same date (April 10), fruit colour was measured using a spectrophotometric colourimeter (CR-400, Konica Minolta, Inc., Tokyo, Japan) on the same ten commercially mature fruits per experimental unit used for quality assessment. The L*, a*, b*, C*, and h° values were obtained, and the total colour difference (ΔE) relative to the control was calculated.

Fruit quality attributes. On the same date (April 10), for each unit experiment, ten commercially mature fruits were analyzed. Single-fruit weight was recorded (BSA423S-CW, Sartorius, Germany; 0.01 g pre-

cision). Firmness was measured using a penetrometer (GY-4, Landian, China) with a 3.5-mm probe. Total soluble solids (TSS) content was determined with a digital refractometer (PAL-1, Atago, Japan). Titratable acidity (TA) and Vitamin C (Vc) content were analysed by automatic potentiometric titration using a ZDJ-4B titrator (Shanghai Yidian Scientific Instrument Co., Ltd., China). The TSS/TA ratio was calculated.

Data analysis. Data were analyzed by Two-way ANOVA with cultivar (three levels: Benihoppe, Yuexiu, and White Snow Princess) and plant promoter (Pp) (seven levels: CK, T1–T6) as fixed factors, including their interaction, followed by Tukey’s HSD post-hoc tests ($P < 0.05$). When a significant interaction was detected, simple effects analysis was conducted separately for each cultivar. Stepwise regression was performed to identify key predictors among cultivar dummy variables, plant promoter dummy variables, and 5-ALA concentration (entry/removal: $P < 0.05$ / $P > 0.10$). All data are presented as means $\pm$ sSEM. SPSS Statistics was used for ANOVA and regression; OPLS-DA was performed with SIMCA-P (v14.1); PLS-SEM was constructed using SmartPLS 4.0; Figures were generated with Origin 2024.

RESULTS

Table 2. Two-way ANOVA and multiple comparison results for various traits of strawberry under cultivar, plant promoter and their interaction

Indicator	Cultivar (C)	Plant promoter (Pp)	C $\times$ Pp	Cultivar of multiple comparisons	plant promoter of multiple comparisons
	<i>F</i>	<i>F</i>	<i>F</i>	(Y) (W)	CK T1 T2T3 T4 T5 T6
Leaf length (cm)	90.884***	7.275***	3.671**	A A B	C AB A BC AB AB BC
Leaf width (cm)	26.083***	3.671**	1.995	A A B	B AB A AB AB B B
Petiole diameter (mm)	86.186***	4.51**	3.575**	A B C	B AB A A AB A A
Petiole length (cm)	125.205***	1.975	1.802	A B C	B AB A AB AB AB AB
Pn ($\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	32.438***	2.858*	3.022**	A B B	B B AB AB AB AB A
Gs ($\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	36.65***	1.756	1.967	C B A	A A A A A A A
Ci ($\mu\text{mol} \cdot \text{mol}^{-1}$)	16.261***	3.827**	2.861**	B B A	B AB A A A AB AB
Tr($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	40.145***	3.763**	4.189***	C B A	C BC ABC ABC AB ABC A
SPAD	95.88***	69.452***	47.263***	B C A	CD EF B F DE BC A
L*	6791.766***	1.373	1.790	C B A	A A A A A A A
a*	4430.718***	3.225*	1.774	A B C	AB AB B A AB AB AB
b*	865.259***	2.717*	2.385*	A B C	A A A A A A A
C*	3633.282***	3.552*	2.161*	A B C	AB ABC C A ABC BC ABC
h°	1479.192***	2.369*	1.441	B B A	AB AB A AB B AB AB
ΔE	0.57	2.457*	1.406	A A A	B AB AB AB A AB AB
TA (%)	295.841***	8.252***	6.089***	A A B	A A A B A A A
TSS (%)	798.689***	127.748***	105.793***	A B C	C CD E B D A E
TSS/TA	104.001***	16.17***	12.191***	B C A	BCD CD D A BC B CD
Firmness ($\text{kg} \cdot \text{cm}^{-2}$)	212.393***	10.287***	33.335***	B A B	AB CD ABC BCD A ABC D
Single fruit weight (g)	1170.587***	327.889***	31.129***	A B A	C B B A B A A
Vc ($\text{mg} \cdot 100 \text{ mL}^{-1}$)	916.238***	6.325***	14.143***	A B C	BC C BC BC AB A BC

Note: Values are F-statistics; *, **, and *** indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; (H) = Benihoppe, (Y) = Yuexiu, (W) = White Snow Princess; multiple comparisons for both cultivar and plant promoter were performed using Tukey’s HSD test ($P < 0.05$); in the multiple comparison columns, uppercase letters are ordered alphabetically from highest to lowest mean value within each row ($A > B > C$); different letters within the same row indicate significant differences, whereas the same letter indicates no significant difference; plant promoter levels codes: CK = water control, T1 = S-abscisic acid, T2 = amino acid fertilizer, T3 = propyl jasmonate, T4 = $10 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA, T5 = $50 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA, T6 = $100 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA

Vegetative growth. Two-way ANOVA revealed that cultivar had highly significant effects on leaf length, leaf width, petiole diameter, and petiole length ($P < 0.001$). Plant promoter significantly affected leaf length, leaf width, and petiole diameter ($P < 0.01$), but not petiole length ($P > 0.05$). The cultivar $\times$ promoter interaction was significant for leaf length and petiole diameter ($P < 0.01$), but not for leaf width or petiole length (Table 2). In Figure 1, in Benihoppe, T2 exhibited the most pronounced growthpromoting effects, increasing leaf length, leaf width, and petiole length by 35.5%, 20.6%, and 17.7%, respectively ($P < 0.05$); T3 also significantly increased petiole diameter by 20.7% ($P < 0.05$). In Yuexiu, T1 significantly enhanced leaf length and petiole length (+25.7–25.9%, $P < 0.05$), while T2 significantly increased leaf width (+32.1% $P < 0.05$). In White Snow Princess, T5 and T6 promoted petiole growth, particularly increasing petiole diameter and petiole length. Overall, optimal growth-promoting treatments were cultivar-specific: T2 for Benihoppe, T1 for Yuexiu, and T5/T6 for White Snow Princess.

Photosynthetic characteristics. Two-way ANOVA revealed that cultivar had highly significant effects on Pn, Gs, Ci, Tr, and SPAD ($P < 0.001$). Plant promoter significantly affected Pn ($P < 0.05$), Ci and Tr ($P < 0.01$), and SPAD ($P < 0.001$), but not Gs ($P > 0.05$). The cultivar $\times$ promoter interaction was significant for Pn, Ci, Tr, and SPAD ($P < 0.01$ to $P < 0.001$), but not for Gs (Table 2). In Figure 2, in Benihoppe, T2–T6 increased Pn, peaking

Figure 1. Growth parameters of three strawberry cultivars under seven plant promoter levels (CK and T1–T6): a) leaf length, b) leaf width, c) petiole diameter, d) petiole length. CK, water control; T1, S-ABA; T2, amino acids; T3, propyl jasmonate; T4–T6, 10, 50, and 100 mg $\cdot$ L⁻¹ 5-ALA, respectively. Data are means $\pm$ SE. Different lowercase letters within each cultivar indicate significant differences among plant promoter levels based on one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). The same applies to subsequent figures

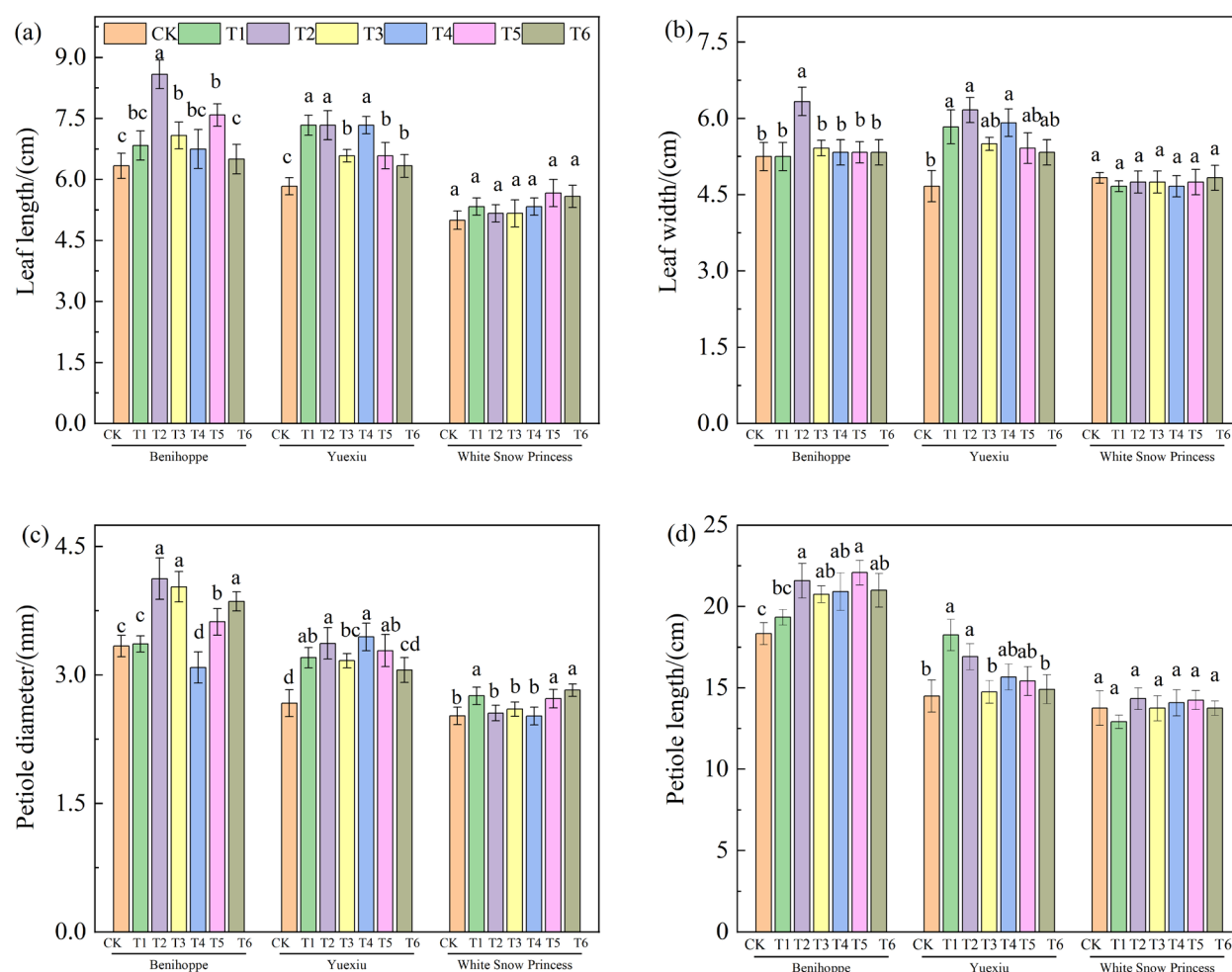


Figure 2. Photosynthetic characteristics and leaf SPAD readings of three strawberry cultivars under various foliar plant promoters (CK, T1–T6): a) net photosynthetic rate, b) intercellular CO₂ concentration, c) stomatal conductance, d) transpiration rate, e) SPAD value

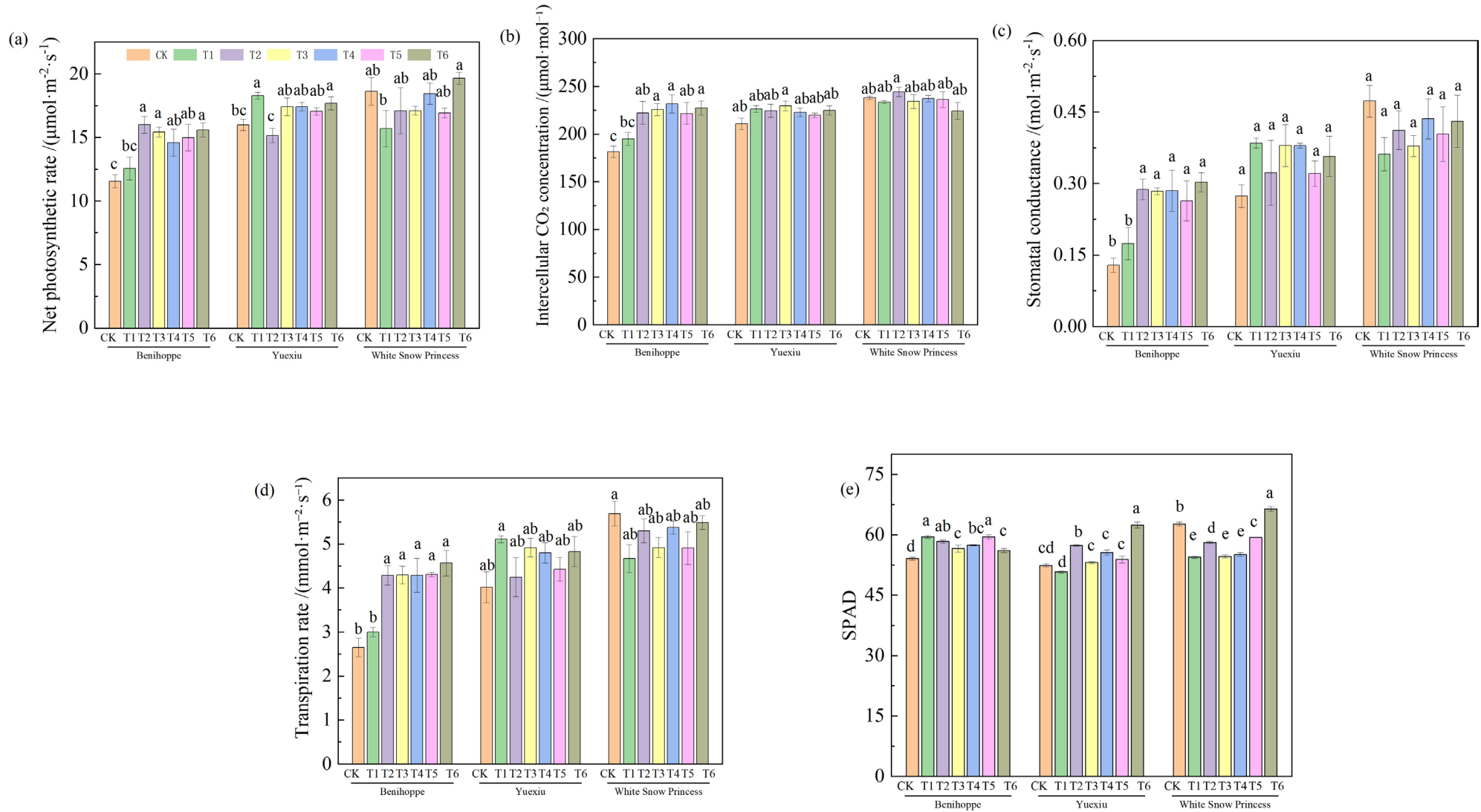
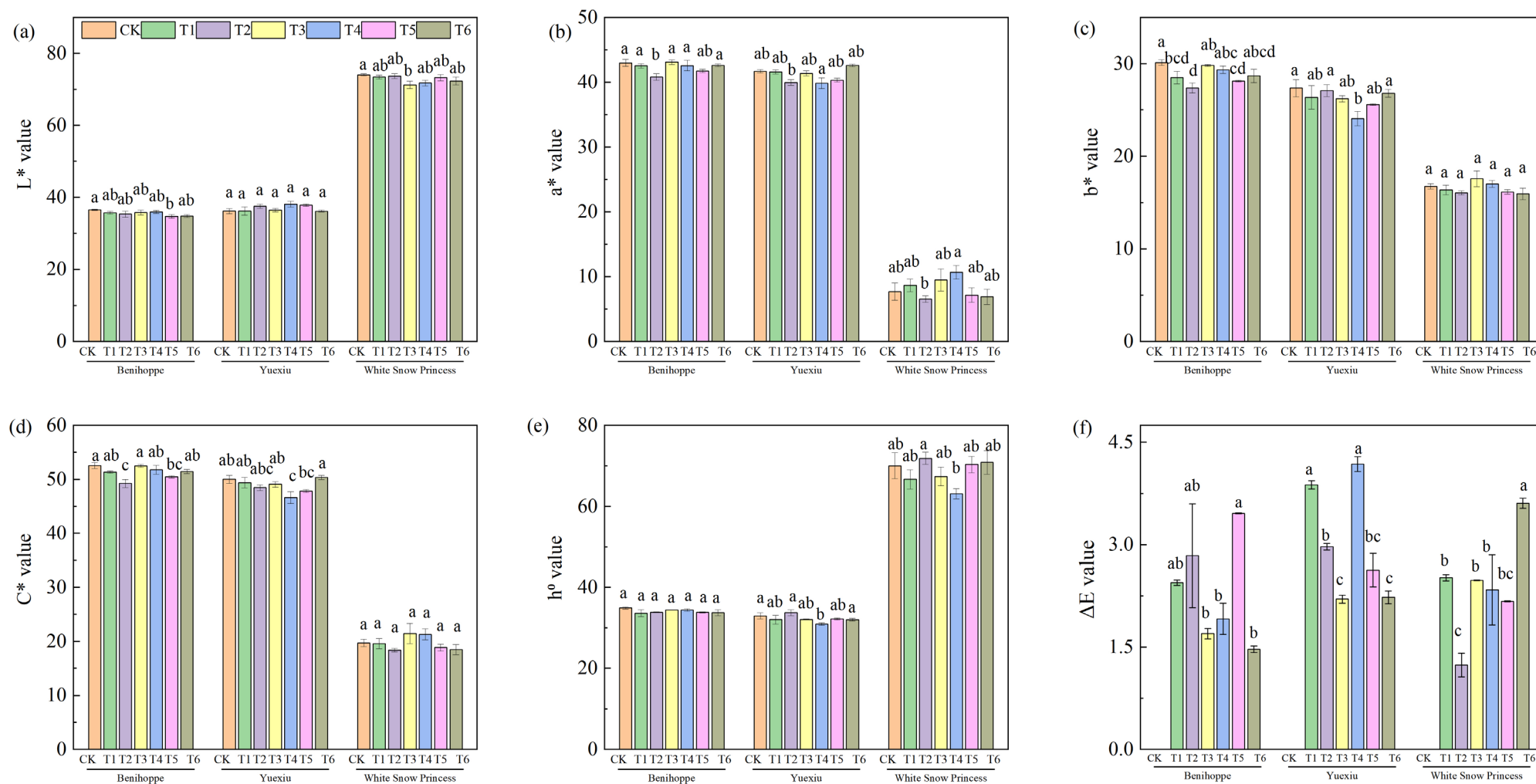


Figure 3. Fruit colour attributes of three strawberry cultivars under six foliar biostimulant treatments (T1–T6) and water control (CK): a) L*, b) a*, c) b*, d) C*, e) h°, f) ΔE



under T2 (+38.5%, $P < 0.05$); T6 produced highest Gs and Tr. T3, T4, and T6 elevated Ci (+24.4–27.7%, $P < 0.05$). SPAD peaked under T1 and T5, exceeding the control; T6 (56.11) also exceeded the control ($P < 0.05$). In Yuexiu, T1 and T6 increased Pn (+14.4% and +10.6%, $P < 0.05$); T6 gave highest SPAD (62.43, +19.2%, $P < 0.05$); T1 reduced SPAD ($P < 0.05$). In White Snow Princess, T6 had the highest Pn (19.66), but not significantly; T1 suppressed Pn (–15.7%, $P < 0.05$). T6 gave highest SPAD (66.45, +6.0%, $P < 0.05$); T1, T3, and T4 reduced SPAD (–13.1% to –12.1%). Responses were cultivar-specific, with 5-ALA effects on SPAD varying by concentration.

Fruit colour attributes. Two-way ANOVA revealed that cultivar had highly significant effects on L^* , a^* , b^* , C^* , and h° ($P < 0.001$), but not on ΔE (Table 2). Plant promoter significantly affected a^* , b^* , C^* , h° , and ΔE ($P < 0.05$), but not L^* ($P > 0.05$). No significant cultivar $\times$ plant promoter interactions were detected for any colour parameter (Table 2). For a^* , post-hoc comparisons revealed that a^* was significantly lower under T2 than under T3 (Table 2). In Figure 3, in Benihoppe, T5 gave greatest ΔE (3.46), reducing L^* (–5.0%) and b^* ($P < 0.05$); T2 reduced a^* and C^* (–5.1% and –6.3%, $P < 0.05$). T1, T3, T4, and T6 showed smaller ΔE (1.47–2.45) without significant changes. In Yuexiu, T4 gave highest ΔE (4.18), reducing a , b , and C^* (–4.5%, –12.1%, –6.8%, $P < 0.05$). T1 ($\Delta E = 3.88$) affected no single parameter significantly. In White Snow Princess, T6 gave highest ΔE (3.61); T4 increased a^* (+38.4%, $P < 0.05$), indicating reddening; T3 reduced L^* (–3.8%) and T4 reduced h° (–9.9%) ($P < 0.05$). No significant changes were observed in b^* or C^* . Responses were cultivar-specific, with white-fruited White Snow Princess showing marked reddening against a low red baseline.

Fruit quality attributes. Two-way ANOVA revealed that cultivar had highly significant effects on TA, TSS, TSS/TA, firmness, single fruit weight, and Vc ($P < 0.001$). Plant promoter also significantly affected all these traits ($P < 0.001$ to $P < 0.01$). The cultivar $\times$ promoter interaction was significant for all quality traits ($P < 0.001$) (Table 2). For traits with significant interactions, simple effects analysis was performed to examine promoter effects within each cultivar. In Figure 4, in Benihoppe, T5 performed best, increasing Vc, firmness, single fruit weight, and TSS by 3.8%, 18.8%, 26.2%, and 13.4%, respectively ($P < 0.05$). T3 improved TSS/TA ratio (+19.6%) and fruit weight (+31.7%, $P < 0.05$), with TSS slightly below T5. T6 had the highest Vc and single fruit weight but reduced TSS by 11.9% ($P < 0.05$). In Yuexiu, T3 increased TSS/TA (+7.8%, $P < 0.05$); T5/T6 raised TSS (+2.1%, $P < 0.05$); T2/T4 enhanced firmness (+24.7%, $P < 0.05$) but reduced TSS; T1 maximised fruit weight (+29.4%, $P < 0.05$). In White Snow Princess, T4 gave highest TSS/TA (20.07) and lowest TA (0.42%, $P < 0.05$); T3 and T6 maximised fruit weight (+31.7%, $P < 0.05$); T2 had highest Vc (+7.1%, $P < 0.05$); all treatments reduced firmness, with T1 lowest (–21.9%, $P < 0.05$).

OPLS-DA using all measured variables. showed excellent predictive ability, with $R^2Y = 0.845$ and $Q^2 = 0.805$ after 200 permutation tests (Figure 5). The first two principal components explained 73.79% of total variance. The 95% confidence ellipses of the three cultivars were completely separated, with no overlap, and replicates clustered tightly, confirming stable cultivar-specific differences.

Using VIP > 1 as a threshold, the top discriminative variables were petiole length (2.235), single fruit weight (2.120), intercellular CO_2 concentration (Ci) (1.725), Vc (1.607), and Pn (1.478). Variables with VIP < 0.5 were considered to have negligible contribution to cultivar separation, including TA (0.004), leaf width (0.058), a^* (0.251), Gs (0.281), fruit firmness (0.313), leaf length (0.315), L^* (0.322), TSS (0.353), fruit hue angle h° (0.376), TSS/TA (0.397), and total color difference ΔE (0.499).

Path analysis via structural equation modelling (SEM). PLS-SEM analysis indicated that the constructed “photosynthesis–growth–fruit colour–fruit quality” model exhibited strong explanatory power, with R^2 values of 0.303, 0.908, and 0.811 for growth, fruit colour, and fruit quality, respectively (Figure 6). Path analysis revealed that photosynthesis exerted a significant direct negative effect on growth (standardised path coefficient = –0.709, $P < 0.05$), suggesting a resource-based trade-off between these two processes. Growth had a strong positive direct effect on fruit colour (0.922, $P < 0.05$) and emerged as the primary driver of colour development. By contrast, photosynthesis showed no significant direct effect on fruit colour (–0.086), exerting its influence only indirectly through growth. Fruit quality was regulated through multiple synergistic pathways.

Figure 4. Fruit quality attributes of three strawberry cultivars treated with six foliar biostimulants (T1–T6) and water control (CK): a) titratable acidity, b) total soluble solids, c) vitamin C, d) TSS/TA ratio, e) fruit firmness, f) single fruit weight

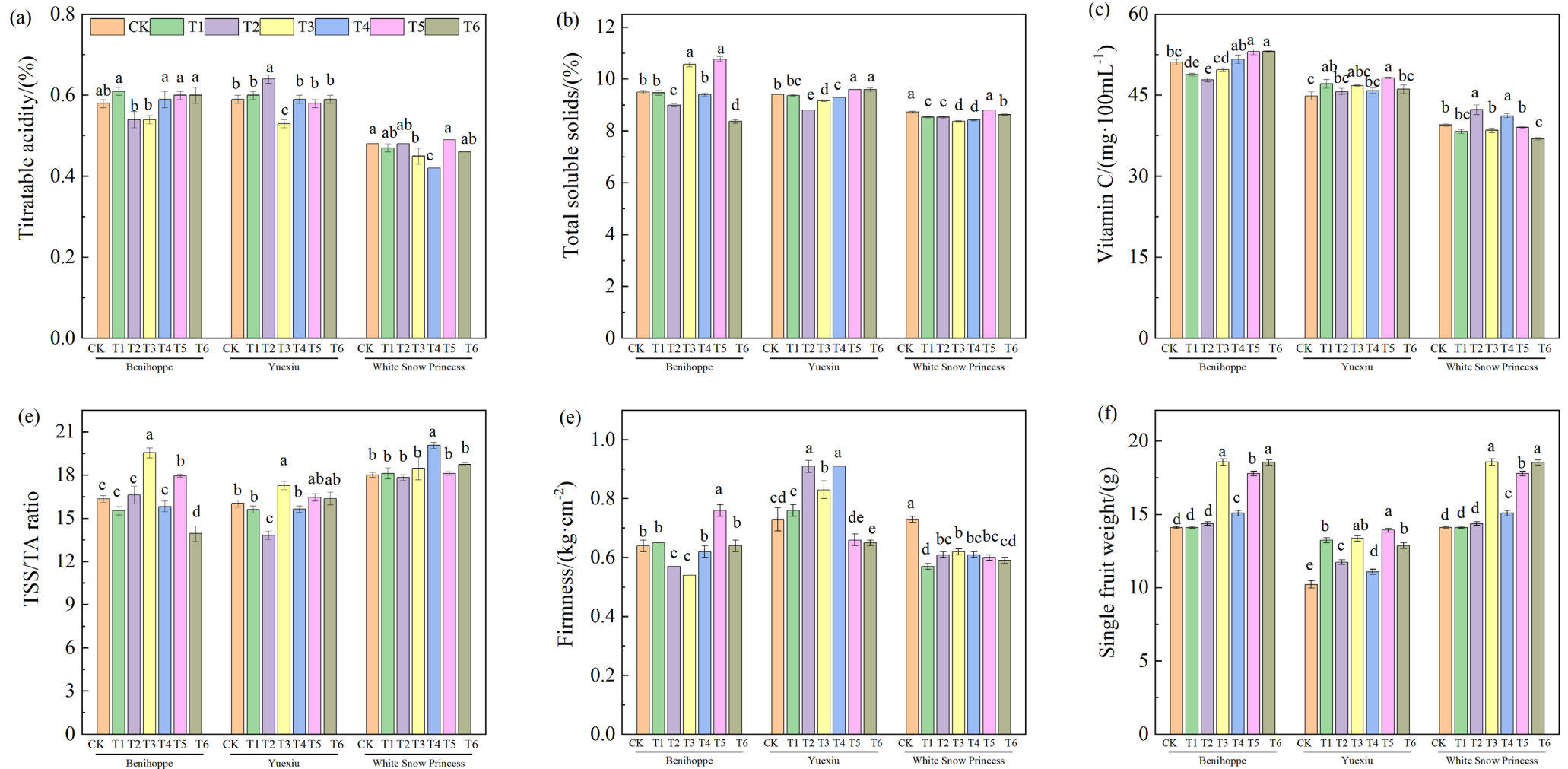


Figure 5. Orthogonal partial least squares discriminant analysis (OPLS-DA) of integrated vegetative, photosynthetic, and fruit quality traits for three strawberry cultivars (Benihoppe, Yuexiu, and White Snow Princess): a) score plot with 95% confidence ellipses showing distinct clustering of each cultivar; b) permutation test (200 random permutations) indicating no overfitting; c) S-plot combined with VIP scores identifying variables with $VIP > 1$ as key discriminative traits. Variables with $VIP > 1$ are highlighted in red

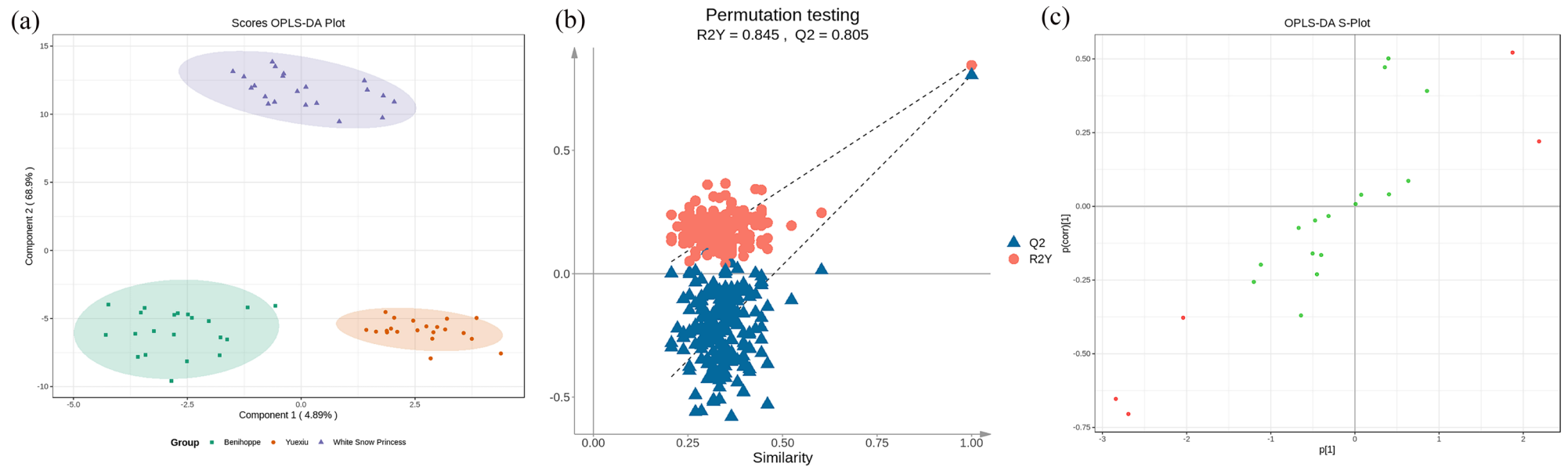


Figure 6. Path diagram of the partial least squares structural equation model (PLS-SEM) illustrating relationships among photosynthesis, vegetative growth, fruit colour, and fruit quality under elevated spring temperatures. Standardised path coefficients are shown; solid lines indicate significant effects ($P < 0.05$), and dashed lines indicate nonsignificant effects. R^2 values for endogenous variables are displayed in parentheses

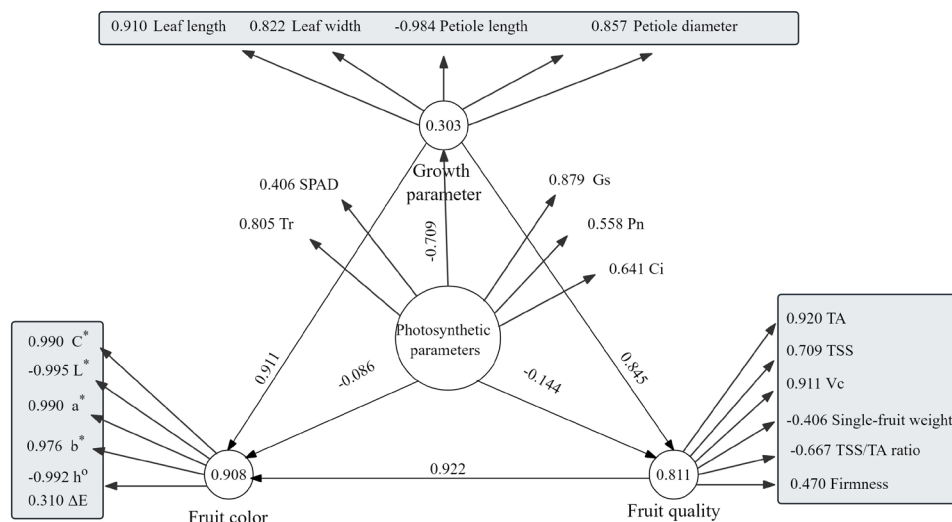


Table 3. Stepwise regression analysis of vegetative, photosynthetic, and fruit quality traits in response to cultivar, foliar treatments, and 5-aminolevulinic acid (5-ALA) concentration

Dependent variable	Variables entered	Final β	R^2	Model P
Leaf length	H, Y, T5, T4	0.782, 0.630, 0.350**, 0.279*	0.606	< 0.001
Leaf width	H, Y	0.845, 0.476	0.510	< 0.001
Petiole diameter	H, Y, 5-ALA	0.845, 0.476, 0.311**	0.601	< 0.001
Petiole length	Y	0.876***	0.760	< 0.001
SPAD	T6, H, Y	0.523, -0.549, -0.468**	0.494	< 0.001
Pn	H, 5-ALA, Y, T4	-0.864, 0.385**, -0.281, 0.236*	0.669	< 0.001
Gs	H, Y	-0.852*, -0.460**	0.518	< 0.001
Ci	—	—	—	>0.05
Tr	H, Y	-0.770*, -0.462**	0.418	< 0.001
L*	H, Y	-1.018, -0.975**	0.995	< 0.001
a*	H, Y	1.015, 0.974**	0.990	< 0.001
b*	H, Y	1.084, 0.816**	0.954	< 0.001
C*	H, Y, T5	1.038, 0.944**, -0.036*	0.989	< 0.001
h°	Y, H, T4	-1.015, -0.954, -0.073	0.976	< 0.001
ΔE	T4	0.408*	0.142	0.013
TA	H, Y	0.963, 0.916**	0.878	< 0.001
TSS	T5, H, Y	0.458, 0.628, 0.603*	0.550	< 0.001
TSS/TA	H, Y	-0.782, -0.751**	0.564	< 0.001
Firmness	Y, 5-ALA	0.451, -0.351*	0.285	< 0.001
Single fruit weight	Y, 5-ALA, T6	-0.763, 1.059, -0.561***	0.970	< 0.001
Vc	H, Y, T5	1.115, 0.605, 0.093*	0.939	< 0.001

Note: Values are standardized regression coefficients (β). (H) = Benihoppe, (Y) = Yuexiu; T4, 10 mg · L⁻¹ 5-ALA; T5, 50 mg · L⁻¹ 5-ALA; T6, 100 mg · L⁻¹ 5-ALA; 5-ALA, 5-aminolevulinic acid concentration as a continuous variable (0, 10, 50, 100 mg · L⁻¹). Only variables that met the entry criterion ($P < 0.05$) are shown; “—” indicates no variable entered the model. Reference levels: White Snow Princess for cultivar; CK (water control) for plant promoter levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Adjusted R^2 values are reported

Stepwise regression. confirmed cultivar was the predominant factor for most traits (Table 3), with cultivar dummy variables entering all final models, predominantly at the first step. Colour parameters (L^* , a^* , b^* , C^* , h°) showed adjusted $R^2 > 0.95$, indicating near-complete cultivar dependence for these traits. For ΔE , only T4 was retained ($\beta = 0.408$, $P = 0.013$, $R^2 = 0.142$).

5-ALA effects were trait-specific. For TSS, T5 entered first ($\beta = 0.458$), preceding cultivar, confirming promoter effect exceeded cultivar effect. In single fruit weight, Yuexiu ($\beta = -0.763$), 5-ALA concentration ($\beta = 1.059$), and T6 ($\beta = -0.561$) were retained ($R^2 = 0.970$), but 5-ALA concentration and T6 were negatively correlated ($r = -0.88$), suggesting high concentration suppressed fruit weight. SPAD: T6 entered first ($\beta = 0.523$), T5 not retained. Firmness: only Yuexiu ($\beta = 0.451$) and 5-ALA concentration ($\beta = -0.351$) were retained ($R^2 = 0.285$). T5 was retained in Vc, but effects were smaller than cultivar effects.

For Gs, Tr, TA, TSS/TA, leaf width, petiole length, and most colour parameters (L^* , a^* , b^*), only cultivar variables were retained. Petiole diameter additionally included 5-ALA concentration. No predictors entered the Ci model.

DISCUSSION

This study examined cultivar-specific responses to foliar biostimulants under spring protected conditions of reduced diurnal temperature range ($< 8^\circ\text{C}$) and episodic daytime high temperatures ($\geq 35^\circ\text{C}$). The key findings, cultivar dominance, broad-spectrum 5-ALA effects with concentration dependency, and a photosynthesis–growth trade-off are discussed below in relation to their physiological mechanisms and implications for precision management.

Cultivar effect. Cultivar was the primary determinant of trait variation, with colour and quality traits almost entirely cultivar-dependent (adjusted $R^2 > 0.95$). This aligns with Kesici et al. [2020], who linked heat tolerance differences to genetic variation in heat shock proteins, antioxidant enzymes, and hormone signalling. The broad response of Benihoppe to multiple treatments suggests greater photosynthetic flexibility, whereas the specific response of Yuexiu to S-ABA may reflect an efficient ABA signalling network, consistent with the observations of Fagherazzi et al. [2021] that initial crown diameter – a proxy for plant vigour – strongly influences yield and quality in strawberry. OPLS-DA completely separated the three cultivars, with VIP analysis identifying traits spanning morphology, fruit quality, and photosynthesis as core discriminators. Notably, T4 entered the stepwise model, indicating colour difference between white-fleshed White Snow Princess and red-fruited cultivars is primarily treatment-induced. This is consistent with Ullah et al. [2025], who reported that anthocyanin synthesis is limited in white strawberries and colour regulation depends mainly on environment and cultivation practices. Cultivar dominance thus requires cultivar-specific, not universal, foliar strategies.

Broad-spectrum effects of 5-ALA, concentration dependency, and compensation for reduced diurnal temperature range. 5-ALA exhibited positive effects in all three cultivars, indicating broad-spectrum efficacy, but its mode of action varied with cultivar and concentration. For Benihoppe, T5 ($50\text{ mg} \cdot \text{L}^{-1}$) was optimal for quality improvement, while T6 ($100\text{ mg} \cdot \text{L}^{-1}$) most enhanced photosynthesis. For Yuexiu, T6 increased sweetness, and T5 and T6 optimised flavour. For White Snow Princess, T4 ($10\text{ mg} \cdot \text{L}^{-1}$) synergistically improved both photosynthesis and quality. Stepwise regression quantified this concentration–effect relationship: T5 entered the TSS model first ($\beta = 0.458$, $P < 0.001$), preceding cultivar variables ($\beta = 0.628$ for Benihoppe, $\beta = 0.603$ for Yuexiu). This indicates that $50\text{ mg} \cdot \text{L}^{-1}$ 5-ALA promotes TSS accumulation more strongly than cultivar effects, providing statistical support for its use as a chemical compensator for sugar accumulation under reduced diurnal temperature range.

The mechanism of 5-ALA is multifaceted. Long et al. [2022] found that 5-ALA enhances stress tolerance by regulating chloroplast ultrastructure, promoting chlorophyll synthesis, and boosting antioxidant capacity. In this study, SPAD responses to 5-ALA were concentration-dependent: Benihoppe responded best at low to moderate concentrations ($10\text{--}50\text{ mg} \cdot \text{L}^{-1}$), whereas Yuexiu and White Snow Princess required high concentrations ($100\text{ mg} \cdot \text{L}^{-1}$) for maximal chlorophyll response, possibly due to differences in uptake efficiency or signal transduction thresholds [Ullah et al. 2024]. Notably, T6 enhanced gas exchange in Benihoppe without significantly increasing SPAD, suggesting that 5-ALA promotes gas exchange inde-

pendently of chlorophyll content, possibly through stomatal regulation or Rubisco activity [Wang et al. 2025], thereby compensating for reduced source supply under reduced diurnal temperature range. Salwa et al. [2025] similarly confirmed that exogenous hormones and chemicals alleviate heat-induced damage by modulating stomatal behaviour and antioxidant systems. However, excessive 5-ALA can be detrimental: in the single fruit weight model, 5-ALA concentration correlated negatively with T6 ($r = -0.88$), indicating that $100 \text{ mg} \cdot \text{L}^{-1}$ suppresses fruit weight, revealing an optimal concentration range. This hormesis effect implies that 5-ALA application must be precisely dosed to avoid metabolic costs, especially when nocturnal respiration is already elevated under reduced diurnal temperature range.

Photosynthesis-growth trade-off under high temperature and its implications for reduced diurnal temperature range. PLS-SEM path analysis revealed a significant photosynthesis-growth trade-off: photosynthetic parameters exerted a direct negative effect on growth (standardised path coefficient = -0.709 , $P < 0.05$), while growth strongly positively affected fruit colour (0.922) and fruit quality (0.845). This challenges the conventional view, articulated by Akram [2024], that enhanced photosynthesis inevitably promotes both growth and quality, highlighting the need to reconsider resource allocation under stress.

The physiological basis of this trade-off lies in resource competition. Manafi et al. [2021] and Ullah et al. [2024] noted that under high temperature, plants increase transpiration to cool leaves, but this adaptive response simultaneously elevates the risk of photoinhibition and reactive oxygen species (ROS) production, triggering costly defence mechanisms. To counter oxidative stress, plants activate energy-intensive defences (e.g., heat shock proteins, antioxidants, osmoprotectants), diverting assimilates from growth to stress responses [Akhtar et al. 2025]. In this study, T5 ($50 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA) induced only a moderate photosynthetic increase (+13.4%) but achieved synergistic gains in fruit weight (+26.2%), Vc (+3.8%), and TSS (+13.4%). This represents a more economical “moderate enhancement” strategy, contrasting with the “aggressive promotion” of T6. This finding aligns with Menzel [2024], who reported that temperatures above 30°C suppress strawberry leaf growth, and with Bieniasz et al. [2022], who found that titanium organic complexes improve yield under heat by enhancing pollination and fruit development, not merely by increasing photosynthesis. Stepwise regression further supports this network for: Gs, Tr, TA, TSS/TA, leaf width, petiole diameter, and most colour parameters, only cultivar variables entered the model; treatment variables did not meet entry criteria. This indicates that these traits are primarily genetically regulated, with limited scope for foliar intervention. Thus, under reduced diurnal temperature range with elevated night respiration, overemphasising single photosynthetic indicators may be counterproductive; instead, synergistic optimisation of the entire photosynthesis-growth-quality pathway through moderate physiological activation is required.

Cultivar-specific responses and implications for precision fertilisation. The practical value of this study lies in revealing cultivar-specific response patterns, providing a framework for precision fertilisation under reduced diurnal temperature range and high temperature in spring protected production. The significant cultivar $\times$ treatment interactions observed for multiple traits demonstrate that the “optimal treatment” is not universal but results from the synergistic matching of cultivar, treatment type, and concentration. This finding underscores the necessity of genotype-aware management strategies in protected strawberry cultivation.

Based on the comprehensive statistical results, recommended cultivar–treatment combinations are: (1) Benihoppe – T5 ($50 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA), which performed best for TSS, fruit weight, and Vc, balancing quality and growth through moderate photosynthetic enhancement; (2) Yuexiu – T1 (S-ABA) for growth, T3 (jasmonic acid) for flavour balance (TSS/TA +7.8%), or T5/T6 for sweetness (TSS +2.1%); (3) White Snow Princess – T4 ($10 \text{ mg} \cdot \text{L}^{-1}$ 5-ALA), which optimises flavour quality with low metabolic cost. Although jasmonic acid improved TSS/TA and fruit weight in Benihoppe, it did not enter any growth model in stepwise regression, suggesting limited promotive effects on vegetative growth. This is consistent with Ullah et al. [2024], who reported that jasmonic acid signalling promotes fruit development while inhibiting vegetative growth, and with Zhang et al. [2024], who demonstrated that plants under high temperature reallocate assimilates from growth to stress defence – a tradeoff that appears to be amplified by jasmonate application. Fruit firmness in White Snow Princess decreased under all treatments, with T1 showing the greatest reduction (-21.9% vs control). This may relate to the unique cell wall polysaccharide composition and

metabolism of its white flesh [Ullah et al. 2025], suggesting that postharvest performance must be considered alongside quality improvement. In summary, this study quantified cultivar, treatment, and interaction effects, revealed a photosynthesis-growth trade-off under spring stress conditions, and established a decision chain for cultivar-specific precision management. Future multiomics studies should elucidate the molecular networks underlying differential cultivar responses.

CONCLUSION

This study established cultivar-specific foliar strategies to mitigate elevated spring temperatures and narrowed diurnal range in protected strawberry cultivation. Cultivar was the predominant factor for most traits, with colour and quality parameters almost entirely cultivar-dependent (adjusted $R^2 > 0.95$). Optimal responses were achieved with 50 mg · L⁻¹ 5-ALA for Benihoppe, S-ABA or 10 mg · L⁻¹ 5-ALA for Yuexiu, and 10 mg · L⁻¹ 5-ALA for White Snow Princess. PLS-SEM revealed vegetative growth as the central driver of fruit colour (0.922) and fruit quality (0.845), while photosynthesis exerted a significant direct negative effect on growth (−0.709, $P < 0.05$), indicating a resourcebased tradeoff under elevated spring temperatures. Stepwise regression confirmed that 5-ALA promoted TSS more strongly than cultivar effects in Benihoppe, whereas the highest concentration (100 mg · L⁻¹) suppressed fruit weight. Inter-cellular CO₂ concentration showed no significant predictors. These findings support cultivar-specific precision management, emphasizing growth promotion over photosynthetic enhancement to sustain fruit quality under spring stress conditions.

AUTHORS CONTRIBUTIONS

Si-Pu Zhang and Jia-Jia Niu contributed equally to this work and share first authorship. Conceptualization, methodology, investigation, formal analysis, writing – original draft, funding acquisition. Wei Cui: data curation, validation, visualization. Ke Zhang: resources, project administration. Yun-Feng Lu: supervision, writing – review & editing. All authors have read and approved the final manuscript.

DECLARATION OF THE USE OF GENERATIVE AI IN THE PROCESS OF PREPARING THE PAPER

The authors declare that no generative AI tools were used in the preparation of this manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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EFFECTS OF DIFFERENT BIOCHAR TYPES ON THE GROWTH AND BIOCHEMICAL CHARACTERISTICS OF BROCCOLI AND PEPPER SEEDLINGS UNDER GREENHOUSE CONDITIONS

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ABSTRACT

This study aimed to verify the effects of biochars produced from chestnut seeds, compost, pine bark, and pine sawdust on the growth and quality of broccoli (*Brassica oleracea* var. *italica* Plenck) and pepper (*Capsicum annuum* L.) seedlings under greenhouse conditions. It was hypothesized that the effects would depend on the biochar feedstock and the plant genotype. Biochars were incorporated into a universal peat substrate at 4.4% (dry weight); an unamended substrate served as the control. Pots experiment was arranged in a completely randomized design, with 10 replicates per treatment. Biochar application generally did not significantly modify the biometric and biochemical parameters of broccoli seedlings. Pine sawdust biochar increased leaf perimeter compared to the control; however, it lowered chlorophyll fluorescence, suggesting a stress response. Compost biochar increased the number of root tips compared to chestnut seed biochar, but roots had the lowest diameter. At the same time, compost biochar-treated broccoli had lower anthocyanin, chlorophyll, and SPAD index contents, contrary to pine bark-treated seedlings. In peppers, pine sawdust biochar provided the best results across plant biometric parameters, compared to pine bark and compost biochar. Plants grown in substrate with chestnut seed biochar had the longest roots and the highest root area, volume, diameter, and number of root tips. Leaf chlorophyll content and the SPAD index were also higher compared to the control. Compost biochar did not positively affect seedling development. Our results suggesting pine sawdust and chestnut seed biochars as promising amendments for peat substrates used in pepper seedling production and highlighting the need to select the appropriate biochar type for each plant species to obtain high-quality planting material.

Keywords: biometric parameters, chestnut seeds, compost, pine bark, pine sawdust, vegetable plants

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INTRODUCTION

Horticulture differs from agriculture in terms of crops (fruit, vegetables, ornamental plants, herbs) and more intensive cultivation systems. Some crops are produced in substrates under protected culture (greenhouses, plastic tunnels), while others are grown in open fields. Currently, the horticultural sector is adopting new strategies to move toward eco-friendly production [Zulfiqar et al. 2022]. One innovative solution gaining particular attention is the use of biochar, which is defined as a renewable fuel that can be produced by heating biomass like farm waste, municipal waste, once considered useless, energy crops, or plants cultivated specifically for this purpose [Patel and Panwar 2023]. Biochar is used primarily for its high content of easily assimilable carbon, in the form of a finely divided, highly porous material. Biochar can increase soil quality and crop yield. It is also an effective, fast way to sequester carbon and reduce greenhouse gas emissions [Patel and Panwar, 2023]. Biochar is most commonly produced by pyrolysis (at temperatures of 350–900 °C) in closed containers with absent or depleted oxygen [Zulfiqar et al. 2022, Khater et al. 2024]. It can be used as a standalone product or as an additive to enhance the properties of growing substrates in horticultural plant cultivation [Amery et al. 2021]. Biochar significantly enhances the physical, chemical, biological, and nutritional qualities of the substrate, prevents fertilizer leaching, and supplies additional nutrients to plants. Biochar's high sorption capacity can also greatly improve water conditions [Zannatul et al. 2023, Simhi Samyukta et al. 2024]. The substrate-biochar mixture is pathogen-free and can transform some heavy metals into less toxic forms for plants. Biochar's disease-control mechanisms include adsorbing virulence factors, inducing systemic resistance in plants, altering soil microbial communities, and/or increasing soil silicon content. However, its final effectiveness varies with factors such as biomass input, pyrolysis conditions, and application rate [Iacomino et al. 2022].

Seedling production is an essential practice in horticulture. High-quality planting material in the initial phase of vegetable plant production is crucial for final yield efficiency and quality. Using seedlings increases shoot and root growth, accelerates maturity, and enhances vegetable yield compared to direct seed sowing. Transplanted seedlings also have higher chlorophyll content than directly seeded plants. Growing seedlings in a controlled environment limits the spread of pests and diseases and reduces the need for plant protection products later on [Gallegos-Cedillo et al. 2024]. Vegetable seedlings should grow uniformly, reach the desired size and growth stage at transplanting, and be free of disease and insect pests. The substrate plays a critical role in producing healthy seedlings [Maynard et al. 2024].

Capsicum annuum L. (pepper) belongs to the Solanaceae family and is the most commonly cultivated plant in the *Capsicum* genus. The species contains both pungent and non-pungent (sweet) fruits. Pepper is one of the most widely used spices worldwide and has valuable medicinal properties due to numerous bioactive compounds [Hernández-Pérez 2020, Liu et al. 2023]. High-quality pepper seedlings should be dark green with sturdy shoots and well-developed root systems. Sweet pepper seedlings have a relatively long growing period (40–60 days), depending on the cultivation conditions and cultivar [Jankauskienė and Laužikė 2023]. Pepper has high nutrient requirements, and the substrate is an important factor affecting seedling quality, plant morphological characteristics, and disease susceptibility [Awuku and Egyir 2018]. Commercial substrates are usually used. Replacing the commercial substrate with alternative substrates can reduce the cost of seedling production [Júnior et al. 2018]. The method of preparing pepper seedlings affects subsequent growth and yield [Jankauskienė and Laužikė 2023]. Container production (also used in this research) provides higher yields and fruit with better characteristics than seedlings produced using the standard method [Markovic et al. 2000].

Broccoli (*Brassica oleracea* L. var. *italica* Plenck), on the other hand, is a representative of the Brassicaceae vegetables and possesses many valuable medicinal properties, high nutritional value, and strong antioxidant activity [Syed et al. 2023]. Its cultivation requires particularly favorable conditions, especially during seedling production, which should ultimately be characterized by a compact habit with no more than five leaves. Strong, healthy seedlings give plants an advantage in competition for water, light, and nutrients, which are essential for proper growth. Well-developed, hardened, and nourished plants have a better chance of adapting to their permanent location and minimizing losses resulting from biotic and abiotic factors [Ptaszek et al. 2021]. According to Moon et al. [2025], 35-day-old broccoli seedlings yield

the best. Generally, earlier-planted seedlings produce higher plants with more leaves and give the lowest share of aberrant curds.

Despite growing interest in biochar as an ecological additive to horticultural substrates, studies rarely consider how diverse, unconventional biochar sources affect the early stages of vegetable plant development. Knowledge remains limited on selecting appropriate biochar types for specific vegetable species and their effects on seedling quality. Previous studies have often focused on the general effects of biochar, without considering differences resulting from feedstock origin and plant genotype. Chrysargyris et al. [2019] tested commercial biochars in cabbage (*Brassica oleracea* L. var. *capitata*) seedling cultivation. Biochars were mixed with peat at 0, 5, 10, 15, and 20% ratios. Forest wood biochar and bamboo biochar at 20% reduced seed germination and plant length. Forest wood, husks and paper fiber, and fresh wood screening biochars applied at 5% and 10% maintained or improved seedling growth (fresh weight and length). Cucumber (*Cucumis sativus* L.) seedlings cultivated in a substrate with 10% corn stover biochar were characterized by the highest shoot and root growth, while the seedlings treated with 25% biochar were less than 22.23% of the growth found in the 10% biochar treatment, and even less than 1.5% of the growth of the control seedlings [Ma et al. 2024].

This study aimed to investigate the impact of biochars derived from chestnut seeds, compost, pine bark, and pine sawdust on the quality of pepper and broccoli seedlings, with particular emphasis on plant biometric and biochemical parameters. The research hypothesis assumed that supplementing peat substrate with biochar could affect the growth and quality of the tested seedlings. Observed effects will vary depending on the type of biochar and plant genotype. This integrated approach fills a knowledge gap on selecting the right biochar type for a specific vegetable species and supports sustainable production of high-quality vegetable seedlings. The novelty of this study lies in using biochars derived from less explored feedstocks, which have rarely been evaluated as substrate amendments for vegetable seedling production.

MATERIALS AND METHODS

Time and location of the experiment

The experiment was performed in a greenhouse at the Faculty of Agriculture and Biotechnology of the Bydgoszcz University of Science and Technology, Poland (53°07'12.0" N 18°00'29.4" E), from July 1, 2024, to August 5, 2024.

Plant material

Seeds of pepper *Capsicum annuum* L. Marta Polka and broccoli *Brassica oleracea* var. *italica* Plenck – Cezar were purchased from W. Legutko Hobby Ltd. (Rawicz, Poland). Marta Polka is an early cultivar suitable for both indoor and outdoor cultivation. Its stem is stiff and reaches 40 cm in length. It produces large, block-shaped, yellow fruit. The cultivar tolerates unfavorable environmental conditions but prefers fertile soil that warms quickly. Cezar is an early cultivar of broccoli, resistant to ground frosts. It produces large, green heads, prefers fertile, moist soil; during drought, regular watering is recommended.

Experimental design

The biochars were prepared via pyrolysis. The feedstock materials for biochar production were obtained from the following sources: compost from the municipal wastewater treatment plant in Piła, Poland; chestnut seeds from green area maintenance; pine bark; and pine sawdust from a local sawmill. The production of biochars was carried out by the low-temperature pyrolysis (400 °C) of air-dried feedstock biomass (under atmospheric pressure) in the muffle furnace FCF 22 M (Czylok Ltd., Jastrzębie-Zdrój, Poland). Process execution time was 1 h. The obtained biochars were ground and homogenized in a PM100 ball mill (Retsch, Düsseldorf, Germany). Milling parameters: 10 min, 400 rpm, 10 zirconium balls in the milling chamber. The mean size distribution of ground biochar particles was as follows:

- chestnut seed biochar – 2.0–0.05 mm: 30.87%, 0.05–0.002 mm: 66.42%, <0.002 mm: 2.71%,
- compost biochar – 2.0–0.05 mm: 47.22%, 0.05–0.002 mm: 47.05%, <0.002 mm: 5.73%,
- pine bark biochar – 2.0–0.05 mm: 12.86%, 0.05–0.002 mm: 84.23%, <0.002 mm: 2.92%,
- pine sawdust biochar – 2.0–0.05 mm: 34.67%, 0.05–0.002 mm: 62.98%, <0.002 mm: 2.35%.

Biochar particle-size distribution was determined using a Malvern Instruments Mastersizer 2000 analyzer equipped with a dispersing device (Hydro 2000MU; Malvern Panalytical, Malvern, UK).

To provide a comprehensive characterization of the materials used, the physicochemical properties of the biochars and substrate were determined, including the content of total organic carbon (TOC) and total nitrogen (TN), pH, electrical conductivity (EC), ash content, as well as the content of micro- and macroelements. The content of TOC and TN was assayed with the Vario Max CN analyzer (Elementar, Langensfeld, Hesse, Germany). The pH was analysed in accordance with the standard PN-ISO 10390 – soil quality, determination of pH. For this purpose, 0.5 g of biochar/substrate was mixed with 25 mL of deionized water (pH 6.06, EC 0.923 $\mu\text{S}\cdot\text{cm}^{-1}$) and shaken for 5 min; then the flask was set aside for 1 h. The pH was determined using a 540 GLP pH-meter (WTW, Washington, DC, USA) with a glass electrode. Electrical conductivity was measured according to the standard PN-ISO 11265 – soil quality, determination of the specific electrical conductivity. For EC analysis, the extract of water and biochar/substrate described above was used. The EC was determined by the CC-501 conductometer (Elmetron, Zabrze, Poland); see Table 1.

The content of K, Ca, and Na was determined by a flame photometer BWB XP (BWB Flame Technologies, Newbury, UK). The mineralization of biochar/substrate samples was performed in sulphuric acid (96%); 1 g of sample was mixed with 10 mL H_2SO_4 . Mineralization was performed in a DK 20 Digestion Unit (VELP Scientifica Srl, Usmate, Italy) at 265 °C for 6 h. After cooling, the mineralization was continued at 375 °C until the solutions turned dark brown. Then the solutions were decolorized with hydrogen peroxide (30%, 5 mL), and the mixtures were further digested for 30 min (375 °C). After cooling, the solutions were poured into 100-mL volumetric flasks and filled up with deionized water. The contents of Cu, Zn, Mn, Mg, and Fe were measured by atomic absorption spectrometry using an AA240FS spectrometer (Varian, Mulgrave, Australia). Biochar/substrate samples were mineralized in a muffle furnace (750 °C, 150 min.). The obtained ash was further mineralized in a mixture containing 30 mL of 65% nitric acid and 10 mL of 38% hydrochloric acid. The Tecator digester open system (160 °C, 120 min.) was used. After cooling, the solutions were poured into 100-mL volumetric flasks and filled up with deionized water. After filtering, the resulting mineralization solutions were assayed using an AAS. The content of Cu, Zn, Mn, Mg, and Fe was calculated as the concentration of individual elements in ash and finally in pure biochar/substrate (Table 2).

Table 1. Biochar and peat substrate characteristics: total organic carbon (TOC) and total nitrogen (TN) contents, pH, electrical conductivity (EC), ash share

Tested materials	TOC ($\text{g}\cdot\text{kg}^{-1}$)	TN ($\text{g}\cdot\text{kg}^{-1}$)	pH	EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	Ash (%)
Chestnut seed biochar	714.5	16.74	8.24	777.8	8.47
Compost biochar	298.9	26.38	7.35	632.1	70.52
Pine bark biochar	699.3	2.30	4.72	127.95	2.10
Pine sawdust biochar	642.2	1.30	3.86	108.05	0.47
Peat substrate	538.40	11.10	6.49	356.7	6.92

Table 2. Content of micro- and macroelements in biochars and peat substrate

Tested materials	Macro- and micro-elements content ($\text{mg}\cdot\text{g}^{-1}$)							
	K	Ca	Na	Cu	Zn	Mn	Mg	Fe
Chestnut seed biochar	119.500	5.523	1.724	1.724	1.416	0.527	53.250	2.279
Compost biochar	7.756	4.122	1.000	0.154	0.604	0.458	5.473	4.659
Pine bark biochar	0.139	0.526	4.166	0.217	0.472	2.337	6.527	4.291
Pine sawdust biochar	0.037	0.734	0.638	0.103	0.368	5.267	2.176	2.094
Peat substrate	5.482	59.440	0.769	0.703	1.974	2.003	101.708	36.032

Biochars were added to a universal peat substrate (Hartmann Polska Sp. z o.o., Poznań, Poland) at

a concentration of 4.4% (dry weight). Substrate without biochar was used as a control. Biochar and peat substrate characteristics are provided in Tables 1 and 2. A single biochar application rate of 4.4% was considered sufficient to modify substrate properties while minimizing the risk of adverse effects associated with excessive biochar addition on plant growth (unpublished data). Based on a review of the scientific literature on the effect of biochar on seedling development, the most frequently recommended biochar addition to the substrate ranges from 0.2% to 10% [Chrysargyris et al. 2019, Ma et al. 2024, Kharytonov et al. 2026]. We used the same mass-based biochar doses to simplify preparing the biochar-substrate mixture for commercial gardening.

Seeds were sown individually into 0.25-L plastic pots to a depth of 2 cm, watered evenly, and allowed to germinate in an automated greenhouse under controlled temperature and humidity conditions. No artificial lighting was applied. Plants were cultivated under a natural 16 h photoperiod and light intensity. Air temperature was maintained at 20 °C $\pm$ 2 °C, and air humidity at 80%. Plants were watered according to a uniform schedule to maintain optimal substrate moisture (70–80%), and no additional fertilization or fertigation was applied.

The experiment was conducted in a completely randomized design (CRD), in which pots representing individual experimental treatments were randomly arranged in the greenhouse to minimize environmental variability. Each combination included 10 replications (plants).

Assessment of plant biometric and biochemical traits

After five weeks of cultivation, the leaf number, shoot length (cm), shoot fresh weight (g), and shoot dry weight (mg) were determined. Morphological parameters of leaf length (cm), width (cm), perimeter (cm), and area (cm²), as well as total length (cm), area (cm²), root system volume (cm³), root diameter (mm), and number of root tips were assessed using a Perfection V800 scanner (Seiko Epson Corporation, Suwa-shi, Nagano, Japan). The WinFOLIA 2016b program was used to analyze leaf architecture, and WinRHIZO 2016a (Regent Instruments, Canada, USA) was applied to analyze root parameters. The five-week evaluation period was selected as a critical stage of seedling development, allowing the assessment of early effects of biochar amendment on biometric and physiological traits before transplanting. Pepper seedlings of early cultivars have about a 40-day growing period [Jankauskienė and Laužikė 2023]. According to Moon et al. [2025], 35-day-old broccoli seedlings yield the best.

The relative content of flavonols, anthocyanins, chlorophyll, and soil plant analysis development (SPAD) leaf greenness index in the fresh leaves was estimated using a portable MPM-100 multi-pigment meter (Opti-Sciences Inc., Hudson, NH, USA). The relative values of F_0 (minimal fluorescence), F_v (variable fluorescence), F_m (maximal fluorescence), F_v/F_m , and F_v/F_0 ratios in the leaves were measured using the OS-30p+ chlorophyll fluorimeter (Opti-Sciences Inc., Hudson, NH, USA). Measurements were performed on the second pair of fully expanded true leaves to ensure uniform physiological status and comparability among treatments.

Data analysis

The results were statistically processed using Statistica 12.0 (StatSoft Polska, Kraków, Poland). Before the analysis, the Shapiro–Wilk test for normality was performed. The results were analyzed using one-way analysis of variance, and the means were compared using Fisher’s least significant difference (LSD) test at $\alpha = 0.05$. Tables and figures present means $\pm$ standard deviations (SD) and are labeled with statistical letters indicating significant differences, separately for each plant species tested.

RESULTS

Biometric traits

Shoot traits. In broccoli, all analyzed shoot biometric traits (leaf number, shoot length, and shoot fresh and dry weights) did not differ statistically with biochar application (Table 3, Figure 1). In pepper, however, adding biochar to the substrate resulted in significant differences in the analyzed traits. Plants grown in

the substrate supplemented with pine bark and compost biochars produced fewer leaves (6.38 and 6.00, respectively) than the control group (7.50), plants cultivated in substrate with pine sawdust biochar (8.17), and chestnut seed biochar (7.43). The application of biochar from compost resulted in a 15.6–25.5% reduction in shoot length compared to control plants and plants cultivated in substrate with biochar from pine sawdust and pine bark. Adding pine sawdust biochar resulted in the highest shoot fresh weight (almost 38% higher than in plants grown with pine bark biochar and 86.0% higher than in plants treated with compost biochar). A similar relationship was observed in the shoot dry weight. The value of this trait was highest with pine sawdust biochar, representing a 37.2% increase compared with the control group, a 42% increase compared to pine bark biochar, and an 85% increase compared to compost biochar (Table 3, Figure 2). Pine sawdust biochar had the most beneficial effect on pepper shoot growth in terms of intensifying and shortening horticultural production. Plants treated with this biochar had a high number of leaves (8.17), length (12.70 cm), and the highest biomass (3.46 g fresh weight and 254.98 mg dry weight).

Table 3. Biometric traits of broccoli Cezar and pepper Marta Polka shoots, depending on the experimental combination

Experimental combination	Number of leaves	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (mg)
broccoli				
Control (substrate without biochar)	4.13 ±0.96 a	11.48 ±2.75 a	1.91 ±1.06 a	153.36 ±88.20 a
Substrate + pine sawdust biochar	3.50 ±0.52 a	11.06 ±1.41 a	1.72 ±0.62 a	140.37 ±49.07 a
Substrate + chestnut seed biochar	3.33 ±1.28 a	10.07 ±3.01 a	1.93 ±1.09 a	152.14 ±91.49 a
Substrate + pine bark biochar	3.38 ±1.02 a	10.06 ±3.03 a	1.72 ±0.93 a	138.34 ±76.06 a
Substrate + compost biochar	3.56 ±0.98 a	9.99 ±3.53 a	1.55 ±0.97 a	124.71 ±81.77 a
pepper				
Control (substrate without biochar)	7.50 ±1.26 a	11.21 ±2.44 a	2.68 ±1.07 ab	185.80 ±83.76 b
Substrate + pine sawdust biochar	8.17 ±1.11 a	12.70 ±1.37 a	3.46 ±0.38 a	254.98 ±33.11 a
Substrate + chestnut seed biochar	7.43 ±0.94 a	11.19 ±1.11 ab	2.89 ±0.58 ab	188.81 ±34.84 ab
Substrate + pine bark biochar	6.38 ±0.50 b	11.28 ±1.11 a	2.51 ±0.49 bc	179.50 ±43.90 b
Substrate + compost biochar	6.00 ±0.84 b	9.46 ±1.78 b	1.86 ±0.67 c	137.56 ±62.30 b

Means ± standard deviation marked with the same letter do not differ significantly at $\alpha = 0.05$ ($n = 10$), separately for each plant species.

Figure 1. Broccoli Cezar seedlings after five weeks of cultivation: A – control (substrate without biochar), B – substrate + pine sawdust biochar, C – substrate + chestnut seed biochar, D – substrate + pine bark biochar, E – substrate + compost biochar (bar = 15 cm)

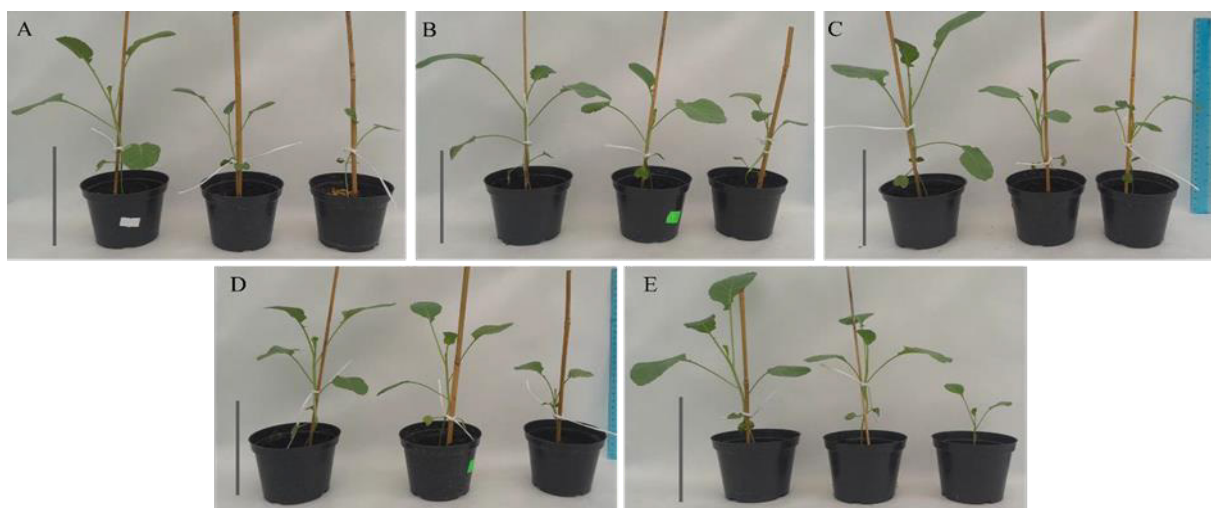


Figure 2. Pepper Marta Polka seedlings after five weeks of cultivation: A – control (substrate without biochar), B – substrate + pine sawdust biochar, C – substrate + chestnut seed biochar, D – substrate + pine bark biochar, E – substrate + compost biochar (bar = 15 cm)

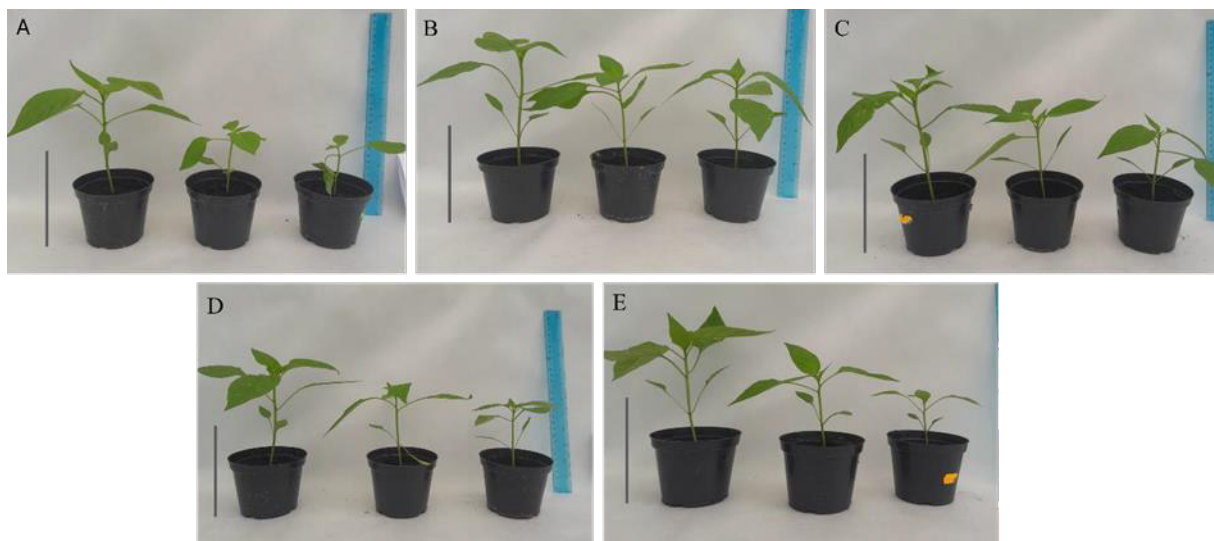


Figure 3. Morphological traits of broccoli Cezar and pepper Marta Polka root systems, depending on the experimental combination; means $\pm$ standard deviation marked with the same letter do not differ significantly at $\alpha = 0.05$ ($n = 10$), separately for each plant species

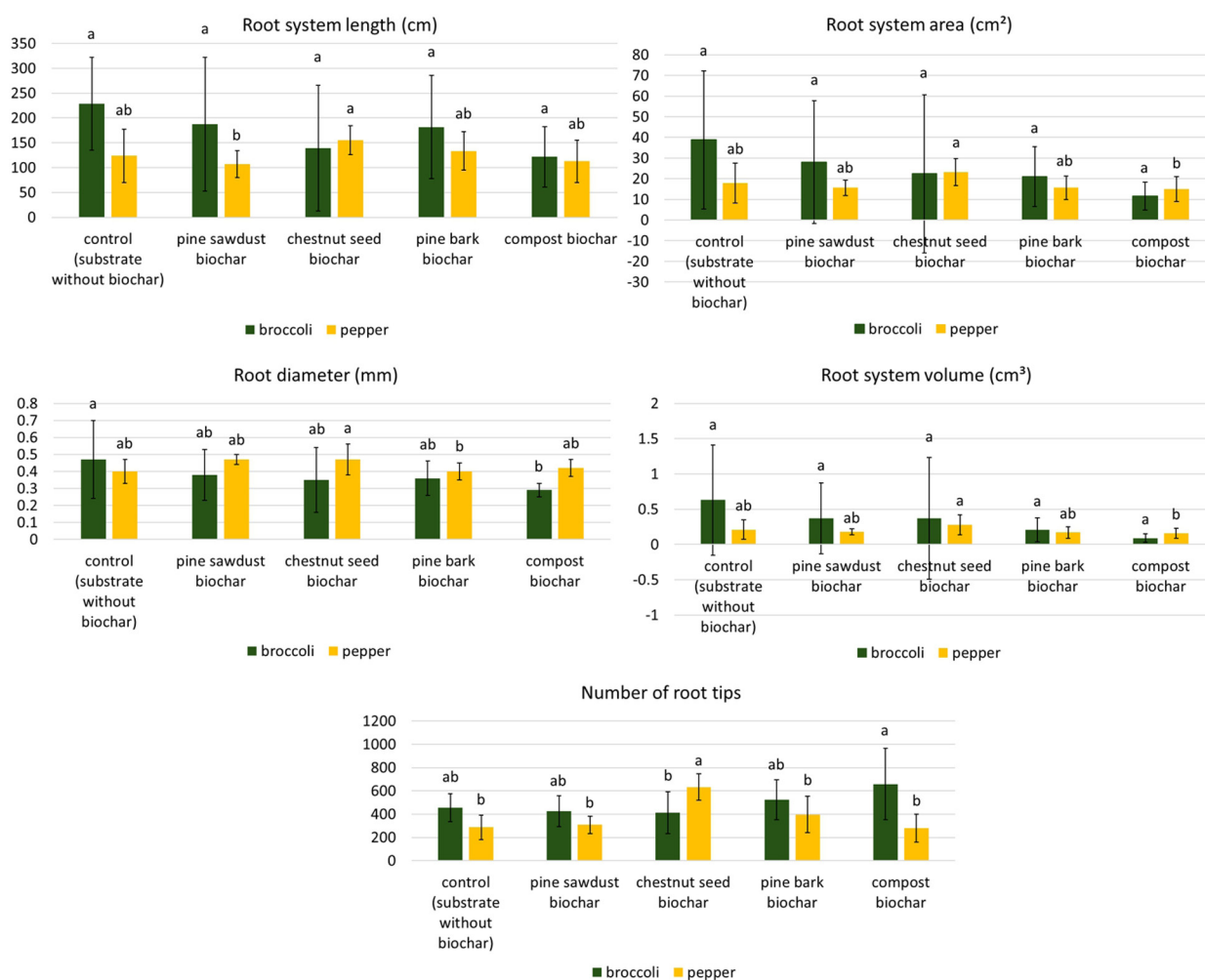
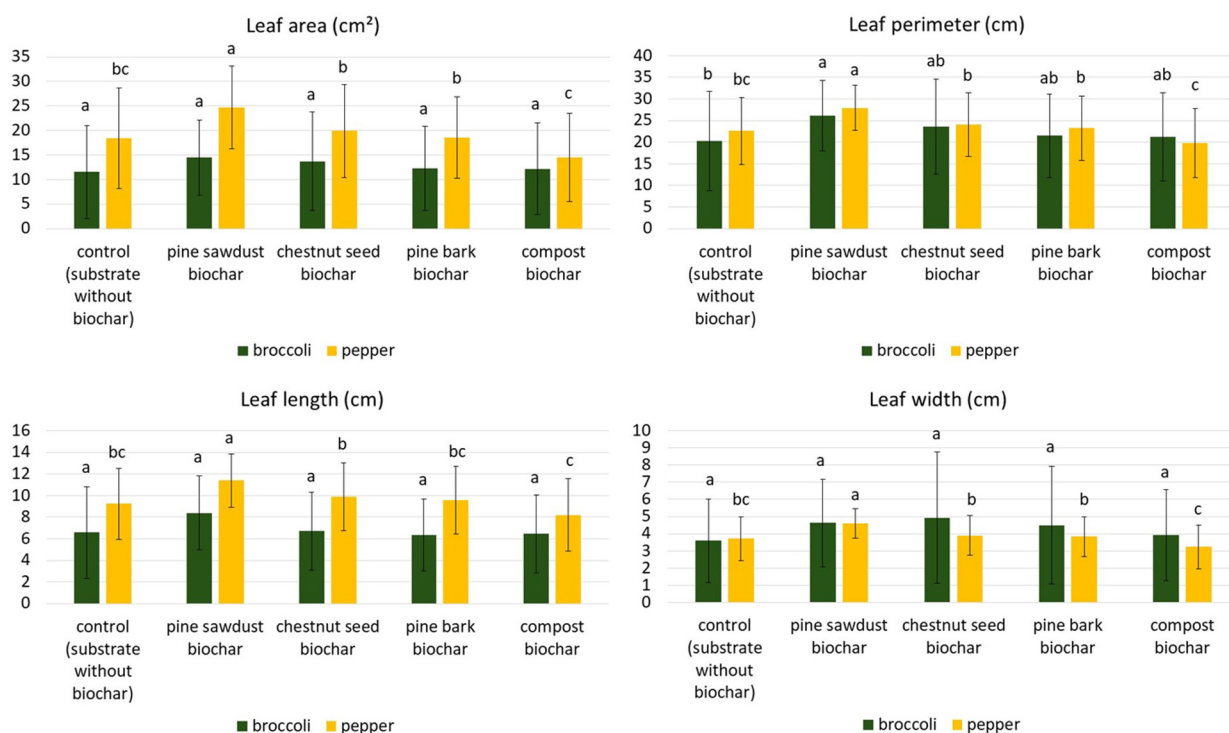


Figure 4. Morphological traits of broccoli Cezar and pepper Marta Polka leaves, depending on the experimental combination; means $\pm$ standard deviation marked with the same letters do not differ significantly at $\alpha = 0.05$ ($n = 10$), separately for each plant species



Root traits. In broccoli, experimental combinations showed no significant differences in root system length, area, or volume. However, control plants had the highest root diameter (0.47 mm), while plants treated with compost biochar had a root diameter reduced by more than 38%. Statistically significant differences were also noted in the number of root tips. The use of compost biochar resulted in the development of the highest number of root tips. This number was reduced by almost 40% in the root systems of plants growing in the substrate with chestnut seed biochar (Figure 3).

Pepper plants cultivated in the substrate supplemented with chestnut seed biochar had the longest root systems (155.40 cm), i.e., a 45.4% increase compared with plants from the substrate with pine sawdust biochar. The root system area and volume in plants treated with chestnut seed biochar were significantly higher than in plants treated with compost biochar, indicating a positive effect of this biochar on root development. The addition of chestnut seed biochar also resulted in a higher root diameter (0.47 mm) than pine bark biochar (0.40 mm), showing a 17.5% increase, and a higher number of root tips compared to other experimental combinations (Figure 3).

The number of root tips showed a different trend across the plant species studied, depending on whether chestnut seed biochar or compost biochar was used. In broccoli, the highest number of root tips was formed in the substrate with compost biochar, and the lowest in the substrate with chestnut seed biochar. In peppers, the relationship was reversed.

Leaf morphology. In broccoli, leaf area, length, and width did not differ across the experimental combinations; however, adding pine sawdust biochar to the substrate significantly increased leaf perimeter (26.12 cm) compared to the control (20.23 cm), representing a 29.1% increase (Figure 4).

In pepper plants, those grown in a substrate with pine sawdust biochar had the highest leaf area (24.68 cm²), perimeter (27.94 cm), length (11.38 cm), and width (4.59 cm), in contrast to plants treated with compost biochar, which presented the lowest values of these traits. Compared to the control plants, leaves of plants grown in the substrate supplemented with pine sawdust biochar had almost 38% higher surface area, almost 24% higher perimeter, and 23% higher length and width (Figure 4).

Physiological parameters

Chlorophyll fluorescence. In broccoli, the highest F_0 value was recorded in plants grown in the substrate supplemented with pine sawdust biochar (139.40), which was 7.5% higher than in plants treated with compost biochar (129.67). Control plants had the highest F_v value (611.33). The F_m in the control plants was significantly higher (744.93) than in the plants supplemented with chestnut seed, pine bark, and compost biochars. The lowest F_v/F_m and F_v/F_0 values were recorded in the plants supplemented with pine sawdust biochar (0.808 and 4.243, respectively) (Table 4).

In pepper, depending on the experimental combination, the F_0 did not differ statistically, ranging from 196.80 to 202.00. At the same time, F_v and F_m in plants grown in a substrate with pine bark biochar were higher than in the control plants, representing increases of 4.3% and 3.8%, respectively. Both the F_v/F_m and F_v/F_0 ratios in plants grown on the chestnut seed biochar substrate were significantly lower than those in plants treated with the other biochars, indicating slightly poorer photosynthetic apparatus condition (Table 4).

Table 4. Chlorophyll fluorescence parameters of broccoli Cezar and pepper Marta Polka plants, depending on the experimental combination

Experimental combination	F_0	F_v	F_m	F_v/F_m	F_v/F_0
broccoli					
Control (substrate without biochar)	133.60 ± 4.98 b	611.33 ± 24.51 a	744.93 ± 27.97 a	0.820 ± 0.005 a	4.577 ± 0.154 a
Substrate + pine sawdust biochar	139.40 ± 4.94 a	591.20 ± 15.49 b	730.60 ± 19.49 ab	0.808 ± 0.004 c	4.243 ± 0.100 c
Substrate + chestnut seed biochar	131.53 ± 4.47 bc	575.93 ± 14.53 c	707.47 ± 18.02 c	0.814 ± 0.004 b	4.380 ± 0.103 b
Substrate + pine bark biochar	130.80 ± 4.09 bc	576.73 ± 19.73 c	707.53 ± 22.15 c	0.815 ± 0.005 b	4.410 ± 0.143 b
Substrate + compost biochar	129.67 ± 5.11 c	592.53 ± 12.01 b	722.20 ± 14.71 bc	0.820 ± 0.006 a	4.577 ± 0.175 a
pepper					
Control (substrate without biochar)	196.80 ± 14.12 a	785.53 ± 56.34 b	982.33 ± 66.54 b	0.799 ± 0.010 ab	3.998 ± 0.229 ab
Substrate + pine sawdust biochar	200.93 ± 7.15 a	812.80 ± 44.83 ab	1013.73 ± 51.43 ab	0.801 ± 0.004 a	4.043 ± 0.109 a
Substrate + chestnut seed biochar	201.67 ± 7.77 a	785.73 ± 31.94 b	987.40 ± 38.06 ab	0.795 ± 0.005 b	3.897 ± 0.115 b
Substrate + pine bark biochar	200.00 ± 9.16 a	819.73 ± 37.25 a	1019.73 ± 45.68 a	0.803 ± 0.003 a	4.099 ± 0.088 a
Substrate + compost biochar	202.00 ± 5.83 a	812.73 ± 27.27 ab	1014.73 ± 31.20 ab	0.800 ± 0.004 a	4.024 ± 0.115 a

Means ± standard deviation marked with the same letter do not differ significantly at $\alpha = 0.05$ ($n = 10$), separately for each plant species. F_0 – minimal chlorophyll fluorescence, F_v – variable chlorophyll fluorescence, F_m – maximal chlorophyll fluorescence

Table 5. Relative pigment content and SPAD index in broccoli Cezar and pepper Marta Polka plants, depending on the experimental combination

Experimental combination	Flavonols	Anthocyanins	Chlorophyll	SPAD index
broccoli				
Control (substrate without biochar)	0.105 ± 0.056 a	0.025 ± 0.012 ab	14.83 ± 2.20 ab	39.17 ± 2.39 ab
Substrate + pine sawdust biochar	0.102 ± 0.043 a	0.027 ± 0.009 ab	14.95 ± 2.78 ab	39.23 ± 2.80 ab
Substrate + chestnut seed biochar	0.089 ± 0.018 a	0.026 ± 0.012 ab	14.16 ± 1.50 ab	38.52 ± 1.70 ab
Substrate + pine bark biochar	0.109 ± 0.053 a	0.028 ± 0.009 a	15.72 ± 2.84 a	40.06 ± 3.27 a
Substrate + compost biochar	0.111 ± 0.051 a	0.020 ± 0.010 b	13.55 ± 2.59 b	37.59 ± 2.61 b
pepper				
Control (substrate without biochar)	0.083 ± 0.053 a	0.017 ± 0.008 ab	7.32 ± 0.89 b	27.95 ± 1.94 b
Substrate + pine sawdust biochar	0.078 ± 0.051 a	0.014 ± 0.012 b	7.78 ± 0.91 b	28.91 ± 1.93 b
Substrate + chestnut seed biochar	0.089 ± 0.069 a	0.021 ± 0.009 a	8.63 ± 0.80 a	30.63 ± 1.54 a
Substrate + pine bark biochar	0.101 ± 0.054 a	0.013 ± 0.007 b	7.61 ± 1.18 b	28.49 ± 2.57 b
Substrate + compost biochar	0.075 ± 0.046 a	0.019 ± 0.013 ab	7.74 ± 0.94 b	28.88 ± 1.82 b

Means ± standard deviation marked with the same letter do not differ significantly at $\alpha = 0.05$ ($n = 10$), separately for each plant species.

Relative pigment content and SPAD index. In both plant species, adding biochar did not affect flavonol content. In broccoli, pine bark biochar application increased anthocyanin, chlorophyll, and SPAD index content to the greatest extent. Plants grown in the substrate with compost biochar had the lowest content of these pigments. Pine sawdust and chestnut seed biochars did not change these parameters compared to the control (Table 5).

In pepper, adding chestnut seed biochar resulted in higher anthocyanin content than pine sawdust and pine bark biochars, as well as the highest chlorophyll content (8.63) and SPAD index (30.63) among the other experimental combinations. Chestnut seed biochar-treated plants accumulated almost 18% more chlorophyll and had an SPAD index higher by almost 10% compared to the control seedlings, confirming better physiological condition and nutrition in chestnut seed biochar-amended plants (Table 5).

DISCUSSION

Plant biometric traits after biochar treatment

A review of scientific publications confirms that biochar modifies plant growth, but the magnitude and direction of the effect depend on the dosage, plant type, feedstock, and soil conditions [Xiang et al. 2017, He et al. 2020, Joseph et al. 2021]. Widowati et al. [2025] recommend using rice husk biochar at 4% for optimal plant growth in wild chili pepper (*Capsicum frutescens* L.) cultivation. Adding biochar adjusted soil pH and significantly increased organic carbon content, water retention capacity, and total N, P, and K content. The use of biochar increased the plant growth rate and leaf number, and improved photosynthesis. In our study with *C. annuum*, four different biochars were added to the substrate at 4.4% concentration, resulting in significant differences in the values of the seedling traits. Biochar from pine sawdust and chestnut seed did not affect the number of leaves compared with the control plants, whereas biochar from pine bark and compost reduced it (on average by 1.31). Furthermore, adding compost biochar shortened shoots compared to control plants (by an average of 1.75 cm), which in turn reduced shoot fresh weight by over 30%. No such relationship was observed in broccoli; none of the biochars used affected shoot growth (Table 3). Leaf area, length, and width were similar, but adding pine sawdust biochar increased their perimeter compared to the control (Figure 4). The results regarding the use of biochars in broccoli are inconclusive. Nayem et al. [2024] demonstrated that corn stover biochar improved broccoli plant growth parameters, including leaf length and width. However, this did not apply specifically to plant length, which is consistent with our study. In the study performed by Graber et al. [2010], pepper growth (leaf area, leaf weight, number of leaves, and yield) was stimulated by wood-derived biochar. In our research, pepper plants cultivated in a substrate amended with pine sawdust biochar also had the highest leaf area, perimeter, length, and width. Effects of biochar on germination and seedling growth range from inhibition to stimulation. Hormesis is commonly observed (high rates of biochar can inhibit, while low rates can stimulate plant growth). The most important factors determining whether biochar impacts seed germination and seedling growth are: (i) release of salts from biochar to the soil solution; (ii) release of phytotoxins; (iii) release of germination-inducing hormones; (iv) change in water capacity and porosity of the soil. Biochar feedstock, production method, and application amount have a range of impacts on germination speed and rate. The specific sensitivity of seeds from different plant species to salinity, toxins, hormone-like compounds, and water availability also leads to highly variable results [Joseph et al. 2021, Bednik et al. 2022].

The use of different biochars resulted in varying responses in pepper root system growth, with the best results after applying chestnut seed biochar (Figure 3). In broccoli, root system length, area, and volume showed no significant differences, and compost biochar reduced root diameter compared with the control. Xiang et al. [2017] conducted a meta-analysis with 2108 paired observations from 136 articles to evaluate root traits under biochar application. Overall, biochar application increased root biomass (+32%), root volume (+29%), and surface area (39%). The biochar-induced increases in root length (+52%) and number of root tips (+17%) were much larger than the increase in root diameter (+9.9%); this result suggests that biochar application benefits root morphological development to alleviate plant nutrient and water deficiency rather than to maximize biomass accumulation.

The physicochemical properties of biochar, such as pH, electrical conductivity, porous structure, and density, are strongly modified by biomass type and thermal parameters of the production process [Ahmad et al. 2021, Tomczyk et al. 2020]. The elemental and mineral composition of biochar depends directly on biomass type, and thermochemical conversion affects nutrient concentrations [Campos et al. 2020, Sayğan et al. 2025]. Biochars derived from biomass rich in organic components exhibit higher potassium and phosphorus contents [Uroić Štefanko and Leszczyńska 2020]. Typical biochar contains 45% to 60% carbon, 2–5% hydrogen, and 10–20% oxygen, although these proportions vary significantly depending on the feedstock [Fakhar et al. 2025]. Biochars derived from agricultural waste exhibit high concentrations of phosphorus (up to 134.6 mg·kg⁻¹) and calcium (up to 649.0 mg·kg⁻¹), as well as magnesium, sodium, and sulfur [Khater et al. 2024]. Much of the N within the biochar C matrix is unavailable to plants, whereas most K in biochar is present in soluble forms, released in the short term after application to soil, and is readily available to plants [Joseph et al. 2021]. In the study by Küçükyumuk et al. [2017], biochar produced from wood debris, combined with chemical fertilizers, increased soil pH, organic matter, N, P, K, Fe, and Mn content, and plant dry weight, while reducing the soil EC value, which suggests that biochar can be used with chemical fertilizers to increase yields and nutrient concentrations in pepper. Laurentino et al. [2025] found that adding biochar to pepper cultivation resulted in higher vitamin C and soluble solids content than mineral fertilization. In our study, pepper shoot dry weight increased significantly (by 37.23%) only after adding pine sawdust biochar, compared with plants grown without biochar (Table 3).

According to Lévesque et al. [2020], adding 5% maple bark and pine chip biochars improved plant water-use efficiency, increased the dry weight of tomato (*Solanum lycopersicum* L.) and pepper fruits, and increased N and P uptake efficiency. Majeed et al. [2022] showed that single-plant biochar derived from corn cobs was insufficient to provide adequate nutrients for optimal pepper growth. While biochar alone increases soil carbon sequestration, most biochars do not contain sufficient available plant nutrients, so the authors suggest the use of biochar in conjunction with mineral or organic fertilizers rich in available plant nutrients. Biochar application alone had no significant effect on most growth parameters, but combining 0.5% biochar with 2% poultry litter resulted in the highest plant height and stem diameter. According to Montoya et al. [2022], using enriched biochar in broccoli cultivation is advisable because it does not cause yield losses and increases nutrient content in inflorescences. In contrast, in our study, biochar use in broccoli cultivation did not cause nearly any changes in plant biometric or biochemical traits (Tables 3–5). In contrast, in pepper, the effect varied, confirming plants' genotypic sensitivity to specific biochar types, as reported by Liao et al. [2024]. This may be attributed to differences in species' tolerances to changing soil/substrate conditions. The results should also be related to the carbon and nitrogen content determined in the tested biochars (Table 1). Compost biochar generally did not increase the analyzed traits of broccoli and pepper plants. This biochar had the lowest total organic carbon content. The other biochars contained more than twice as much carbon. However, compost biochar had the highest total nitrogen content. The high nitrogen content of the biochars does not appear to be a key factor determining the growth of pepper and broccoli in this experiment. Pine sawdust and chestnut seed biochar had similar total carbon content, but chestnut seed biochar had thirteen times more nitrogen (Table 1). While pine sawdust, characterized by low pH (3.86) and EC (108.05 µS·cm⁻¹), stimulated more intensive shoot development in pepper seedlings, chestnut seed biochar with high pH (8.24) and EC (777.8 µS·cm⁻¹) stimulated more intensive development of the root system (Table 3, Figure 3). A large portion of the nutrients, including N, P, K, and Ca, in the feedstocks can be retained in the biochar's porous structure during pyrolysis. These nutrients promote soil fertility and plant growth. The elemental composition of biochar plays an important role in modulating plant growth [Guo et al. 2023]. The chemical analysis of the tested biochars showed that, compared to peat substrate, chestnut seed biochar was rich in K, Na, Cu and Zn. The high content of these elements in chestnut seed biochar may be associated with its alkaline pH and high EC value. Moreover, compared to the other types of biochar tested, chestnut seed biochar contained the highest levels of K, Ca, Cu, Zn, and Mg – elements that stimulate root system development. On the other hand, pine sawdust biochar had very low content of K, Ca, Na, Cu, Zn, Mg, and Fe, but it was characterized by a high Mn content. Manganese is an essential micronutrient for pepper plants, playing a major role in growth, enzyme functions, and stress responses [Silber et al. 2009, Yasuor et al. 2015].

Plant physiological parameters after biochar treatment

He et al. [2020] synthesized 74 publications to evaluate the overall tendency of plant photosynthesis and growth following biochar amendment. They found that biochar significantly increased photosynthetic rate by 27.1% and improved stomatal conductance, transpiration rate, water use efficiency, and chlorophyll concentration by 19.6%, 26.9%, 26.8%, and 16.1%, respectively. Corn stover biochar applied at 2–8 ton per ha improved chlorophyll content in broccoli [Nayem et al. 2024]. In our study, chlorophyll content in broccoli did not differ in treatments with biochar compared to the control. In contrast, pepper showed a higher chlorophyll content only in plants treated with chestnut biochar (Table 5). Chestnut seed biochar contained 53.25 mg·g⁻¹ Mg, 5–8 times higher than the other biochars tested, and half the content found in the peat substrate. Magnesium is a central building block of chlorophyll. Without it, plants cannot produce chlorophyll, capture sunlight, and carry out photosynthesis. Magnesium (Mg²⁺) is pivotal for the vitality, yield, and quality of horticultural crops. Central to plant physiology, Mg²⁺ stimulates growth and biomass accumulation [Ishfaq et al. 2022, Ahmed et al. 2023]. Under Mg deficiency, pepper plants show decreased relative growth rate, total dry weight, stem and root mass fractions, and leaf area, whereas the shoot-to-root ratio increased [Riga and Anza 2003].

The broccoli plants treated with pine sawdust, chestnut seeds, and pine bark biochars had slightly lower F_v/F_m and F_v/F_0 ratios. As for pepper, the lowest F_v/F_m and F_v/F_0 values were found for chestnut seed biochar (Table 4). The ratio of variable to maximum fluorescence (F_v/F_m) represents the maximum potential quantum efficiency of open photosystem II centers. Its average optimal value for most plant species is 0.79 to 0.83, with lowered values indicating plant stress [Maxwell and Johnson 2000]. The values obtained in our study fall within the reference range, confirming the absence of stress resulting from the application of the tested biochars. In contrast, F_v/F_0 is a more sensitive plant stress detector, normalized over the minimum fluorescence measurement. It defines the efficiency of the water-splitting reaction (photolysis) and the performance of the electron donor for photosystem II following dark adaptation of the plant. It highlights even minor disruptions in electron transport and damage to the oxygen-evolving complex, for example, by drought, salinity, or toxins. For healthy plants, this parameter typically falls within the range of 4–6 [Baker and Oxborough 2004, Banks 2018]. In our study, all F_v/F_0 values in all broccoli plants were above 4, whereas in pepper, only plants treated with chestnut seed biochar and the control group had F_v/F_0 values below 4. Therefore, biochar treatment did not induce stress in the studied plants.

CONCLUSION

In broccoli, using various biochars during seedling production had an ambiguous effect on plant cultivation. None of the tested biochar treatments significantly stimulated plant growth for horticultural production, allowing for shortening the seedling cultivation period or increasing the commercial quality of the plants. The use of pine sawdust, chestnut seeds, pine bark, and compost biochars at a concentration at 4.4% in peat substrate does not seem justified in the production of broccoli seedlings under greenhouse conditions. Perhaps higher concentrations of the tested biochars would have positive effects, which is worth testing in future studies.

Pepper plants grown in peat substrate supplemented with pine sawdust biochar showed improved shoot growth parameters. Chestnut seed biochar positively affected root system development. This biochar also stimulated the highest accumulation of anthocyanins and chlorophyll in leaves. Chlorophyll fluorescence parameters of biochar-treated pepper seedlings were statistically similar to those in the control. The use of biochars from pine sawdust and chestnut seeds may therefore be a beneficial modification in pepper seedling production. The observed stimulation of plant growth can shorten the seedling production period, thereby ensuring high-quality seedlings. The obtained results are worth implementing in horticultural production.

Overall, various biochar types affect shoot and root development differently. Plant genotype is an important factor in the mechanism of plant response to biochar treatment. Future research should focus on broader testing of different vegetable species and cultivars, as well as examining different combinations of

biochars at higher concentrations. It is important to bear in mind the relationship between the chemical composition of biochars and their impact on plants. An important research direction is also to determine the microbiological and biochemical activity of substrates supplemented with biochars.

DECLARATION OF THE USE OF GENERATIVE AI TOOLS IN THE PROCESS OF PREPARING THE PAPER

The authors did not use genAI tools during the research and manuscript preparation.

AUTHOR CONTRIBUTIONS

A.T. – developing a concept, methodology, conducting research, data curation, visualization, writing, review, supervision; D.K. – methodology, conducting research, data curation, review; P.W. – methodology, conducting research, data curation, review; A.K. – conducting research, visualization; A.M. – conducting research; M.A. – software, analysing the results, visualization, writing, review.

PATENTS

The elaborated method of pine sawdust biochar and chestnut seed biochar use in pepper seedlings cultivation was sent to the Patent Office of the Republic of Poland as a patent application P.455823 [WIPO ST 10/C PL455823]: “Method for producing pepper seedlings using biochar from pine sawdust or chestnut seeds” (Polish title: “Sposób produkcji rozsady papryki rocznej z wykorzystaniem biowęgla z trocin sosnowych lub kasztanów”); in Polish.

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COMPARATIVE ASSESSMENT OF RASPBERRY CULTIVARS FROM ORGANIC AND CONVENTIONAL CULTIVATION SYSTEMS USING IMAGE ANALYSIS AND TRADITIONAL MACHINE LEARNING ALGORITHMS

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ABSTRACT

The objective of this study was to evaluate the usefulness of image analysis and traditional machine learning algorithms for the discrimination of raspberry cultivars originating from organic and conventional cultivation systems. Images of the whole fruit of four raspberry cultivars: Polana, Polesie, Poemat, and Poranek, were acquired using a digital camera. Texture descriptors were extracted from images. The mean values of selected image features were compared, and the artificial intelligence models were developed. The mean comparison revealed significant differences in image texture descriptors among raspberry cultivars within both cultivation systems and between organic and conventional samples of the same cultivar. For cultivar discrimination of Polana, Polesie, Poemat, and Poranek in organically grown raspberries, the highest accuracy of 89.2% was achieved using the Subspace Discriminant classifier, followed by Quadratic Support Vector Machine (88.1%) and Wide Neural Network (86.0%). In conventionally cultivated raspberries, the highest accuracy of distinguishing cultivars reached 91.7% for the Linear Discriminant model, followed by Subspace Discriminant (91.3%) and Medium Neural Network (88.9%). All models were characterized by high area under the Receiver Operating Characteristic (ROC) curve (AUC) values, indicating excellent discrimination performance. Complete separation of organic and conventional raspberries for all analyzed cultivars with an accuracy of 100% was obtained. The results demonstrate that image texture analysis combined with traditional machine learning algorithms provides a rapid, objective, and non-destructive approach for raspberry cultivar identification and cultivation-system authentication. The proposed methodology shows considerable potential for applications in quality control, traceability, and verification of production practices in the raspberry industry.

Keywords: raspberries, production, image texture features, artificial intelligence algorithms, discrimination

INTRODUCTION

The common raspberry (*Rubus idaeus* L.) is one of the most economically important berry crops cultivated worldwide. Its fruit is highly valued by consumers due to its attractive appearance, distinctive flavor and aroma, as well as its high nutritional and health-promoting value. Raspberries are a rich source of bioactive compounds, including polyphenols, anthocyanins, vitamin C, and other antioxidants, which

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may play an important role in reducing oxidative stress and supporting the prevention of various chronic diseases [Lopez-Corona et al. 2022, Lo Piccolo et al. 2020]. Growing consumer awareness of the relationship between diet and health, together with increasing interest in high-quality foods and organically produced products, has contributed to greater interest in berry fruits, including raspberries [Sangiorgio et al. 2021, Dudziak and Kocira 2022, Kazimierczak et al. 2024]. Compared with conventional production, organic farming employs different fertilization, plant protection, and soil management strategies, which may affect plant growth and fruit quality [Sangiorgio et al. 2021, Kotuła et al. 2022].

Previous studies indicate that production systems may influence fruit chemical composition, bioactive compound content, and selected quality attributes important to consumers [Kotuła et al. 2022]. Therefore, the identification of traits associated with different production systems represents an important area of research from both scientific and practical perspectives. Fruit appearance is among the most important factors determining market attractiveness and consumer acceptance. Raspberry cultivars differ in morphological characteristics such as fruit size, shape, drupelet number, and other traits affecting market quality. These characteristics are determined by genetic factors, environmental conditions, and cultivation practices [Titirică et al. 2023]. Their objective evaluation is of considerable importance in breeding programs, quality control procedures, and studies of fruit marketability.

Traditional fruit quality assessment is based primarily on manual measurements and visual evaluation performed by specialists. Although widely used, these methods are time-consuming and may be affected by subjectivity. The rapid development of digital imaging technologies has created new opportunities for fast, objective, and non-destructive evaluation of fruit characteristics. Computer vision systems enable the acquisition and analysis of information related to color, texture, shape, and other visual features, providing valuable data for quality assessment and plant material classification [Bhargava et al. 2021].

Advances in artificial intelligence have further expanded the potential applications of image analysis in agriculture and horticulture. Algorithms such as support vector machines (SVM), decision trees, artificial neural networks, and deep learning models enable the automatic detection of patterns in image data and effective object classification [Kamilaris and Prenafeta-Boldú 2018, Liakos et al. 2018, Bird et al. 2022, Knott et al. 2023]. These methods have been applied to cultivar identification, ripeness assessment, damage detection, and fruit quality classification.

Numerous studies have demonstrated the usefulness of image-analysis-based approaches for assessing the quality of berry fruits. Ropelewska and Masny [2025] achieved high classification accuracy in the discrimination of strawberry cultivars and breeding materials using image analysis and machine learning techniques. Similar approaches have also been applied to raspberries. Rodríguez-Pulido et al. [2017] demonstrated the feasibility of assessing raspberry ripeness using colorimetric and near-infrared imaging techniques. Wang et al. [2022] demonstrated the effectiveness of combining hyperspectral imaging with chemometric methods for geographical origin identification and prediction of selected quality parameters of raspberry fruits. Quintero Rincón et al. [2022] developed a specialized RGB image database intended for the automatic quality assessment of raspberries using computer vision techniques.

Despite the growing number of studies on the application of image analysis and artificial intelligence in horticultural sciences, relatively little attention has been devoted to differentiating raspberry fruit originating from different production systems. In particular, studies investigating the possibility of distinguishing fruit of the same cultivars grown under organic and conventional production systems solely based on digital image features remain limited. A better understanding of these relationships may provide valuable insights into the effects of production technologies on fruit appearance and contribute to the development of tools supporting the identification of fruit origin.

The aim of this study was to evaluate the potential of image analysis and artificial intelligence methods for differentiating fruit of four raspberry cultivars grown under organic and conventional production systems.

MATERIALS AND METHODS

Raspberry samples

The study was conducted on fruit of four autumn cultivars of common raspberry (*Rubus idaeus* L.): Polana, Poemat, Polesie, and Poranek. The fruit samples were harvested at full harvest maturity from plants grown at two locations differing in production systems. Material from organic cultivation was obtained from the Organic Experimental Orchard in Nowy Dwór-Parcela, Polska while fruit samples from conventional cultivation were harvested at the Experimental Orchard in Brzezna, Polska. The Organic Experimental Orchard represented an established organic production system and contained all four cultivars included in the study. Therefore, when selecting the conventional orchard, particular attention was paid to obtaining fruit from the same four cultivars in order to minimize cultivar-related variation between the two production systems. An orchard located in the same or a geographically close region was also sought to reduce differences in climatic and atmospheric conditions between the two sites. The two experimental orchards are located within the same general climatic region, which was considered when selecting the study sites. The same four cultivars were therefore evaluated under both production systems, and fruits were collected at full harvest maturity. This approach was intended to minimize the potential influence of differences in cultivar composition and developmental stage and to facilitate comparison of the production systems across a common set of cultivars. Nevertheless, because the organic and conventional fruits were obtained from two different orchards, the effects of production system and orchard location cannot be completely separated in the present experimental design. Site-specific factors, including soil properties, microclimate, environmental conditions, and management history, may have contributed to the observed differences. Thus, the results should be interpreted as differences between fruit samples obtained under the two production systems at the respective experimental sites, rather than as definitive evidence that the cultivation system alone caused all observed differences.

Crop cultivation in both the organic and conventional systems was conducted in accordance with current agrotechnical recommendations for the respective production methods. Management practices included fertilization, weed control, irrigation, and plant protection in accordance with the principles applicable to the respective cultivation systems. For each cultivar and production system, 100 fruits were harvested. Subsequently, fruits were selected from the harvested material for image acquisition and further analysis. Only healthy fruits were selected for the study – those free from mechanical damage, disease symptoms, and pest feeding, uniform in size and shape, and at full harvest maturity. Overripe, deformed, or damaged fruits were excluded from further analysis. Immediately after harvesting, the fruit samples were transported to the laboratory, where it was cleaned and prepared for photography. The selected raspberries were characterized by uniform coloring typical of the cultivar.

Image acquisition

The whole raspberries representing belonging to four cultivars, Polana, Polesie, Poemat, and Poranek, from organic and conventional cultivation systems were imaged with 60 repetitions for each cultivar and cultivation system on a white background. Image acquisition was performed immediately after preparation of the selected fruit samples under laboratory conditions. The raspberry fruits were photographed using an Apple iPhone SE (2nd generation) (Apple Inc., Cupertino, CA, USA). The images were acquired at a resolution of 4032 × 3024 pixels, corresponding to a 4:3 aspect ratio, and stored in JPEG format. According to the EXIF metadata available in the original image files, the camera settings for the documented images included an aperture of f/1.8, an exposure time of 1/100 s, ISO 32, an exposure compensation of 0 EV, and a focal length of 4 mm.

The fruits were placed individually on a uniform, light-colored background and arranged without overlapping. The camera was mounted on a tripod at a height of 24 cm above the sample surface and positioned vertically above the fruit to obtain a consistent view of the fruit surface. The same camera position,

distance from the samples, sample arrangement, background, and imaging procedure were maintained throughout the image acquisition process in order to minimize variation unrelated to the external characteristics of the fruit. The photographs were acquired under laboratory conditions using a consistent imaging setup. However, detailed information concerning the exact illumination intensity and the white-balance setting was not available in the archived image metadata. Therefore, these parameters were not retrospectively specified.

The same image acquisition procedure was applied to the fruits from the different cultivars and production systems to ensure the comparability of the photographs used for subsequent image processing and classification.

Image processing

For mean comparison of image features and development of discriminative models using traditional machine learning algorithms, the obtained images were subjected to image processing using MaZda 4.7 software (Łódź University of Technology, Institute of Electronics, Łódź, Poland) [Szczypiński et al. 2007, Szczypiński et al. 2009, Strzelecki et al. 2013]. The procedure included conversion of images into individual color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S*, segmentation, and determination of regions of interest (ROI), each corresponding to a whole raspberry fruit. For each ROI, 2172 texture parameters were computed.

Mean comparison

The statistical analysis of image texture features was performed to identify differences in fruit surface structure among the studied raspberry cultivars originating from an organically managed mother plantation or a conventional cultivation system, and to compare fruit samples from organic and conventional systems. For comparative analysis between samples, mean values of textures computed on the basis of the histogram (HMean) for individual color channels were selected, as RHMean, GHMean, BHMean, LHMean, aHMean, bHMean, XHMean, YHMean, ZHMean, UHMean, VHMean, SHMean. Mean comparison of image textures was performed using Statistica 13.3 (Tibco Software Inc., PaloAlto, CA, USA). Prior to the analysis, the normality of the data distribution was assessed using the Shapiro–Wilk test, and the homogeneity of variances was evaluated using Levene’s test. When the assumptions were satisfied, differences among means were assessed using Tukey’s honestly significant difference (HSD) test. The significance level was set at $p < 0.05$.

Discriminant analysis

The discriminant analysis was performed in the MATLAB environment (MathWorks, Inc., Natick, MA, USA). The initial image database contained 60 images of individual fruits for each cultivar in each cultivation system. The procedures were carried out in several steps to distinguish the following samples:

- 1) raspberry cultivars Polana, Polesie, Poemat, and Poranek from an organically managed mother plantation, with 60 images acquired for each cultivar, resulting in a total dataset of 240 images;
- 2) raspberry cultivars Polana, Polesie, Poemat, and Poranek from a conventional cultivation system, with 60 images acquired for each cultivar, resulting in a total dataset of 240 images;
- 3) Polana from an organically managed mother plantation vs. Polana from a conventional cultivation system, with 60 images acquired for each cultivation system, resulting in a total dataset of 120 images;
- 4) Polesie from an organically managed mother plantation vs. Polesie from a conventional cultivation system, with 60 images acquired for each cultivation system, resulting in a total dataset of 120 images;
- 5) Poemat from an organically managed mother plantation vs. Poemat from a conventional cultivation system, with 60 images acquired for each cultivation system, resulting in a total dataset of 120 images;
- 6) Poranek from an organically managed mother plantation vs. Poranek from a conventional cultivation system, with 60 images acquired for each cultivation system, resulting in a total dataset of 120 images.

Each image represented a separate fruit. Therefore, multiple images of the same fruit were not present in the datasets. Consequently, images of the same fruit could not be assigned to both the training and validation subsets. For each classification step, feature selection was performed using the MRMR

(Minimum Redundancy Maximum Relevance) feature-selection method. Image texture descriptors were selected from the complete set of 2172 extracted attributes and subsequently used to classify raspberry samples using traditional machine-learning methods. The MRMR procedure is a supervised feature-selection method that evaluates the relevance of each feature to the predefined class labels while simultaneously minimizing redundancy among the selected features. Features were ranked according to the MRMR criterion, which is based on the trade-off between maximum relevance to the target class and minimum redundancy with respect to the other selected features. The 30 highest-ranked texture attributes were selected for each classification task and used as input variables for the subsequent classification models. The feature-selection procedure was performed separately for each classification task, using the corresponding class labels (i.e., cultivar or cultivation system) as the target variable. The same MRMR procedure, parameter settings, and selection criterion were applied consistently across all classification tasks, with the number of selected attributes fixed at 30. This standardized procedure ensures reproducibility of the feature-selection process. Therefore, the models were developed using the selected features with the highest discriminatory power, which contributed to improved classification accuracy and reduced model development time. Subsequently, discrimination was performed using a 10-fold cross-validation procedure. In 10-fold cross-validation, each dataset was divided into 10 subsets (folds). In each iteration, 9 folds were used for model development, and the remaining fold was used for validation. This process was repeated 10 times so that each image was used once as part of the validation set. Model performance was assessed using accuracy and Receiver Operating Characteristic (ROC) curve (AUC) values.

In the case of the classification of raspberry cultivars derived from the organically managed mother plantation, three algorithms representing different machine learning approaches were selected, as providing the highest accuracies: Subspace Discriminant, Quadratic Support Vector Machine (SVM), and Wide Neural Network. The Subspace Discriminant model was trained using an ensemble of discriminant learners operating in randomly selected subspaces of the feature space. The model utilized 30 learners with a subspace dimension of 30. The prediction speed was approximately 290 observations/second, and the training time was 27.94 seconds. The Quadratic SVM classifier employed a quadratic kernel function and a one-versus-one multiclass strategy. The kernel scale was automatic. The box constraint level was set at 1. The standardize data option was used. The prediction speed was equal approximately 1200 observations/second, and the training time reached 10.77 seconds. The Wide Neural Network model consisted of a fully connected neural architecture with one hidden layer containing 100 neurons. The network was trained using the ReLU activation function, and the training process was performed with a validation-based optimization procedure. The iteration limit was 1000, and the regularization strength (Lambda) was 0. The model was characterized by the prediction speed of approximately 2200 observations/second and the training time of 55.48 seconds.

For the classification of raspberry cultivars sampled from the conventional system, Linear Discriminant, Subspace Discriminant, and Medium Neural Network algorithms were chosen. The Linear Discriminant classifier was constructed using a linear discriminant function for multiclass recognition. The covariance structure was full. The model was characterized by the prediction speed reaching approximately 1000 observations/second and the training time of 4.94 seconds. The Subspace Discriminant approach employed an ensemble learning strategy based on multiple discriminant learners operating on randomly generated feature subspaces. The ensemble consisted of 30 learners with a subspace dimension equal to 43. The prediction speed was approximately 240 observations/second, and the training time was 66.27 seconds. The Medium Neural Network model was characterized by a single fully connected hidden layer with 25 neurons, ReLU activation function, iteration limit of 1000, regularization strength (Lambda) of 0, prediction speed of approximately 760 observations/second, and the training time of 74.77 seconds.

For distinguishing raspberry samples of each cultivar from an organically managed mother plantation vs. a conventional cultivation system, Subspace Discriminant was selected. The classifier consisted of 30 learners, with the subspace dimension set to 14. The prediction speed and the training time were for Polana – approximately 98 observations/second and 59.31 seconds, Polesie – approximately 110 observations/second and 61.40 seconds, Poemat – approximately 100 observations/second and 54.95 seconds, and Poranek – approximately 100 observations/second and 53.00 seconds, respectively.

RESULTS

Distinguishing raspberry cultivars from the organically managed mother plantation

Mean comparison of selected image textures of raspberry cultivars from the organically managed mother plantation. Analysis of the mean values of selected texture parameters computed based on histogram (HMean) for images in color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S* revealed significant differences among the selected investigated raspberry cultivars originating from organic cultivation (Table 1). The cultivar Poemat was characterized by a clearly statistically significantly higher RHMean value (79.83) than Polana (73.56). Whereas Polesie and Poranek showed intermediate values, not significantly different from the other cultivars. In the case of GHMean, significantly higher values were observed for Polesie and Poemat compared with Polana and Poranek. The BHMean parameter also differentiated the cultivars, with Poranek exhibiting the lowest value (27.49), whereas the remaining cultivars formed a statistically homogeneous group. Poemat was characterized by the highest LHMean value (76.27), significantly differing from Polana and Poranek. The aHMean parameter had the lowest value for Polesie, while the other cultivars showed significantly higher and not statistically significantly different values. For bHMean, three homogeneous groups were observed, with the highest value for Poranek. Clear differences in image textures were also observed for ZHmean, where Polesie and Poemat had the highest values, and Polana and Poranek had the lowest values. Distinct differences among cultivars were additionally observed for the parameter UHMean, with the lowest value for Poranek, the intermediate for Polesie and Poemat, and the highest for Polana. Conversely, in the case of VHMean and SHMean, the highest value was found for Poranek in one homogeneous group with Poemat, while Polesie exhibited the lowest values in one homogeneous group with Polana. This confirmed cultivar-dependent differences in fruit surface structure.

Table 1. Comparison of selected image textures among raspberry cultivars from an organically managed mother plantation

Image texture	Polana	Polesie	Poemat	Poranek
RHMean	73.56 a	75.06 ab	79.83 b	78.19 ab
GHMean	24.25 a	30.02 b	29.76 b	24.48 a
BHMean	32.60 b	34.40 b	34.37 b	27.49 a
LHMean	71.15 a	74.44 bc	76.27 c	72.91 ab
aHMean	144.99 b	143.26 a	144.80 b	145.91 b
bHMean	132.62 a	133.37 ab	134.39 b	136.66 c
XHMean	9.39 a	10.50 ab	11.51 b	10.36 ab
YHMean	6.23 a	7.48 bc	7.92 c	6.86 ab
ZHMean	4.31 a	5.08 b	4.99 b	3.75 a
UHMean	122.86 c	121.58 b	120.84 b	119.42 a
VHMean	151.00 ab	149.18 a	151.69 bc	153.64 c
SHMean	45.28 ab	42.47 a	47.44 bc	51.50 c

a, b, c – the same letters within rows indicate no statistically significant differences among cultivars

Cultivar discrimination of raspberries from the organically managed mother plantation using traditional machine learning algorithms. The models for distinguishing raspberry cultivars from the organically managed mother plantation based on image texture descriptors using traditional machine learning algorithms demonstrated high effectiveness. The applied algorithms achieved an accuracy of 89.2% in the case of Subspace Discriminant. The confusion matrix (Figure 1a) obtained for the model built using this algorithm indicated that the cultivar Polana was classified with the highest correctness of 96.9%. While Polesie was characterized by the lowest accuracy of 82.0%. Misclassifications were observed mainly between cultivars Polesie and Poemat. As many as 11.5% of cases belonging to the true class Polesie were misclassified as Poemat, and 6.2% of cases from the true class Poemat were incorrectly classified as Polesie.

Figure 1. Cultivar discrimination of raspberries from the organically managed mother plantation based on image textures by Subspace Discriminant using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)

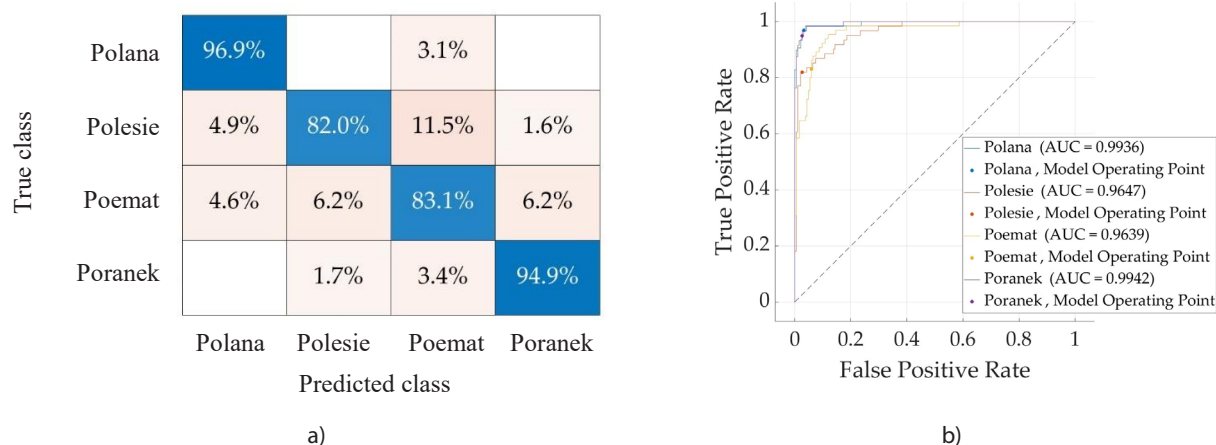
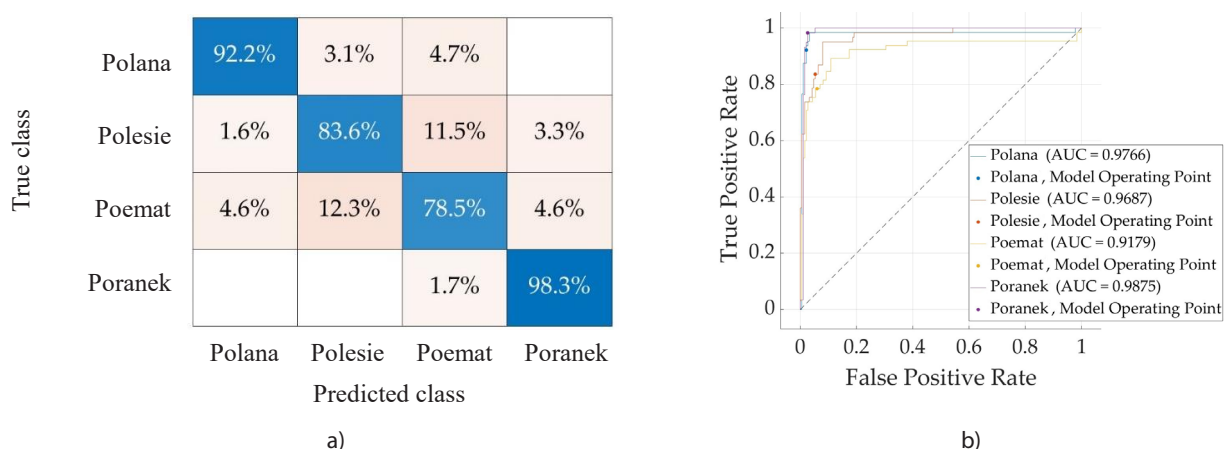


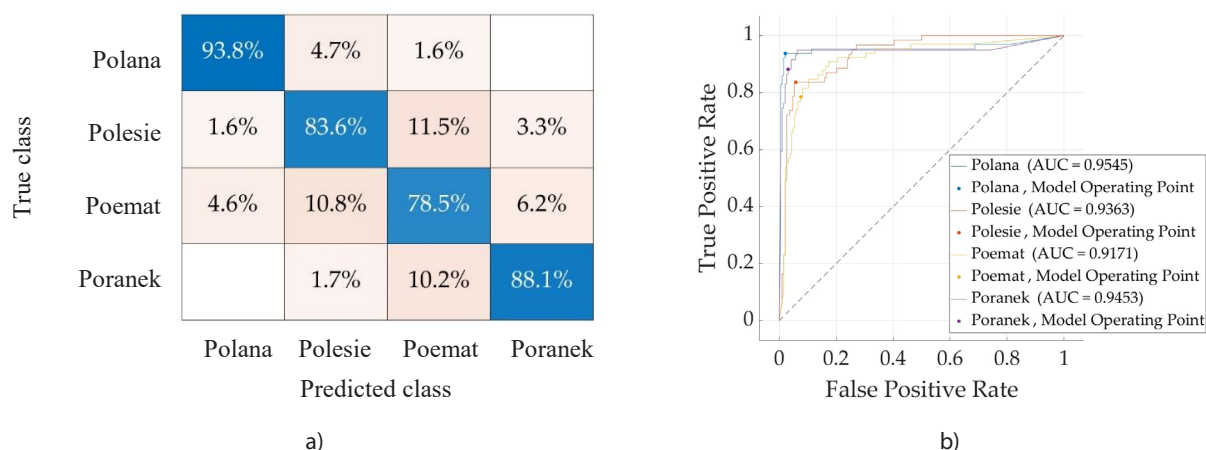
Figure 2. Discrimination of raspberry cultivars from the organically managed mother plantation by Quadratic SVM algorithm based on image texture features using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)



The Receiver Operating Characteristic (ROC) curves presented in Figure 1b demonstrated high discrimination performance of the Subspace Discriminant algorithm for cultivar identification of raspberries originating from the organically managed mother plantation based on image-derived features. All evaluated cultivars achieved very high area under the curve (AUC) values, confirming the strong predictive capability of the developed classification approach. Among the tested cultivars, Poranek and Polana achieved the highest classification performance with an AUC of 0.9942 and 0.9936, respectively. The slightly lower AUC values were observed for Polesie (0.9647) and Poemat (0.9639). The ROC curves located near the upper-left corner demonstrate the strong discriminatory power of the developed models. The operating points selected for the models further confirm balanced performance with low false positive rates and high true positive rates.

The Quadratic SVM classifier also provided satisfactory results, with an accuracy of 88.1%. The highest classification accuracy was achieved for the cultivar Poranek, with 98.3% of cases correctly classified (Figure 2a). The lowest classification performance was observed for Poemat (78.5%). Misclassifications

Figure 3. Classification performance for distinguishing raspberry cultivars originating from an organically managed mother plantation by the Wide Neural Network model using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)



occurred mainly between Polesie and Poemat, indicating the greatest similarity of their image-derived characteristics.

The ROC analysis demonstrated very good discrimination performance of the applied Quadratic SVM algorithm for raspberry cultivars originating from the organically managed plantation (Figure 2b). The highest predictive performance was obtained for Poranek (AUC = 0.9875).

The discriminant analysis performed using the Wide Neural Network model achieved an accuracy of 86.0%. The confusion matrix shown in Figure 3a revealed that the cultivars were generally well differentiated. Among the analyzed cultivars, Polana exhibited the highest classification accuracy (93.8%). Whereas Poemat and Polesie had the lowest accuracies of 78.5% and 83.6%, respectively, with the greatest misclassifications between these cultivars.

All raspberry cultivars achieved high areas under the ROC curve (AUC) exceeding 0.91 (Figure 3b). The cultivar Polana showed the highest AUC of 0.9545, while Poemat was characterized by the lowest AUC of 0.9171.

Distinguishing raspberry cultivars from the conventional cultivation system

Mean comparison of selected image textures of raspberry cultivars from the conventional cultivation system. It was observed that some analyzed raspberry cultivars originating from the conventional cultivation system differed significantly in image texture descriptors (Table 2). The cultivar Poranek had the statistically significantly highest values of RHMean (85.01), aHMean (146.71), XHMean (12.16), VHMean (154.65), and SHMean (52.88), as well as the lowest BHMean (34.39), whereas all the remaining cultivars were in one homogeneous group. For UHMean, raspberry Poranek was also distinguished by the lowest value of 120.26. However, the highest value of 124.28 was noted for Polana, and cultivars Polesie and Poemat were in one homogeneous group with intermediate values. This proves the greatest differentiation of the analyzed image texture features between Polana and Poranek.

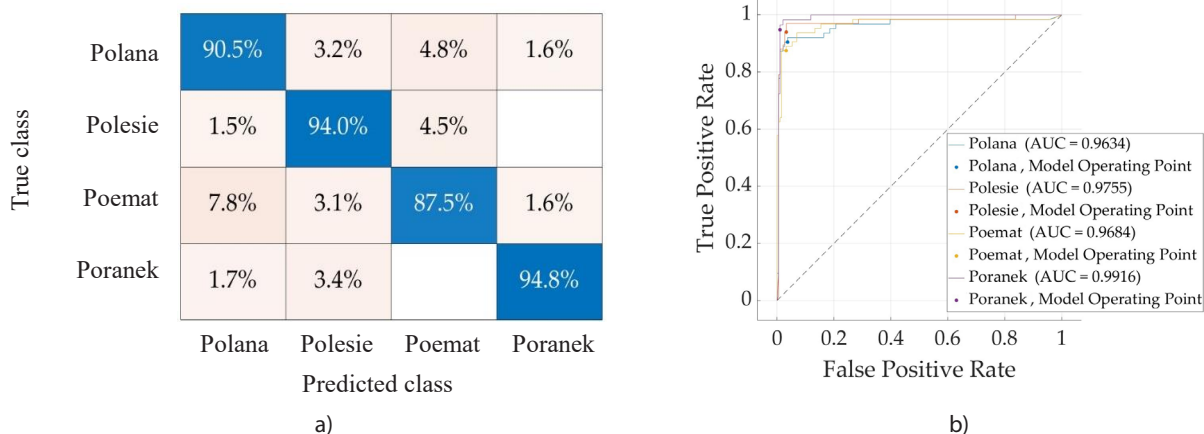
Cultivar discrimination of raspberries from the conventional cultivation system using traditional machine learning algorithms. The discrimination of raspberry cultivars originating from a conventional cultivation system performed using a traditional machine learning approach based on selected image texture parameters revealed very high accuracy of up to 91.7% for the Linear Discriminant model. The highest class-specific accuracy was observed for Poranek (94.8%) and the lowest for Poemat (87.5%). Misclassifications were relatively limited and mainly occurred between Poemat and Polana (Figure 4a).

Table 2. Comparison of selected image textures among raspberry cultivars originating from a conventional cultivation system

Image texture	Polana	Polesie	Poemat	Poranek
RHMean	71.18 a	74.58 a	74.91 a	85.01 b
GHMean	30.44 ab	32.50 b	31.93 b	28.86 a
BHMean	38.72 b	38.58 b	39.49 b	34.39 a
LHMean	73.54 a	75.63 ab	75.57 ab	78.05 b
aHMean	142.21 a	142.38 a	142.80 a	146.71 b
bHMean	130.17 a	131.47 b	130.92 ab	135.26 c
XHMean	9.21 a	10.33 a	10.27 a	12.16 b
YHMean	6.55 a	7.40 ab	7.30 ab	8.02 b
ZHMean	5.19 ab	5.42 b	5.52 b	4.65 a
UHMean	124.28 c	122.95 b	123.54 b	120.26 a
VHMean	146.71 a	147.55 a	147.89 a	154.65 b
SHMean	36.92 a	38.84 a	39.38 a	52.88 b

a, b, c – the same letters within rows indicate no statistically significant differences among cultivars

Figure 4. Classification performance for raspberry cultivar discrimination using Linear Discriminant algorithm based on selected image texture parameters using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)



The Receiver Operating Characteristic (ROC) curves (Figure 4b) further confirmed the robustness of the model, with area under the curve (AUC) values ranging from approximately 0.9634 (Polana) to 0.9916 (Poranek). This indicates excellent separability and strong discriminative power of the selected texture descriptors when combined with the Linear Discriminant classifier. The optimal operating points demonstrated high true positive rates with low false positive rates, supporting the reliability of the developed classification framework.

The Subspace Discriminant ensemble classifier provided a classification accuracy of 91.3%, confirming its high effectiveness in distinguishing between the analyzed cultivars. The highest classification accuracies were observed for Polesie (95.5%) and Poranek (93.1%), while the lowest performance was noted for

Poemat with an accuracy of 85.9%. The greatest misclassifications were observed between Poemat and Polana (Figure 5a).

The ROC analysis (Figure 5b) confirms the robustness of the Subspace Discriminant model, with AUC values reaching 0.9927 for Polesie and 0.9919 for Poranek. This indicates excellent discriminative ability and strong consistency in separating cultivar-specific texture patterns.

The classification of raspberry cultivars obtained from conventional cultivation conditions using a Medium Neural Network based on selected image texture descriptors reached an accuracy of 88.9%, indicating a strong, although slightly lower, discriminative capability compared to linear and ensemble-based approaches. The raspberry cultivar Poranek was distinguished with the highest accuracy of 96.6%, and Poemat had the lowest accuracy of 82.8%. The most incorrectly classified cases were between Poemat and Polesie, as well as between Poemat and Polana (Figure 6a).

Figure 5. Classification performance metrics for raspberry cultivar discrimination using Subspace Discriminant based on selected image texture parameters using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)

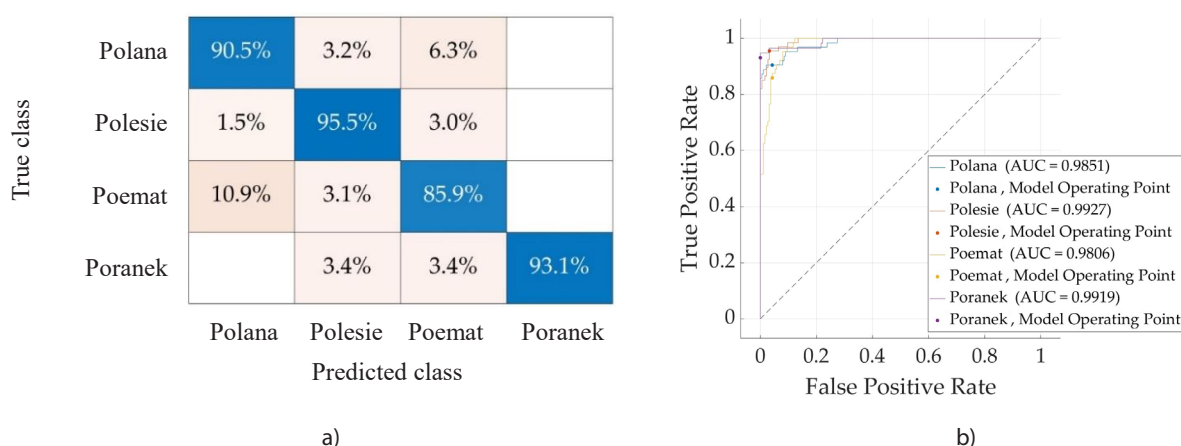
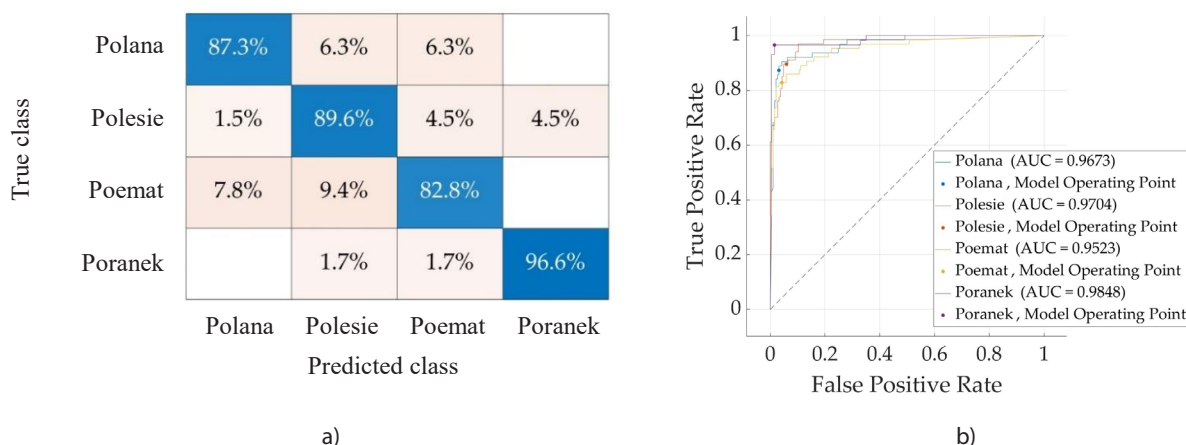


Figure 6. Classification results for raspberry cultivar recognition by a Medium Neural Network based on selected image texture features using a confusion matrix (a), where shades of blue – correctly classified cases with higher percentages of correct classification represented by more intense blue color and other colors – misclassified cases; and Receiver Operating Characteristic (ROC) curves (b), where AUC – area under the curve and the diagonal dashed line represents random classification (AUC = 0.5)



The ROC curves presented in Figure 6b confirm satisfactory classification performance of the neural network model. The AUC values remained high for all cultivars, reaching 0.9848 for Poranek (Figure 6b).

Comparison of raspberries from conventional and organic cultivation systems

Mean comparison of selected image textures between raspberries from conventional and organic cultivation systems. Raspberries of the Polana cultivar exhibited differences in selected image textures between samples from organic and conventional cultivation systems (Table 3). Raspberries from the conventional cultivation system were characterized by statistically significantly higher values of GHMean, BHMean, LHmean, ZHMean, and UHMean compared with fruit from the organic system. In contrast, organically cultivated raspberries showed significantly higher values of aHMean, bHMean, VHMean, and SHMean. No statistically significant differences were found for RHMean, XHMean, and YHMean. The results confirmed that the cultivation system significantly affected selected image texture parameters of Polana raspberries.

Table 3. Comparison of selected image textures between raspberries of the Polana cultivar from conventional and organic cultivation systems

Image texture	Cultivation system	
	organic	conventional
RHMean	73.56 a	71.18 a
GHMean	24.25 a	30.44 b
BHMean	32.60 a	38.72 b
LHMean	71.15 a	73.54 b
aHMean	144.99 a	142.21 b
bHMean	132.62 a	130.17 b
XHMean	9.39 a	9.21 a
YHMean	6.23 a	6.55 a
ZHMean	4.31 a	5.19 b
UHMean	122.86 a	124.28 b
VHMean	151.00 a	146.71 b
SHMean	45.28 a	36.92 b

a, b – the same letters within rows indicate no statistically significant differences between samples

According to the data presented in Table 4, conventionally cultivated raspberries Polesie had statistically significantly higher GHMean, BHMean, and UHMean values. In turn, organic samples were characterized by significantly higher bHMean, VHMean, and SHMean values. No statistically significant differences in terms of cultivation systems were observed for RHMean, LHMean, aHMean, XHMean, YHMean, and ZHMean.

The results shown in Table 5 revealed numerous statistically significant differences between cultivation systems for raspberries Poemat. Fruit from the organic system showed significantly higher RHMean, aHMean, bHMean, XHMean, VHMean, and SHMean values, whereas conventionally cultivated raspberries were characterized by significantly higher GHMean, BHMean, and UHMean values. No significant differences were observed for LHMean, YHMean, and ZHMean.

In the case of Poranek raspberry cultivar (Table 6), organically cultivated raspberries had statistically significantly higher bHMean. Whereas the conventionally cultivated samples were characterized by higher values of image texture parameters, such as RHMean, GHMean, BHMean, LHMean, aHMean, XHMean, YHMean, ZHMean and UHMean. No statistically significant differences were observed for VHMean and SHMean.

Table 4. Mean comparison of selected image textures between raspberries of the Polesie cultivar originating from conventional and organic cultivation systems

Image texture	Cultivation system	
	organic	conventional
RHMean	75.06 a	74.58 a
GHMean	30.02 a	32.50 b
BHMean	34.40 a	38.58 b
LHMean	74.44 a	75.63 a
aHMean	143.26 a	142.38 a
bHMean	133.37 a	131.47 b
XHMean	10.50 a	10.33 a
YHMean	7.48 a	7.40 a
ZHMean	5.08 a	5.42 a
UHMean	121.58 a	122.95 b
VHMean	149.18 a	147.55 b
SHMean	42.47 a	38.84 b

a, b – the same letters within rows indicate no statistically significant differences between samples

Table 5. Comparison of selected image texture features between raspberries of the Poemat cultivar from conventional and organic cultivation systems

Image texture	Cultivation system	
	organic	conventional
RHMean	79.83 a	74.91 b
GHMean	29.76 a	31.93 b
BHMean	34.37 a	39.49 b
LHMean	76.27 a	75.57 a
aHMean	144.80 a	142.80 b
bHMean	134.39 a	130.92 b
XHMean	11.51 a	10.27 b
YHMean	7.92 a	7.30 a
ZHMean	4.99 a	5.52 a
UHMean	120.84 a	123.54 b
VHMean	151.69 a	147.89 b
SHMean	47.44 a	39.38 b

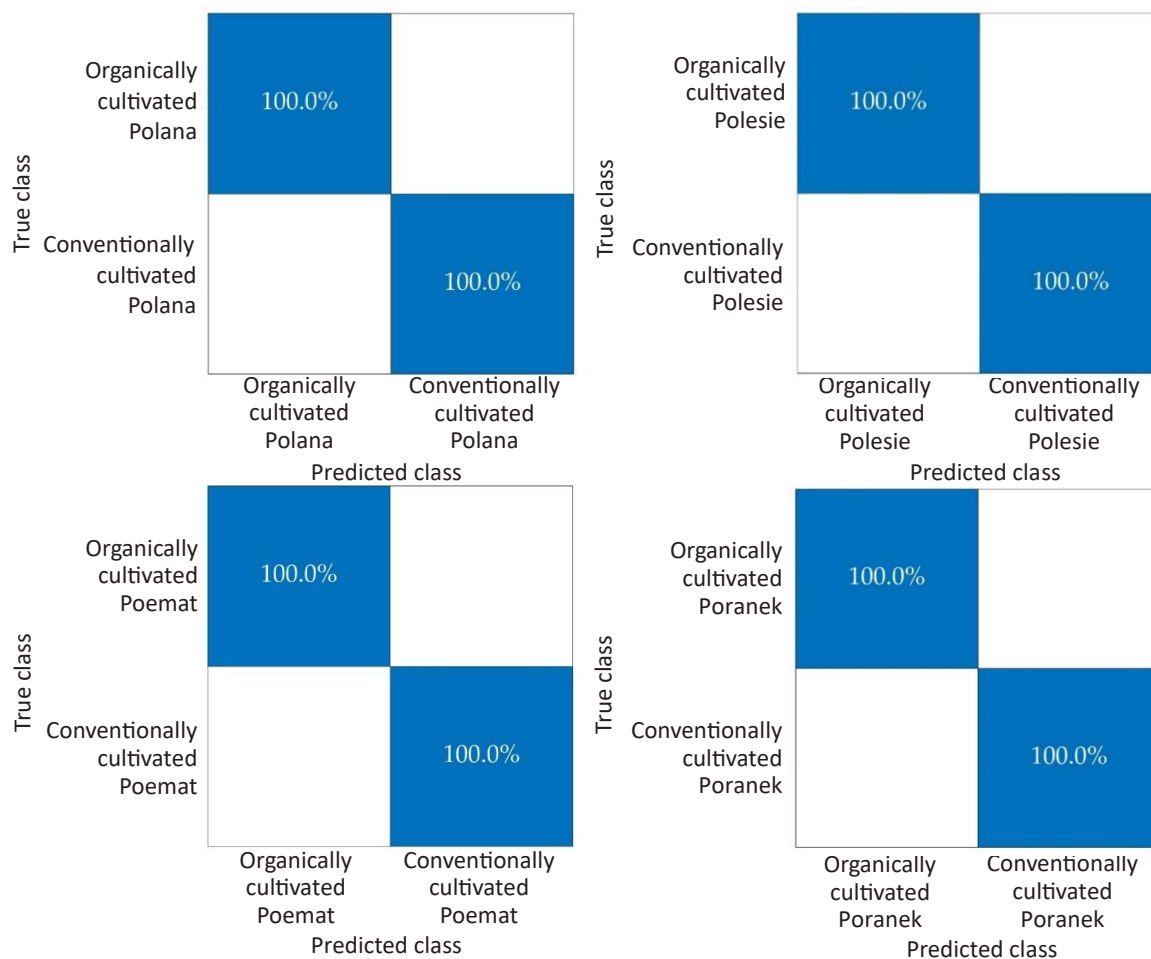
a, b – the same letters within rows indicate no statistically significant differences between samples

Table 6. Comparison of selected image textures between raspberries of the Poranek cultivar from conventional and organic cultivation systems

Image texture	Cultivation system	
	organic	conventional
RHMean	78.19 a	85.01 b
GHMean	24.48 a	28.86 b
BHMean	27.49 a	34.39 b
LHMean	72.91 a	78.05 b
aHMean	145.91 a	146.71 b
bHMean	136.66 a	135.26 b
XHMean	10.36 a	12.16 b
YHMean	6.86 a	8.02 b
ZHMean	3.75 a	4.65 b
UHMean	119.42 a	120.26 b
VHMean	153.64 a	154.65 a
SHMean	51.50 a	52.88 a

a, b – the same letters within rows indicate no statistically significant differences between samples

Figure 7. Distinguishing raspberries from conventional and organic cultivation systems using a model built by Subspace Discriminant based on selected image textures



Discrimination of raspberries from organic and conventional cultivation systems

Samples from organic and conventional cultivation systems were correctly distinguished in 100% based on image features selected from a whole set of 2172 attributes in the case of all analyzed raspberry cultivars (Figure 7). This indicates the existence of fruit image texture sets that allow for the complete differentiation of samples from organic cultivation from those from conventional cultivation.

DISCUSSION

The present study demonstrated that image analysis combined with traditional machine learning algorithms is an effective approach to discriminating raspberry cultivars originating from different cultivation systems. Both the statistical comparison of selected image texture descriptors and the classification results confirmed that raspberry fruit may have cultivar-specific and cultivation-system-dependent image characteristics that can be successfully extracted using computer vision techniques. Surface texture descriptors can reflect differences in the structural and physiological characteristics of fruit surfaces, including surface uniformity and spatial variation in colour and texture. These characteristics can be influenced by growing conditions, as fertilization and nutrient availability affect fruit ripening, phenolic metabolism, and the activity of enzymes involved in antioxidant responses [Frías-Moreno et al. 2021]. Frías-Moreno et al. [2021] demonstrated that organic and conventional fertilization regimes affected antioxidant enzyme activity as well as the content of anthocyanins and other bioactive compounds in raspberry fruit. At the same time, the effects of cultivation practices may depend on the cultivar, highlighting the importance of genotype × environment interactions [Anjos et al. 2020]. Therefore, differences in texture descriptors observed in images of fruit surfaces may be partly associated with changes in ripening-related metabolism, pigment accumulation, and plant responses to growing conditions.

The mean comparison of image texture parameters revealed significant differences among raspberry cultivars from organic and conventional cultivation systems. The observed variability indicates that each cultivar exhibits a unique combination of surface appearance attributes, reflecting genetic differences. The findings indicate that image texture analysis can capture subtle phenotypic differences in fruit. Similar observations have been reported in studies focused on cultivar identification across various fruit species, in which image-derived color, texture, and morphological features enabled successful discrimination of genotypes. For example, strawberry cultivars were effectively differentiated with an accuracy reaching 68% using multiple appearance attributes, such as color, shape, and size, extracted from fruit images, demonstrating the presence of cultivar-specific visual features [Yamamoto et al. 2015]. Ropelewska and Masny [2025] obtained an accuracy of up to approximately 96% of distinguishing cultivars and advanced clones of strawberry in the case of machine learning models developed based on image textures. Likewise, successful identification of plum cultivars based on endocarp size, color, shape, and texture features confirmed the usefulness of image-derived features for distinguishing closely related cultivars [Sarigu et al. 2017]. The effectiveness of stone texture descriptors in characterizing genetic variability of plum was also demonstrated by Ropelewska [2022], where machine learning models achieved high classification accuracy, reaching 96.67% based solely on image texture parameters. In blueberries, image texture parameters combined with both traditional machine learning and deep learning algorithms enabled highly accurate cultivar authentication with accuracy reaching above 91% for both approaches [Ropelewska and Koniarski 2025]. Whereas Ropelewska and Szymajda [2026] reported lower classification accuracies for distinguishing plum cultivars when using deep learning models compared to traditional machine learning. Deep learning also has limitations, such as the need for larger amounts of input data to build models, which can affect results [Ropelewska et al. 2022].

In our study, the cultivars Polesie and Poemat, particularly in the organic cultivation system, exhibited similar image texture characteristics. Nevertheless, despite this similarity, the developed models maintained high overall classification performance. This observation reflects one of the major challenges of visual recognition, namely the presence of small inter-class differences combined with considerable intra-class variability. Previous studies on plant recognition have highlighted that closely related cultivars often exhibit subtle visual differences that require advanced classification approaches for reliable iden-

tification [Šulc and Matas 2017]. The successful discrimination achieved in the present study suggests that the selected texture descriptors were sufficiently sensitive to capture these subtle differences among raspberry cultivars. In contrast, cultivars such as Polana and Poranek were generally recognized with the highest accuracy, suggesting the presence of more distinctive image attributes. Additionally, the comparison between organic and conventional cultivation systems revealed significant differences in image texture descriptors within each cultivar. Complete discrimination between organic and conventional raspberries was achieved for all analyzed cultivars. These findings suggest that cultivation practices influence fruit appearance to a degree that can be detected using computer vision techniques.

The effectiveness of the proposed methodology can be attributed to several factors. The analysis was based on a large set of image texture descriptors extracted from multiple color channels, allowing comprehensive characterization of fruit appearance. Furthermore, the feature selection procedure enabled identification of the most informative attributes while reducing data dimensionality. Moreover, the use of cross-validation ensured reliable model evaluation and reduced the risk of overfitting. Together, these elements contributed to the high predictive performance observed across all classification tasks. Similar strategies have proven to be effective in most recent research [e.g., Ropelewska and Szymajda 2026, Ropelewska et al. 2026a, 2026b] and as well as earlier ones [Ropelewska 2021, Ropelewska and Rutkowski 2023].

From a practical perspective, the obtained results demonstrate the potential of image analysis as a rapid, objective, and non-destructive tool for authentication and quality control of raspberry production systems. Visual assessment systems have the potential to reduce reliance on subjective evaluation, time consumption, the requirement for appropriate equipment, and highly experienced specialists. While increasing processing speed and consistency [De Nart et al. 2024]. Moreover, recent reviews emphasize that artificial intelligence-assisted non-destructive technologies are becoming increasingly important components of fruit quality monitoring, offering opportunities for real-time decision support throughout the production and supply chain [Fu and Sun 2026].

Nevertheless, several limitations of the approach applied in this study should be considered. The analyzed fruit originated from specific cultivation conditions and a limited number of cultivars. Therefore, further studies including additional cultivars, production locations, growing seasons, and environmental conditions would be beneficial to verify the generalizability of the developed models. The challenge of maintaining classification performance under variable environmental conditions has also been highlighted in other studies, where model robustness often decreases when evaluated on external datasets [De Nart et al. 2024]. Moreover, integrating image analysis with other non-destructive sensing techniques, such as hyperspectral imaging or fluorescence imaging, may further improve classification performance and provide deeper insight into the factors underlying the observed visual differences. Furthermore, in addition to traditional machine learning algorithms, deep learning models can also be applied. Therefore, multimodal approaches integrating various imaging systems with traditional machine learning and deep learning algorithms may represent a promising direction for future research on raspberry cultivar authentication and cultivation system verification.

CONCLUSIONS

Image analysis combined with traditional machine learning algorithms proved to be an effective approach for distinguishing raspberry cultivars and cultivation systems. Significant differences in selected image texture descriptors were observed among the analyzed cultivars within both organic and conventional production systems, confirming the existence of cultivar-specific visual characteristics. The developed machine learning models achieved high classification accuracies for cultivar discrimination. For raspberries originating from the organically managed mother plantation, the highest accuracy reached 89.2% using the Subspace Discriminant classifier, while for conventionally cultivated raspberries, the highest accuracy of 91.7% was obtained using the Linear Discriminant algorithm. The cultivation system significantly influenced raspberry image texture characteristics. Statistically significant differences between organic and conventional samples were identified within each cultivar, indicating that cultivation practices affect fruit

appearance and surface properties. The classification accuracy of organic and conventional raspberries reached 100% for all analyzed cultivars. This demonstrates the strong potential of image-derived texture features for the authentication of cultivation systems. Future studies could validate the developed approach using larger datasets, additional cultivars, and fruit originating from different growing regions and seasons.

CONFLICT OF INTEREST

The authors declare that they do not have any known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DECLARATION OF THE USE OF GENERATIVE AI IN THE PROCESS OF PREPARING THE PAPER

The authors declare that no generative AI tools were used in the preparation of this manuscript.

SOURCE OF FUNDING

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OCCURRENCE AND PROPERTIES OF ENDOPHYTIC FUNGI OF THYME (*Thymus* spp.)

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ABSTRACT

Endophytic fungi constitute an important yet still poorly explored component of the microbiome of thyme (*Thymus* spp.). This review summarizes the current knowledge on their diversity, ecological functions, and biotechnological potential based on literature published up to April 2026. Reported endophytes represent a diverse taxonomic community, comprising 49 genera and 53 species most of which belong to common ascomycete genera, such as *Alternaria*, *Fusarium*, *Colletotrichum*, *Aspergillus*, *Penicillium*, *Phoma*, and *Trichoderma*. Current evidence from experiments carried out *in planta* indicates that these microorganisms may contribute to plant growth promotion, enhanced tolerance to abiotic stress, modulation of essential oil production, and protection against plant pathogens through the synthesis of bioactive metabolites and volatile organic compounds. Moreover, in *in vitro* assays several isolates have demonstrated antimicrobial, antioxidant, cytotoxic, and enzyme-producing activities, highlighting their promising applications in sustainable agriculture and biotechnology. Although significant progress has been achieved, many aspects of the ecology, host specificity, and functional interactions of thyme-associated fungal endophytes remain insufficiently understood.

Keywords: thyme, Lamiaceae, endophytes, essential oils, defensive mutualism, growth promotion

INTRODUCTION

Endophytic fungi are polyphyletic microorganisms that inhabit plants without inducing overt negative effects on their health, eventually establishing mutualistic relationships with the host. For a long time, the widespread assumption that pathogens basically were the only microorganisms capable of colonizing living plant tissues has delayed recognition that endophytic fungi are constantly associated with plants and can significantly influence their ecological fitness. Currently, they are regarded as a reservoir of unexplored biodiversity, awaiting to be exploited as plant protection agents or growth promoters, as well as for a variety of biotechnological applications [Nicoletti and Fiorentino 2015, Yadav et al. 2022]. Indeed, many studies have shown that these microbial associates may improve plant fitness through various interactions with agents of biotic adversities, such as antibiosis, parasitism, competition for space and nutrients, and the promotion of tolerance and resistance mechanisms [Rabiey et al. 2019, Morelli et al. 2020]. Moreover, their recognized role in growth promotion supports their consideration as genuine and effective plant probiotics [Pandey et al. 2022]. Beyond the intrinsic effects/properties of single strains or species, a more comprehensive ecological approach should consider that endophytes may perform these functions in the form of interacting consortia with other components of the plant microbiome [Adeleke et al. 2022].

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As with other medicinal plants, the cultivation of thyme (*Thymus vulgaris* and other *Thymus* spp.) has recently expanded, owing to its increasing use in gastronomy and the cosmetic industry [Nieto 2020, Halat et al. 2022, Waheed et al. 2024]. This ongoing boost especially concerns the organic farms, where adherence to the principles of integrated pest management is particularly important. Thus, insights into the organization of the plant associated microbes are crucial to improve our understanding of their ecological role, as well as to promote practical applications. Unlike endophytic bacteria, whose interactions with thyme have recently been the subject of several studies [Checcucci et al. 2017, Mohamad et al. 2020, 2022, Musa et al. 2020, Abdel-Hamid et al. 2021, Delbari et al. 2023, Zhao et al. 2023], the impact of endophytic fungi is less studied and recognized. In addition to endophytism in the strict sense, some fungal species have been reported to participate in a tritrophic association including gall midges of the genus *Asphondylia* (Diptera, Cecidomyiidae) [Malagaris 2011, Zimowska et al. 2017, 2020, 2021]. This paper provides an overview of the occurrence and properties of the endophytic symbionts associated with thyme, based on the examination of the literature published until April 2026. To this end, we searched the Google Scholar and PubMed databases using the terms ‘thyme endophytic fungi’ and ‘*Thymus* endophytic fungi’, without any time restriction and excluding only unpublished dissertations and degree theses.

Botanical notes on *Thymus* L.

The genus *Thymus* L. is one of the most diverse and taxonomically challenging groups within the family Lamiaceae. Belonging to the tribe Mentheae and subtribe Menthinae, the genus comprises approximately 220–250 species of perennial aromatic herbs and dwarf shrubs distributed primarily throughout the Mediterranean basin and adjacent regions of Europe, North Africa, Macaronesia, and temperate Asia extending eastwards to the Himalayas [Morales 2002, Drew and Sytsma 2012]. The Mediterranean basin represents the principal center of diversification of *Thymus*, where repeated geological, climatic, and ecological processes have promoted extensive speciation and local endemism. Major diversity hotspots occur in the Iberian peninsula, southern France, Italy, the Balkan peninsula, Greece, Anatolia, and the Caucasus, while secondary centers of diversification extend into the Irano-Turanian region and western Asia [Morales 2002, Celep and Dirmenci 2017, Bartolucci and Nachychko 2025, Bahadori et al. 2026].

Modern molecular studies generally support the monophyly of *Thymus*, although relationships among infrageneric groups remain incompletely resolved and continue to be refined through phylogenomic analyses [Soorni et al. 2019, Nieto-Lugilde et al. 2026].

The complexity of *Thymus* taxonomy arises from a combination of extensive morphological variability, ecological specialization, local endemism, hybridization, polyploidization, and the proliferation of names introduced during the eighteenth and nineteenth centuries. As botanical exploration intensified across Europe and the Mediterranean region, numerous taxa were described based on subtle differences in vegetative or floral morphology, generating extensive synonymy and uncertain species circumscriptions. Consequently, modern taxonomic studies have focused on stabilizing nomenclature through typification and critical revision of historical names. Important advances in this regard were achieved by Bartolucci et al. [2013], who clarified the application of several names through lectotypification and taxonomic annotations, and by Bartolucci and Nachychko [2025], whose comprehensive revision for the Euro+Med Plant-Base provided updated nomenclature, typifications, synonymies, and distributional data for the genus.

Morphological plasticity is particularly pronounced and frequently obscures taxonomic boundaries. Plant size, growth form, leaf morphology, pubescence, and inflorescence architecture may vary substantially in response to environmental conditions including altitude, substrate, grazing pressure, drought intensity, and exposure. Consequently, populations belonging to a single species may exhibit remarkable phenotypic divergence across ecological gradients, whereas unrelated taxa occupying similar habitats may converge morphologically [Morales 2002, Federici et al. 2013].

Hybridization represents another major source of taxonomic complexity. Natural hybrids have been documented throughout the genus, particularly in regions where closely related species occur sympatrically. Hybrid populations often display intermediate morphological characteristics and mixed phytochemical profiles, complicating species identification and obscuring evolutionary relationships. Furthermore, several species complexes exhibit extensive polyploid variation, including diploid, tetraploid, and higher polyploid cytotypes. Polyploidization has long been recognized as an important evolutionary mechanism

within *Thymus*, but recent genomic investigations have demonstrated that interploidy gene flow may be far more widespread than previously assumed

The advent of molecular systematics has greatly improved understanding of evolutionary relationships within the genus, revealing the limitations of the traditional morphology-based classifications. Federici et al. [2013], using DNA barcoding approaches, demonstrated that standard plant barcode markers often fail to discriminate closely related species of *Thymus*, reflecting recent evolutionary divergence, incomplete lineage sorting, and shared ancestral polymorphism. Their results emphasized the need for integrative approaches combining molecular and morphological evidence. More recently, advances in phylogenomics have provided unprecedented insights into species relationships and diversification processes. A comprehensive genomic study of *Thymus* sect. *Mastichina* demonstrated that patterns of morphological variation, ecological niche differentiation, and genomic structure do not always coincide, revealing extensive historical introgression and complex evolutionary histories among the currently recognized taxa [Nieto-Lugilde et al. 2026]. These findings suggest that species boundaries within some groups of *Thymus* are considerably more dynamic and permeable than previously assumed.

The genus is also renowned for its extraordinary chemical diversity. *Thymus* species produce a wide range of essential oils (EOs), including thymol, carvacrol, linalool, geraniol, borneol, γ -terpinene, and *p*-cymene. These compounds play important ecological roles in defense against herbivores and pathogens and determine the economic significance of thyme species in medicine, food preservation, cosmetics, and perfumery [Etri and Pluhár 2024]. Chemotaxonomic studies have traditionally been used to support species delimitation; however, growing evidence indicates that phytochemical variation does not necessarily correspond to taxonomic boundaries. Najar et al. [2021] demonstrated the persistence of distinct thymol and linalool chemotypes within cultivated populations of *T. vulgaris* in central Italy, while Del Valle et al. [2026] showed that EO composition is shaped by a complex interaction between genetic background and environmental conditions. Consequently, although phytochemical profiles remain valuable taxonomic characters, they should not be used independently for species recognition.

Occurrence of endophytic fungi in *Thymus* spp.

A list of the fungal species so far identified as endophytic associates of *Thymus* spp. is provided in Table 1, along with details on the geographical locations and the part of the plant from which isolations were carried out. Most of the findings refer to direct isolation in axenic cultures, after external sterilization of the tissues excised from the plants. However, in one case the report was based on direct microscopic observation; particularly, the arbuscular mycorrhizal fungi *Acaulospora gedanensis* and three *Glomus*-like species (namely, *Entrophospora claroidea*, *Planticonsortium tenue* and *Rhizophagus fasciculatus*) were identified in the roots of *T. vulgaris* plants growing at the Garden of Medicinal Plants of the Jagiellonian University in Kraków (Poland), along with resting spores of *Olpidium* sp. and dark septate endophytes [Zubek and Błaszowski 2009]. Taxonomic identification of the accessions in Table 1 was based exclusively on morphological characters, with two exceptions: an isolate ascribed to *Trichoderma samuelsii* (Sordariomycetes, Hypocreales), as resulting from the sequence homology of three selected molecular markers (ITS, *tef1* and *rpb2*) [Ma et al. 2023], and an isolate classified as *Neopestalotiopsis aotearoa* (Sordariomycetes, Amphisphaeriales) based on 18S rDNA sequence homology [Abdelhamid et al. 2024]. However, in the latter case it must be considered that 18S is insufficient as a marker for a reliable species identification [López et al. 2026].

The available data do not allow us to make conjectures on any specific or systematic association with *Thymus* spp. of a defined fungal species. Within a sample of 11 endophytic fungi recovered from *T. vulgaris* at the Garden of Medicinal and Aromatic Plants of the Agriculture Research Centre in Giza (Egypt), *Aspergillus terreus* was the most frequently recovered species, represented by five isolates [Ali et al. 2018]. In an investigation carried out in Austria *Colletotrichum gloeosporioides* and *Ascochyta* sp. were found to be the dominant endophytic fungi, respectively in leaves and stems of *Thymus serpyllum* and *Thymus polytrichus* (currently *Thymus praecox* subsp. *polytrichus*), while *Alternaria alternata* was the most frequent species recovered from *Thymus capitatus* (syn. *Thymbra capitata*), *Thymus herba-barona* and *Thymus richardii* at the island of Mallorca, Spain [Fisher et al. 1992]. As a whole, more than 400 isolates from about 30 species were obtained in this study carried out over 30 years ago, which still represents the largest survey of endophytic fungi conducted on thyme.

Table 1. Fungi reported for endophytic occurrence in *Thymus* spp.

Fungal species	<i>Thymus</i> species *	Location	Reference
<i>Acaulospora gedanensis</i>	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Allophoma tropica</i> **	<i>T. eriocalyx</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Alternaria alternata</i>	<i>T. capitatus</i> L-S, <i>T. herba-barona</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
	<i>T. eriocalyx</i> L-S, <i>T. fallax</i> L, <i>T. kotschyanus</i> S, <i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
	<i>T. vulgaris</i> S	Alsharqia, Egypt	Olama et al. 2023
	<i>T. vulgaris</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Alternaria atra</i> **	<i>T. vulgaris</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Alternaria franseriae</i>	<i>T. eriocalyx</i> L, <i>T. kotschyanus</i> L, <i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Alternaria gossypina</i> ** (<i>A. tangelonis</i>)	<i>T. eriocalyx</i> L-S, <i>T. kotschyanus</i> L-S, <i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Alternaria</i> sp.	<i>T. capitatus</i> S, <i>T. herba-barona</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
	<i>T. polytrichus</i> L	Altaussee, Austria	
	<i>T. eriocalyx</i> L, <i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
	<i>T. algeriensis</i> R	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
	<i>T. vulgaris</i> S	Alsharqia, Egypt	Olama et al. 2023
<i>Anthostomella</i> sp.	<i>T. capitatus</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Ascochyta</i> sp.	<i>T. polytrichus</i> L-S, <i>T. serpyllum</i> L-S	Altaussee, Austria	Fisher et al. 1992
	<i>T. richardii</i> S	Mallorca, Spain	
<i>Aspergillus japonicus</i>	<i>T. vulgaris</i> L	Giza, Egypt	Ali et al. 2018
<i>Aspergillus</i> sp.	<i>T. fallax</i> L	Hamedan, Iran	Masumi et al. 2015
	<i>T. algeriensis</i> R	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
<i>Aspergillus terreus</i>	<i>T. vulgaris</i> L	Giza, Egypt	Ali et al. 2018
<i>Asteromella</i> sp.	<i>T. polytrichus</i> L	Altaussee, Austria	Fisher et al. 1992
<i>Aureobasidium pullulans</i>	<i>T. serpyllum</i> L	Altaussee, Austria	Fisher et al. 1992
<i>Aureobasidium</i> sp.	<i>T. capitatus</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Boeremia exigua</i> var. <i>exigua</i> **	<i>T. vulgaris</i> L-R-S	Fajslawice, Poland	Machowicz-Stefaniak et al. 2002
<i>Botrytis cinerea</i>	<i>T. serpyllum</i> S	Altaussee, Austria	Fisher et al. 1992
<i>Chaetosphaeronema</i> sp.	<i>Thymus</i> sp. S	Hamedan, Iran	Mirzaei et al. 2023
<i>Cladosporium cladosporioides</i>	<i>T. eriocalyx</i> L, <i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Cladosporium oxysporum</i>	<i>T. serpyllum</i> L	Altaussee, Austria	Fisher et al. 1992
<i>Cladosporium tenuissimum</i>	<i>T. polytrichus</i> L-S	Altaussee, Austria	
	<i>T. capitatus</i> S, <i>T. herba-barona</i> L	Mallorca, Spain	Fisher et al. 1992
<i>Cladosporium variabile</i>	<i>T. eriocalyx</i> L, <i>T. kotschyanus</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Colletotrichum dematium</i>	<i>T. polytrichus</i> S	Altaussee, Austria	Fisher et al. 1992
	<i>T. herba-barona</i> S	Mallorca, Spain	
<i>Colletotrichum gloeosporioides</i>	<i>T. serpyllum</i> L-S	Altaussee, Austria	Fisher et al. 1992
<i>Colletotrichum</i> sp.	<i>T. kotschyanus</i> L-R	Hamedan, Iran	Masumi et al. 2015
<i>Coniochaeta tetraspora</i>	<i>T. capitatus</i> L-S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Curvularia akaii</i>	<i>T. eriocalyx</i> S, <i>T. kotschyanus</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Cylindrocarpon</i> sp.	<i>T. eriocalyx</i> R	Hamedan, Iran	Masumi et al. 2015
dark septate endophytes	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Diaporthe</i> sp.**	<i>T. polytrichus</i> L, <i>T. serpyllum</i> L	Altaussee, Austria	Fisher et al. 1992
<i>Didymella glomerata</i> **	<i>T. vulgaris</i> L-R-S	Fajslawice, Poland	Machowicz-Stefaniak et al. 2002
	<i>T. eriocalyx</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Didymella pomorum</i> **	<i>T. capitatus</i> L-S	Mallorca, Spain	Fisher et al. 1992
<i>Diplodia</i> sp.	<i>T. capitatus</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Ectophoma multirostrata</i> **	<i>T. vulgaris</i> L-R-S	Fajslawice, Poland	Machowicz-Stefaniak et al. 2002
<i>Ectophoma pomi</i> **	<i>T. kotschyanus</i> L-S	Hamedan, Iran	Masumi et al. 2015
<i>Entrophospora clarioidea</i> **	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Fusarium acuminatum</i>	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium equiseti</i>	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium javanicum</i>	<i>T. fallax</i> R	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium lateritium</i>	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium oxysporum</i>	<i>T. daenensis</i> L, <i>T. kotschyanus</i> S	Hamedan, Iran	Masumi et al. 2015

Fungal species	<i>Thymus</i> species *	Location	Reference
<i>Fusarium reticulatum</i>	<i>T. eriocalyx</i> F-S	Hamedan, Iran	Masumi et al. 2015
	<i>T. eriocalyx</i> L, <i>T. kotschyanus</i> R	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium</i> sp.	<i>T. algeriensis</i> R	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
	<i>T. vulgaris</i> S	Alsharqia, Egypt	Olama et al. 2023
<i>Fusarium subglutinans</i>	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Fusarium tricinctum</i>	<i>T. polytrichus</i> S	Altaussee, Austria	Fisher et al. 1992
<i>Gilmaniella humicola</i>	<i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Godronia</i> sp.**	<i>T. herba-barona</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Heterophoma nobilis</i> **	<i>T. vulgaris</i> L-R-S	Fajstawice, Poland	Machowicz-Stefaniak et al. 2002
<i>Hormonema</i> sp.	<i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Hypoxylon</i> sp.**	<i>T. capitatus</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
	<i>T. polytrichus</i> L, <i>T. serpyllum</i> L-S	Altaussee, Austria	Fisher et al. 1992
	<i>T. capitatus</i> S, <i>T. herba-barona</i> L-S, <i>T. richardii</i> L-S	Mallorca, Spain	
<i>Mycelia sterilia</i>	<i>T. vulgaris</i> L	Giza, Egypt	Ali et al. 2018
	<i>T. daenensis</i> R, <i>T. eriocalyx</i> F-L-R-S, <i>T. kotschyanus</i> R-S, <i>T. lancifolius</i> L, <i>T. vulgaris</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Neopestalotiopsis aotearoa</i>	<i>T. vulgaris</i> S	Mansoura, Egypt	Abdelhamid et al. 2024
<i>Olpidium</i> sp.	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Paraboeremia selaginellae</i> **	<i>T. eriocalyx</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Paraphoma chrysanthemicola</i> **	<i>T. eriocalyx</i> F	Hamedan, Iran	Masumi et al. 2015
<i>Penicillium chrysogenum</i>	<i>T. vulgaris</i> L	Giza, Egypt	Ali et al. 2018
<i>Penicillium</i> sp.	<i>T. algeriensis</i> S	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
<i>Phialophora</i> sp.	<i>T. richardii</i> L-S	Mallorca, Spain	Fisher et al. 1992
<i>Phoma haematocycla</i>	<i>T. daenensis</i> L	Hamedan, Iran	Masumi et al. 2015
	<i>T. polytrichus</i> L-S, <i>T. serpyllum</i> L-S	Altaussee, Austria	Fisher et al. 1992
<i>Phoma</i> sp.	<i>T. capitatus</i> S, <i>T. herba-barona</i> L-S, <i>T. richardii</i> L-S	Mallorca, Spain	
	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
	<i>T. algeriensis</i> L	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
<i>Planticonsortium tenue</i> **	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Plenodomus pimpinellae</i> **	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Pyrenophora dematioidea</i> **	<i>T. daenensis</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Pyrenophora tetrarrhenae</i> **	<i>T. eriocalyx</i> S	Hamedan, Iran	Masumi et al. 2015
<i>Rhizophagus fasciculatus</i> **	<i>T. vulgaris</i> R	Kraków, Poland	Zubek and Błaszowski 2009
<i>Rhizopus</i> sp.	<i>T. algeriensis</i> R	Bordj Bou Arreridj, Algeria	Zerroug and Sadrati 2022
<i>Sclerophomella glaucii</i> **	<i>T. vulgaris</i> L-R-S	Fajstawice, Poland	Machowicz-Stefaniak et al. 2002
	<i>T. polytrichus</i> L-S, <i>T. serpyllum</i> L-S	Altaussee, Austria	Fisher et al. 1992
<i>Septoria</i> sp.	<i>T. capitatus</i> S, <i>T. richardii</i> L	Mallorca, Spain	
<i>Sordaria fimicola</i>	<i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Sporormiella intermedia</i>	<i>T. richardii</i> L	Mallorca, Spain	Fisher et al. 1992
<i>Stemphylium</i> sp.	<i>T. lancifolius</i> L	Hamedan, Iran	Masumi et al. 2015
<i>Stemphylium vesicarium</i> **	<i>T. capitatus</i> S, <i>T. richardii</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Subplenodomus valerianae</i> **	<i>T. eriocalyx</i> R, <i>T. kotschyanus</i> L-S	Hamedan, Iran	Masumi et al. 2015
<i>Taeniolella stilbospora</i>	<i>T. herba-barona</i> S	Mallorca, Spain	Fisher et al. 1992
<i>Trichoderma harzianum</i>	<i>T. serpyllum</i> S	Altaussee, Austria	Fisher et al. 1992
<i>Trichoderma samuelsii</i>	<i>T. mongolicus</i>	Yinchuan, China	Ma et al. 2023
unidentified yeasts	<i>T. eriocalyx</i> L-S, <i>T. fallax</i> S, <i>T. lancifolius</i> S	Hamedan, Iran Central and Western Iran	Masumi et al. 2015 Masumi et al. 2015b
	<i>T. vulgaris</i> L-R-S	Fajstawice, Poland	Machowicz-Stefaniak et al. 2002
<i>Westerdykella capitulum</i> **	<i>T. eriocalyx</i> L	Hamedan, Iran	Masumi et al. 2015

* *T. lancifolius* and *T. polytrichus* are now regarded as subspecies of *T. daenensis* and *T. praecox*, respectively.

** For these records, the species names were updated according to Mycobank.

F: flower; L: leaf; R: root; S: stem

Another large set of isolates (95) was collected in a study carried out in the Hamedan province of Iran considering six *Thymus* species: once more, *A. alternata* was the most common endophyte with 16 isolates recovered from all the above-ground plant parts, followed by several species identified within the genera *Fusarium* and *Phoma sensu lato* [Masumi et al. 2015a]. Fungi belonging to the above three genera were again the most frequent in an investigation carried out on *Thymus algeriensis* in Algeria [Zerroug and Sadrati 2022], while *Alternaria* and *Fusarium* were the only genera represented in a small sample of five isolates recovered from *T. vulgaris* in Egypt [Olama et al. 2023]. Moreover, earlier investigations on field-grown *T. vulgaris* in the Lublin region of Poland documented a diverse assemblage of *Phoma sensu lato* fungi colonizing shoots, leaves and, less frequently, roots of *T. vulgaris*. Several species were identified that have been recently renamed after taxonomic revision, with the new binomial indicated in brackets: *Phoma capitulum* (= *Westerdykella capitulum*), *P. glaucii* (= *Sclerophomella glaucii*), *P. dictamnica* (= *Heterophoma nobilis*), *P. glomerata* (= *Didymella glomerata*), *P. multirostrata* var. *microspora* (= *Ectophoma multirostrata*), and *P. exigua* var. *exigua* (*Boeremia exigua* var. *exigua*) [Machowicz-Stefaniak et al. 2002]. Subsequent pathogenicity studies demonstrated that, among these taxa, only *P. exigua* var. *exigua* was confirmed as an occasional (facultative) pathogen of thyme, causing reduced seed germination, seedling damping-off, and necrosis of roots and hypocotyls following artificial inoculation [Machowicz-Stefaniak et al. 2008], whereas the remaining species were reported only as endophytic colonizers, without any evidence of pathogenicity. Besides *Phoma* [Zimowska 2022], *Alternaria* and *Fusarium* are also under revision with reference to the recent concepts in fungal taxonomy [Li et al. 2022, Crous et al. 2022]; hence, a re-interpretation according to the updated nomenclature is desirable and appropriate for the available data concerning these genera, which have also been reported among the most common endophytic associates of many other plant species [Ahmed et al. 2023, Nicoletti et al. 2020, Manathunga et al. 2024, Nicoletti and Zimowska 2023].

Biochemical and ecological properties of endophytic fungi in *Thymus* spp.

It is a key issue in mycological research to accurately investigate the biochemical properties of endophytic fungi in view of possible biotechnological exploitation [Gupta et al. 2023, Agrawal and Bhatt 2023]. Indeed, secondary metabolites are regarded as the main factor influencing the interspecific relationships established with both the plant host and all components of its associated microbiome, with substantial effects on plant growth and protection. With reference to *Thymus* spp., it has been shown that the EO contained in the leaf glandular tissues represent one of the primary selective factors shaping the endophytic community composition [Checcucci et al. 2017].

As also demonstrated on several crop species, the beneficial effects of two well-known endophytic basidiomycetes belonging to the Sebaciales, namely *Serendipita* (= *Sebacina*) *vermifera* and *Serendipita* (= *Piriformospora*) *indica* [Ray and Craven 2016, Saleem et al. 2022], have been documented on *T. vulgaris* plants cultured *in vitro* and *in vivo*. Besides an increase in height and root length, as well as in fresh and dry weights of shoots and roots, oil yield was found to be enhanced in all the inoculated plants, and its analysis carried out by means of gas chromatography coupled with mass spectrometry (GC-MS) showed a notably increased content of thymol along with a decrease in its isomer carvacrol [Dolatabadi et al. 2011].

At least in part, the growth-promoting effects induced by Sebaciales are attributed to the direct and indirect production of auxins and other phytohormones. In this respect, more circumstantial evidence has been obtained for an isolate of *N. aotearoa* from *T. vulgaris*, which was found to produce indole-3-acetic acid (IAA) at a concentration of 11.16 µg mL⁻¹ [Abdelhamid et al. 2024]. On the other hand, the inoculation with fungal endophytes isolated from other Lamiaceae species was found to significantly improve growth, chlorophyll, and carotenoid content in thyme plants under drought stress. Moreover, lipid peroxidation levels were significantly reduced, and the higher activity of antioxidant enzymes and improved redox state of glutathione were considered to be crucial for the increased resistance to drought [Kamyab et al. 2024].

Besides influencing EO production and composition in the host plants, endophytic fungi of thyme may also directly synthesize these compounds. This is the case of a strain of *A. alternata* isolated from *T. vulgaris* in Egypt producing an array of volatile organic compounds (VOCs), some of which were common to the host plant [Olama et al. 2023]. Moreover, ethyl acetate extracts from culture of a strain of *A. terreus*, again from *T. vulgaris* in Egypt, were found to contain carvacrol and thymol in higher concentrations than

those determined in the host plant. Other compounds characteristic of the EOs were detected in the GC-MS analysis, such as terpineol, α -phellandrene, camphene, α -pinene, isopregol, dihydrocarveol, borneol, citronellal, humulene, ascaridole, geraniol, caryophyllene, cedrenol, along with some mycotoxins, namely aflatoxin G2, cyclopaldic acid, nivalenol, and typical plant secondary metabolites, such as glaucine, scoparone, scoprophulein, trispherine, luteolin, trimethylquercetin and other flavonoids. This compound assortment accounts for the notable antioxidant properties determined for the culture extract of this strain, as well as for the cytotoxicity against HEPG-2 hepatoma cells, with an IC_{50} value of $3.53 \pm 0.4 \mu\text{g mL}^{-1}$ [Ali et al. 2018]. In addition, high antibacterial and antifungal activities against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus flavus* and *Candida albicans* were documented for the culture extract of this strain by the same research group [El-Ela Sayed et al. 2020].

An endophytic isolate of *T. samuelsii* recovered from the stem of *Thymus mongolicus* displayed antagonistic activity in a co-culture experiment towards a panel of eight pathogenic fungi, including *A. alternata*, *Alternaria solani*, *Botrytis cinerea*, *Cytospora mali*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium sambucinum*, and *Neocosmospora solani*, with inhibition rates ranging between 31.0% and 68.5%. However, the antifungal activity of its fermentation broth and crude extract was low. GC-MS analysis showed that VOCs were the key antifungal compounds released in the co-cultures, with the main component identified as 6-pentyl-2H-pyran-2-one [Ma et al. 2023].

In a study including 89 endophytic isolates from several *Thymus* spp., some strains stood out for their antimicrobial effects, as assayed either directly or through their culture extracts; that is, strains of *Fusarium subglutinans*, *Fusarium equiseti*, *Fusarium javanicum*, *Fusarium lateritium*, *F. oxysporum*, *A. alternata*, *Allophoma tropica*, *Subplenodomus valerianae* and *Chaetosphaeronema* sp. displaying inhibitory effects against *B. cinerea*. The latter strain was the only one to show clear antibacterial effects in assays carried out against a panel of four strains belonging to *E. coli*, *S. aureus*, *Xanthomonas arboricola* pv. *juglandis* and *Streptomyces scabies*; conversely, a higher effectiveness was displayed by the culture extracts of *F. equiseti* and *F. lateritium* against *S. aureus*, and of *A. alternata*, *F. equiseti*, *F. oxysporum*, *F. subglutinans* and *S. valerianae* against *S. scabies* [Mirzaei and Masumi 2023]. In a previous study by the same research group, six strains of unidentified yeasts collected from three *Thymus* spp. also showed limited antifungal effects (14.70–37.25% growth inhibition) against *B. cinerea* [Masumi et al. 2015b].

Additional data on the antimicrobial properties have been collected for several endophytic isolates which were only identified at the genus level. This list includes a panel of 16 isolates belonging to the genera *Aspergillus*, *Alternaria*, *Fusarium*, *Penicillium*, *Phoma* and *Rhizopus*, which exhibited varying degrees of antibacterial and antifungal activities *in vitro* against *Bacillus cereus*, *Enterococcus faecalis*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *S. aureus*, *Fusarium caeruleum*, *F. oxysporum* and *Phytophthora infestans* [Zerroug and Sadrati 2022].

Finally, some extent of antimicrobial properties were documented for the ethyl acetate culture extracts of isolates of *Alternaria* and *Fusarium* collected from *T. vulgaris* in Egypt; in particular, this refers to antibacterial activity in assays carried out against *Bacillus subtilis*, *P. aeruginosa* and *S. aureus*, as well as to antifungal activity against *Aspergillus niger* and *C. albicans*. Moreover, these extracts were determined to possess antioxidant properties in assays based on the diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, with IC_{50} values up to $36.80 \mu\text{g mL}^{-1}$ [Olama et al. 2023].

Besides the implications related to the antimicrobial, cytotoxic and antioxidant properties, potential biotechnological exploitation may also concern the production of enzymes. In this respect, in the screening of a set of 89 endophytic isolates from several *Thymus* spp. collected in Western Iran high levels of asparaginase and amylase production were recorded from strains of *F. subglutinans* and *Curvularia akaii*, respectively [Masumi et al. 2014].

CONCLUSIONS

This paper provides an overview of the current knowledge on the diversity, ecological functions, and biotechnological potential of endophytic fungi associated with *Thymus* species. These fungi represent a taxonomically diverse community comprising 49 genera and 53 species, mostly belonging to common

ascomycete genera such as *Alternaria*, *Fusarium*, *Colletotrichum*, and *Phoma*; considering that most identifications were based on morphological characters, a more rigorous taxonomic reassessment would be desirable. The currently available data are limited, and do not allow to establish if any species has developed a mutualistic association reflecting ecological significance. Nevertheless, some of these microorganisms have proved to be able to modulate production of EOs and contribute to plant growth promotion, enhanced tolerance to abiotic stress, and protection against plant pathogens through the synthesis of bioactive metabolites and volatile organic compounds. Moreover, several isolates have been demonstrated antimicrobial, antioxidant, and cytotoxic activities, deserving further assessments with a view to their effective application in sustainable agriculture and biotechnology. On the other hand, the occurrence of potential pathogens and species known as mycotoxin producers calls for additional work aimed at verifying their safety with respect to both plant and human health.

Future research in the field should focus particularly on the culture-independent characterization of the fungal endophytic communities, the standardization of sampling and isolation methods, the use of appropriate molecular markers for the isolate identification and the deposition of the obtained DNA sequences in recognized public repositories, and a more detailed investigation of host and organ specificity. Moreover, it is desirable that the experimental protocols be designed to more diffusely include controlled inoculation assays, field validation, and biosafety screening.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF THE USE OF GENERATIVE AI TOOLS IN THE PROCESS OF PREPARING THE PAPER

The authors declare that no genAI tools were used in the process of preparing the paper.

AUTHORS CONTRIBUTION

The authors contributed in equal parts to collecting information and writing this paper.

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