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Article

Biostimulant Effects of Mycorrhiza and Duckweed Extract on Growth and Biochemical Composition of Lettuce

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Abstract

The use of biostimulants offers a sustainable strategy to improve crop quality. This study assessed the effects of an arbuscular mycorrhizal fungi inoculum and an aqueous extract of *Landoltia punctata* on the growth and biochemical composition of lettuce (*Lactuca sativa* L. cv. ‘Dubáček’) under indoor conditions. Four variants were tested: control (C), mycorrhiza (M), *L. punctata* extract (L), and their combination (M+L), with biometric, physiological, and biochemical parameters evaluated. While biostimulant treatments did not affect above-ground biomass, *L. punctata* extract (L and M+L) significantly raised chlorophyll a (by 15.9 % and 16.0 %) and chlorophyll b (by 55.5 % and 42.8 %) compared to the control. The combined treatment (M+L) achieved the highest total phenolic content (254.28 mg/kg). All treated variants significantly reduced leaf nitrate content, with M and M+L being most effective (−35.1 % and −33.6 %). Amino acid metabolomic analysis showed that the extract is rich in γ-aminobutyric acid, valine, phenylalanine, tryptophan, and other proteinogenic amino acids that may drive its biostimulant effects. Microscopy confirmed successful root colonisation in mycorrhizal variants (58 % in M, 42 % in M+L). Overall, while biometric parameters remained unaffected, the applied biostimulants enhanced lettuce nutritional quality and physiological status by boosting photosynthetic pigments and phenolic compounds while lowering nitrate content, highlighting their potential for producing high-quality, safe crops.

Keywords: *Landoltia punctata*; arbuscular mycorrhizal fungi; pigments; antioxidants; nitrates

1. Introduction

Since the 1950s, agriculture has primarily aimed at intensifying production by increasing crop yields. This goal has largely been pursued through heavy use of mineral fertilizers and pesticides (Boincean, 2024), whose global consumption continues to rise (IFA, 2025; FAO, 2024, 2025). However, the overuse of these agrochemicals contributes to soil degradation, water contamination and greenhouse gas emissions (Silva et al., 2019; Zhang et al., 2022; Bebbler & Richards, 2022; Uddin & Kurosawa, 2011; Zhou et al., 2022; Lee & Jones-Lee, 2005; Menegat et al., 2022). For example, global consumption of mineral fertilizers (N, P, K) increased from 93.1 to 119.9 kg/ha of arable land between 2001 and 2022, while global pesticide use rose from 1.5 to 2.4 kg/ha over the same period (IFA, 2025; FAO, 2024, 2025).

Therefore, there is an urgent need to seek and apply natural-based alternatives that will not further burden arable soils environmentally, while supporting the development of beneficial microorganisms and maintaining or even increasing the yield and nutritional parameters of cultivated crops. This definition includes a group of substances collectively referred to as plant biostimulants. According to Regulation (EU) 2019/1009 of the European Parliament, the group of plant biostimulants is divided into microbial and non-microbial categories. Microbial products contain beneficial fungi, bacteria, or their combination. Non-microbial products include those based on 1) humic and fulvic acids, 2) protein hydrolysates and other N-containing compounds, 3) chitosan and other biopolymers, 4) inorganic substances (beneficial elements), and 5) seaweed and plant extracts, or combinations of these (du Jardin, 2015; European Parliament and Council (EU), 2019).

Rossini, Ruggeri, and Rossini (2025) found that a mixture of seaweed extracts and microorganisms applied with a reduced nitrogen fertilizer dose (100 kg/ha) achieved a wheat yield comparable to the standard dose (150 kg/ha), thus saving 33% of nitrogen. A similar trend was observed by Fall et al. (2023), who found that the combination of mycorrhizal inoculum with 50% doses of N, P, and K outperformed the 100% dose of N, P, and K in terms of growth and nutrient content in maize grown both in the greenhouse and in the field.

The group of arbuscular mycorrhizal fungi (AMF) consists of an obligate symbiotic class of fungi, the *Glomeromycetes*, which currently includes five orders: *Archeosporales*, *Diversisporales*, *Entrophosphorales*, *Glomerales*, and *Paraglomerales* (Spatafora et al., 2016; Schoch et al., 2020). These fungi encompass hundreds of species that colonize a diverse range of plants — from liverworts, ferns, and gymnosperms to angiosperms, with the exception of certain families such as *Brassicaceae* and *Araceae* (Gryndler, 2004; Smith & Read, 2008). In economically important crops, the presence of AMF is associated with increased nutrient and water uptake, often leading to higher yields, as well as positive changes in the plants' primary and secondary metabolomes, which enhance their resistance to stress factors and improve the nutritional value of the crops (Rasouli et al., 2022; Keller-Pearson et al., 2020; Nedorost & Pokluda, 2012).

Landoltia punctata belongs to the family *Araceae*, subfamily *Lemnoideae*, and is an aquatic plant freely floating on or just below the water surface. Its body consists of a frond measuring 1–3 mm and 2–5 roots (Lee, Choi & Shiga, 2020; UF IFAS, 2025). Most studies on representatives of *Lemnoideae* focus on their ecological significance, especially wastewater bioremediation, where they effectively remove heavy metals, dyes, nanomaterials, microplastics, organic pollutants, pesticides, and pharmaceuticals (Xu et al., 2018; Neag, Malschi & Măicăneanu, 2018; Yue et al., 2018; Pop et al., 2021; Sikorski et al., 2019; Drobniowska et al., 2024). Some studies also examine the use of duckweed biomass as an alternative fertilizer for cereals (Ahmad et al., 1990; Hong et al., 2024; Pulido et al., 2021), tomatoes (Mahofa, Kapenzi & Masaka, 2013), and leafy vegetables (Baldi et al., 2025; Pratiwi, Aji & Sumbudi, 2022; Chikuvire, Muchaonyerwa & Zengeni, 2019). However, few studies have so far focused on the biostimulant effects of these plants, and all have concerned only *Lemna minor*. The aqueous extract of *L. minor* has been tested on maize under optimal (Del Buono et al., 2022) and stress conditions of increased NaCl (Priolo et al., 2024) and Cu (Miras-Moreno et al., 2022), on olive trees under optimal (Regni et al., 2021) and stress conditions of increased NaCl (Regni et al., 2024), and also on tomatoes (Priolo et al., 2024).

This study examined the effects of a well-established and commonly used group of biostimulants—arbuscular mycorrhizal fungi—alongside a newly emerging biostimulant derived from an aqueous extract of the aquatic plant *L. punctata* (clone no. 5562). According to available information, no previous study has tested the biostimulant effects of an extract from this plant. The main aims of this research were (i) to evaluate the effects of individual and combined applications of a mycorrhizal inoculum and an aqueous extract of *L. punctata* on lettuce biometric parameters, physiological status and biochemical composition, (ii) to assess mycorrhizal root colonization, and (iii) to conduct a metabolomic analysis of amino acids in the aqueous extract of *L. punctata*.

2. Materials and Methods

Plant Material and Growing Conditions

The experiment was conducted under indoor conditions at the Faculty of Agronomy, Mendel University, located at Zemědělská 1, 61300 Brno, Czech Republic. Seeds of leaf lettuce cultivar 'Dubáček' (MORAVOSEED CZ S.A., Mikulov, Czech Republic) were sown into a universal peat substrate (BAHAG AG, Mannheim, Germany) in plastic trays on March 3, 2025. Two of the four prepared variants were treated with mycorrhizal inoculum before sowing (see the chapter Biostimulant Preparation and Application).

The prepared trays were placed inside an indoor tent, type BudBox PRO Titan I+ HL 200 (BudBox™ Ltd., Harrogate, United Kingdom) and were cultivated under constant conditions until harvest. Throughout the entire experiment, a temperature regime of 23/20 °C day/night was maintained, the relative humidity was maintained in the range of 60–80 % using exhaust fans and humidifiers. Just above the edge of the trays, the photosynthetic photon flux density (PPFD) was measured, averaging $218.6 \pm 27.27 \mu\text{mol}/\text{m}^2/\text{s}$ with a red to blue light ratio of 20:1. The photoperiod was set to 18 hours of light and 6 hours of darkness. Irrigation was carried out daily – 1.5 litres of settled tap water per tray. During the short-term cultivation of lettuce, no additional fertilization was required.

Experimental Design

The experiment included 4 variants, each represented by one plastic tray (87×124×43 cm). The trays were distributed among the variants according to a completely randomised design. Each variant contained 12 plants. The experiment included the following variants:

- (1) C – control variant without any treatment;
- (2) M – variant treated with mycorrhizal inoculum;
- (3) M+L – variant treated with a combination of mycorrhizal inoculum and *Landoltia punctata* (clone no. 5562) extract;
- (4) L – variant treated with *L. punctata* (clone no. 5562) extract.

Biostimulant Preparation and Application

Two types of biostimulant products were used in this experiment. The first was an AMF inoculum containing *Claroideoglomus claroideum* BEG96, *Claroideoglomus etunicatum* BEG92, *Funnelformis geosporum* BEG199, *Funnelformis mosseae* BEG95, and *Rhizophagus irregularis* BEG140. This mix contained 145 spores per g, and was obtained from the company Symbiom, Ltd., Sázava, Czech Republic. The other composition of this product, as declared by the manufacturer, is as follows: natural clay carriers, natural humates, seaweed extracts, ground minerals, and hydrogel particles. The mycorrhizal inoculum was applied in two variants (M and M+L) by adding 1 gram of the mycorrhizal inoculum into each sowing hole prior to seed sowing.

The second biostimulant product used in this experiment was an extract from duckweed, *L. punctata* clone no. 5562 (a pure clone purchased from the Friedrich Schiller Institute, Jena, Germany). The *L. punctata* clone was cultivated in an indoor growing system at the Faculty of Horticulture, Mendel University, located at Valtická 337, 69144 Lednice, Czech Republic. *L. punctata* was grown in a hydroponic nutrient solution Solinure GT 7 (ICL Group Ltd., Tel Aviv, Israel) at a concentration of 0.855 g of fertilizer per litre of tap water. The solution was acidified using 15 % HNO₃ to reach a pH close to neutral. Nutrient solution parameters at the time of *Landoltia* harvest were: temperature 20.1 °C, pH 7.36, EC 1590 µS, TDS 1.11 ppt, salinity 0.5 g/L, NH₄⁺-N 180.0 mg/L, NO₃⁻-N 86.5 mg/L, PO₄³⁻ 65.3 mg/L, K⁺ 43.1 mg/L. Ambient air temperature at harvest was 21.6 °C, with a relative humidity of 51.1 %. *L. punctata* was cultivated under a PPFD of 119.5 µmol/m²/s with a 16/8 light/dark photoperiod and a daily light integral (DLI) of 6.9. The extract was obtained following the methodology of Priolo et al. (2024). For the purpose of extract preparation, 50 g of fresh biomass was harvested, thoroughly

washed with tap water, and drained for 5 minutes in a sieve. The biomass was then dried at 40 °C for 72 hours, and 2 g of the dry biomass was mixed with 400 mL of deionized water (pH = 7.0). The mixture was ground together in a mortar for 5 minutes and transferred into a vial placed on a shaker for 12 hours at room temperature and 150 rpm. The suspension was then filtered and brought up to a volume of 400 mL with deionized water, resulting in a 0.5 % duckweed aqueous extract (dry weight/water volume – wt/v). Lettuce leaves in variants M+L and L were treated with an extract from *L. punctata* at two time points – at the 5–7 true leaf stage (on March 21, 2025) and at the >10 true leaf stage (on March 28, 2025). In the first application, a dose of 2.5 mL per plant was used, and in the second, 5 mL per plant. The extract was freshly prepared for each treatment and applied using the FarmBot, version V1.7 (FarmBot, Inc., San Luis Obispo, United States), a robotic system with a standard spray watering nozzle. The dose per plant was adjusted by modifying the movement speed of the robotic arm. The remaining portion of the extract was used for metabolomic analyses.

Harvest of Plants and Sampling

Lettuces were harvested on April 7, 2025, for the purpose of evaluating biometric parameters. Each of these parameters (see below) was measured on all 12 plants per variant (n = 12). For subsequent analyses of biochemical compounds, composite samples were prepared from three plants per variant, resulting in four replicates for analytical measurements (n = 4).

Assessment of Photosynthetic, Biometric, and Biochemical Parameters of Lettuce

Prior to harvest, Normalized Difference Vegetation Index (NDVI) and Quantum Yield (Qy) values were measured at two time points during the experiment – on March 28, 2025 (before the second application of the *Landoltia* extract), and on April 4, 2025 – on all 12 plants per variant (n = 12). After harvest, various biometric parameters were evaluated. Above-ground biomass was measured as the weight of the edible part using a scale with 0.1 g accuracy. Leaf area was measured using the mobile application LeafScan (developed by Carlos Anderson), with a white reference square of 30 cm edge length as a background. Plant height and leaf rosette diameter were measured with a ruler accurate to millimeters. Additionally, the total number of leaves per plant was recorded. Root parameters were not evaluated because the complete root system could not be extracted from the peat substrate without damage. However, fragments of roots from all of the variants were used for subsequent microscopic observation.

Among the biochemical parameters, the following were determined: the percentage of dry matter in the edible aboveground part, content of vitamin C, chlorophyll a and b, carotenoids, total phenolic compounds, flavonoids, DPPH antioxidant capacity, and nitrates.

The dry matter content of the aboveground plant biomass was determined gravimetrically at 105 °C until a constant weight of the weighing dishes was reached (Zbiral, 2005).

Vitamin C was analysed using high-performance liquid chromatography (ECOM Ltd., Prague, Czech Republic) with an ARION® Polar C18 column (5 µm, 150 mm × 4.6 mm) and a mobile phase consisting of tetrabutylammonium hydroxide, oxalic acid, and water in a ratio of 10:20:70.

The determination of plant pigments (chlorophyll a, chlorophyll b, and carotenoids) was carried out according to the method of Holm (1954). According to this method, the pigments are extracted in acetone and subsequently measured spectrophotometrically at wavelengths of 662, 644, and 440 nm, in that order.

For the determination of total phenolic compounds, flavonoids, and DPPH antioxidant capacity, a methanolic extract of the samples was first prepared. Homogenized samples were extracted in 75% methanol for 24 hours, filtered, and then adjusted to a final volume of 50 mL with 75% methanol. Total phenolic content was determined using the Folin–Ciocalteu reagent and a 7% sodium carbonate solution, with spectrophotometric measurement at 765 nm, according to Zloch, Čelakovský & Aujezdská (2004). The total content of flavonoids was determined using a 5% sodium nitrite solution, a 10% aluminium chloride solution, a 1 M sodium hydroxide solution, and a 1 mM catechin standard solution, with spectrophotometric measurement at 510 nm (Zloch, Čelakovský & Aujezdská, 2004).

The total antioxidant capacity was determined using the DPPH spectrophotometric method, which is based on the scavenging of the DPPH⁺ radical cation (2,2-diphenyl-1-picrylhydrazyl). Under standard conditions, this cation exhibits a purple colour, which turns yellowish upon reduction. For the reaction, a 100 µM solution of DPPH and a 0.5 mM solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used. The resulting absorbance was measured at a wavelength of 515 nm (Zloch, Čelakovský & Aujezdská, 2004).

Nitrate content in the samples was determined according to Zbiral (2005), using an ion-selective electrode (type 07–35) and a mercury sulphate reference electrode (type RME 121), both by Monokrystaly Ltd., Přepeře, Czech Republic.

Amino Acid Metabolomic Profiling of the Landoltia punctata Extract

For the purpose of metabolome analysis in the Landoltia extract sample, 200 mL of a 0.5% extract was frozen at -18 °C and subsequently lyophilized using the AMARU device from the manufacturer Lyotrade Freeze Dryers Ltd., Prague, Czech Republic. Within the lyophilizer, the extract was first frozen for 2 hours at -40 °C, then dried at a pressure ranging from 100 to 300 Pa, with the temperature not exceeding 35 °C for a duration of 36 hours. The lyophilized sample was rinsed with HPLC-grade methanol (99.9%) and then diluted with the same methanol to a final volume of 100 mL.

For the analysis of the duckweed extract, high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) was used. Acetonitrile hypergrade for LC-MS LiChrosolvR was supplied by Merck KGaA (Darmstadt, Germany). Acetic acid was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The Purelab Classic (ELGA LabWater, High Wycombe, Bucks, UK) was used to generate high purity water for preparation of aqueous mobile phase.

Samples were analysed for metabolomics twice on HPLC-HRMS with positive and negative polarity of MS-orbitrap. Both records from DAD detector and from high resolution mass spectrometer were monitored and saved for check of system and evaluation results.

For the high-performance liquid chromatography part, a Dionex Ultimate 3000 (ThermoFisher Scientific, USA/Dionex RSLC, Dionex, USA) was used. The column used was a Hypersil Gold column (C18) 150 mm × 2.1 mm, 3 µ (ThermoFisher Scientific, USA). Flow rate of mobile phases was 0.3 mL/min and column temperature was set to 30 °C. The HPLC mobile phase consisted of (A) acetonitrile and (B) water containing 0.1 % acetic acid. Both mobile phases (A) and (B) were filtrated and degassed for 10 min in an ultrasonic bath prior to use. Gradient elution chromatography was performed starting with 10 % acetonitrile (A) and 90 % water (0.1 % acetic acid) (B) and held 5 minutes. Within time interval (5-20 minutes), A % composition was increased to 90 %. This composition was then maintained for 5 min. after which the system was 5 min. equilibrated to initial conditions (10 % acetonitrile (A) and 90 % water (0.1 % acetic acid)). The following wavelengths 254, 272, 274, 331 nm were monitored.

MS and MSn were performed using LTQ Orbitrap XL-high resolution mass spectrometer (ThermoFisher Scientific, USA) equipped with a HESI II (Heated electrospray ionization) source. The high-resolution mass spectrometer (orbitrap) was operated in Full Scan with resolution 60 000. Full scan spectra were acquired over mass range m/z 50-1000 in positive mode and 65-1000 in negative mode. The resolution and sensitivity of the Orbitrap were controlled by injection of mixed standard (phenolic compounds) after analysing of every 25 samples and resolution was also checked by the help of lock masses (phthalates). Blanks were also analysed during sequence. The compounds were searched in the mass library which was created from measurement of standards in MS and MSn modes of orbitrap.

Root Sample Processing for Microscopy

Sample preparation for microscopy was carried out according to methodology of Vierheilig, Schweiger & Brundrett (2005). Root samples were thoroughly rinsed with tap water, following strict procedures to avoid any cross-contamination between variants (including disinfection of tools and

frequent replacement of disposable gloves). From each variant, at least 10 root segments approximately 1 cm in length were placed into Eppendorf tubes filled with PBS solution. The PBS solution was subsequently replaced with FAA fixative (formaldehyde, ethanol, acetic acid in a 10 %:50 %:5 % ratio, plus 35 % water). Samples were incubated in this fixative for 7 days.

After fixation, the samples were rinsed three times with deionized water. A pre-prepared mixture of fluorescent dyes (WGA, concanavalin A, and acid fuchsin in a 1:1:1 ratio) was then added to the samples. The root segments were incubated in this staining solution for 5 hours at room temperature.

After staining, the samples were washed four times for 3 minutes each using PBS. During the final wash, the samples were left in PBS overnight (12 hours). Prior to mounting, the PBS solution was replaced with deionized water, and the samples were gently rinsed.

Five root segments per variant were then mounted on microscope slides (root segments were aligned side by side). The roots were covered with VECTASHIELD mounting medium with DAPI and sealed with a cover slip.

The evaluation of mycorrhizal colonization of the roots was performed according to the method described by Alarcón & Cuenca (2005). For each of the 5 segments per variant, the percentage of mycorrhizal colonization was assessed, and a corresponding score was assigned on a scale from 0 to 5 (see Table 1).

Confocal microscopy was completed using the LSM 800 (Carl Zeiss AG, Oberkochen, Germany) microscope at 405/420-480 nm, 488/500-550 nm, 590/617 nm, and 650/668 nm excitation/emission. Lens used were 20x/0.8 NA. Processing of pictures was conducted in Zen Blue 3.0 software (Carl Zeiss AG, Oberkochen, Germany).

Table 1. Evaluation of the degree of root colonization by mycorrhizal fungi according to Alarcón & Cuenca (2005).

Value	Proportion of mycorrhiza in observed plant tissue samples
0	Without targeted mycorrhiza
1	Incidence trace
2	Targeted mycorrhiza present in less than 10% of plant tissue
3	From 11 to 50%
4	From 51% to 90%
5	More than 90%

Statistical Analyses

The data were processed and statistically analysed using Statistica 14 software package (DataBon Ltd., Prague, Czechia). Initially, extreme and outlying values were identified and removed using box plot analysis with a coefficient of 1.5. Subsequently, all monitored variables were tested for normality using the Shapiro-Wilk test at a significance level of $\alpha = 0.05$. Simultaneously, the homogeneity of variances was assessed using Levene’s test at the same significance level ($\alpha = 0.05$). For the data sets that met the assumptions of normality and homogeneity of variances, one-way analysis of variance (ANOVA) was employed. Statistically significant differences between groups were further evaluated using Fisher’s Least Significant Difference (LSD) post-hoc test ($\alpha = 0.05$). In cases where the data did not meet the assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis ANOVA was applied. Statistically significant differences were subsequently identified through multiple comparisons of mean ranks across all groups ($\alpha = 0.05$).

To investigate the relationships between measured traits, a correlation analysis was performed. Pearson’s correlation coefficient (r) was calculated to determine the strength and direction of the linear relationship between pairs of variables, conducted separately for the biometric and the analytical datasets. Statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Plant Physiological Status

NDVI

Early in the plant's development (March 28th), no significant differences in the NDVI were observed among the treatments. The *Landoltia* extract-only (L) treatment resulted in the highest mean NDVI of 0.604. The control group (C) and the combined treatment (M+L) showed similar mean values of 0.600 and 0.601, respectively. Interestingly, the mycorrhiza-only (M) treatment resulted in the lowest mean NDVI of 0.576.

On the second measurement date (April 4th), the differences in NDVI between the treatments were also statistically insignificant. The mean NDVI values slightly decreased, with the control measuring 0.464, the M 0.507, the M+L 0.527, and the L treatment 0.509. The overall decrease in NDVI values across all groups could reflect a change in leaf physiology as the plants entered a new growth phase. Between March 28 and April 4, the NDVI value in the control variant decreased by 0.136, while in the treated variants the decrease was noticeably lower, ranging from 0.095 to 0.069. This suggests that the application of biostimulants contributed to maintaining higher physiological activity and overall plant vitality compared to the untreated control.

Quantum Yield of Photosystem II

The quantum yield of photosystem II (Qy), which reflects the efficiency of the light-harvesting process, remained high and stable throughout the entire experiment. Mean Qy values consistently ranged from 0.81 to 0.83 across all treatment groups at both measurement dates. Critically, no statistically significant differences were found between the variants in the first date. In spite of significantly higher (0.82) result at M+L treatment in the second term, plants did not fundamentally alter or impair the core efficiency of the photosynthetic machinery. All plants, regardless of treatment, were photosynthetically healthy.

3.2. Biometric Parameters at Harvest

Above-ground Biomass

The above-ground fresh biomass was not significantly affected by the biostimulant applications. The control plants (C) produced a mean biomass of 214.3 g. The application of mycorrhiza alone (M) resulted in a modest insignificant increase to 223.4 g. Both *Landoltia* treatments (L and M+L) showed a slight but not-significant decrease in mean biomass compared to the other variants, with mean values of 199.3 g and 199.2 g, respectively.

Leaf Area, Plant Height and Diameter, Number of Leaves

According to statistical evaluation, the applied treatments had no significant effect on leaf area, which ranged from 956.6 cm² (in variant C) to 916.5 cm² (in variant L). Lettuce height was also not affected by the treatment variant. It ranged from 166.8 mm (in variant M+L) to 161.8 mm (in variant M). The largest average rosette diameter was recorded in the control variant at 270.9 mm, which was significantly higher than the mean diameter in variant L (248.3 mm). All variants had an average of 28.9 leaves, with no statistically significant differences between treatments.

3.3. Qualitative and Compositional Analysis

Dry Matter Content

The mean percentage of leaf dry matter was highest in the *Landoltia* variant (4.89 ± 0.32 %) and the mycorrhiza + *Landoltia* (M+L) variant (4.39 ± 0.81 %). The control and mycorrhiza variants showed lower mean values of 3.93 ± 0.26 % and 3.83 ± 0.11 %, respectively. The assumption of homogeneity of variance was violated for this parameter ($p < 0.05$); therefore, the Kruskal-Wallis test was

employed. Multiple comparison of p-values test revealed no statistically significant differences in the dry matter of leaves among the four treatment groups.

Vitamin C Content

The concentration of vitamin C (ascorbic acid), a key nutritional quality indicator, was not significantly altered by the biostimulant treatments. The highest mean concentration of vitamin C was recorded in the M+L variant (55.7 ± 4.00 mg/kg), followed by the *Landoltia* (49.5 ± 10.51 mg/kg) and control (49.1 ± 2.41 mg/kg) variants. The mycorrhiza variant exhibited the lowest mean value (44.1 ± 7.30 mg/kg).

Photosynthetic Pigments

The concentrations of photosynthetic pigments were significantly influenced by the treatments. For chlorophyll a, the post-hoc test identified two distinct groups (Figure 1). The M+L (2001.69 ± 204.54 mg/kg) and L (2000.48 ± 101.57 mg/kg) variants showed significantly higher concentrations compared to both the control (1725.66 ± 70.15 mg/kg) and mycorrhiza (1713.91 ± 218.33 mg/kg) variants.

For chlorophyll b, the analysis revealed a gradient of effects across three overlapping homogenous groups (Figure 2). The L variant (561.05 ± 36.24 mg/kg) and the M+L variant (515.51 ± 68.41 mg/kg) had significantly higher concentrations than the control variant (360.92 ± 58.39 mg/kg). The mycorrhiza variant (453.70 ± 81.20 mg/kg) did not differ significantly from either the control or the M+L and *Landoltia* variants, holding an intermediate position.

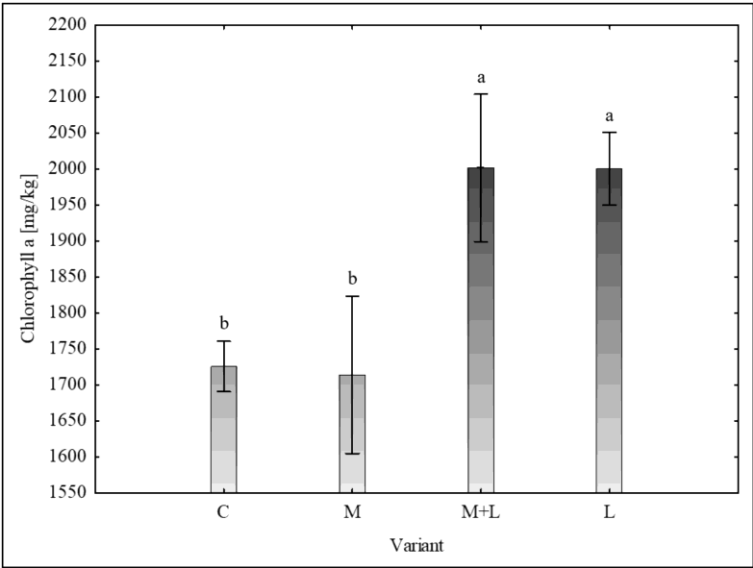


Figure 1. Effect of treatment (C – control; M – mycorrhiza; M+L – mycorrhiza and *Landoltia*; L – *Landoltia*) on total chlorophyll a content in lettuce leaves; error bars represent standard error (n = 4); different letters above the bars indicate statistically significant differences according to Fisher’s LSD post-hoc test ($\alpha = 0.05$).

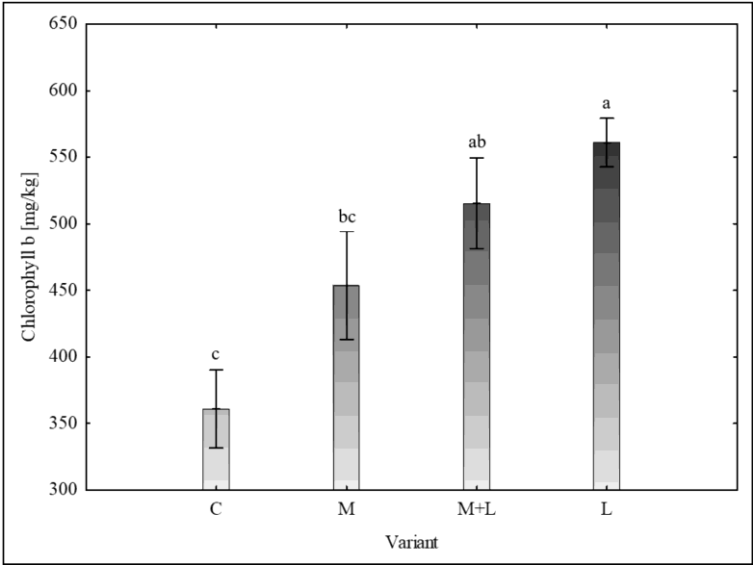


Figure 2. Effect of treatment (C – control; M – mycorrhiza; M+L – mycorrhiza and *Landoltia*; L – *Landoltia*) on total chlorophyll b content in lettuce leaves; error bars represent standard error (n = 4); different letters above the bars indicate statistically significant differences according to Fisher’s LSD post-hoc test ($\alpha = 0.05$).

Carotenoids

Analysis of carotenoid content (Figure 3) revealed that the control group (561.67 ± 72.13 mg/kg) had a significantly higher concentration than the mycorrhiza variant (414.02 ± 39.63 mg/kg) and M+L variant (483.30 ± 24.40 mg/kg). The *Landoltia* variant (520.27 ± 19.30 mg/kg) was not significantly different from the control or from M+L variant.

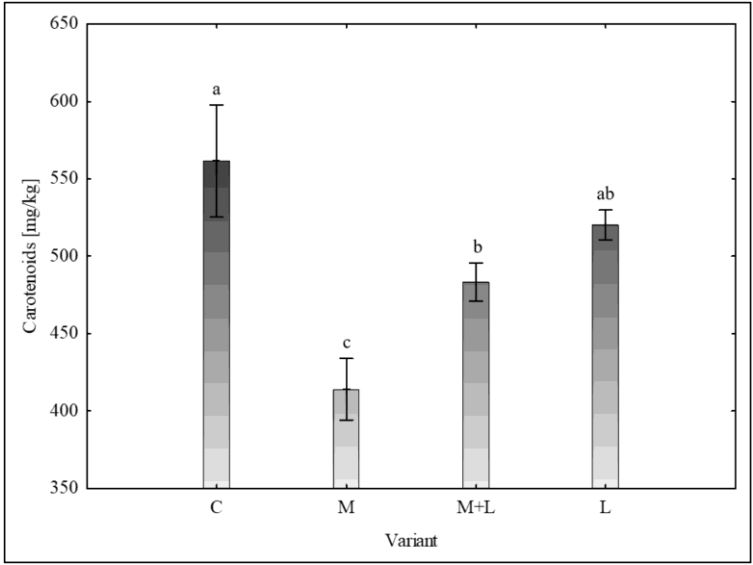


Figure 3. Effect of treatment (C – control; M – mycorrhiza; M+L – mycorrhiza and *Landoltia*; L – *Landoltia*) on the content of carotenoids in lettuce leaves; error bars represent standard error (n = 4); different letters above the bars indicate statistically significant differences according to Fisher’s LSD post-hoc test ($\alpha = 0.05$).

Phenolic and Flavonoid Content, Total Antioxidant Capacity

A statistically significant effect was observed on the accumulation of antioxidant compounds. The total amount of phenolic compounds (Figure 4) in control plants was 198.00 ± 16.14 mg /kg. This was significantly elevated in M+L treatment (254.28 ± 23.63 mg /kg). The rest of treatments showed similar level to the control – M (185.77 ± 34.61 mg /kg), L (207.84 ± 20.84 mg /kg).

The highest mean flavonoid content was recorded in variant M (110.22 ± 24.00 mg/kg), followed by variant M+L (105.83 ± 4.73 mg/kg), while variants C and L reached only 97.67 ± 8.79 mg/kg and 90.21 ± 20.87 mg/kg, respectively. However, no statistically significant differences were observed among the data.

The total antioxidant capacity was not significantly different among the four treatment groups. Although there were slight variations in the mean values, the post-hoc tests confirmed that all variants belonged to a single homogenous group. The control group exhibited the highest mean antioxidant capacity, with a value of 126.68 ± 23.04 mg/kg, in contrast, variant M showed the lowest value of 113.14 ± 40.64 mg/kg.

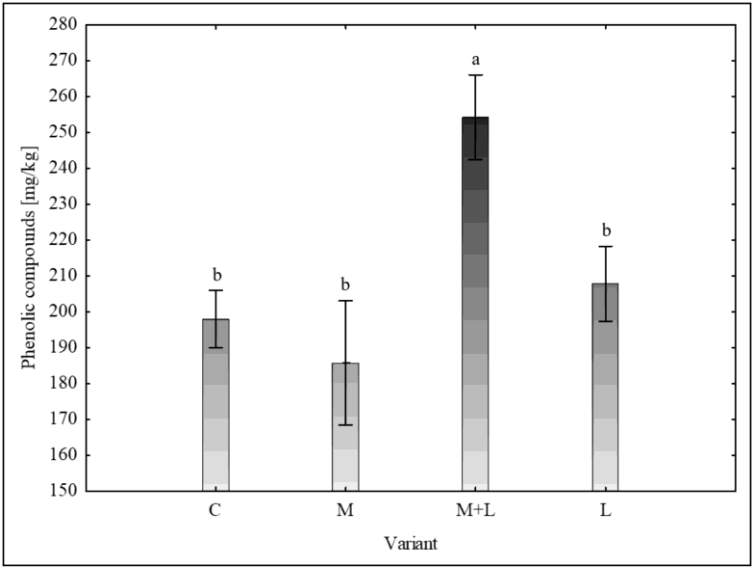


Figure 4. Effect of treatment (C – control; M – mycorrhiza; M+L – mycorrhiza and *Landoltia*; L – *Landoltia*) on the content of phenolic compounds in lettuce leaves; error bars represent standard error (n = 4); different letters above the bars indicate statistically significant differences according to Fisher’s LSD post-hoc test ($\alpha = 0.05$).

Nitrate Content

The concentration of nitrates in the leaves (Figure 5), a critical parameter for food safety, was significantly affected by the treatments. The control group showed the highest mean nitrate level at 2402 ± 377 mg/kg. All biostimulant treatments significantly reduced this value, with the most effective being the M and M+L variant, which lowered the nitrate content to 1559 ± 192 and 1596 ± 137 mg/kg, respectively. Significant decrease was confirmed at L treatment alone, too.

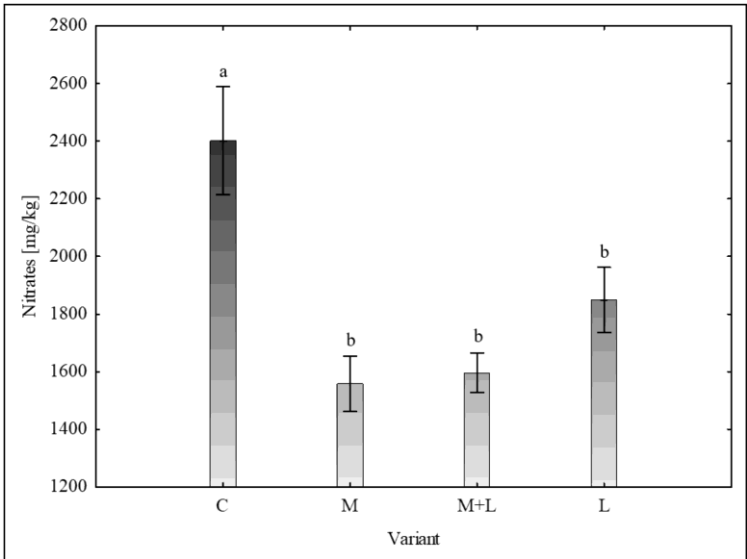


Figure 5. Effect of treatment (C – control; M – mycorrhiza; M+L – mycorrhiza and *Landoltia*; L – *Landoltia*) on the content of nitrates in lettuce leaves; error bars represent standard error (n = 4); different letters above the bars indicate statistically significant differences according to Fisher’s LSD post-hoc test ($\alpha = 0.05$).

Correlation Analysis

Correlation analyses were performed within the groups of biometric and analytical parameters, and Pearson correlation coefficients were calculated for all pairs of variables. Among the more significant relationships, a strong positive correlation was found between chlorophyll a and b parameters (Figure 6), with a Pearson correlation coefficient of $r = 0.875$. Furthermore, a strong positive correlation was also observed between carotenoid and nitrate content parameters (Figure 7), with $r = 0.772$.

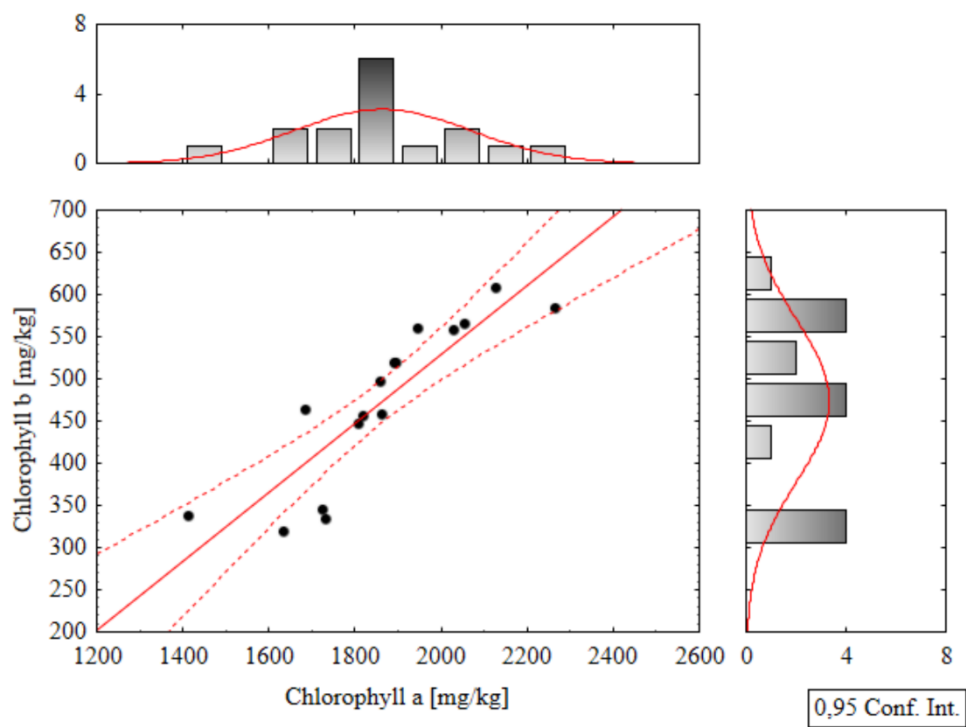


Figure 6. Correlation graph of chlorophyll a and b content in lettuce leaves; red lines indicate the 95 % confidence interval boundaries.

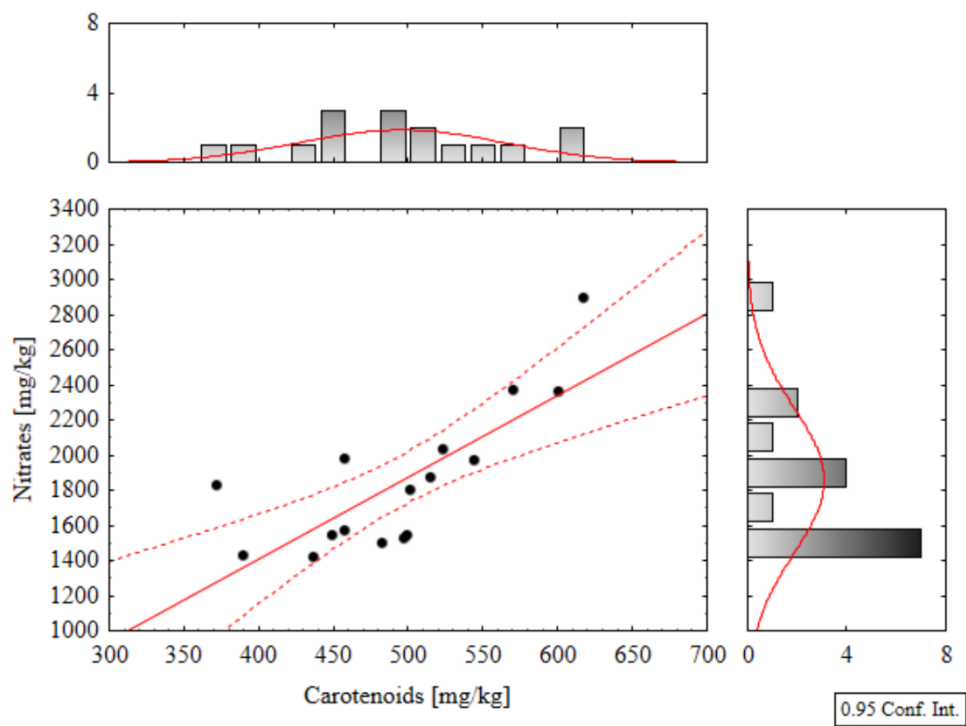


Figure 7. Correlation graph of carotenoid and nitrate content in lettuce leaves; red lines indicate the 95 % confidence interval boundaries.

3.4. Identified Amino Acids in the Aqueous Extract of *Landoltia punctata*

After performing a metabolomic analysis of a 0.5 % aqueous extract of *L. punctata* (clone no. 5562), all proteinogenic amino acids (AAs) were detected, as well as two non-proteinogenic ones: gamma-aminobutyric acid (GABA) and hydroxyproline. The concentrations of individual amino acids are summarized in Table 2. The most abundant AAs were GABA and valine, with concentrations of 1548 and 1527 µg/mL, respectively, followed by phenylalanine (751 µg/mL) and tryptophan (551 µg/mL). Other highly represented AAs included alanine, leucine, arginine, aspartic acid, isoleucine, asparagine, glutamic acid, tyrosine, proline, and histidine, with concentrations ranging from 500 to 100 µg/mL. Glycine, threonine, glutamine and lysine were present only in the tens of micrograms per mL. Minor amounts of methionine, hydroxyproline, serine, and cysteine (in units or less than one µg/mL) were also detected in the duckweed extract.

Table 2. Amino acid composition in a 0.5 % aqueous extract of *Landoltia punctata* (clone no. 5562).

AA	Mean concentration [µg/l] ± SD
GABA	1547.68 ± 4.208
Valine	1526.97 ± 1.842
Phenylalanine	750.93 ± 3.787
Tryptophan	550.65 ± 3.999
Alanine	471.19 ± 4.164
Leucine	418.51 ± 3.179
Arginine	346.03 ± 4.005
Aspartic acid	285.20 ± 0.408
Isoleucine	259.16 ± 0.490
Asparagine	235.54 ± 0.116
Glutamic acid	219.85 ± 0.334
Tyrosine	200.70 ± 0.164
Proline	124.65 ± 0.283
Histidine	112.37 ± 0.276

Glycine	74.31 ± 0.213
Threonine	66.18 ± 0.418
Glutamine	33.98 ± 0.418
Lysine	12.63 ± 0.028
Methionine	4.16 ± 0.036
Hydroxy-proline	1.09 ± 0.005
Serine	0.32 ± 0.002
Cysteine	0.08 ± 0.001

3.5. Mycorrhizal Colonisation

The inoculation with AMF was confirmed as successful and treatment-specific. Microscopic analysis of the root systems revealed a complete absence of mycorrhizal structures in both the control (C) and *Landoltia* extract-only (L) variants, confirming a zero-colonisation rate. In contrast, plants from the combined treatment group (M+L) treatment exhibited an evident mean colonisation of 42 %. An even higher colonisation intensity was noted in the mycorrhiza-only (M), which achieved a mean rate of 58 %. This successful establishment of the symbiosis is fundamental for interpreting the subsequent results.

The Figures 8 and 9 display a longitudinal section of a lettuce root cortex colonised by an arbuscular mycorrhizal fungus. The plant cell walls are distinguished by their blue autofluorescence, whereas the fungal structures are visualized in orange. The arrow labelled A indicates a mature, finely branched arbuscule occupying a cortical cell. Arrow M points to a developing arbuscule or a hyphal coil. The thread-like orange structures are the intercellular hyphae that proliferate within the root apoplast before penetrating host cells to form the arbuscules. Figure 8 (left and right) show mycorrhization in the M variant, while Figure 9 (left and right) in M+L variant.

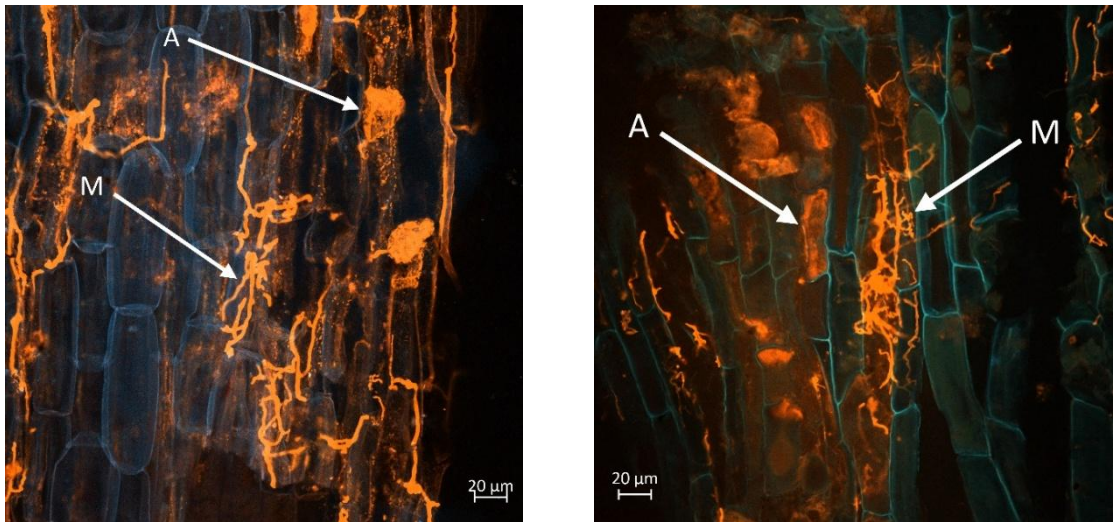


Figure 8. (left). An arbuscular mycorrhizal fungus colonising the root cortex of lettuce. Finely branched arbuscule (A) and a developing arbuscule or hyphal coil (M) within host cells. Fungal structures are visualised in orange, against the blue autofluorescence of the plant cell walls. Scale bar = 20 µm. **(right).** Mature arbuscules of mycorrhizal fungus (A) developing in the cortical cells of a lettuce root. Newly developing arbuscule from mycelial hyphae (M). Scale bar = 20 µm.

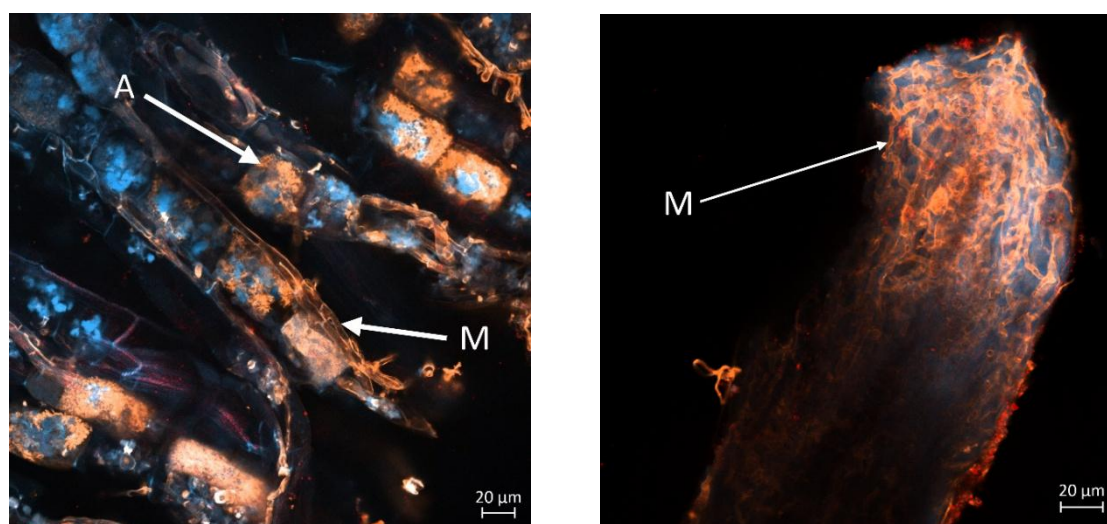


Figure 9. (left). Intraradical structures of an arbuscular mycorrhizal fungus within the root cortex of lettuce. The image displays intercellular hyphae forming the mycelium (M) and a developing arbuscule (A) inside a cortical cell. Scale bar = 20 µm. **(right).** An extensive network of extramatrical mycelium (M) of an arbuscular mycorrhizal fungus colonising the tip of a lettuce root. This hyphal network is responsible for exploring the soil substrate and absorbing nutrients. Scale bar = 20 µm.

4. Discussion

The experiment tested four treatment variants (1. control, 2. treatment with a mixed mycorrhizal inoculum, 3. treatment with a 0.5 % aqueous extract of *L. punctata*, and 4. a combination of the two previous) in a growth trial with lettuce (*Lactuca sativa* L. cv. 'Dubáček'). During plant growth and after harvest, photosynthetic, biometric, and qualitative parameters were evaluated.

Since this is a previously untested preparation, a metabolomic analysis of AAs in the aqueous extract of *L. punctata* was also conducted in order to link compositional information with potential stimulatory effects on lettuce plants. The results revealed particularly high levels of GABA, valine, phenylalanine, and tryptophan, including a complete set of proteinogenic amino acids. These data complement the findings of Regni et al. (2021) and Buono et al. (2022), who reported a metabolomic profile of *Lemna minor* extract containing, in addition to AAs, phytohormones, low-molecular-weight phenolic compounds (phenylpropanoids and their glycosides, flavonoids, and simple phenolic acids), glucosinolates, alkaloids, isoprenoids, tetraterpenes, and chlorophylls.

Furthermore, mycorrhizal symbiosis establishment was microscopically examined to confirm its potential influence on lettuce culture. Both mycorrhizal treatments showed average standard colonization rates of 58 % (M) and 42 % (M+L). AMF are known to be host-specific, meaning that different AMF species interact with different host plants at varying colonization levels, ranging from just a few percent to nearly 100 % (Carrenho et al., 2007). Microscopic assessment of AMF colonization confirmed that the inoculum was well suited for this plant species and can therefore be associated with the observed effects.

Photosynthetic Parameters

Present study did not demonstrate significant effects of individual treatments on photosynthetic parameters. However, the substantially lower decline in NDVI values between the monitored dates across all treated variants suggests prolonged vegetation vitality and delayed chlorophyll degradation. A statistically significant increase in the quantum yield of photosystem II was observed only in the combined treatment with mycorrhiza and *L. punctata* extract (M+L) at the second measurement, whereas the extract alone did not show this effect.

Priolo et al. (2024) reported a stronger response in tomato, where the effective quantum yield of photosystem II (Φ_{II} , Φ_{II2}) was highest with 0.5 % and 1 % aqueous extracts of *L. minor*, although no

differences were observed in the maximum quantum yield (Fv/Fm). Our results therefore suggest that the effect of *L. punctata* extract may be more evident in combination with mycorrhizal inoculation, though it cannot be excluded that a significant effect on Φ_{II} could also appear under single extract application, similar to the findings of Priolo et al. (2024) with *L. minor*.

Regni et al. (2021) studied the effects of *L. minor* aqueous extract on olive trees, where 0.5 % and 1 % concentrations significantly increased net photosynthesis (Pn), stomatal conductance (gs), and substomatal CO₂ concentration compared to the untreated control. A positive influence on photosystem II efficiency was also reported by Asadi et al. (2022) after combined application of AMF (specifically *Funneliformis mosseae*, also included in our inoculum) with foliar application of 3 g/L seaweed extract. Moreover, Regni et al. (2024) observed improved photosynthetic activity in olive trees treated with *L. minor* extract under increased salinity, similar to the findings of Miras-Moreno et al. (2022) in maize exposed to excessive Cu concentrations.

Although current evidence supports the positive impact of duckweed extracts on the photosynthetic apparatus of plants, the specific effects of *L. punctata* extract alone and of mycorrhizal inoculation still require further clarification. Our results indicate that the observed increase in the quantum yield of photosystem II may result from their synergistic action as well as from the potential individual contributions of both treatments.

Biometric Parameters

A number of studies have confirmed the positive effects of mycorrhizal inoculation on the growth and yield parameters of lettuce. For example, Zrig et al. (2025) reported a 38.8 % increase in lettuce yield following inoculation with *F. mosseae* compared to the control. Similarly, Han et al. (2023) observed a 30 % increase in lettuce plant height in variants inoculated with AMF (*F. mosseae*) compared to the control. In another experiment led by Rasouli et al. (2022), co-application of AMF (*F. mosseae*) with foliar application of seaweed extract (3 g/L) increased the number of leaves by 44 %, the average head diameter 3.6-fold, and head yield 3.4-fold compared to the control.

Although present study did not confirm statistically significant differences in biometric parameters, a similar trend was observed for above-ground biomass. The mean value in the M variant was slightly higher (223.4 g) compared to the untreated control (214.3 g). Previous studies with aqueous extracts of *L. minor* have also reported positive effects on biometric parameters such as fresh shoot weight, shoot dry matter, plant height, leaf area, and number of leaves, with the optimal concentration being 0.5 % (the same concentration was used in this study) (Buono et al., 2022; Regni et al., 2021; Priolo et al., 2024).

Regni et al. (2024) further showed that salt stress induced by NaCl reduced several growth parameters in olive trees (number of leaves, lateral shoots, and plant height). However, after treatment with *L. minor* extract, these parameters recovered to levels comparable with unstressed controls. A similar trend was observed by Miras-Moreno et al. (2022), where excess Cu caused a decline in growth parameters, but treatment with *L. minor* extract restored or even exceeded control values for plant height, root length, and fresh shoot weight.

The positive influence of duckweed extract on vegetative growth reported by these authors is likely related to a combination of factors: increased chlorophyll content (see below), enhanced photosynthesis, uptake of organic N in the form of AAs via foliar nutrition (see amino acid profile results), and thus accelerated protein biosynthesis.

Photosynthetic Pigments and Carotenoids

The study showed that variants treated with the *Landoltia* extract (L and M+L) had significantly higher concentrations of chlorophyll a than both the control and the mycorrhizal variant, while chlorophyll b was significantly higher only compared to the control. This suggests that compounds present in the extract may either promote chlorophyll synthesis or slow down its degradation. These findings are consistent with those of Priolo et al. (2024) and Regni et al. (2021).

In contrast, mycorrhizal application alone (M) in our study did not increase chlorophyll levels compared to the control, which contradicts the results of Cela et al. (2022), who reported a 35 % increase in chlorophyll in lettuce treated with *F. mosseae* at 35 days after treatment and a 50 % increase at 53 days compared to the untreated control.

In our study, the sum of chlorophyll a + b was highest in the L variant (2561.53 mg/kg), followed by M+L (2517.2 mg/kg), M (2167.61 mg/kg), and the lowest in the control C (2086.58 mg/kg). An opposite trend was observed for carotenoids, which may indicate more intensive photosynthetic processes accompanied by reduced stress and thus a lower need for protective carotenoid pigments. In contrast, Priolo et al. (2024) found that treatment of NaCl-stressed maize plants with *L. minor* extract had no effect on carotenoid content.

Qualitative Parameters

Vitamin C content was not significantly affected by the treatments. Although a slight trend towards higher values was observed in the combined M+L treatment and in the L variant alone, the data did not follow a normal (Gaussian) distribution, and the variability within groups was too high to establish a statistically significant effect.

The treatments had different impacts on the antioxidant profile of lettuce. A clear synergistic effect was observed in the combined M+L variant, which resulted in a significantly higher content of total phenolic compounds compared to all other treatments. A similar synergistic effect was also reported by Rasouli et al. (2022) with AMF application in combination with 3 g/L of seaweed extract. In contrast, no statistically significant differences were found in flavonoid content or in total antioxidant capacity as measured by the DPPH assay.

From a food safety perspective, one of the most valuable outcomes of this study is the significant reduction of nitrate concentrations in all biostimulant-treated variants. The M and M+L treatments were the most effective, reducing nitrate levels by 35 % and 34 %, respectively, compared to the control. This result is consistent with the known ability of AMF to improve nitrogen use efficiency in host plants (Aini, Yamika & Ulum, 2019). Nitrate reduction can generally be interpreted as their more efficient assimilation into ammonia and subsequently into organic nitrogen. This theory is supported by the findings of Priolo et al. (2024), who reported a significant increase in protein content in NaCl-stressed maize treated with *L. minor* extract, reaching levels comparable to unstressed control plants. Thus, future testing of this potentially biostimulant preparation from duckweed should also focus on total protein content.

Importantly, all measured values were well below the maximum permissible limits set by the European Commission (Regulation 2023/915), confirming the high quality and safety of the produce.

5. Conclusions

The application of arbuscular mycorrhizal fungi and an aqueous extract of *Landoltia punctata* did not significantly change the fresh biomass of lettuce under the experimental conditions. However, the treatments induced significant and beneficial changes in the plant's biochemical profile. Notably, the *Landoltia* extract, applied both individually and in combination with mycorrhiza, led to a substantial increase in chlorophyll a and b concentrations. A clear synergistic effect was observed in the combined treatment (M+L), which was the only variant to significantly elevate the content of total phenolic compounds. Crucially, all biostimulant applications resulted in a significant reduction of nitrate levels in the leaves, a key parameter for food safety. These findings demonstrate that whilst these biostimulants may not serve as primary growth promoters in non-stressed, short-term cultivation, they are highly effective in modulating plant metabolism to enhance nutritional quality. The use of *L. punctata* extract, therefore, represents a promising strategy for producing lettuce with improved phytochemical content and greater consumer value.

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