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Effect of Protein Gel Treatments on Biometric and Biochemical Attributes of Tomato Seedlings in Greenhouse Condition

Daniela Balan [1], Gabriela Luţă [1,*], Maria Stanca [2,*], Ovidiu Jerca [1], Mihaela Niculescu [2], Carmen Gaidau [2], Stefana Jurcoane [1] and Antoaneta Mihalcea [3]

[1] Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăşti Blvd, 011464 Bucharest, Romania
[2] Research and Development National Institute for Textiles and Leather-Division Leather and Footwear Research Institute, Leather Research Department, 93, Ion Minulescu Str., 031215 Bucharest, Romania
[3] SC MARCOSER SRL, Str. Principala, Nr. 1A1, 807185 Matca, Romania
* Correspondence: glutza@yahoo.com (G.L.); maria.alexandu@gmail.com (M.S.); Tel.: +40-374-022-802 (G.L.); +40-021-340-4200 (M.S.)

Abstract: Protein hydrolysates are widely used in agricultural crops for improving plant nutrient uptake, growth, yield, and fruit quality. Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetables consumed around the world both for its good taste and rich content in vitamins, minerals, lycopene, and β-carotene. The objective of the present study was to assess the effect of new stimulant products based on protein hydrolysates obtained from animal tissue by-products on tomato seedlings. Given the increased intake of amino acids, it is expected that this treatment will exert beneficial effects on the development of certain vigorous seedlings, representing the premise for obtaining superior tomato plants and the improvement of the production and quality of tomato fruit. Two variants of protein gels based on gelatin and keratin hydrolysates were obtained by processing bovine hide and wool and were used for periodical root applications on tomato seedlings cultivated in a greenhouse. During the experiment, the biometric characteristics of seedlings were measured weekly. The content of photosynthetic pigments, dry weight, sugars, and polyphenols were analyzed, and the antioxidant activity was assessed in the leaves. The research performed showed that applied biostimulant treatments increased the content of photosynthetic pigments by 10%, the content of sugars by 75%, and the content of polyphenols by 16% compared to the control untreated variant. Between the variants of protein gels tested, the best results were obtained by applying a mixture of bovine gelatin and keratin.

Keywords: protein gels; tomato seedlings; photosynthetic pigments; polyphenols; antioxidant activity

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1. Introduction

In recent years, plant biostimulants have been considered a sustainable approach to improve the growth and productivity of field crops, being an alternative to chemical fertilizers. Protein hydrolysates represent a category of plant biostimulants defined as mixtures of polypeptides, oligopeptides, and amino acids obtained by the partial hydrolysis of protein sources [1]. Increased interest in protein hydrolysates was noted due to their beneficial effects on crop performances, especially under environmental stress conditions [2]. Essentially, more than 90% of the products based on protein hydrolysates used in horticulture are obtained through the chemical hydrolysis of proteins from animal residues (such as collagen from leather by-products in Europe, India, and China or fish by-products in the United States), and the market size for biostimulants is estimated to reach USD 4.14 billion by 2025 [3,4].

The effects of foliar and root applications of protein hydrolysates on plants include the stimulation of carbon and nitrogen metabolism, an increase in nutrient uptake, and an improvement of water and nutrient use efficiencies [3,5]. The higher nutrient uptake in

protein hydrolysates-treated plants has been attributed to an increase in nutrient availability in the soil solution resulting from the complexation of nutrients by peptides and amino acids along with enhanced microbial and enzymatic activities in soil [6]. Furthermore, modifications noticed in the root of plants, in terms of their length, density, and the number of lateral roots, as an effect of protein hydrolysates application, are supposed to contribute to the improvement of the nutrient supply in the plants [3,7].

Research carried out in recent years concluded that, due to the presence of specific peptides and precursors of phytohormone, such as tryptophan and proline, protein hydrolysates could interfere with the phytohormone balance of the plant [3]. Francesca et al. [8] also reported growth-stimulating effects exerted by protein hydrolysates on tomato plants subjected to combined abiotic stress, likely by promoting endogenous phytohormones biosynthesis.

It can be stated that the chemical properties of fruit and vegetables are positively affected by biostimulant application. The use of several types of plant-derived biostimulants in research performed on different plant species, such as tomato [9], rocket [10], zucchini [11,12], and lettuce [13] has shown potential effects on their growth and quality.

The current research was performed on tomato seedlings, focusing attention on the effects of protein hydrolysates on plant metabolism and physiology.

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops cultivated and consumed around the world both for its attractive taste and for its high content of health-benefiting compounds. In addition to their role in the human diet, tomatoes also represent a food source of chemical compounds that may positively influence health since they are rich in vitamins, minerals, lycopene, β-carotene, and anticancer agents [14,15].

Positive results have been observed in terms of yield and fruit quality by using biostimulants in tomato crops, especially under abiotic stress conditions [8,16,17].

Taking this into account, the objective of the present study was to assess the effect of tomato crop treatment with new stimulant products based on protein hydrolysates obtained from animal tissue by-products. Bovine hide and wool were processed by hydrolysis, obtaining some gelatin and keratin gels (protein hydrolysates), which were used for periodical root applications on tomato seedlings cultivated in a greenhouse. During the development of experiments, observations and measurements of plant growth were performed in different stages: Biometric characteristics of seedlings were measured weekly, while biochemical determinations in the leaves were performed when the seedlings reached the optimal point for successful planting in the field. The content in photosynthetic pigments, dry weight, sugars, and polyphenols were analyzed, and the antioxidant activity was assessed. The applied biostimulant treatments determined differences regarding the growth and biochemical parameters of tomato seedlings. This innovative treatment is expected to induce the development of vigorous seedlings, representing the premise for obtaining superior tomato fruits in terms of production and nutritional qualities.

2. Materials and Methods

2.1. Biological Material

Tomato seeds (*Solanum lycopersicum*) of the Qualitet F1 hybrid supplied by Marcoser SRL (Matca, Galati, Romania) were selected for the research.

The experiment was installed in an experimental greenhouse of the USAMV Bucharest (44°26′ N and 26°06′ E latitude and longitude, respectively). It was established as a monofactorial experiment with 3 variants, considering the application of stimulant gels on the tomato seedlings. The experimental variants were as follows: V1—untreated seedlings (control); V2—seedlings treated with bovine gelatin gel; V3—seedlings treated with a combination of bovine gelatin and keratin gels. Sowing was performed on 10 February. Fourteen days later, on February 24, the transplant operation was performed in two phases: I—in alveolar pallets (alveolar ø = 5 cm) of 100 mL volume (60 seedlings); II—on 17 March (thirty-five days after sowing), 27 seedlings were selected and transplanted in polyethylene

plastic pots of 300 mL volume (diameter 10 cm and height 9.5 cm). Both types of containers were filled with a professional nutrient substrate Kekkila BP peat, characterized by a high content of mineral nitrogen and phosphorous and optimum content in potassium. The ratio of N:P:K was 1:0.6:0.7. Plants were arranged in a completely randomized block design with nine biological replicates (in this experiment, a biological replicate means a plant) for each experimental variant (treatment). During the growth period, specific agrotechnical practices for seedling production were applied: Daily ventilation, watering, and weeding weeds. The temperature was maintained at 24–26 °C to 30 °C during the day and 22–24 °C at night, and the air relative humidity was 65–80%. During the experiment, the light that prevailed was only natural (11–12 h and 13–14 h of daylight in March and April, respectively). Watering of the seedlings was performed once a day to maintain the soil at the appropriate humidity, starting with 100 mL of water/plant/day in the alveolar pallets. After transplanting the seedlings into the pots, we started with 100 mL/plant/day, and then the amount of water was progressively increased during the growth period of the seedlings in the pots, finally reaching 500 mL/plant/day. No other fertilizing applications were administered except for biostimulant treatments with protein gels in all the experimental variants. The application of the treatment started one week after the seedlings' transplantation into the pots. The protein gels were applied as a substrate-drenching treatment with diluted solutions of gels (30 g in 60 mL of water) freshly prepared at a rate of 10 mL per plant during the experiment on 24 March (forty-two days after seeds sowing), 31 March (forty-nine days after seeds sowing), and 7 April (forty-nine days after seeds sowing).

2.2. Obtaining Gels (Protein Hydrolysates)

Bovine hide and wool were bought from a local slaughterhouse and a sheep farmer from Romania, Lumina village, district of Constanta (44°18′09.2″ N and 28°33′31.1″ E latitude and longitude, respectively) For the treatment of the tomato plants, we used two variants of protein gels: Bovine gelatin and a combination of bovine gelatin with keratin mixed in a 1:1 ratio.

Bovine gelatin was obtained by thermal hydrolysis as previously described [18]. Bovine delimed hide mixed with a certain amount of water was heated at 80 °C for five hours. pH was adjusted between 5.5 and 6 using a solution of 1 M acetic acid. pH was controlled and adjusted every hour. The obtained gelatin was filtrated and dried in an oven at 60 °C.

Keratin hydrolysates were obtained by alkaline hydrolysis at 80 °C by a previously described method [19]. Briefly, after washing and degreasing using NaOH 4% *w*/*w*, Na_2CO_3 1% *w*/*w*, and Boron SE 0.6% *w*/*w* for 2 h at 40 °C, wool was washed to neutral pH, cut into small pieces using a grinding machine, and then mixed with a solution of NaOH 2.5% *w/w* in a stainless-steel vessel equipped with automatic temperature control and a mechanical stirrer for four hours at 80 °C. The obtained keratin hydrolysates were filtrated and dried in an oven at 40 °C.

The obtained gels (bovine gelatin and a combination of bovine gelatin with keratin mixed in a 1:1 ratio) were analyzed regarding certain chemical characteristics. Standards in force were used for the determination of the dry substance [20], total ash [21], total nitrogen and protein content [22], pH [23], bloom test [24], and viscosity [25].

2.3. Determination of Free Amino Acids in Protein Gels

Determination of the free amino acids content was performed by the ninhydrin assay [26,27], a spectrophotometric method based on the reaction between α-amino acids and ninhydrin involved in the development of a purple-colored compound known as Ruhemann's purple. The color intensity of the formed complex is proportional to the concentration of amino acids in the solution, so absorbance was measured at 570 nm.

2.4. Biometric Measurements of the Tomato Seedlings

Biometric measurements of the seedlings were performed after seven days of each treatment on the 9 biological replicates from each experimental variant (treatment).

During the development of experiments, observations and measurements on seedling growth were performed after the treatments were applied, as follows: *Plant height*; *the number of true leaves,* and *leaves frequency* (number of leaves/plant height).

2.5. Biochemical Analysis of the Tomato Seedlings

Biochemical determinations in the leaves were performed when the seedlings had reached the optimal point for successful planting in the field. The robustness of seedlings was conferred by a large root system capable of exploring deep soil layers so the tomato seedlings can be transplanted into the field when the root system is sufficiently developed and can be seen outside the pot. Four biological replicates from each experimental variant were selected in order to perform biochemical determinations at the end of the experimental period (7 April).

Determination of photosynthetic pigment content was performed after *chlorophyll* and *carotenoid pigment* extraction in 80% acetone and spectrophotometrically measured at 663 nm, 647 nm, and 480 nm with a UV-VIS Spectrophotometer ThermoHelios Alpha purchased from Thermo Spectronic, Cambridge, UK. The extinction coefficients and equations described by Schopfer (1989) [28] were used for calculation. The results were expressed in mg/100 g fresh weight.

The dry substance content was analyzed by the gravimetric method: Samples had been dried to a constant mass in a Venticell oven purchased from the MMM-Group, Germany, at 105 °C, and the loss of weight was used to calculate the dry substance content of the sample.

The determination of total soluble sugars was performed according to the Somogyi–Nelson method described by Iordachescu et al. [29]. Non-reducing sugars were first transformed into reducing sugars by hydrolysis with hydrochloric acid. The reducing sugars heated with alkaline copper tartrate reduced the copper, thus cuprous oxide was formed. The addition of arsenomolybdenic acid led to a reduction of molybdic acid to molybdenum blue. The measurements of absorbance were achieved at 510 nm with a UV-VIS Spectrophotometer ThermoHelios Alpha purchased from Thermo Spectronic, Cambridge, UK. The results were expressed in g% fresh weight.

The total polyphenols content was determined according to the modified Folin–Ciocalteu assay described by Singleton et al. [30]. The method consists of the chemical reduction of the Folin–Ciocalteu reagent and measurement of the intensity of the obtained blue color at 750 nm. The measurements were achieved with a UV-VIS Spectrophotometer ThermoHelios Alpha purchased from Thermo Spectronic, Cambridge, UK. Total polyphenols values were expressed in terms of gallic acid equivalents (mg GAE/g fresh weight).

The total antioxidant capacity (radical scavenging activity) was determined using the stable free radical diphenylpicrylhydrazyl (DPPH) method according to the Blois [31] procedure adapted by Brand-Williams et al. [32] for complex matrices. Briefly, a 100 µM solution of DPPH in methanol was prepared, and 2 mL of this solution was mixed with 1 mL of different concentrations of tomato leaf extract in 80% aqueous methanol. After 30 min incubation in the dark at room temperature, absorbance (A) was measured at 515 nm. The percentage of radical scavenging activity (RSA) was calculated as follows:

$$\% \text{ RSA} = (1 - [A_{\text{sample}}/A_{\text{control t=0}}])/100$$

DPPH solution in 80% methanol was used as a control. The EC_{50} parameter for each sample, defined as the concentration of the sample required to scavenge 50% of DPPH free radicals, was calculated from the linear regression curve of the sample extracts (mg/mL) against the percentage of the radical scavenging activity.

2.6. Statistical Analyses

All measurements were carried out in at least three repetitions, and the results are presented as means ± standard deviation (S.D.). The significance of the influence of the biostimulant treatment on the measured parameters was assessed by one-way ANOVA. The

comparisons of means were calculated using the Duncan test at the 5% level of significance ($p < 0.05$). Regression analysis was performed to fit a linear equation to the data of the tomato seedling parameters examined using Microsoft Excel Office 2019 for Windows.

3. Results and Discussions

3.1. Protein Gels Characterization

Two protein gels, bovine gelatin and a combination of bovine gelatin with keratin, mixed in a 1:1 ratio were obtained and chemically characterized according to the standard in force. The physico-chemical characteristics of protein gels are presented in Table 1. It can be seen that the results are very similar. The differences in the bloom test and viscosity are due to the combination of gelatin with keratin.

Table 1. Physico-chemical characteristics of the used gels.

Characteristics	Bovine Gelatin	Bovine Gelatin and Keratin (1:1)
Dry substance (%)	2.26	1.71
Total ash (%)	0.44	0.34
Total nitrogen (%)	0.24	0.21
Protein content (%)	1.35	1.18
pH	7.08	7.21
Bloom test (g)	130	<50
Viscosity (mPa·s)	1.25	1

3.2. Determination of Free Amino Acids Content in Used Gels (Protein Hydrolysates)

Animal-derived protein hydrolysates usually contain a high amount of total amino acids, but the major amino acids differ according to the source of proteins. Collagen-derived protein hydrolysates' composition is dominated by amino acids such as glycine and proline [3,5].

The analysis performed on the tested protein gels shows a higher content of free amino acids in the variant containing a mixture of bovine gelatin and keratin (1:1) than in the variant with bovine gelatin only (Table 2). Significant differences between variants were noted in this regard.

Table 2. Total free amino acids content in protein gels.

Variant	Total Free Amino Acids (mg/g)
Bovine gelatin	0.35 ± 0.02^{a}
Bovine gelatin + keratin (1:1)	1.49 ± 0.05^{b}

Data expressed as mean values ($n = 3$) ± standard deviation; different superscript letters indicate significant differences between variants ($p < 0.05$).

Amino acids are involved in plant physiology, playing roles in stimulating growth and the biosynthesis of important molecules such as auxin and chlorophyll [33]. Furthermore, amino acids and amides represent the principal transport form for organic nitrogen in most plants, and they can be used directly for protein synthesis [34], therefore providing ready-for-uptake amino acids and representing an advantage for plants. The research performed in this study aimed to test whether protein gels containing a high amount of free amino acids exert positive effects on tomato seedlings' growth.

3.3. Influence of the Treatment on the Biometric Parameters of the Tomato Seedlings

During one month, treatments were made once a week via root applications of the solutions of protein gels. Biometric measurements of the seedlings were performed seven days after each treatment. Tomato seedlings showed uniform behavior regarding growth

and development one week after the first treatment (Table 3). Regarding the number of leaves, no significant differences were noted between variants at this analysis stage. Instead, the plant height registered a significant increase in variant V3 compared to the control plants. Moreover, in relation to the untreated variant V1, the frequency of leaves showed no large variation in variant V2 but did show significant differences compared to variant V3.

Table 3. Growth of tomato seedlings at one week after the first treatment.

Variant	Number of Leaves	Plant Height (cm)	Leaves Frequency (Number of Leaves/Plant Height)
V1 (Control)	5.22 ± 0.44^{a}	16.53 ± 1.13^{a}	0.32 ± 0.03^{a}
V2	5.11 ± 0.33^{a}	17.32 ± 0.74^{a}	$0.30 \pm 0.03^{a,b}$
V3	5.00 ± 0.00^{a}	18.09 ± 0.56^{b}	0.28 ± 0.01^{b}

Data expressed as mean values ($n = 9$) ± standard deviation; different superscript letters within the same column indicate significant differences between variants ($p < 0.05$).

To determine the overall effect of the treatment applied to the tomato seedlings, observations and measurements were also made one week after the second application. Similar results were obtained regarding the growth parameters of all experimental variants (Table 4). In fact, no significant differences were noted between the control plants (untreated variant V1) and the variants treated with protein gels. An exception is the V3 variant, treated with the mixture of bovine gelatin and keratin, which recorded a significantly increased plant height in relation to the other two variants.

Table 4. Growth of tomato seedlings one week after the second treatment.

Variant	Number of Leaves	Plant Height (cm)	Leaves Frequency (Number of Leaves/Plant Height)
V1 (Control)	6.44 ± 0.53^{a}	26.67 ± 2.08^{a}	0.24 ± 0.03^{a}
V2	6.11 ± 0.33^{a}	27.88 ± 1.48^{a}	0.22 ± 0.02^{a}
V3	6.44 ± 0.53^{a}	29.88 ± 1.34^{b}	0.22 ± 0.02^{a}

Data expressed as mean values ($n = 9$) ± standard deviation; different superscript letters within the same column indicate significant differences between variants ($p < 0.05$).

Comparing the two variants of treatment used, the data accumulated through biometric measurements show that tomato seedlings treated with a mixture of bovine gelatin and keratin were found to have increased plant height and number of leaves in relation to the variant in which only bovine gelatin was used. These experimental results are consistent with the findings of previous studies. Popko et al. [35] evaluated the influence of new products stimulating plant growth based on amino acids on crop yield in winter wheat and also reported no statistically significant differences between the control and treated plots. In contrast, protein hydrolysate and other protein-based product applications were found to enhance plant growth in greenhouse tomatoes [36]. Mironenko et al. [37] also reported an increase in the biometric indicators of growth in spring wheat plants after treatment with a fish protein hydrolysate.

3.4. Influence of the Treatment on the Photosynthetic Pigments Content

Chlorophylls are the most important photosynthetic pigment in plants, therefore changes in the chlorophyll content will influence photosynthetic efficiency and, hence, plant growth. Furthermore, carotenoids play an essential role in photosynthesis, and in addition to this, they are an important class of antioxidants involved in the protection of plants against photo-oxidative processes [38,39]; their destruction through oxidation may reduce the efficiency of the antioxidant defense system [40]. Generally, increased amounts of N and other macroelements (P, K) in the leaf are positively correlated not only with leaf dry weight but also with leaf bioactive contents [41], so it is expected that the application

of protein hydrolysates, representing sources of amino acids, will improve the attributes of the leaves.

The research performed shows an increase in photosynthetic pigment content in plants grown under protein gel treatment relative to the control plants in all experimental variants (Figure 1). In relation to the values obtained in the control variant (0.95 ± 0.06 mg/g for chlorophyll A and 0.44 ± 0.04 mg/g for chlorophyll B), the effect of gel application was not significant on the chlorophyll B content (0.45 ± 0.04 mg/g in variant V2 and 0.48 ± 0.02 mg/g in variant V3), but the accumulation of chlorophyll A improved most in variant V3 (bovine gelatin + keratin) (1.08 ± 0.01 mg/g). Therefore, significant differences regarding the content of chlorophyll A and total chlorophyll were noted for the experimental variants treated with protein gels in relation to the control plants. Regarding carotenoids, the obtained results indicated no significant differences between variants after the applied treatments.

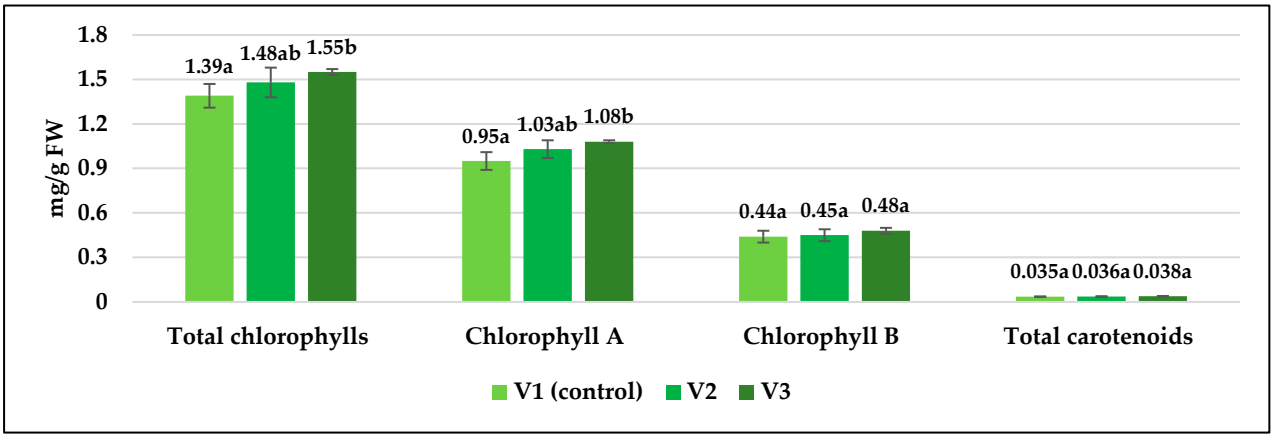

Figure 1. Variability of photosynthetic pigments in the tested seedlings. Data were calculated as mean values (n = 4) ± standard deviation; bars marked with the same letters between variants are not significantly different ($p < 0.05$).

A common effect of the application of plant biostimulants containing amino acids, macro, and microelements is the increase in photosynthetic pigments such as chlorophyll A, chlorophyll B, and carotenoids, which play a significant role in chlorophyll biosynthesis [13,42]. The increase in the content of chlorophyll A and B in plants can be attributed to the high percentage of free amino acids in biostimulants [43]. Amino acids perform numerous functions in plants: Stimulating growth, precursors of auxin and chlorophyll, and the stimulation of seeds germination [33]. Plants are able to synthesize amino acids, but this process consumes a great deal of energy; therefore, the application of ready-to-use amino acids allows plants to save energy and intensify their development [44].

The present study revealed the highest chlorophylls content in the tomato seedlings treated with the protein gel containing keratin added to bovine gelatin (V3), which releases higher amounts of amino acids compared to the other variant (V2 bovine gelatin). Previous studies reported increased amounts of assimilatory pigments under applications of certain growth biostimulators in tomato leaves [45], wheat plants [46], and rosemary cuttings [47]. Moreover, Xu and Mou [48] found that fish-derived protein hydrolysates significantly enhanced chlorophyll content in lettuce leaves, which could be responsible for an increase in their photosynthetic activity plant and consequently to greater production and quality of fruits [8,45].

The total amount of leaf chlorophyll content directly influences the photosynthetic capacity of plants, making it an important parameter for measuring plant photosynthesis and growth potential.

3.5. Influence of the Treatment on the Biochemical Compounds Accumulation in the Leaves

Sugars are considered important metabolites, not only as the first organic complex compounds formed in the leaves as a result of photosynthesis but also as a major respiratory substrate. Furthermore, sugars are involved in plant protection against wounds and infections, as well as in cell detoxification [49].

A significant effect ($p < 0.05$) of protein gel treatment was noted on the dry weight and total soluble sugar content of tomato seedling leaves (Table 5). The results showed that the values obtained were affected by the different treatments with the protein gels used. Both treatments with bovine gelatin (V2) and with a mixture of bovine gelatin and keratin (V3) increased the dry weight and sugar content of the leaves, but the effect was higher for variant V3 in relation to variant V2.

Table 5. Variability of some biochemical parameters in the tested seedlings.

Variant	Dry Weight (g%)	Total Soluble Sugars (mg/100g FW)	Total Polyphenols (mg GAE/100 g FW)
V1 (Control)	19.91 ± 0.05 ^{a}	19.23 ± 0.61 ^{a}	214.54 ± 11.83 ^{a}
V2	20.43 ± 0.27 ^{b}	49.03 ± 3.02 ^{b}	225.56 ± 8.11 ^{a}
V3	21.49 ± 0.60 ^{c}	81.22 ± 10.84 ^{c}	255.29 ± 12.98 ^{b}

Data expressed as mean values ($n = 4$) ± standard deviation; different superscript letters within the same column indicate significant differences between variants ($p < 0.05$).

Biochemical analyses performed revealed the lowest content of total soluble sugars in the leaves of the untreated variant of tomato seedlings (19.23 ± 0.61 mg/100 g FW), while the highest value was noted as an effect of the mixture of bovine gelatin + keratin application (81.22 ± 10.84 g/100 g FW) (Table 5).

Treatment with the tested protein gels induced a high content of total chlorophyll and exerted a positive influence on plant capacity of photosynthesis and sugar biosynthesis. The best result in this regard was obtained after the application of a protein gel containing a mixture of bovine gelatin and keratin. The higher content of free amino acids compared to the other variant may help to increase the biosynthesis of chlorophyll in the leaves and achieve better accumulation of the metabolites in plants.

The biostimulant products allow for achieving significant increases in the quality and quantity of yield by improving the efficiency of fertilizer nutrient uptake. The enhancement in growth in tomato plants treated with protein hydrolysates was attributed to the stimulation of nitrogen uptake and assimilation, which may improve the CO_2 assimilation and enhance the translocation of photosynthates (i.e., soluble sugars) via the phloem to potential sinks [5,50]. This way, the biostimulant-mediated positive effects on plant nutrition, photosynthesis, and secondary metabolism can increase vegetable quality [51].

The results reported in the present study are in accordance with Ertani et al. [52], who noted an increase in content in sugars, antioxidant activity, lycopene, phenolic content, and ascorbic acid for greenhouse chili pepper (*Capsicum chinense* L.) as a result of the foliar application of certain protein hydrolysates.

Polyphenols are bioactive compounds with potent antioxidant activity widespread in many vegetables. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species involved in producing oxidative stress, leading to cellular damage.

Table 5 shows that the application of the gel protein treatments improved the total polyphenol content of tomato seedling leaves compared to the control variant. The obtained values were between 214.54 ± 11.83 and 255.29 ± 12.98 mg GAE/100 g FW. Significant differences were found between the V3 variant, which accumulated the highest amount of polyphenols, and the other experimental variants.

The polyphenol content in the leaves of tomato seedlings increased as a result of the tested protein gel treatments. These findings are in accordance with the report of Rouphael et al. [53], which found increased total phenolic contents, antioxidant activity, and nutrient

contents of tomato plants compared to the control as a result of a legume-derived protein hydrolysate foliar application. Furthermore, these results are in accordance with those previously reported on spinach, broccoli, and zucchini [12,54,55], which also concluded that the use of biostimulants enhanced the polyphenol content.

3.6. Influence of the Treatment on the Antioxidant Activity

The rich content of polyphenols and others compounds with antioxidant potential, such as chlorophylls and carotenoids, could induce the high antioxidant capacity of tomato seedling leaves. Samples of leaves from all experimental variants were screened for their possible radical scavenging activity, and the EC_{50} values were calculated for further comparison, indicating significant differences between all analyzed variants (Table 6).

Table 6. EC_{50} values of DPPH scavenging activities of the analyzed variants.

Variant	EC50 (mg/mL)
V1 (Control)	48.19 ± 3.96^{a}
V2	43.09 ± 0.17^{b}
V3	33.03 ± 2.16^{c}

Data expressed as mean values ($n = 4$) $\pm$ standard deviation; different superscript letters indicate significant differences between variants ($p < 0.05$).

The highest antioxidant activity was noted for the plants treated with the bovine gelatin + keratin mixture (33.03 ± 2.16 mg/mL expressed as the EC_{50} value), confirming the expectations due to their higher content in total polyphenols compared to the other experimental variants. The lowest scavenging capacity was recorded in the leaves of the untreated variant, which required a higher concentration (48.19 ± 3.96 mg/mL) to scavenge 50% of DPPH free radicals.

Furthermore, a correlation study (using Pearson's correlation coefficient) was performed between the radical scavenging activity (expressed as EC_{50}) and the content of total polyphenols, chlorophylls, and carotenoids in order to reveal the contribution of these biochemical compounds to the total antioxidant capacity of the tomato seedling leaves (Table 7). The antioxidant capacity of tomato plants varies in inverse proportion to the content of biochemical compounds.

The total biochemical compounds analyzed have a very strong correlation with the EC_{50} values in the seedling leaves ($R = -0.9112$, $p < 0.05$). The strongest correlation among biochemical compounds was noted for total polyphenol content ($R = -0.8659$, $p < 0.05$), indicating that these phytochemicals are the major contributors to antioxidant capacity. The results also revealed strong correlations between the antioxidant activity of the leaves and their contents in total chlorophyll (-0.7412), largely due to the content of chlorophyll A and less so due to chlorophyll B. For carotenoids, we found a lower value of the coefficient of correlation of -0.4424, which indicates a moderate correlation between carotenoid content and antioxidant capacity.

Table 7. Statistical dependence between biochemical compounds and antioxidant activity (EC_{50}) of tomato seedlings extracts.

Biochemical Compounds	R (Coefficient of Correlation)	R^2 (Determining Coefficient)
Chlorophyll A	−0.6804	0.4629
Chlorophyll B	−0.5236	0.2742
Total chlorophylls	−0.7412	0.5494
Total carotenoids	−0.4424	0.1957
Total polyphenols	−0.8659	0.7498
Total biochemical compounds	−0.9112	0.8302

As in the case of polyphenols, DPPH scavenging activity was significantly influenced by the protein gel application. Colla et al. [6] noted that the biostimulant effects of protein

hydrolysates on antioxidant enzyme biosynthesis may be responsible for increasing the antioxidant activity in the host plant. These findings are in accordance with research performed on several plant species, such as cowpea [56], pepper [57], zucchini [12], and lettuce [13], which reported higher antioxidant activity after biostimulant treatments. Moreover, the linear correlation of the increase in polyphenol content with antioxidant capacity found in the present study is supported by previous research [13].

The high activity of antioxidant defense enzymes is correlated with high stress tolerance [58,59]. Previous research on that topic reported that treatment with growth regulators containing free amino acids and microelements enhanced antioxidant activity and increased tolerance to various abiotic stresses [59–61]. Similarly, it seems that the application of protein hydrolysates can alleviate the negative effects of abiotic plant stress [6,62,63].

4. Conclusions

Protein hydrolysates are widely used in agricultural crops as a promising treatment that increases plant growth and the efficient use of available soil resources.

The research performed in this study showed the beneficial effects of the biostimulant product application on the analyzed parameters in tomato seedling leaves. The content in photosynthetic pigments and in sugars, primary metabolites, were increased consequently the treatments with the tested protein gels. The content of polyphenols and the antioxidant capacity were higher in the treated tomato seedlings compared to the control variant (untreated), and are expected to improve the ability of plants to cope with abiotic stress.

Between the variants of protein gels tested, the best results were obtained by applying the mixture of bovine gelatin and keratin, which can be attributed to a higher amount of amino acids released in the soil compared to the variant containing only bovine gelatin.

Further investigations are considered to confirm the positive effect of biostimulants based on protein gels on the production and quality of tomato fruits.

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