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Research Article

Synergistic effect of cultivation methods and seaweed-based biostimulations on iceberg lettuce in sustainable agriculture

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Abstract

This research, conducted over two consecutive years in the production fields of the Iceberg Lettuce Centar in Belgrade, aimed to investigate the impact of biostimulants on the morphological and phytochemical characteristics of iceberg lettuce in two seasons (spring and autumn), using greenhouse and open field cultivation. The biostimulator was derived from a seaweed (Kelpak). The effects of different applications (seedling soaking and foliar treatment once, twice, and three times during the growing season) on plant growth (total plant mass, leaf mass, and stem mass) and phytochemical properties, including pigments (chlorophyll a, chlorophyll b, carotenoids), as well as antioxidant activity (TAC, polyphenols), were investigated. The results showed that the application of biostimulators, particularly through the seedling soaking method, contributed to an increase in plant mass and greater biomass accumulation, whereas foliar treatments enabled better development in both seasons. Plants grown in the greenhouse had more stable growth and better quality, while the spring season proved to be more favourable than autumn. Biostimulators also had a positive effect on the content of photosynthetic pigments and antioxidants, which improved the nutritional value and stress resistance of plants.

Keywords: biostimulator; greenhouse; iceberg lettuce; open field; seaweed extracts;

Introduction

Seaweed extracts have multiple positive effects on plant growth, including the improvement of physiological processes such as growth, photosynthesis, and nutrient absorption. Their application increases plant resistance to stress conditions and contributes to yield enhancement, making them an important tool in sustainable agriculture.

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The application of biostimulators in lettuce cultivation was highlighted by Carillo *et al*., (2020), who found several specific effects and benefits that biostimulators bring to this vegetable such as increased growth and yield (growth stimulation and greater yield), improved quality (increased nutrient content and maintained freshness, reducing quality loss), stress resistance (enhanced resistance and reduced oxidative stress), improved root system (allowing better water and nutrient absorption), and ecological sustainability through reduced need for chemical fertilizers.

When lettuce is grown in an open field, the plants are more exposed to weather changes, so biostimulators are used to enhance the resistance of lettuce to extreme conditions (drought and high temperatures). Under these conditions, there is a greater need to treat plants with biostimulators to stabilize yields. Under controlled greenhouse conditions, biostimulators help optimize nutrient uptake, promote faster growth and fruit quality, and reduce stress caused by high humidity and temperature (Rouphael and Colla, 2020).

The morphological characteristics of plants, including total plant mass, leaf mass and stem mass, are key indicators of growth and development. These parameters are influenced by various factors such as environmental conditions, nutrient availability and the application of biostimulants. In lettuce cultivation, morphological characteristics are particularly important as they correlate directly with yield and marketability. Total plant mass is a comprehensive measure of overall plant growth and reflects the cumulative effect of biostimulants on biomass accumulation. Studies have shown that the application of algal extracts and cyanobacterial biostimulants can significantly increase total plant mass by improving nutrient uptake, photosynthesis and metabolic efficiency (Calvo *et al*., 2014; du Jardin, 2015). Leaf mass is a key factor in the quality of lettuce, as the leaves are the most important edible part of the plant. Biostimulants have been shown to increase leaf mass by stimulating cell division and expansion as well as improving chlorophyll content and photosynthetic efficiency (Rouphael and Colla, 2020). Although stem mass is less emphasized in lettuce compared to leaf mass, it plays a supporting role in plant structure and nutrient transport. Biostimulants can influence stem mass by promoting vascular development and strengthening the structural integrity of the plant. This is particularly important for lettuce varieties such as iceberg lettuce, where a robust stem is necessary for head formation and stability (Rouphael and Colla, 2020). In addition, a larger stem mass can contribute to better water and nutrient transport, which in turn promotes overall plant growth and productivity.

Chlorophyll a is the primary driver of photosynthesis, while chlorophyll b helps expand the spectrum of light that can be utilized. Their joint role enables plants to use solar energy efficiently for food and energy production. The role of chlorophyll a: 1. Primary photosynthetic pigment - the main pigment in photosynthesis that directly participates in converting light energy into chemical energy. 2. Light absorption - this energy is used to initiate reactions in photosystems I and II in chloroplasts. 3. Electron transfer is the first step in the electron transport chain, and is necessary for the synthesis of ATP and NADPH, which store energy. The role of chlorophyll b: 1. Accessory pigment - plays a role in expanding the light spectrum that the plant can utilize for photosynthesis. 2. Light harvesting - transfers the absorbed energy to chlorophyll a, which initiates the chemical reactions of photosynthesis. In this way, chlorophyll b increases photosynthesis efficiency. 3. Adaptability to different light conditions - Plants growing in lower light conditions (greenhouse) often have a higher ratio of chlorophyll b to chlorophyll a, enabling them to utilize weaker light and maintain photosynthetic efficiency (Hopkins and Hüner, 2008; Taiz *et al*., 2015).

Total carotenoids are also important in photosynthesis as they absorb light in the spectrum where chlorophyll is not effective against oxidative stress, safeguarding the plant from damage. The roles of total carotenoids in plants are as follows: 1. Light absorption (in parts of the spectrum that chlorophyll cannot effectively absorb); 2. Protection against photooxidative stress (protecting photosynthetic systems from damage caused by excess light energy - photoprotection. This process prevents the formation of reactive oxygen species that can damage cellular structures); 3. Neutralization of free radicals (acting as antioxidants that can neutralize free radicals and reactive oxygen species (ROS) under stress conditions due to excess light, drought or other unfavourable environmental conditions); 4. Synthesis of vitamin A - plant carotenoids play an important role in its synthesis; 5. Pigmentation of fruits and flowers (Young, 1991; Demmig-Adams and Adams III, 1996; Hopkins and Hüner, 2008; Taiz *et al*., 2015).

Lettuce contains a wide range of polyphenolic compounds, including flavonoids and phenolic acids, which contribute significantly to its antioxidant activity. The types of polyphenols in lettuce include flavonoids-quercetin, kaempferol, and luteolin-that help plants protect against UV radiation and oxidative stress, and phenolic acids-chlorogenic acid and caffeic acid. Phenolic acids contribute to the antioxidant protection of plants by neutralizing reactive ROS generated under stress conditions (Nicolle *et al*., 2004). Cultivation conditions can affect the polyphenol content in lettuce, as they depend on external factors: Light: Can increase the synthesis of polyphenols that act as protection against UV damage and oxidative stress. Llorach *et al*. (2008) found that lettuce plants grown under UV radiation had significantly higher levels of polyphenols compared to those grown under controlled conditions (greenhouse). Stress: Abiotic stresses can also stimulate increased polyphenol synthesis. Oxidative stress caused by water deficiency encourages plants to produce more antioxidant compounds, including polyphenols, as part of their defence mechanism (Kulbat, 2016).

Research on the Total Antioxidant Capacity (TAC) of lettuce grown under treatments with cyanobacterial biostimulators aims to determine how these treatments affect plant health and product quality, enhancing growth, stress resistance, and the nutritional value. Differences between greenhouse and open field cultivation presented that plants produce more antioxidant compounds in response to stress conditions, while in greenhouses, controlled conditions may reduce the stress levels on plants, leading to different results compared to open fields. Koca and Karadeniz (2011), studied the antioxidant properties of lettuce and compared the total antioxidant capacity under different cultivation conditions. Various studies showed that different factors, including greenhouse treatments, affect the quality and antioxidant capacity of lettuce after harvest (Koukounaras *et al*., 2007) and how different agricultural practices influence antioxidant activity and quality in lettuce, including total antioxidant capacity (TAC), in both organic and conventional farming (Muscolo *et al*., 2022).

In iceberg lettuce cultivation, biostimulators can significantly contribute to increased yield and the plant's resistance to abiotic stresses (e.g., drought, high salinity). Combining photoperiod (growing time) with biostimulators allows even better control of plant growth (Calvo *et al*., 2014). The application of biostimulators can enhance photosynthetic efficiency and accelerate the plant's metabolism, which is especially important when plants receive optimal light conditions (du Jardin, 2015). The combination of proper photoperiodism and biostimulators leads to a synergistic effect that enables stable plant growth, improved photosynthesis, and optimal head formation, which are crucial for the commercial production of iceberg lettuce (Rouphael and Colla, 2020).

This research aimed to examine the effects of biostimulators derived from cyanobacteria and their application methods on the commercial production of iceberg lettuce in open fields and greenhouses during growing periods with different photoperiods: spring (a regular growing period) and autumn (a period needed to meet commercial demands for this vegetable).

Materials and Methods

Experimental design and plant material

During 2022 and 2023, a simultaneous experiment was set up in both a greenhouse and an open field with iceberg lettuce on the production fields of Iceberg Salat in Belgrade. The experiment was conducted in two time periods, during spring and autumn (August 25th to October 16th, 2022, and March 17th to May 27th, 2023).

The total area of the experimental plot was 480 m^2, and it was divided into five equal plots (60 m x 5 m), with a planting scheme of 33 cm x 33 cm. The variety of iceberg lettuce used in this experiment was 'Umbrians' F1 owned by the seed house Rijk Zwaan.

Treatments

During the two production seasons (autumn and spring), the effect of biostimulators derived from seaweed (Kelpak) on the morphological (total plant mass, leaf mass, and stem mass) and phytochemical properties (pigments: chlorophyll a, chlorophyll b, and total carotenoids, as well as antioxidant activity: total antioxidant capacity and total polyphenol content) of iceberg lettuce was investigated. Lettuce plants were treated as follows:

1. 35-day-old seedlings were soaked in a biostimulator solution (P);
2. Foliar treatment once-10 days after transplanting (T1);
3. Foliar treatment twice during the growing season (T2);
4. Foliar treatment three times during the growing season (T3);
5. Control-untreated plants (CO).

Plants in the seedling soaking treatment were immersed in a 1% solution of Kelpak for 24 hours prior to transplanting, while iceberg lettuce plants in the foliar treatment were treated with a 0.3% solution of Kelpak from one to three times every 10 days.

Experimental design and replications

Before setting up the experiment, the soil was analysed chemically at two depths (0-30 cm and 30-60 cm) in both the open field and the greenhouse. Basic soil cultivation was conducted to a depth of 30 cm, along with the implementation of fundamental agronomic measures-fertilization with 500 kg ha^{-1} of NPK mineral fertilizer (formulation 15:15:15) and an additional 500 kg ha^{-1} of pelleted chicken organic manure (Fertipolina). The combination of these fertilizers optimized the soil quality. Pre-sowing land preparation for the autumn season was performed in July, whereas for the spring season, it was conducted in November.

The experiment in the open field and greenhouse was set up as a two-factorial completely randomized block design with three replications. For each replicate 30 plants were measured for each treatment. Phytochemical analyses were conducted on 10 plants in triplicate.

Environmental conditions

For this experiment, temperature and relative humidity were measured over a 24-hour period using a data logger (RC-4HC Data Logger, Elitech Technology, Inc., USA), (Figure 1, 2, 3 and 4).

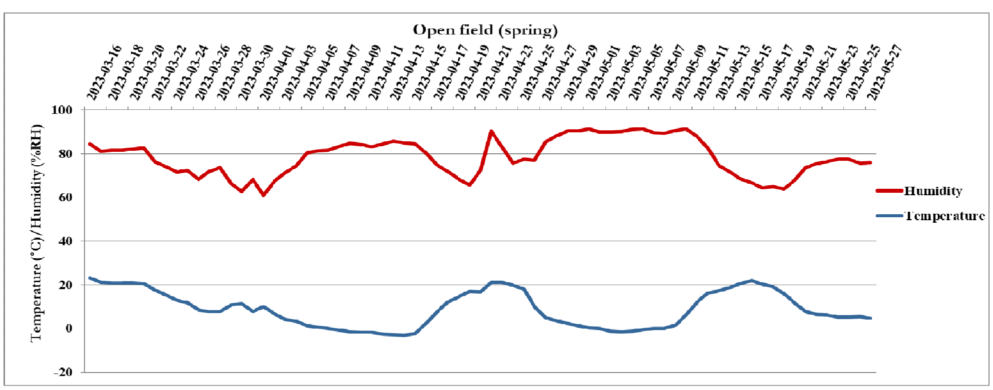

Figure 1. Temperature and humidity fluctuations during the spring period (March, May) in the open field

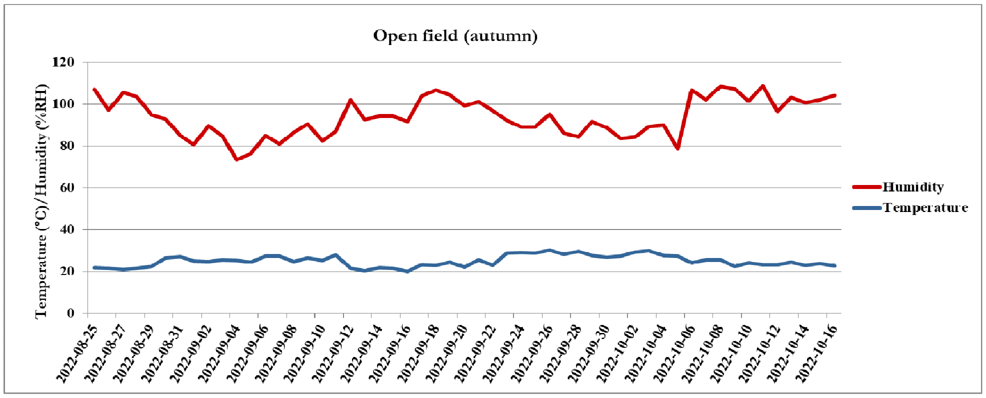

Figure 2. Temperature and humidity fluctuations during the autumn period (September, October) in the open field

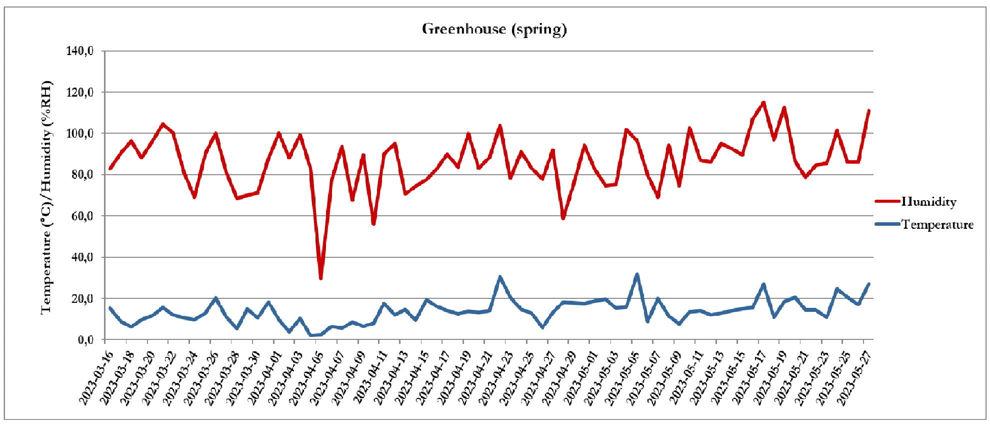

Figure 3. Temperature and humidity fluctuations during the spring period (March, May) in the greenhouse

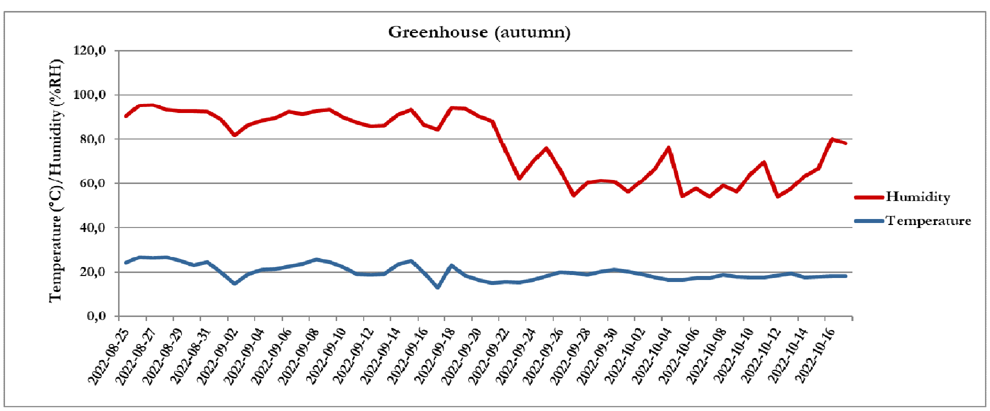

Figure 4. Temperature and humidity fluctuations during the autumn period (September, October) in the greenhouse

Measured parameters

At technological maturity, the heads of iceberg lettuce were harvested, and defined parameters were measured. This study presents and analyses the morphological properties:

- Total plant mass (g)
- Leaf mass (g)
- Stem mass (g)

Phytochemical analyses were determined:

- Chlorophyll a (mg g^{-1})
- Chlorophyll b (mg g^{-1}
- Total carotenoids (mg g^{-1})
- Polyphenols (mg g^{-1})
- TAC (mg g^{-1}

Phytochemical analyses were performed at the Belgrade Faculty of Agriculture laboratory.

Total antioxidant capacity (TAC)

The TAC analysis was performed applying the method described by Prieto *et al*. (1999). The antioxidant capacity was calculated according to the calibration curve prepared with ascorbic acid as the standard. Results were expressed as mg of ascorbic acid equivalents (AAE) per g of fresh weight (FW).

The content of chlorophyll a and b

The determination of chlorophyll a and b content was performed by using a spectrophotometer (UV-1800, Shimadzu USA Manufacturing Inc., Canby, OR, USA), measuring the absorbance of the samples at 646 nm and 663 nm in 2 ml of undiluted plant extract. The obtained values for the mentioned wavelengths were used to determine chlorophyll a and b content using the following formulas proposed by Laware (2015):

- Chlorophyll a (mg mL^{-1}) = 12.21 A663 - 2.81 A646
- Chlorophyll b (mg mL^{-1}) = 20.13 A646 - 5.03 A663

Where A645 is the absorbance of the sample at a wavelength of 645 nm; A663 is the absorbance of the sample at a wavelength of 663 nm. The results obtained are expressed as mg g^{-1} FW.

Total polyphenol content (TPC)

The Folin-Ciocalteu (FC) method was used for TPC determination as described in the literature (Ng *et al.,* 2000). TPC quantification was done based on the calibration curve prepared with ferulic acid (FA) as the standard (Simin *et al*., 2013; Asemani *et al*., 2019). Results are expressed as mg of ferulic acid equivalents (FAE) per g of fresh weight (FW).

Statistical analysis

Differences between means (between morphological and phytochemical properties of lettuce grown in two production cycles in open fields and in greenhouses, as well as those treated with biostimulators in four ways compared to the untreated control) were determined using a two-factor ANOVA model, and significant differences were expressed as an LSD test. Principal component analysis was used to identify the sources and structure of variation, as well as their contribution to the overall variability of the properties. Principal components were extracted when the eigenvalue was greater than 1. Statistical analyses were conducted using the STATISTICA 10 software package (StatSoft, Inc., Tulsa, OK, USA). The connection of genotypes and traits was done by multi-variation technique of PCA-Principal Component analysis using Statistical software: XLSTAT Version 2012.4.02 Copyright Addinsoft 1995-2012. The analysis was performed according to average values of the researched parameters.

Results

Analysing results for the chlorophyll a content in the leaves of the iceberg lettuce, a statistically significant difference was observed between the autumn and spring growing periods, in both greenhouse and open-field conditions. In the spring growing period, a higher chlorophyll a content was found, particularly in greenhouse cultivation, whereas opposite results were in autumn cultivation. For the biostimulator treatments, a significant difference in chlorophyll content was found between the untreated control and treatments of the biostimulator during both growing periods. In spring cultivation, in chlorophyll content the treatment with two sprayings of the biostimulator was at the control level, while other treatments differed significantly among themselves and from control. In autumn cultivation, there were no differences between the seedling soaking and one-spray treatments, while other treatments were significantly different, as well as the control treatment, which had the lowest chlorophyll a value observed (Table 1).

Table 1. Average values of pigment content - chlorophyll a, chlorophyll b, and total carotenoids of individual biostimulator treatments, in open field and greenhouse of iceberg lettuce

Treatments		Chlorophyll a (mg g^{-1})		Chlorophyll b (mg g^{-1})		Carotenoids (mg g^{-1})	
		Autumn	Spring	Autumn	Spring	Autumn	Spring
Biostimulant (A)	Control (CO)	0.01204c	0.00516d	0.00296d	0.00005c	0.00637d	0.00329e
	Soaking (P)	0.00852d	0.00671c	0.00140e	0.00172b	0.00524e	0.00468b
	T1	0.01361b	0.00643c	0.00407c	0.00177b	0.00691c	0.00405d
	T2	0.01187c	0.01174a	0.00455b	0.00309a	0.00788b	0.00566a
	T3	0.02626a	0.00894b	0.00819a	0.00125b	0.01118a	0.00445c
Location (B)	Open field	0.01640a	0.00737b	0.00476a	0.00154	0.00799a	0.00402b
	Greenhouse	0.01252b	0.00823a	0.00371b	0.00161	0.00704b	0.00483a
ANOVA							
A		*	*	*	*	*	*
B		*	*	*	ns	*	*
AXB		*	*	*	*	*	*

The values denoted with different small letters within columns for every treatment are significantly different ($p \leq 0.05$) in accordance with the LSD test

The interaction between the treatments and cultivation methods are presented in Figure 5 (A - autumn and B - spring growing period). The autumn growing period in the open field showed a difference in chlorophyll a content compared to greenhouse cultivation in the control sample at a significant level. Treatment of seedling soaking with biostimulant solution induced a decrease in chlorophyll a content in the open field. The treatments with two and three sprays had different effects on the chlorophyll a content in the open field and greenhouse. One spray treatment did not cause significant differences in chlorophyll content in the open field and greenhouse. In three-sprayings treatment in the open field, a surge in chlorophyll a content was noted, which was not the case in greenhouse production (Figure 5A). In spring production, there was a tendency for an increase in chlorophyll a content. Overall, significant differences in chlorophyll a content were observed everywhere except in the open field between the one-spray and two-spray treatments (Figure 5B).

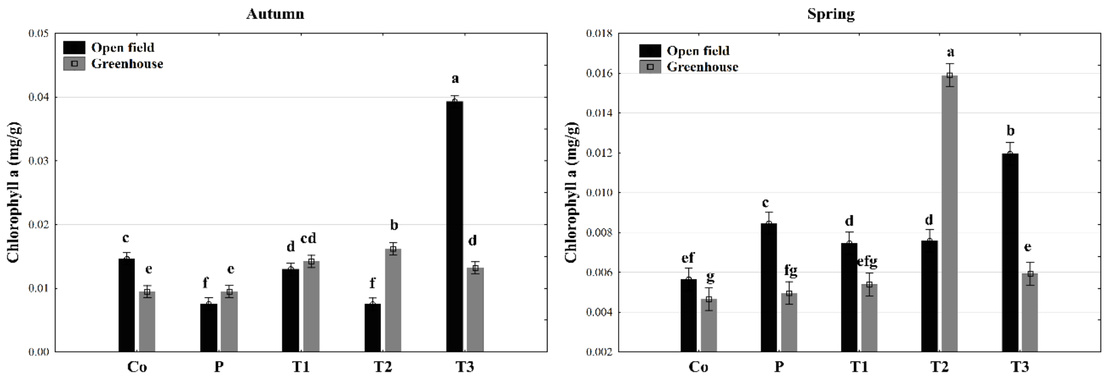

Figure 5. Interactions: A) Chlorophyll a content depending on the treatment method in the autumn and B) in the spring growing period

Values characterised by different lower-case letters differ significantly (p ≤ 0.05) according to the LSD test

Analysing the results for the chlorophyll b content in the leaves of iceberg lettuce, a statistically significant difference was observed concerning the biostimulator treatments, as well as cultivation methods during both growing periods (autumn and spring). The average values (Table 1) indicate that in the spring growing period, higher chlorophyll b content was found in the greenhouse compared to the open field. On the other hand, in the autumn growing period, the content of this pigment was slightly higher in the open field than in the greenhouse. Significant differences were recorded between the control treatment (CO) and the treated samples for the biostimulator treatments. In the spring season, treatments T1 and T2 resulted in a significantly higher chlorophyll b content compared to the control (CO) (Table 1). In the fall season, the lowest chlorophyll b content was observed in the dip treatment (P), while all other biostimulant treatments, especially T2 and T3, significantly increased the pigment content. The interaction of factors (treatment and cultivation method) was statistically significant in both growing seasons.

These results are presented graphically (Figure 5A and 5B), where it can be clearly seen that the greenhouse provides the highest chlorophyll b values in the spring in combination with treatment T2, while the open field with treatment T3 dominates in the fall.

Figure 6A shows that the greenhouse provides significantly higher chlorophyll b values compared to the open field for all treatments. Treatment T1 (1x spraying) resulted in the highest chlorophyll b content in the greenhouse, while the dipping treatment (P) had the lowest pigment content in both cultivation methods, especially in the open field. A gradual increase in pigment content can be observed in treatments T1 and T2, with the greenhouse having an advantage over the open field, while treatment T3 in the open field yielded the highest chlorophyll b values in both cultivation methods. The interaction of treatment and cultivation method is clearly expressed: the greenhouse and treatment T3 provide a synergistic effect on increasing chlorophyll b content.

Figure 6B shows the chlorophyll b content during the spring growing season for the same treatments and cultivation methods. Unlike in the fall, the open field shows a slight advantage over the greenhouse for most treatments. The highest pigment content was found in the T3 treatment in the greenhouse, while the control (CO) had the lowest values in both cultivation methods. Treatment T1 shows similar values in both cultivation methods, indicating its stability under different conditions. The lack of a statistically significant interaction between the cultivation methods in the spring season indicates that the treatments affect the chlorophyll b content independently of the cultivation method.

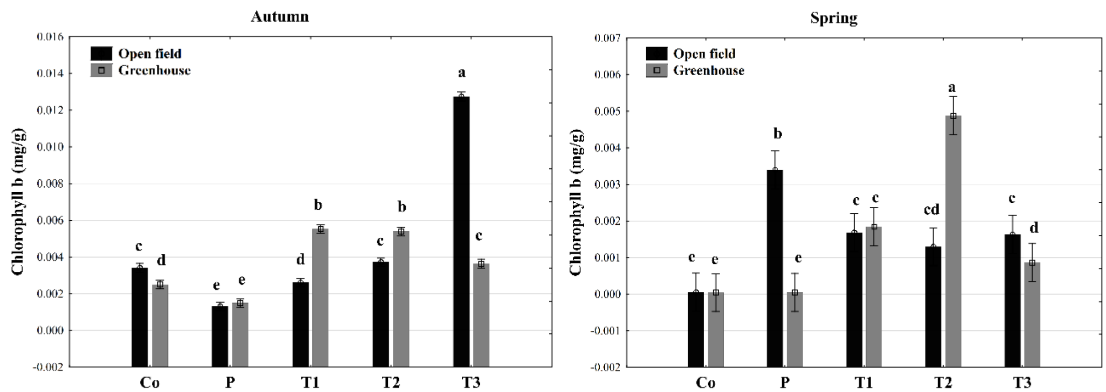

Figure 6. Interactions: A) Chlorophyll b content depending on the biostimulant treatment method in the autumn growing period B) in the spring growing period
Values characterised by different lower-case letters differ significantly ($p \leq 0.05$) according to the LSD test

Results for the total carotenoid content in the leaves of iceberg lettuce also show significant differences depending on the treatment and cultivation method (Table 1). In the spring growing period, average values indicate higher carotenoid content in the greenhouse compared to the open field. Conversely, in the autumn period, the open field shows slightly higher content compared to the greenhouse. As for the biostimulant treatments, the control treatment (CO) had the lowest carotenoid content in the spring growing season, while this was the case for the dip treatment (P) in the fall season. In the autumn period, treatments T2 and T3 stood out with significantly higher content, while treatment T1 had slightly lower content. In the spring period, the highest carotenoid content was recorded in treatment T2, while the control treatment (CO) had the lowest. The interaction between treatment factors and cultivation methods was significant in both growing periods, indicating that the combination of treatment T2 and greenhouse in the spring period, and the open field with treatment T3 in the autumn period, provides the highest carotenoid contents. Based on Figures 7A and 7B, there is a clear increase in carotenoid content with soaking (P) and spraying treatments (especially in T3), while the control treatment (CO) remains mostly below the levels of all treatments in both growing periods.

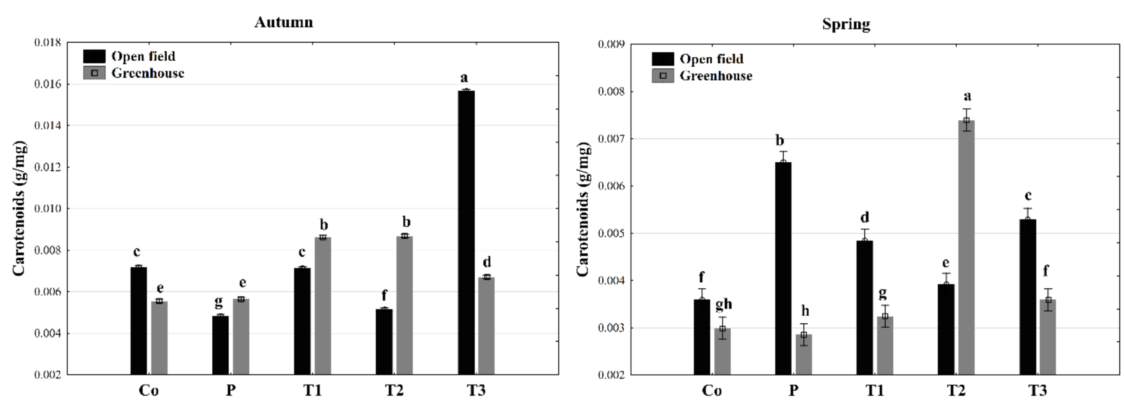

Figure 7. Interaction: A) Total carotenoid content depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period
Values characterised by different lower-case letters differ significantly ($p \leq 0.05$) according to the LSD test

Figure 7A shows the total carotenoid content during the autumn growing period for different biostimulator treatments and cultivation methods. In the greenhouse, carotenoid levels are slightly higher in the spring season than in the field, with the exception of treatment T3, which is significantly higher in the field. The control treatment (CO) shows significantly lower values in the greenhouse, while this is the case in the field for the dipping treatment, which clearly indicates the positive effect of the biostimulants. Treatment T1 (1x spraying) shows a steady increase in carotenoid content in both cultivation methods, while T2 (2x spraying) shows a similar trend, but slightly lower values in the open field. The combination of T3 and open field shows the greatest potential for increasing the carotenoid content in spring. Figure 7B presents the total carotenoid content during the autumn growing period. Unlike spring, the open field has a slight advantage over the greenhouse in most treatments. The highest carotenoid content was found in the T2 treatment in the greenhouse, while the open field showed slightly lower values in the dipping treatment (P), but stood out the most. Treatment T3 also shows a significant increase in carotenoid content in both cultivation methods, while the control (CO) had the lowest values. The interaction of factors was more pronounced in the fall season, where the open field in combination with the dip treatment and the greenhouse in combination with the T3 treatment showed a synergistic effect in increasing carotenoid content.

Analysing the results for total antioxidant capacity (TAC) and polyphenol content, statistically significant differences were found between biostimulator treatments and cultivation methods (greenhouse and open field) during both the autumn and spring periods. In the autumn growing period, a higher TAC value was observed with the "soaking" treatment (P) compared to the control treatment (CO), while other treatments, including spraying with biostimulants (T1, T2, T3), also showed statistically significant differences compared to the control. In the spring period, the highest TAC was recorded in treatment T2, which was statistically significant compared to all other treatments. For polyphenol content in the autumn period, treatment T3 yielded the highest value, while spraying treatments (T1 and T2) showed similar results that significantly differed from the control treatment. In the spring period, the highest concentration of polyphenols was recorded in treatment T2, which significantly differed from other treatments and the control group (CO). The differences in cultivation methods indicate that plants grown in the open field during the autumn period had a higher average TAC compared to those grown in the greenhouse, while in the spring period, there were no significant differences between the cultivation methods. There were no statistically significant differences in polyphenol content between open field and greenhouse in the fall season, while in the spring the plants from the greenhouse had a higher polyphenol concentration than those from the open field, but the differences were not statistically significant. The interaction between the biostimulant treatments and the cultivation methods (greenhouse and open field) was statistically significant for both growing seasons, both for TAC and polyphenols (Table 2).

Table 2. Average values of polyphenol content and total antioxidant activity (TAC) of individual biostimulant treatments, in open field and greenhouse iceberg lettuce

Treatments		TAC (mg g^{-1})		Polyphenols (mg g^{-1})	
		Autumn	Spring	Autumn	Spring
Biostimulant (A)	Control(CO)	2.15c	1.87d	0.309	0.648b
	soaking (P)	2.57a	2.19c	0.413	0.708b
	T1	2.40ab	2.48b	0.288	0.775b
	T2	2.36b	2.79a	0.283	1.432a
	T3	2.46ab	2.35bc	0.431	0.644b
Location (B)	Open field	2.45a	2.34	0.363	0.776
	Greenhouse	2.33b	2.33	0.326	0.906
ANOVA					
A		*	*	ns	*
B		*	ns	ns	ns
AXB		*	*	*	*

The values denoted with different small letters within columns for every treatment are significantly different ($p \leq 0.05$) in accordance with the LSD test

Analysis of the results for total antioxidant capacity (TAC) and total polyphenols depending on treatments and cultivation methods (open field or greenhouse) revealed significant differences during both the autumn and spring periods. For TAC, during autumn (Figure 8A), dip treatment (P) in the open field showed the highest value, statistically significantly higher than the control (CO) and other treatments in both ways of cultivation. On the other hand, there is a lower increase in TAC in the greenhouse, with treatment T3 being significantly different from the control (CO), while the other treatments are close to the control values. The spring season (Figure 8B) shows a similar trend in the open field, where treatment T1 in the open field has the highest TAC compared to other treatments, while the values of treatment T2 in the greenhouse are the highest in both growing seasons and other treatments are significantly different from the control. The interaction between the treatments and the cultivation methods shows that treatment T2 in spring in the greenhouse and treatment P in fall in the field have the highest TAC values, which could indicate a seasonal effect on antioxidant synthesis.

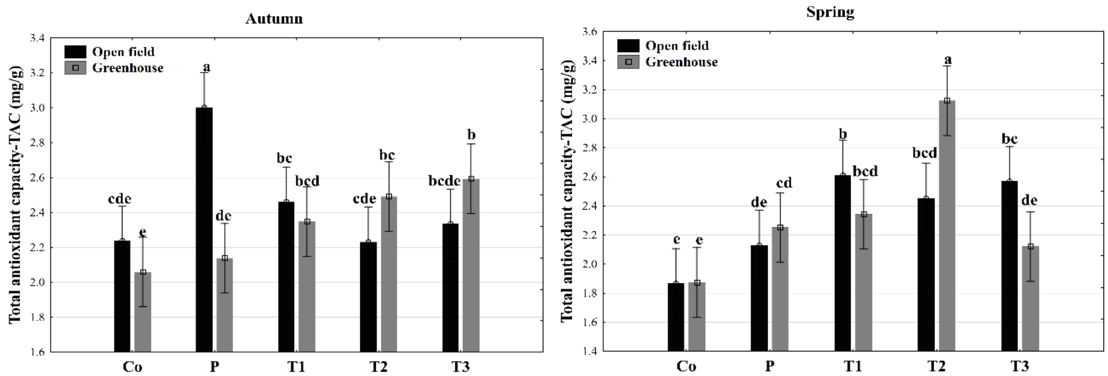

Figure 8. Interaction: A) Total antioxidant capacity depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period
Values characterised by different lower-case letters differ significantly (p ≤ 0.05) according to the LSD test

Total polyphenol content was the highest during autumn (Figure 9A), treatment T3 in the open field, while the control (CO) and other treatments had lower values of TPC. In the greenhouse, the differences between treatments were less expressed, although the dip treatment (P) showed a slight increase compared to the control (CO). In the spring period (Figure 9B), treatment T2 in the open field peaked in TPC, with significantly higher values comparing to the control (CO) and other treatments. In the greenhouse, polyphenol values were similar to the control, with no statistically significant differences among treatments.

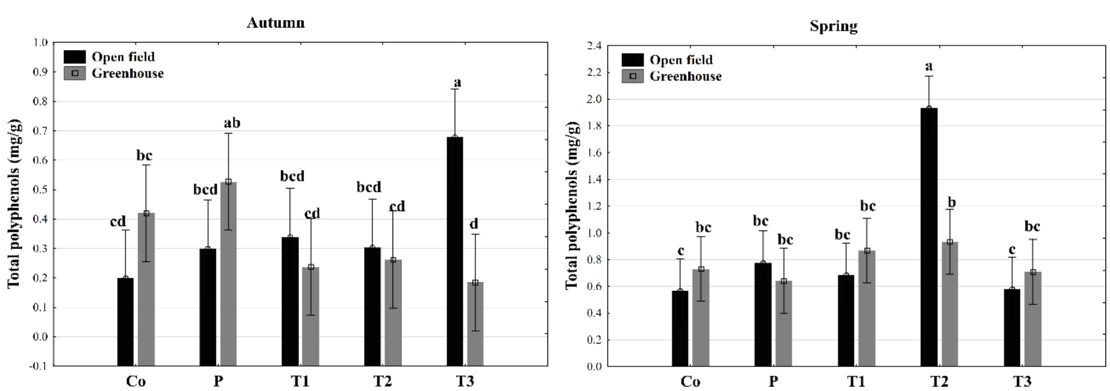

Figure 9. Interaction: A) Total polyphenol content depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period
Values characterised by different lower-case letters differ significantly (p ≤ 0.05) according to the LSD test

These results indicate that treatments, season, and cultivation method significantly affect the accumulation of antioxidant compounds. The highest TAC values were measured in the T2 treatment in the greenhouse in spring, while the highest polyphenol content was obtained in the T2 treatment in the field in spring, suggesting that certain treatments could improve the nutritional potential of plants grown in the field under certain seasonal conditions.

The table 3 presents the morphological parameters for the three components of plants under different treatments and cultivation conditions during autumn and spring.

Table 3. Average values of morphological parameters, total plant mass, leaf mass, and stem mass of individual biostimulant treatments, in open field and greenhouse of iceberg lettuce (3 replications)

Treatments		Fresh plant mass (g)		Leaf mass (g)		Stem mass (g)	
		Autumn	Spring	Autumn	Spring	Autumn	Spring
Biostimulant (A)	Control (CO)	558d	896b	357d	706c	28.2a	35.5d
	soaking (P)	797a	970a	583a	773a	28.3a	43.5a
	T1	737c	954a	521c	732b	28.9a	38.7c
	T2	766b	956a	551b	736b	25.9b	36.5d
	T3	784ab	965a	546b	735b	25.8b	41.3b
Location (B)	Open field	761a	891b	529a	797a	28.0a	38.4b
	Greenhouse	696b	1005a	494b	676b	26.9b	39.8a
ANOVA							
A		*	*	*	*	*	*
B		*	*	*	*	*	*
AXB		*	*	*	*	*	*

The values denoted with different small letters within columns for every treatment are significantly different ($p \leq 0.05$) in accordance with the LSD test

During autumn growing season (Table 3), the control group (CO) (558 g) had the lowest total plant mass, indicating a poor effect of cultivation without additional biostimulator treatments. The soaking treatment (P) had the highest mass (797 g), suggesting that this treatment was the best for increasing total plant mass during autumn. The mass of plants in the greenhouse was smaller than in the open field for all treatments, indicating better growth conditions in the open field during this period. Statistically significant differences confirm that treatment and cultivation conditions significantly affect plant mass. Generally, the mass of plants is higher in spring than in autumn for all treatments and cultivation conditions. The control group (CO) showed an increase to 896 g but was still less than the treated plants. The soaking treatment (P) again had the highest mass (970 g), confirming its effectiveness.

The greenhouse is now provided better growth conditions compared to the open field. ANOVA analysis indicates that all factors are significant (treatments, cultivation method, and their interaction).

In the open field, the soaking treatment (P) and treatment T3 significantly increases plant mass compared to control (Table 3). In the autumn growing period in the greenhouse (Figure 10A), the differences between treatments are even more expressed, indicating additional benefits of the treatments under protected conditions. Statistically significant results confirm that treatments differ in efficiency, where treatment T3 dominating. In the spring growing period (Figure 10B), plant mass increases across all treatments. The greenhouse provides better results than the open field, but the differences between treatments are more pronounced than in the autumn period. The soaking treatment (P) in the open field still provides the highest plant mass, while in the greenhouse treatments T2 and T3 dominate.

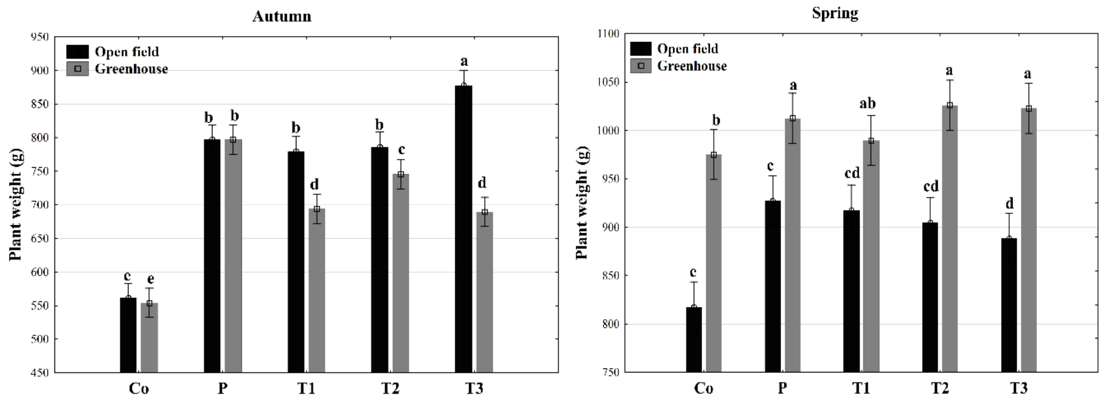

Figure 10. Interaction: A) Total plant mass depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period
Values characterised by different lower-case letters differ significantly ($p \leq 0.05$) according to the LSD test

In the fall growing season (Table 3), the highest leaf mass was observed in the treatment (P) with soaking (583 g), which could indicate that the treatments contribute more to the development of the stem and total plant mass than to the leaves. The lowest leaf mass was recorded in control (CO) (357 g). Statistically significant differences were present, confirming the impact of treatment and cultivation methods. Leaf mass increases in spring for all groups. The soaking treatment (P) (773 g) is still the leader, while the control has the lowest mass (706 g). In both cases, the open field shows superiority over the greenhouse. The ANOVA analysis shows that all factors are significant (treatments, cultivation method and their interaction). In the diagram of the fall season (Figure 11A), treatment T3 has the strongest influence on leaf mass in the open field. In the greenhouse, the differences are less pronounced, but the "watering" treatment (P) has an advantage. The control (CO) has the lowest leaf mass, which confirms the importance of the additional treatments. In spring (Figure 11B), the differences between the treatments are more pronounced than in fall, especially in the field. The "soaking" treatment (P) in the field and the T3 treatment in the greenhouse lead to a significant increase in leaf mass, while the control (CO) shows the lowest growth, which is particularly noticeable in the field (Figure 11B). Statistically significant results confirm that the treatments were effective for stimulating growth.

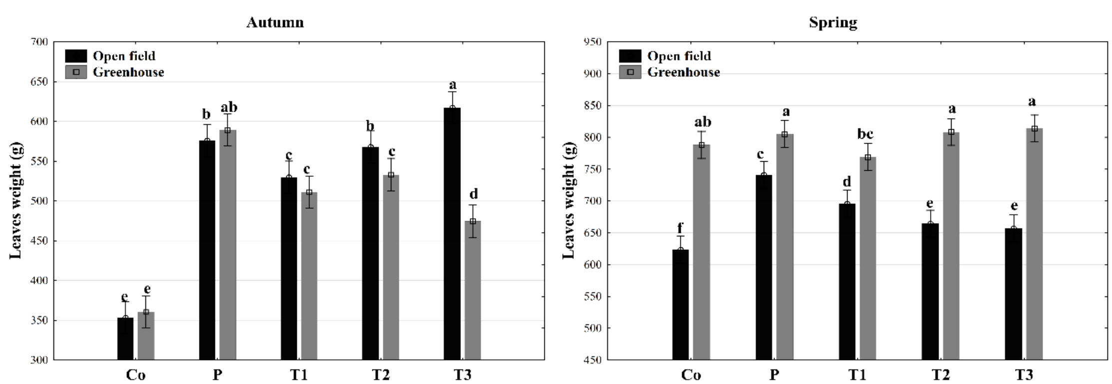

Figure 11. Interaction: A) Leaf mass depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period
Values characterised by different lower-case letters differ significantly ($p \leq 0.05$) according to the LSD test

In the autumn season, stem mass in the control group (CO) (28.2 g) was greater than in treatment T3 (25.8 g) (Table 3). The treatment T1 (28.9 g) had a slight advantage over the control group (CO). Statistically significant differences show a clear advantage of the treatments and the conditions in the field for stem development compared to the greenhouse. In the spring season, a significant increase in stem mass was observed in all groups, indicating better growth in warmer conditions. The "soaking" treatment (P) records the highest stem mass (43.5 g), while the control (CO) had noticeably lower values (35.5 g). The greenhouse shows clear advantages over the open field. ANOVA analysis proved that all factors were significant (treatments, cultivation method, and their interaction). In the autumn season, stem mass varied significantly depending on the treatment (Figure 12). The highest stem mass was recorded in the treatment T1 in the open field, while in the greenhouse the mass was lower, but the greatest values are recorded by treatment soaking (P). In treatment T3 (three-spray treatment), stem mass was the lowest, regardless of growing conditions. Differences between treatments were statistically significant. During the spring period (Figure 12A), stem mass was generally greater compared to the autumn period (Figure 12B). The highest stem mass value was recorded in the greenhouse in the soaking treatment (P). The control (CO), both in the open field and greenhouse, showed significantly lower stem mass values. Generally, plants grown in the greenhouse had higher stem mass values compared to the ones grown in the open field.

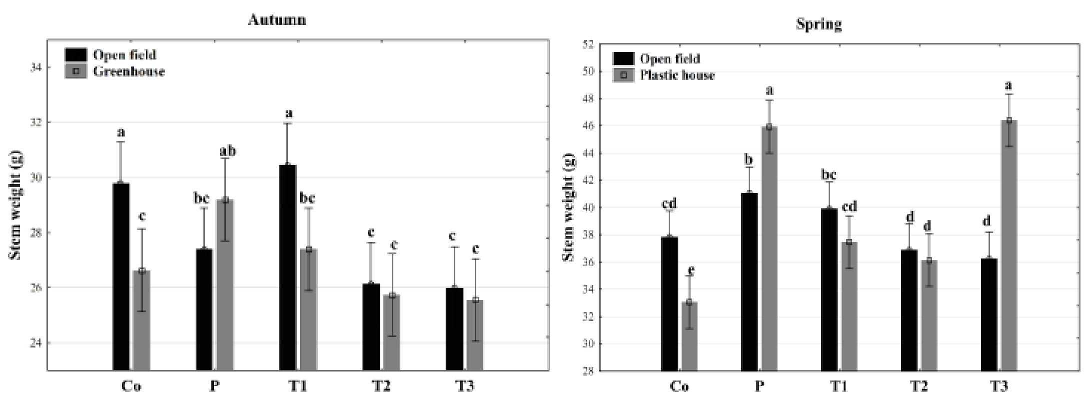

Figure 12. Interaction: A) Stem mass depending on the biostimulant treatment method in the autumn growing period and B) in the spring growing period

Values characterised by different lower-case letters differ significantly (p ≤ 0.05) according to the LSD test

The soaking treatment (P) had the greatest effect in increasing the total plant mass, leaf mass, and stem mass in both growing periods (autumn and spring). The greenhouse provides better growing conditions for the plants compared to open field. Statistically significant results confirm clear differences between treatments and cultivation conditions.

PCA is a used to determine the differences in lettuce growing periods (autumn, spring), cultivation technologies (greenhouse, open field) based on their morphological traits (leaf mass, plant mass, and stem mass), pigments (chlorophyll a, chlorophyll b, and total carotenoids), and antioxidant indicators (total polyphenols and total antioxidant capacity - TAC) influenced by biostimulators (five treatments). The first two variables (PC1 and PC2) indicate that 74.8% of the total variation corresponds to these variables (PC1 = 54.6% and PC2 = 20.2%), as shown in Figure 13.

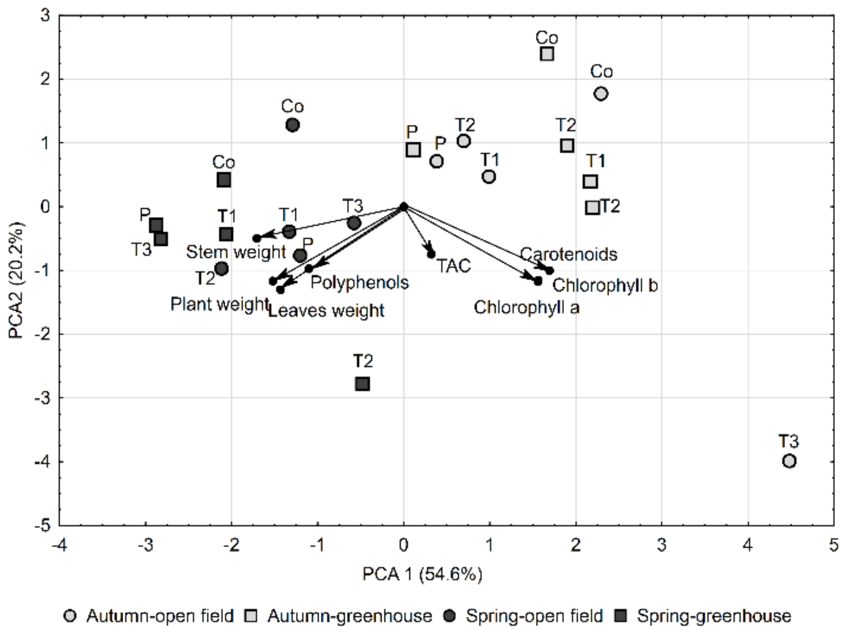

Figure 13. PCA analysis of phytochemical and morphological parameters of iceberg lettuce

Figure 13 further explains that the high correlation was found in morphological traits and polyphenols were grouped in one quadrant. They had close vector orientations, indicating a high correlation between them across all biostimulator treatments. The control treatments (CO) without biostimulants were distant from the origin in both open field and greenhouse lettuce production. The grouping of parameters in the spring growing period based on the treatments significantly reflected on the morphological traits and antioxidant activity largely depended on the biostimulator treatments. The autumn growing period grouped into a separate category where no influence of individual treatments was observed on morphological and antioxidant parameters. Instead, the content of pigments (chlorophyll a, chlorophyll b, and carotenoids) in the form of a reduction in total quantity compared to the spring growing period varied significantly. However, the group of pigment characteristics (chlorophyll a, chlorophyll b, and total carotenoids, and to some extent TAC - total antioxidant capacity) on the graph is positioned in a way that can be attributed to both cultivation methods, reflecting an increase in the spring growing period regardless of the cultivation technology (greenhouse, open field) (Figure 13).

Discussion

Chlorophyll a

In the analysis of chlorophyll a content in the leaves of iceberg lettuce, the results showed a significant difference between treated and untreated plants with biostimulator, as well as a difference between production in greenhouse and open field. Our research indicates that greenhouse conditions contribute to a higher chlorophyll a content compared to the open field, which may be a result of more stable climatic factors in greenhouse production. Similar results were obtained by Llorach *et al.* (2008), who state that greenhouse production allows for greater stability of chlorophyll due to reduced exposure to UV radiation and controlled temperature conditions. Additionally, the impact of biostimulators on chlorophyll a content observed in our study aligns with the research of Rouphael *et al.* (2017), who emphasize that biostimulators enhance the photosynthetic efficiency of plants through improved nutrient uptake and activation of metabolic processes.

Specifically, seaweed-based biostimulators have shown the ability to increase chlorophyll levels in the leaves of various crops (Calvo *et al*., 2014), confirming that the treatments used in our experiment contribute to better photosynthesis and increased lettuce productivity. These results can be further explained by the protective role of carotenoids, which safeguard the photosynthetic apparatus from oxidative damage due to intense light (Llorach *et al*., 2008; Keyhaninejad *et al*., 2012, and Taiz *et al*., 2015). This protective role is crucial in greenhouse production conditions, where stress of light is typically lower, enabling better preservation of pigments.

Chlorophyll b

The chlorophyll b content in the leaves of iceberg lettuce showed significant differences between biostimulator treatments and cultivation methods, with greenhouse conditions significantly contributing to the increase of this pigment compared to the open field. Our research confirms that greenhouses provide more stable conditions, reducing stress caused by unfavourable weather factors, which aligns with the results of Simkin *et al*. (2003), Llorach *et al*. (2008) and Keyhaninejad *et al*. (2012), who showed that controlled conditions in greenhouses allow better preservation of photosynthetic pigments, including chlorophyll b. Furthermore, our finding regarding the synergistic effect of biostimulators on increasing chlorophyll b content is supported by the research of Calvo *et al*. (2014) and Rouphael *et al*. (2017), which emphasize that biostimulators, such as those based on plant extracts (seaweeds) and amino acids, significantly increase the concentration of photosynthetic pigments through the activation of plant metabolic processes. Specifically, seaweed-based biostimulators have shown a consistent positive impact on increasing chlorophyll b content in the leaves of various crops, which corresponds to the results of treatment T3 in our experiment. Research by Rouphael and Colla (2020), and Carillo *et al*. (2020) indicate that biostimulators not only increase chlorophyll b levels but also enhance plant resilience to stress conditions, such as drought, as observed in our study through treatments T1 and T2. These treatments showed a gradual increase in chlorophyll b content, indicating their stability and effectiveness under different cultivation conditions. These observations further confirm the importance of greenhouse conditions and biostimulator treatments for sustainable vegetable production, providing a strong foundation for the application of these methods in modern agriculture.

Carotenoids

Our results revealed significant differences in the total carotenoid content in iceberg lettuce leaves depending on biostimulator treatments and cultivation methods (greenhouse and open field). The greenhouse conditions during the spring period proved to be more favorable for increasing carotenoid content. This aligns with the research by Russo and Howard (2002), Simkin *et al.* (2003), and Keyhaninejad *et al*. (2012), who emphasized that greenhouse conditions allow for better preservation of carotenoids, as plants are protected from extreme light and temperature conditions that can reduce the stability of these pigments. Biostimulator treatments, particularly combined treatments such as T3, demonstrated the greatest potential for increasing carotenoid content, which was consistent with the findings of Bulgari *et al*. (2019). Their research showed that seaweed-based biostimulators significantly influence carotenoid synthesis in leafy vegetables, contributing to their antioxidant activity and stress resistance. The interaction of treatment factors and cultivation methods recorded in our study confirms the synergistic effect of greenhouse conditions and biostimulators on increased carotenoid content. Similar results were reported by Rouphael and Colla (2018) and Rouphael and Colla (2020), who highlight that the combination of controlled conditions and biostimulators contributes to the accumulation of photosynthetic pigments and antioxidant compounds, making plants more resilient to abiotic stress. The absence of statistically significant differences in the autumn period between open field and greenhouse for certain treatments suggests that ecological factors such as lower temperatures and reduced light during this period may have a more pronounced effect on carotenoid synthesis, as noted in the research by Keyhaninejad *et al*. (2012). These results confirm that the combination of biostimulator treatments and greenhouse conditions provides significant advantages in increasing carotenoid content, especially under spring growing conditions.

TAC and polyphenols

Significant differences in total antioxidant capacity (TAC) and total polyphenol content (TPC) were found in iceberg lettuce leaves based on biostimulator treatments and cultivation methods (greenhouse and open field), emphasizing the role of these factors in optimizing the plant's phytochemical properties. Our results indicate a higher TAC in the soaking treatment (P) during the spring period compared to control samples (CO), while biostimulators in the autumn period showed a statistically significant effect on increasing TAC. These findings align with the research by Koca and Karadeniz (2011), which emphasize that biostimulator treatments enhance the antioxidant response of plants through the activation of metabolic processes involved in neutralizing free radicals. The highest level of polyphenols in the autumn period was recorded in treatment T2 in the open field, which corresponds with results from Drobek *et al.* (2019), who demonstrated that open field cultivation and the application of biostimulators can synergistically increase the accumulation of secondary metabolites, including polyphenols (Valencia *et al.*, 2018). In the spring period, treatment T2 shows a similar trend with high accumulation of polyphenols in the open field, which agrees with the research by Rouphael and Colla (2018), emphasizing the role of biostimulators in increasing phenolic compounds through the regulation of carbohydrate and phenylpropanoid metabolism. The absence of statistically significant differences in the greenhouse for most treatments during the autumn period can be explained by the homogenization of growing conditions, which is in accordance with studies conducted by Llorach *et al.* (2008) and Rouphael and Colla (2020), stating that greenhouse conditions reduce stress factors such as UV radiation and temperature fluctuations, leading to more uniform plant development.

Morphological Parameters

Total Plant Mass

Significant differences in total plant mass were found between treatments and cultivation methods in our research. The soaking treatment (P) achieves the highest plant mass in both terms (autumn and spring), and greenhouse conditions further increase total plant mass compared to the open field. El-Nemr *et al.* (2012) and Rouphael and Colla (2017), demonstrated on cucumbers that the application of biostimulators significantly increases vegetable biomass, including lettuce. Biostimulators affect the growth of the entire plant by enhancing nutrient absorption and activating metabolic processes that accelerate protein synthesis and increase photosynthetic activity. Research conducted by Gruda *et al.* (2024) showed that production in greenhouses provides more stable microclimatic conditions, including temperature and humidity, leading to greater biomass accumulation. This explains why the results obtained in this study in the greenhouse are significantly better than those in the open field. Van Oosten *et al.* (2017) note that the spring season is more favorable for biomass accumulation under greenhouse conditions due to more intense light radiation, which aligns with our findings that plant mass is greater in the spring period compared to the autumn period.

Leaf Mass

In the experiment, it was determined that leaf mass was greater under greenhouse conditions, particularly with the soaking treatment (P). Leaf mass is a crucial parameter as it is directly related to photosynthesis and the nutritional value of the plant. El-Nemr *et al.* (2012), Xu and Leskovar (2015), Povero *et al.* (2016) and Abd El-Mageed *et al.* (2017), highlight that biostimulators enhance leaf growth through improved photosynthesis and increased chlorophyll content. Seaweed-based treatments improve nitrogen retention, which is essential for the growth of leaf mass. These findings are consistent with our results, where treatments with biostimulators show a significant increase in leaf mass. Research by Zou *et al.* (2019) indicates that greenhouses protect plants from stress caused by extreme weather conditions, allowing for optimal leaf development. This explains the higher values of leaf mass in the greenhouse compared to the open field in our study. Seasonal variations in leaf mass were evidenced in the work of Calvo *et al*. (2014), which noted that leaves are thicker and more robust in the spring season due to longer light periods (Lee *et al*., 2015), contributing to greater photosynthetic activity. Similarly, in our study, leaf mass in the spring period is greater than in the autumn.

Stem mass

The stem mass was the highest in the "soaking" treatment (P) in the greenhouse, while the open field and control group achieved lower values. This may be related to the effects of biostimulators on biomass accumulation through increased activity of growth hormones, such as auxins and cytokinins. Such effects were confirmed in the study by du Jardin, (2015), Bisen (2020), which explains that biostimulators promote cell elongation and increase stem growth by activating hormones like auxins and cytokinins, which aligns with our results, where the "soaking" treatment (P) shows the greatest stem mass due to better synthesis of growth hormones. Numerous studies have confirmed the positive effect of seaweed-based extracts on the stem, fruit, root, and leaves in crops such as corn (Jeannin *et al.,* 1991), tomato (Crouch and van Staden, 1992), strawberry (Alam *et al.,* 2013), and others. The results of our research indicate that stem mass varies between treatments and cultivation methods, with greenhouse conditions and the "soaking" treatment (P) being the most effective. Rouphael and Colla (2020) demonstrate that greenhouses facilitate constant stem growth by reducing abiotic stress. This research also shows that greenhouse conditions optimize resource distribution within the plant, leading to increased stem mass, which has also been observed in our results. Studies by Waycott (1995), Dufault *et al*. (2006) and Lee *et al*. (2015), have demonstrated that seasonal variations in stem mass and length are a result of temperature changes and day length. In our work, stem mass is greater in the spring period in the greenhouse, which is consistent with these conclusions.

Our results indicate that the combination of greenhouse cultivation and biostimulator application significantly increases stem mass in iceberg lettuce. The "soaking" treatment (P) showed the best results, while seasonal differences suggested a greater potential for spring cultivation.

PCA method

Principal Component Analysis (PCA) is a process for identifying hypothetical variables that encompass the majority of variance in a data set (Hammer *et al*., 2001). In our study, a strong correlational dependence was established between polyphenols and morphological characteristics (leaf, plant, and stem mass), as well as between the pigments themselves (chlorophyll a, chlorophyll b, and carotenoids), based on the clustering in the PCA graph (Figure 13). High correlations of biochemical traits with antioxidant activity support previous publications (Razzaghi-Asl *et al*., 2013) and more importantly, the content of most metabolites in green lettuce increases with the aging of leaves (Assefa *et al*., 2019).

Iceberg lettuce is a plant that responds to the length of daylight, as its flowering and head formation depend on the length of the day. Long-day conditions (more than 12 hours of light per day) can lead to undesirable bolting, which decreases the quality of heads and accelerates the end of the vegetative phase. In this regard, regulating light conditions is important in controlled production environments (such as greenhouses), where photoperiods can be optimized (Leskovar and Cantliffe, 1992), which was not the case in our study. This investigation was divided into autumn-the period of reduced light intensity-and the spring growing period, when light intensity increases under both open field and greenhouse production conditions, which can explain the clear separation of parameters on the PCA graph. The intensity, spectrum, and duration of light directly affect the morphological characteristics and nutritional values of green lettuce. Higher light intensity, particularly in the red and blue spectrum, enhances photosynthetic activity, leading to increased growth and synthesis of pigments such as chlorophyll and carotenoids (Liu *et al*., 2019). Extended light exposure can contribute to higher antioxidant content, such as phenols and flavonoids, which increase the nutritional value of lettuce. In controlled conditions in greenhouses, an optimal photoperiod can improve the morphological status of the plant, which is important for commercial production (Zou *et al*., 2019). The combination of proper photoperiod management and the use of biostimulants represent an effective way to improve the quality and yield of iceberg lettuce. Optimal light combined with the application of biostimulants can significantly enhance plant growth, increase stress resistance, and contribute to sustainable and environmentally friendly production (Rouphael and Colla, 2020).

Conclusions

The results of our research showed that the application of biostimulators and greenhouse cultivation significantly contributed to the enhancement of the growth and development of iceberg lettuce. The combination of these factors (application of biostimulators, greenhouse cultivation and seasonal factors) resulted in increased total plant mass, leaf mass, and stem mass, with emphasized differences between the spring and autumn growing periods. Biostimulator treatments, particularly the "soaking" method yielded the best results, allowing optimal plant development in spring and autumn seasons of the production. The greenhouse proved to be the best environment for growing lettuce, providing stable conditions that reduced plant stress and allowed greater biomass accumulation. Seasonal differences were also evident, with the spring period showing better results than autumn, which can be attributed to the longer duration of light and more favorable temperature conditions.

Biostimulators enabled more efficient photosynthesis, resulting in increased total plant mass, leaf mass, and stem mass. In addition to the quantitative increase in biomass, the application of biostimulators also contributed to qualitative improvements, through higher content of antioxidants and photosynthetic pigments, enhancing their nutritional value and resistance to adverse conditions.

The combination of biostimulators and greenhouse cultivation represents a sustainable approach to iceberg lettuce production, with significant potential for increasing yield and quality while reducing the use of chemical inputs.

Authors' Contributions

Conceptualization: ĐM; Investigation: AK, JP; Methodology: SK; Software: VZ; Writing - original draft: JP; Writing - review and editing: NP, SV.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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