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The Antioxidant Profile of Some Species of Microgreens Cultivated on Hemp and Coconut Substrate Under the Action of a Biostimulator Based on Humic Acids

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Abstract: Microplants are vegetables, grains and aromatic herbs that are consumed in the stage of young plants, without roots, developed after the germination stage, in the stage of cotyledons and which have a high content of nutrients (antioxidants, vitamins, minerals, fatty acids, lutein, β-carotene, proteins and fibers, etc.), which makes them functional, concentrated foods capable of feeding the world's ever-growing population. The significant amounts of antioxidants in microgreens have the role of neutralizing free radicals and reducing their harmful impact on human health. The microgreens studied were spinach (*Spinacia oleracea*) cultivar 'Lorelay', mustard (*Sinapis alba*) cultivar 'White' and radish (*Raphanus sativus)* cultivar 'Red Rambo', tested on hemp and coconut substrates and under the influence of the organic biostimulator Biohumussol, based on humic acids. The antioxidant content of the plants was determined by analyzing total carotenoids, lycopene, chlorophyll, β-carotene, polyphenols and flavonoids, as well as the antioxidant activity by ABTS and DPPH methods. The obtained results indicated that the reaction of the plant material depends on the composition of the substrate and the presence of the applied biostimulator. The highest contents of substances with an antioxidant role were obtained from the microgreens on the hemp substrate, especially mustard and radishes, and the biostimulator proved to be compatible with the spinach microgreens.

Keywords: microgreens; antioxidants; substrate; biostimulator; humic acid

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1. Introduction

Microplants, also known as 'vegetable confetti', are shoots developed from various commercial crops, such as vegetables, grains and aromatic herbs, that are consumed in the stage of young, rootless plants and developed after the first stage, namely that of germination. From the appearance of the first cotyledons and true leaves, the plants can be ready for consumption depending on the variety [1–4]. Used as such, microplants have been noted over time for the benefits they can bring to the human body. These include a high nutrient intake and significant amounts of antioxidants [5–8].

Towards the end of the 2010s, the problem of providing food and nutrients globally, for the entire population of the planet, became more and more accentuated, and microplants began to be seen as an important part of the solution to this problem. Microgreens are new crops gaining popularity nowadays [3,9–11].

Mustard (*Sinapis alba*), spinach (*Spinacia oleracea*) and radish (*Raphanus sativus*) microgreens are among the 10 most cultivated species [12]. They are rich in vitamins (C, A, K, B complex), minerals (calcium, iron, magnesium, potassium, zinc, copper, manganese, phosphorus), fatty acids (omega-3 and omega-6), lutein, β-carotene, antioxidants and glucosinolates, compounds with anticancer potential. They are also an important source of

protein and fiber. Plants provide high concentrations of nutrients due to their early stage of development, contributing to cardiovascular, digestive and immune health [13–18].

Of particular importance with microgreens is the significant amounts of antioxidants they provide. By neutralizing free radicals, antioxidants effectively reduce their damaging impact on cellular structures [19]. Exogenous antioxidants, such as carotenoids, flavonoids, tocopherols and phenolic compounds, have a role both in mitigating oxidative stress and in modulating signaling pathways associated with oxidative stress and inflammation [20]. Among the different types of antioxidants in microgreens, carotenoids and chlorophylls play a crucial role, with β-carotene and lycopene showing the strongest antioxidant effects. These pigments in microgreens have been associated with the reduction of chronic diseases, including some types of cancer [10]. Polyphenols and flavonoids are known for their anti-inflammatory effects, contributing to a lower risk of age-related diseases [21].

Microgreens can be grown in a variety of systems, from home production to plant factories using hydroponics. Thus, different types of substrates specially designed for this type of culture have been developed [22–24]. The most well-known organic substrates are peat, compost, vermicompost, coir, jute and hemp [2,25,26]. Coir fiber is a waste product of the coconut industry (*Cocos nucifera*) and provides a favorable balance of air and water to plant roots [27,28]. Hemp fibers have been proven to be strong, durable and resistant to decay. Hemp fiber has acceptable levels of moisture content, under ambient conditions has good absorption capacity, does not affect the pH or EC of the solution, and does not contain any compounds that either inhibited or delayed seed germination [23,27,29,30]. Cowan (1999) reported that hemp fiber contains β-resercyclic acid, a compound with activity against a wide range of gram-positive bacteria, gram-negative bacteria and fungi [31].

As the scientific community calls for more sustainability and ecological systems in agricultural production practices, research on resources such as plant biostimulant extracts has increased in recent years [30]. The functions and benefits of biostimulants have attracted considerable interest due to their potential as environmentally sustainable resources for production [32]. One of the most well-known and accepted definitions of biostimulants was given by Jardin in 2015; a plant biostimulant is any substance or microorganism applied to plants with the aim of improving nutrition, stress tolerance and/or crop quality, regardless of nutrient content [25]. Among the various types of biostimulants, those based on humic acids stand out for their ability to support plant growth and development by stimulating nutrient uptake and improving photosynthetic efficiency [33,34]. Also, these biostimulators influence the secondary metabolism of plants, activating biochemical pathways that lead to increased production of antioxidant compounds, such as phenols and vitamins, even in the absence of abiotic stress factors. This effect is due to the action of humic acids on the expression of genes involved in the Krebs cycle and other essential metabolic pathways, thus favoring the natural accumulation of compounds with an antioxidant role in plants [35,36].

The aim of the present study is to determine the influence of different types of substrate on the antioxidant content of hydroponic radish, mustard and spinach cultures under the influence of a biostimulator based on humic acids.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was carried out within the University of Life Sciences, Iași, Romania. Commercial seeds of spinach (*Spinacia oleracea*) 'Lorelay' cultivar, mustard (*Sinapis alba*) 'White' cultivar and radish (*Raphanus sativus*) 'Red Rambo' cultivar were used to obtain the plant material, sold by MP SEEDS | Microgreens & Sprouting, Poland. Plant material was obtained according to the protocol proposed by Corrado et al. (2022), with minor modifications [29]. Equal-sized seeds were rinsed with tap water and then sterilized with 3% H_2O_2 (hydrogen peroxide) for 15 min on a rotary shaker and rinsed with distilled water. These were immediately sown in trays specially designed for the culture of microgreens (14 × 19 × 6 cm), in which coir and hemp fiber substrates were introduced. The density

was 3 seeds per square centimeter [29]. The three varieties studied were sown on hemp fiber substrate as well as on coconut fiber substrate. For each substrate used, the reaction of the varieties to the Biohumussol biostimulator was monitored. Thus, the experiment was bifactorial, the two factors studied being the substrate used and the treatment with the biostimulator. Each variation featured three repetitions. The experiment was carried out in a closed-circuit vegetation chamber at a temperature of 24 °C during the day and 18 °C at night, a lighting regime of 16 h during the day and 8 h at night and a relative humidity of 70%. They were watered once a day, and the trays were relocated daily to ensure uniformity of experience. The biostimulator was administered in a concentration of 1.5%. The plants were harvested 14 days after sowing in the cotyledonary phenophase, at a height of 5 cm for spinach and 7 cm for mustard and radish.

Biohumussol is used in organic agriculture, made in accordance with the SR EN ISO 9001:2015 quality management system, implemented and certified by SRAC and IQNET with no. RO-10145. The biostimulator is in the form of a dark brown liquid, a density of 1 mL per 1 g and a water solubility of 100%. Its composition contains 60% organic matter, 5% amino acids and billions of microorganisms. Among the active components are humic acids and fulvic acids, which contribute to the creation of soil structure and increased fertility. The formula is also enriched with essential micro and macro elements, as well as with live bacteria and other easily absorbable substances. The conductivity of the solution is between 3 and 5 mS/cm, and the pH is 6.5, highlighting a favorable environment for biological activity in the soil.

2.2. Extraction of Biologically Active Compounds from Plant Extracts

An ultrasound-assisted extraction method was used to extract phytochemicals from plant samples. Briefly, 1 g of the sample was mixed with 10 mL of n-hexane/acetone solvent mixture (3:1, *v*/*v*, for antioxidant activity using the ABTS method, but also total carotenoids, lycopene, chlorophyll) or with 10 mL ethanol 70% (for the extraction of total polyphenols, total flavonoids and antioxidant activity using the DPPH method) and was subjected to an ultrasound treatment for 35 min at a maximum of 30 °C and a frequency of 40 kHz. After recovering the resulting crude extract, it was then centrifuged for 10 min at 6000 rpm and 4 °C. The supernatant was collected after separation and then analyzed to determine the amount of β-carotene, total carotenoids, chlorophyll and total polyphenols but also the antioxidant activity (ABTS and DPPH).

2.3. Total Carotenoid, β-Carotene and Lycopene Content

To determine the total concentration of carotenoids, lycopene and β-carotene in the extract, UV-Vis spectrophotometry was used according to the protocol presented by Rațu et al. in 2024 [37]. A 0.2 mL sample was added to the extraction solvent mixture and placed in a 1 mL quartz cuvette. A Libra S22 UV-VIS spectrophotometer—Biochrom Ltd. (Cambridge, UK) was used for the analysis. Absorbance was read at λ = 450 nm for total carotenoids, λ = 470 nm β-carotene and λ = 503 nm for lycopene (Biochrom, Cambridge, UK). The results were expressed in $mg \cdot g^{-1}$ dry weight (DW), and the calculations were made based on the following equation:

$$\text{Contents } (mg \cdot g^{-1} \text{ DW}) = (A \times Mw \times Df)/(m \times L \times Ma)$$

A—absorbance of the sample;
Mw—molecular weight;
Df—sample dilution rate;
m—mass/weight of concentrated extract;
L—length of the optical path of the cuvette (1 cm);
Ma—molar absorptivity, which is 2500 L mol^{-1} cm^{-1} for carotenoids, 2590 L mol^{-1} cm^{-1} for β-carotene and 3450 L mol^{-1} cm^{-1} for lycopene.

2.4. Total Flavonoid Content

The flavonoid content of the microgreen samples was determined according to the method proposed by Dewanto et al. (2002) [38]. An amount of 0.25 mL of the sample is mixed with 0.075 mL of 5% sodium nitrite solution ($NaNO_2$) and 2 mL of distilled water. The obtained mixture is left to rest for 5 min, after which 0.15 mL of 10% aluminum chloride solution ($AlCl_3$) is added. After another 6 min of rest, add 0.5 mL of 1 M sodium hydroxide solution (NaOH) and 0.775 mL of distilled water. Readings were taken at λ = 510 nm. Calculations were performed based on a calibration curve with catechin standards (R^2 = 0.9968), and the results were expressed in equivalent milligrams of catechins per gram of dry weight (mg EC/g DW).

2.5. Antioxidant Activity by the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Method

Antioxidant activity was evaluated using the DPPH method, and the results were expressed as µmol of Trolox equivalents per gram of dry weight (µmol TE/g DW) [37]. A calibration curve using Trolox as a standard was used. Absorbance was read at λ = 515 nm for the blank by using 3.9 mL of 0.1 M DPPH in methanol (A0). The PPP extract was added to the mixture and left in the dark for 90 min.

The variation of the antioxidant capacity corresponding to the different samples was studied by determining the inhibition (I%) for each sample to be analyzed using the equation below.

$$I(\%) = (A_{blank} - A_{sample})/A_{blank} \times 100$$

where

A_{blank} = is the absorbance of the blank sample; and

A_{sample} = is the absorbance of the sample.

A calibration curve using Trolox as a standard solution was used, and the results were expressed in µmol TE/g DW. The equation for calculating the antioxidant activity using the standard curve is y = 0.45x + 0.0075, R^2 = 0.993.

2.6. Total Polyphenol Content

The analysis of the total content of polyphenols was carried out using the Folin–Ciocalteau reagent, according to the protocol described by Martis et al. (2021) [39].

Briefly, 1.0 mL of Folin–Ciocalteau solution and 0.2 mL of PPP extract were placed in tubes containing 15.8 mL of distilled water. A quantity of 3 mL of 20% Na_2CO_3 was added to the mixture after 10 min. The resulting combination was kept at room temperature and in the dark for 60 min before the absorbance at λ = 765 nm was measured against a control (pure ethanol). To determine the amount of total polyphenols in the sample, a calibration curve using gallic acid as a standard was used. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.7. Photosynthetic Pigments

Chlorophyll extraction was performed with a mixture of acetone and water in a ratio of 80%/20% (*v*/*v*). A quantity of 2 g of plant tissue was homogenized with 25 mL of 80% acetone solution using a laboratory blender (BLENDER 8010E, MODEL38BL40)—Waring Commercial (Stamford, CT, USA) for 2 min. Filtration was followed (MN G15µ 125 mm), and the filtrate was transferred to a 100 mL volumetric flask covered with aluminum foil to avoid oxidation of chlorophyll from light and filled to the mark with 80% acetone solution. Absorbance was measured at 663 and 645 nm using a Specord 120 spectrophotometer. Total chlorophyll concentration was expressed as $mg \cdot g^{-1}$ fresh weight. The determination of total chlorophyll was carried out using the formula

$$\text{Total chlorophyll } (mg \cdot g^{-1} \text{ DW}) = (20.2\ A645 + 8.02\ A663) \times X/1000 \times n$$

where A645 = absorbance value at 645 nm,

A663 = absorbance value at 663 nm,

X = total volume to filter, and
n = tissue weight.

2.8. Antioxidant Activity with ABTS Method

The antioxidant activity of the obtained extracts was measured using the ABTS [2,20] azinobis (3-ethylbenzothiazole-6-sulfonic acid)] radical decolorization test. The determination of the antioxidant activity was carried out using the ABTS$\bullet^+$ radical method. Dissolve 19.2 mg of ABTS in deionized water to a concentration of 7 mM in a 10 mL volumetric flask. ABTS$\bullet^+$ is generated by oxidizing ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) with 2.45 mM potassium persulfate. The ABTS radical formation reaction proceeds in the dark at room temperature for at least 16–22 h. Thus, the ABTS radical stock solution is obtained. The ABTS stock solution is diluted with ethanol until an absorbance of 0.700 ± 0.020 at 734 nm is obtained (usually 1/90). To determine the antioxidant activity, 0.20 mL of extract dissolved in the extraction solvent was added over 1.80 mL of ABTS$\bullet^+$ reagent. The reaction takes place in the dark for two hours, then the absorbance is read at 734 nm. To express the final results of the antioxidant activity, a standard curve was used and the final results were expressed in µmol Trolox/g DW, using the equation y = 6.1297X, R^2 = 0.9974, where X = A − B (the difference between the absorbance of the control sample and analyzed samples).

The percent inhibition of the ABTS+ radical was evaluated against the parent sample using the equation

$$\text{ABTS inhibition (\%)} = (A - B)/A \times 100$$

where

A is the absorbance of the sample, and
B is the absorbance of the analyzed sample.

2.9. Statistical Analysis

For statistical analysis, Tukey's test, Student's *t*-test and Pearson's correlation coefficient were performed. Tukey's post hoc test compares all pairs of means after an ANOVA analysis to identify significant differences between groups, controlling for the risk of statistical error [40]. Tukey's test was performed after applying a one-way ANOVA test. The Student's *t*-test compares the means of two groups to determine whether the differences between them are statistically significant, taking into account variability and sample size [41]. The Student's *t*-test was performed in Excel to compare the values obtained on the two substrates used as well as to determine the influence of the biostimulator. The Student's *t*-test was performed for the values obtained at each analysis performed, separately for the three species of microgreens used. For the graphical representation of the *t*-test, a heat map was created for the analyses performed on each species. The heatmap was made using Origin Pro 2021 software. The Pearson correlation coefficient measures the strength and direction of the linear relationship between two numerical variables, having values between −1 (perfect negative correlation) and 1 (perfect positive correlation) [42]. The Pearson correlation coefficient was plotted using a correlation matrix obtained in Origin Pro software. This was performed to analyze the correlation between carotenoid and chlorophyll pigments.

3. Results

3.1. Total Carotenoids

The highest content of total carotenoids was obtained in mustard (Figure 1a), in the untreated variants, in the case of the substrate with hemp fibers (14.94 mg/100 g DW), followed by the version on coconut (10.75 mg/100 g DW). A large amount of total carotenoids was also noted in spinach; in this species, the variants treated with the Biohumussol biostimulator presented double values compared to the untreated ones (9.99 mg/100 g DW on hemp and 9.62 mg/100 g DW on coconut). The lowest values were obtained for radishes and were uniform for all analyzed variants.

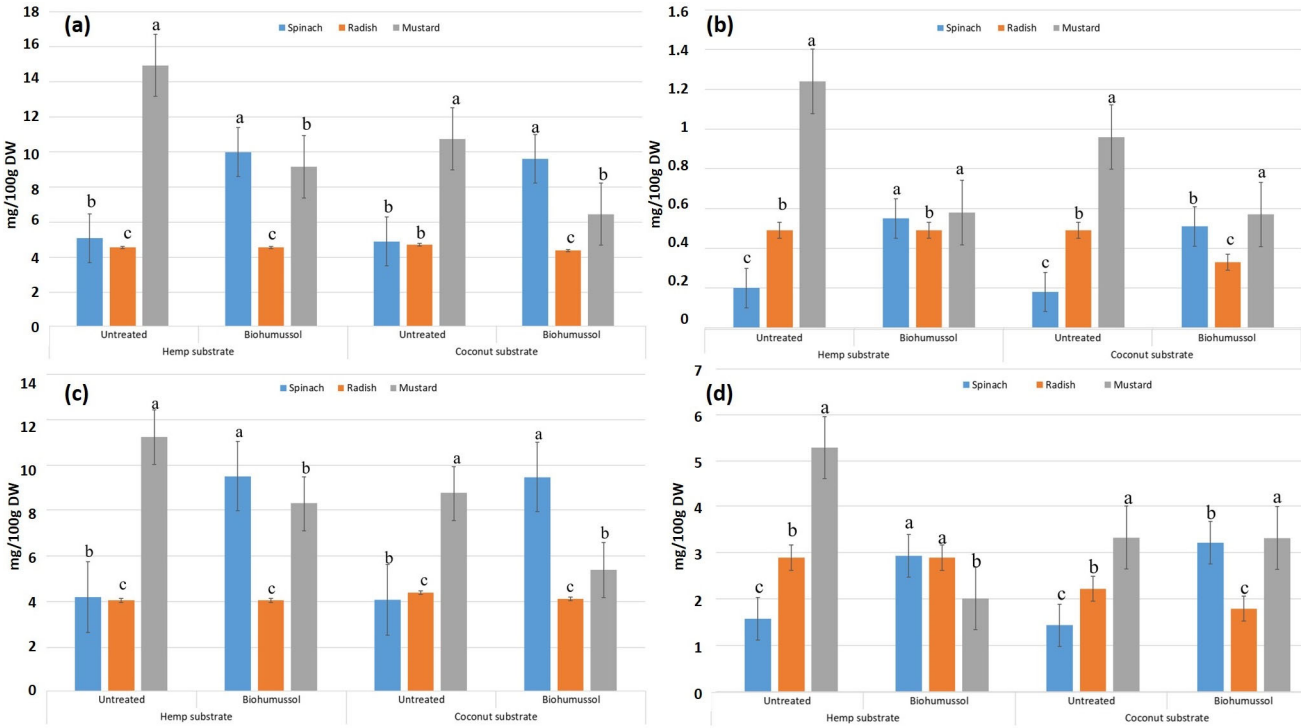

Figure 1. Analysis of mustard, radish and spinach microgreens grown on hemp substrate and coconut substrate under the influence of Biohumussol biostimulator for (**a**) total carotenoid content (mg/100 g DW); (**b**) lycopene content (mg/100 g DW); (**c**) β-carotene content (mg/100 g DW) and (**d**) total content of chlorophyll pigments (mg/100 g DW). Error bars on the graphs represent the standard deviation, and letters on the graph were placed according to Tukey's test with a significance level of 0.05. The raw data can be found in Table S1 of the Supplementary Information. For each analysis, five replicates were performed (n = 5).

3.2. Lycopene

The lycopene content had maximum values in untreated mustard (Figure 1b), both on hemp (1.24 mg/100 g DW) and coconut (0.96 mg/100 g DW), and much lower, almost half, in the versions treated with Biohumussol. In radishes, the values were average and very uniform in all the variants analyzed, neither the substrate nor the treatments influencing the lycopene content. In the case of spinach, the lowest value was noted in the substrate made of coconut fibers in the untreated version (0.18 mg/100 g DW), and the highest value was noted in the substrate with hemp fibers in the case of treatment with biostimulator.

3.3. β-Carotene

β-Carotene was obtained in the highest amount in mustard grown on hemp (Figure 1c), with maxima in untreated variants both on hemp and coconut (11.24 mg/100 g DW on hemp and 8.74 mg/100 g on coconut). High amounts were also found in Biohumussol-treated seedlings grown on hemp (8.29 mg/100 g DW). Spinach had a more special reaction, which synthesized a large amount of β-carotene in the variants treated with Biohumussol, both on hemp (9.5 mg/100 g DW) and on coconut (9.46 mg/100 g DW), and with half values in the untreated variants. The lowest content of β-carotene was recorded in radishes in all the analyzed variants, regardless of substrate or treatment.

3.4. Total Chlorophyll

The total chlorophyll content was greatest in mustard grown on the hemp substrate, untreated (5.29 mg/100 g DW), followed by the variants on coconut substrate (3.32 mg/100 g DW untreated variant and 3.31 mg/ 100 g DW variant with Biohumussol) (Figure 1d). In radish, higher values were obtained for the variants on the hemp substrate

and lower on the coconut substrate. In the case of spinach, a beneficial influence of the biostimulator was observed in the variants cultivated both on hemp and coconut, compared to the untreated ones that had the lowest content of chlorophyll pigments.

3.5. Total Polyphenols

The highest content of total polyphenols was found in radish and mustard microgreens on a hemp substrate (Figure 2a), with maximums in the variants treated with Biohumussol (7.31 mgEAG/g DW in radish on hemp and 7.18 31 mgEAG/g DW in mustard on hemp). Lower values were obtained for the variants grown on coconut substrate. Spinach had a low content of total polyphenols in all analyzed variants, regardless of the substrate used.

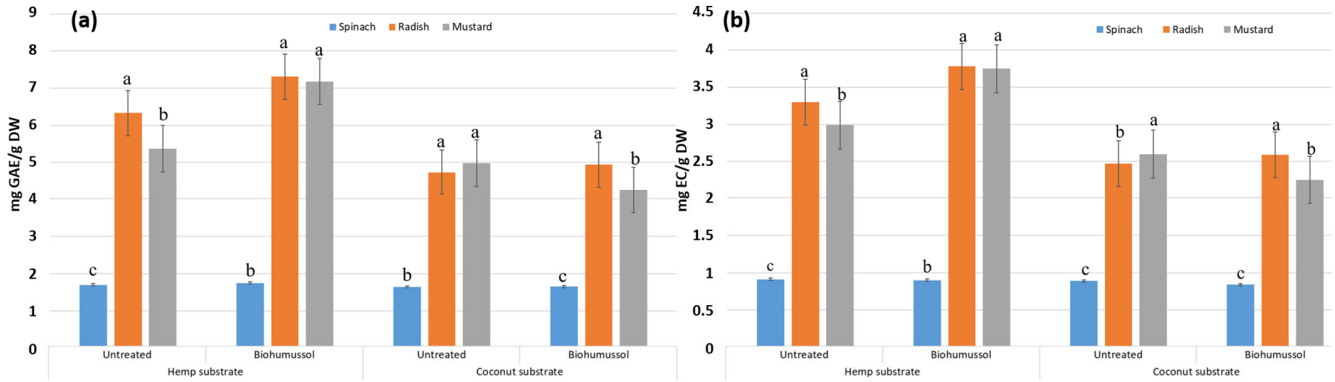

Figure 2. Analysis of mustard, radish and spinach microgreens grown on hemp substrate and coconut substrate under the influence of Biohumussol biostimulator for (**a**) total polyphenol content (mg GAE/g DW) and (**b**) total flavonoid content (mg EC/g DW). Error bars on the graphs represent the standard deviation, and letters on the graph were placed according to Tukey's test, with a significance level of 0.05. The raw data can be found in Table S1 of the Supplementary Information. For each analysis, five replicates were performed (n = 5).

3.6. Total Flavonoids

The species with the highest content of flavonoids were mustard (Figure 2b) (3.75 mg CE/g DW) and radish (3.78 mg CE/g DW) grown on hemp substrate and positively influenced by the treatment with Biohumussol. The variants on coconut substrate had high values for both radishes and spinach. The lowest content of total flavonoids was recorded in the spinach seedlings of all tested variants. They did not present significant differences between the four variants studied (untreated hemp substrate, hemp substrate treated with biostimulator, untreated coconut substrate, coconut substrate treated with biostimulator).

3.7. Antioxidant Activity (DPPH)

Through the DPPH method, the results of the antioxidant activity of the three species of microgreens were completely different compared to the ABTS method. The maximum antioxidant activity was recorded in radish microgreens in all analyzed variants, with a maximum in those grown on untreated hemp substrate (35.34 µmol trolox/g DW), followed by those on coconut substrate (Figure 3a). For mustard, the variants cultivated on the hemp substrate had higher values, while on the coconut substrate the antioxidant activity of the seedlings was much lower. Spinach microgreens showed a special behavior, which had a higher antioxidant activity in the variants treated with Biohumussol (13.42 µmol trolox/g DW on hemp and 13.07 µmol trolox/g DW on coconut) and approx. five times lower in untreated variants.

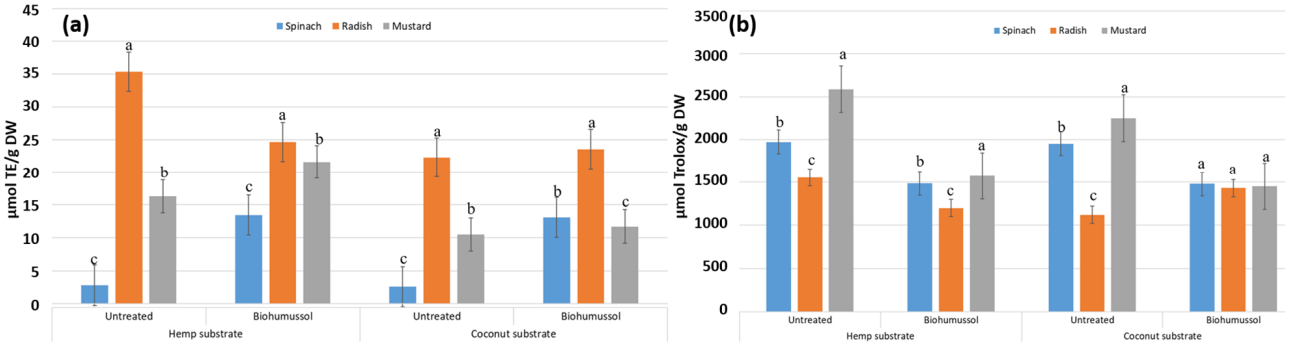

Figure 3. Analysis of mustard, radish and spinach microgreens grown on hemp substrate and coconut substrate under the influence of Biohumussol biostimulator for (**a**) antioxidant activity by the DPPH method (µmol TE/g DW) and (**b**) antioxidant activity by the ABTS method (µmol Trolox/g DW). Error bars on the graphs represent the standard deviation, and letters on the graph were placed according to Tukey's test, with a significance level of 0.05. The raw data can be found in Table S1 of the Supplementary Information. For each analysis, five replicates were performed (n = 5).

3.8. Antioxidant Activity (ABTS)

The antioxidant activity of the three species of microgreens showed the same behavior as that observed in the previous analyses, with maximum values in the untreated mustard both on the hemp substrate (2587.66 µmol trolox/g DW) and on the coconut substrate (2248.34 µmole trolox/g DW). The same behavior was also noted in spinach, where higher values were recorded in the untreated variants, cultivated both on hemp (1972.81 µmole trolox/g DW) and coconut (1953 µmole trolox/g DW) and lower in the variants with Biohumussol. Radish seedlings had higher amounts of total antioxidants in the varieties grown on untreated hemp substrate (1553.45 µmole trolox/g DW) and those treated with Biohumussol on coconut substrate (1428.57 µmole trolox/g DW). In the case of the other variants, the recorded values were considerably lower.

Student's *t*-test (Figure 4) was performed for each analysis carried out in this paper. Thus, the results obtained for total carotenoids (TCs) were compared (Lycopene—Lyc; β-carotene—β-carotene; antioxidant activity by ABTS—ABTS method; total flavonoid content—TF; the total content of polyphenols—TpP; total chlorophyll—TCl) and content and antioxidant activity by DPPH-DPPH method for each species of microgreens was studied. Each of the four plant variants of each species were scored as follows: the variant grown on untreated hemp substrate, I; the variant cultivated on hemp substrate treated with biostimulator, II; the variant grown on untreated coconut substrate, III; the variant cultivated on coconut substrate treated with biostimulator, IV. The values were compared making each possible combination (I–III; I–II; III–IV; II–III; II–IV). The heatmap colors range from deep yellow, which is the equivalent of a null hypothesis, to deep purple, which represents a highly significant statistical difference. Following the analysis of the Student's *t*-tests, statistically significant differences were observed between the four varieties of mustard plants (a). The only situations in which no statistical differences were observed was between the variant grown on untreated coconut and the treated one in the case of the total chlorophyll content, which highlights the fact that the biostimulator did not influence the total chlorophyll content in this substrate. Another situation where no statistical differences were observed was in the case of lycopene between the varieties grown on hemp and coconut, both treated with biostimulator. This highlights the fact that the plants behaved identically to the biostimulator treatment, without the substrate influencing the amount of lycopene. In the case of radish (b) and spinach (c), a more pronounced non-uniformity of the statistical results is observed, a visible effect in the graphs. In the case of radish, the lack of statistical differences could be observed in the case of lycopene, both between the two substrates used and between the variants on the untreated and treated hemp substrate. It can thus be concluded that lycopene in the case of this species was not significantly influenced by the two culture substrates, and in the case of hemp, neither by

the treatment with the applied biostimulator. In spinach, it was observed that the total flavonoids were very little influenced by the substrate on which the plants were grown or by the biostimulator treatment, an aspect that can also be observed in the case of the total content of polyphenols. The close correlation between the two analyses and their similar behavior can thus be highlighted.

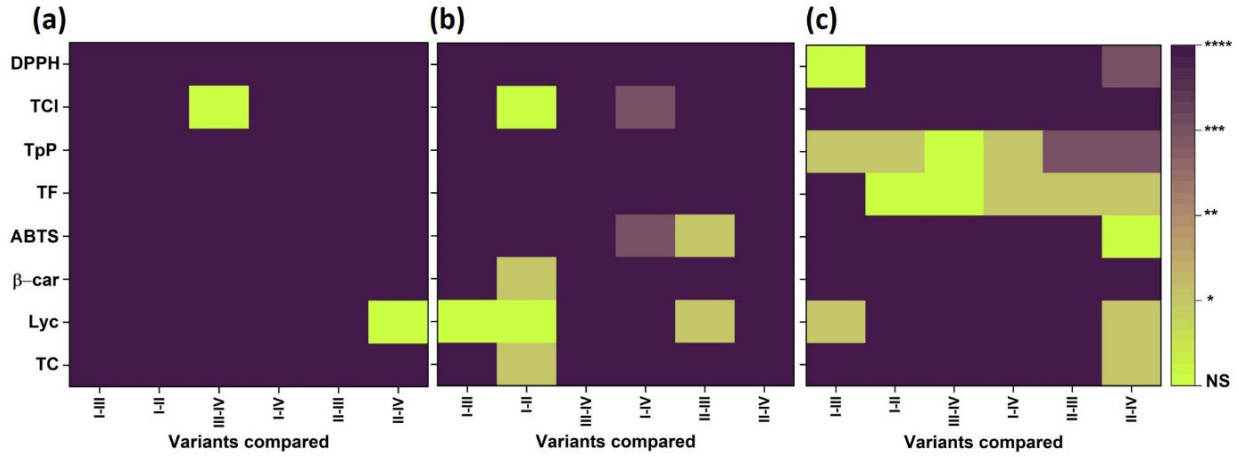

Figure 4. Graphic representation of Student's *t*-tests performed for mustard (**a**), radish (**b**) and spinach (**c**). NS—$p > 0.05$; *—$p < 0.05$; **—$p < 0.01$; ***—$p < 0.005$; ****—$p < 0.001$. The graphic representation was made with the help of a heat map. Specific notations explained in the text were made to create the heat map. The results of the performed *t*-tests can be found in Table S2 of the Supplementary Information.

The physiological growth process was determined by analyzing the average height of microgreens and the fresh weight resulting from 10 g of seeds. The results showed that the highest values were obtained for mustard grown on hemp, treated with Biohumussol (13.8 cm), followed by the untreated one on the same substrate (12.3 cm). In mustard grown on coconut substrate, the highest values were observed in the biostimulator treatment (11.8 cm). In radish, the height of microgreens behaved similar to mustard, showing an average height of 11.9 cm in the seedlings on the hemp substrate fertilized with Biohumussol and smaller in the non-fertilized ones (11.1 cm). Spinach had lower heights in all the variants analyzed, showing higher values in the hemp substrate treated with biostimulator (5.26 cm) and lower in the variants on the coconut substrate (Figure 5a).

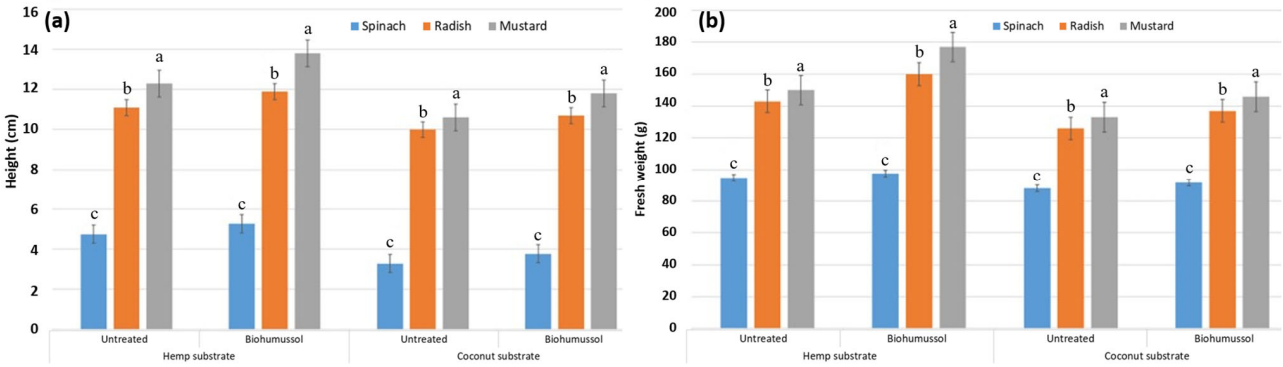

Figure 5. Analysis of mustard, radish and spinach microgreens grown on hemp substrate and coconut substrate under the influence of Biohumussol biostimulator for (**a**) height measured in cm and (**b**) weight measured in g. Error bars represent standard deviation, and letters are assigned using Tukey's 0.05 significance level test. Averages of 10 analyses ($n = 20$) were performed, and values are in Supplementary Information Tables S3 and S4.

The fresh weight of the seedlings from the germination of 10 g of seeds was correlated with the height of the microgreens, the highest values being obtained for the mustard on the hemp substrate treated with Biohumussol (177 g), followed by the untreated variants on the same substrate (150 g). On the coconut substrate, higher weight values were observed in the biostimulator treatment (146 g). Radish plants behaved similarly, with 160 g fresh weight in the variants treated with Biohumussol on hemp substrate and 143 g in the untreated variants on the same substrate. Lower values were obtained in the seedlings on the coconut substrate (Figure 5b).

4. Discussion

4.1. Total Carotenoids

Many carotenoid extracts are used as therapeutic agents in ocular and cardiovascular diseases, to treat cancer, etc. [3,18].

Following the analysis of the values obtained, a different behavior of the three species of microgreens studied was noted. Mustard presented the highest values of carotenoids in all four analyzed situations, which underlines its potential to generate large amounts of carotenoids, a relevant aspect for food and therapeutic applications. Among these, the highest value of total carotenoids was observed in mustard plants grown on untreated hemp (14.94 mg/100 g DW), which highlights the ability of these microgreens to produce significant amounts of carotenoids (Figure 1a). Similar values were recorded in the specialized literature [27,43–45]. The influence of the substrate on the obtained values can also be highlighted, the values recorded for the coconut substrate being significantly lower. Another aspect that stood out is the negative influence of the biostimulator. This decrease in total carotenoid content was strongly correlated with an increase in both height and fresh weight, demonstrating the role of humic acids in the growth of mustard microgreens. Compared to mustard, spinach did not show fluctuations in carotenoids depending on the substrate used but showed significant increases in carotenoids in the variants treated with the biostimulator. Roomi et al. (2018) noted that plants can react differently to humic acid treatments due to a property of the acid that modifies the expression of certain genes responsible for the synthesis of secondary metabolites [36]. The values of carotenoids observed in the radish microgreens did not show significant differences, as they were not affected either by the culture substrate or by the use of the Biohumussol biostimulator.

The close relationship between carotenoids and chlorophyll pigments is evidenced by the high values of photosynthetic pigments in the case of mustard in hemp substrate without biostimulator (Figure 1d). The same positive correlation was observed for all variants tested. The relationship was highlighted by the Pearson correlation matrix (Figure 4).

4.2. Lycopene

Lycopene is a natural chemical compound that is known for its antioxidant properties and potential health benefits.

A very strong positive linear correlation was observed between lycopene and total carotenoid content ($r = 0.9693$), which highlights a similar behavior. As in the case of total carotenoids, both the influence of the culture substrate and that of the Biohumussol biostimulator was observed in mustard (Figure 1b). The high amount of lycopene recorded in mustard grown on the hemp substrate without biostimulator (1.24 mg/100 g DW) is consistent with values recorded in the specialized literature [46–48]. Mustard microgreens grown on coconut substrate showed 24% lower lycopene values compared to those on hemp substrate. According to the study by Kyriacou et al. (2020), the chemical and physical characteristics of the substrates significantly influenced the response of microgreen production [11]. In a study by Gabriele Paglialunga and colleagues (2023), the authors highlighted lower antioxidant values in radish and cabbage microgreens grown on coconut substrate compared to other substrates due to the poor aeration provided by this substrate [22]. In radishes, the values were average and very uniform in all the variants analyzed, neither the substrate nor the treatments influencing the lycopene content. As in the case of total

carotenoids, spinach presented higher values of lycopene in the variants treated with biostimulator compared to the untreated ones. A similar situation was highlighted by Laëtitia Jannin and colleagues (2012) who observed that humic substances modify the expression of genes responsible for photosynthesis and general cellular metabolism [49].

4.3. β-Carotene

β-Carotene, an important source of vitamin A for the human body, was observed in the largest amount in mustard grown on hemp, in the variant not treated with Biohumussol (11.24 mg/100 g DW) (Figure 1c). In the case of β-carotene, the similar behavior of the microgreens to that observed for total carotenoids was also noted. According to the review by Trina Ekawa-ti Tallei et al. (2023), mustard was presented as one of the microgreen species very rich in β-carotene, a fact that is confirmed by the analyses carried out in this paper [26]. The values obtained in the present work for mustard grown on hemp substrate without biostimulator treatment showed higher values than in the case of mustard microgreens tested in various other conditions [44–48]. These values highlight a good compatibility of mustard with the hemp fiber substrate. Marios C. Kyriacou et al. (2020) reported differences in β-carotene content between different substrates. Lower values were recorded on the coconut substrate. The authors explained that the variability of lutein and β-carotene levels was greater in relation to species than in response to substrate [11]. The observation of the authors can also be noted in this work. In the case of radish, the differences between the values are insignificant. The lowest β-carotene content was recorded in radishes for all analyzed variants, regardless of substrate or treatment, but they fell within the literature values [22,46,47].

The same effect described by the authors was also noticed in the case of spinach, which showed a positive reaction to the biostimulator treatment, causing a significant increase in β-carotene compared to the untreated variants.

4.4. Total Corophyll

Chlorophyll, also called 'green blood' for the human body, contributes to increasing the level of energy and oxygen in the body, accelerates the healing process of wounds and helps to prevent anemia, as well as to relieve the symptoms of some medical conditions.

The total chlorophyll content was greatest in the mustard grown on hemp substrate in the variant not treated with Biohumussol, the values falling within the upper limit of those presented in the literature in similar studies [11,46,50]. The behavior of total chlorophyll is correlated with that of total carotenoids due to the dependency relationship between the pigments. The positive linear correlation between the values of the two determinations was highlighted by means of the Pearson correlation matrix (Figure 6). In spinach, a beneficial influence of the biostimulator was observed in both the hemp and coconut variants, compared to the untreated ones. Humic substances, existing in the Biohumussol biostimulator, are known for their beneficial effects on photosynthesis, stimulating chlorophyll synthesis and improving the efficiency of the photosynthesis process in plants [39]. According to the Pearson tests, a strong correlation is observed between the values obtained for each variant of the analyzed species. Thus, it can be concluded that, statistically, carotene and chlorophylls are positively related. Following the analysis of the matrix, the strongest correlations are observed in the case of mustard, where the differences between the treated and untreated variants are the most significant (Figure 6). A positive correlation was recorded between total chlorophyll content and growth in this case. Thus, among the microgreens analyzed in the case of spinach, both an increase in weight and height and a strong increase in the activity of secondary metabolism were observed.

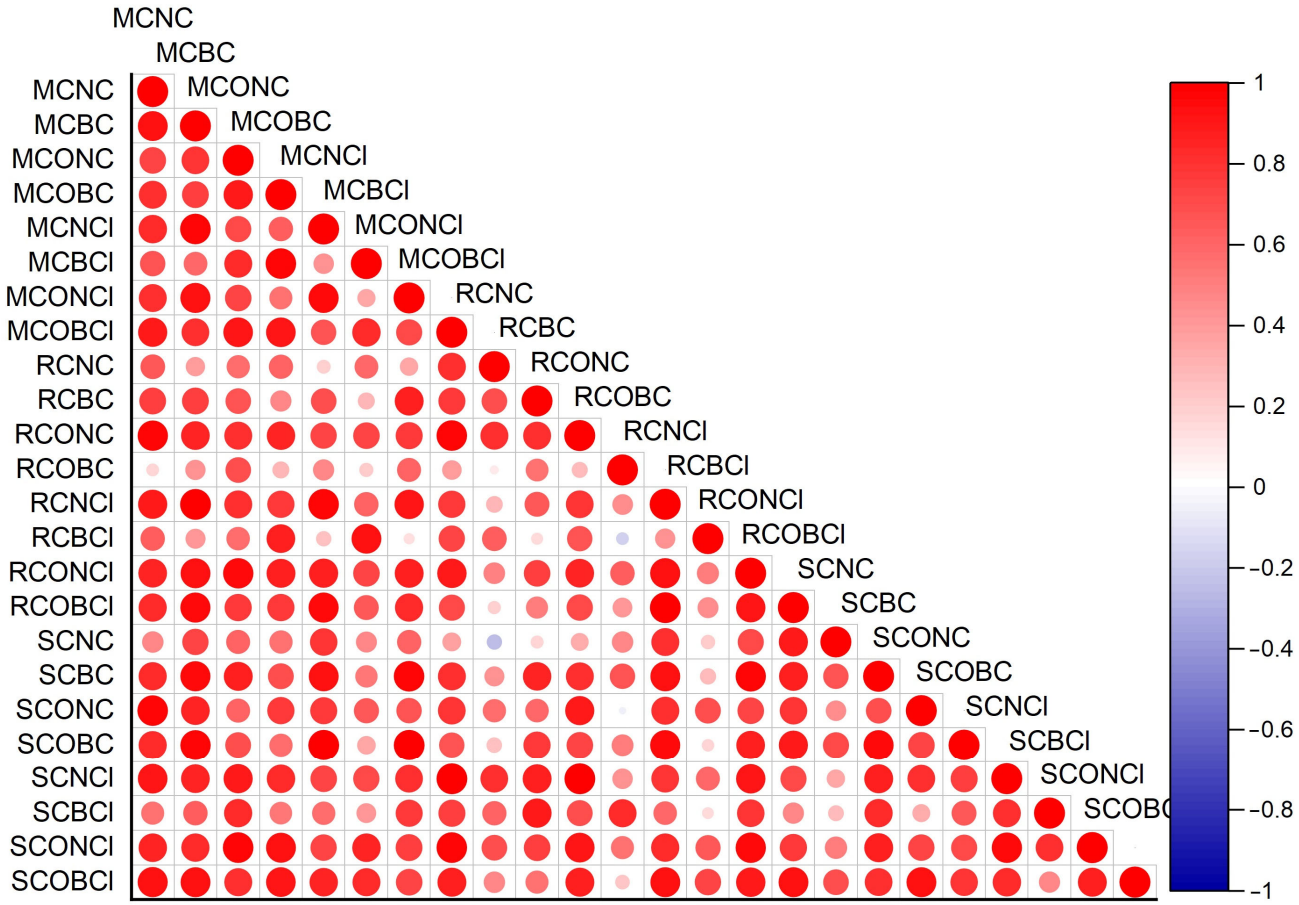

Figure 6. Pearson correlation matrix between the total carotenoid content and the total chlorophyll content for the three species of microgreens tested grown on the two substrates (hemp and coconut) under the action of the Biohumussol biostimulator. The notations present on the matrix are formed as follows: Initial of the species: mustard (M), radish (R) and spinach (S); C—Hemp, CO—Coconut, to which the letters N—Untreated and B—Biohumussol were added, depending on whether or not the treatment was applied. The last letters were C—Total Carotenoids or Cl—Total Chlorophyll.

4.5. Total Polyphenols

Polyphenols are natural chemical compounds found in plants and have been associated with a number of health benefits due to their antioxidant and anti-inflammatory properties. Significant values of polyphenols were recorded in both radish and mustard. In the case of the two microgreens, a similar behavior of the amount of polyphenols was observed. A positive influence of the hemp substrate compared to the coconut one can be noted and also an interaction of the substrates with the applied treatment. Thus, a decrease in values was noted in the case of microgreens grown on coconut substrate and treated with biostimulator. The biggest differences between control and treated plants were recorded in radish grown on hemp, where the biostimulator caused an increase in total polyphenol content by 15.48%, and in mustard grown on hemp, with an increase of 33% compared to the untreated control.

In a study by Tongyin Li et al. (2021) [23] on several types of hydroponic substrates on which they grew microgreens, including radish and mustard, they observed the highest heights in the species grown on hemp. The authors explained this behavior as a strong interaction between the species and the substrate. Another effect observed by the authors was the high amount of calcium and magnesium in plants grown on hemp substrate [23]. Both calcium and magnesium may indirectly influence polyphenol content, which could explain the observed increases. A lower content of polyphenols on the coconut substrate was also obtained by Kyriacou et al. (2020) [11], and values similar to those in the present

paper can also be found in studies from the specialized literature [3,17,22,27,47]. Spinach had the lowest content of total polyphenols of all the variants analyzed, regardless of the substrate used, thus highlighting a lack of influence of the substrate and the biostimulator on this species. This aspect was highlighted by statistical analysis (Figure 4c).

4.6. Total Flavonoids

Total flavonoids represent a significant fraction of the total polyphenol content, showing similar behaviors to other plant phenolic compounds [51]. Thus, the identical behavior of total flavonoids with total polyphenols can be explained.

The highest content of flavonoids observed in mustard and radishes grown on hemp substrate and treated with biostimulator highlights a positive reaction and a good compatibility of microgreens both with the culture substrate and with Biohumussol. The values obtained in this work were in accordance with the specialized literature [17,50,52–57]. Significant differences between the values obtained for mustard and radish were highlighted by Student's *t*-test (Figure 4a,b). The lack of differences in the case of spinach were also observed in the case of statistical analysis (Figure 4c).

4.7. ABTS and DPPH Antioxidant Activity

Because antioxidants can act through different mechanisms, such as free radical scavenging and peroxide decomposition, to exert their antioxidant properties, it is important to use multiple assays to evaluate the antioxidant activity of plant extracts with complex mixtures of different antioxidant compounds [58]. In this work, the use of ABTS and DPPH methods was chosen. The antioxidant activity of the three species of microgreens by the ABTH method showed maximum values in the untreated mustard grown on the hemp substrate. This value highlights a negative effect produced by the coconut fiber substrate compared to the hemp one on antioxidant activity. The biostimulator negatively influenced the antioxidant activity, correlating positively with the results obtained following the analysis of carotenoid and chlorophyll pigments. A similar correlation was reported by Slađana Stajčić et al. (2024) [59]. They performed Pearson correlations for the content of pigments analyzed in Japanese radish, Chinese radish, onion and pea [59]. The correlation between the two analyses can be explained by the antioxidant activity presented by carotenoid and chlorophyll pigments for radish and mustard.

Using the DPPH method, the antioxidant activity results of the three species of microgreens were different. The maximum antioxidant activity was recorded in radish microgreens in all analyzed variants, with a maximum in those grown on untreated hemp substrate (35.34 µmol trolox/g DW). In the case of plants grown on hemp and treated with biostimulator, there was a decrease in antioxidant activity by 30% compared to the control plants, in contrast to the coconut substrate where the values increased compared to the control by 5%. In mustard, the variants on the hemp substrate had higher values, while on the coconut substrate, the antioxidant activity of the seedlings was lower, with slight positive influences from the organic fertilizer.

The spinach microgreens showed a special behavior, showing a higher antioxidant activity in the versions treated with Biohumussol and approximately five times lower in the untreated versions; in this case, the effect of the organic fertilizer used is obvious. In the case of spinach, a direct correlation was observed between the values recorded in the analyses of carotenoid and chlorophyll pigments. The obtained values are in accordance with the results from the specialized literature [43,50,56,59].

4.8. Growth

The application of the Biohumussol biostimulator had a positive impact on the growth in height and weight of the three species of microgreens studied. The humic substances present in the Biohumussol biostimulator favor plant growth through different mechanisms, such as stimulation of nutrient absorption and modification of root architecture [60–62]. This effect is consistent with the observations of Canellas et al. (2015) who suggest that

HS stimulates cell elongation by activating ATPase at the root plasma membrane, which facilitates better water and nutrient uptake, thus favoring vertical growth [63]. The results show a pronounced increase in fresh weight in the treated microgreens, especially mustard, where the weight on the treated hemp substrate was 177 g compared to 150 g in the control. These data are consistent with the study by Rose et al. (2014), showing that exogenously applied humic acids can increase aerial biomass by up to 22%. The positive effect on biomass can be explained by the ability of humic acids to activate auxin-like hormonal responses, contributing to cell division and expansion [64]. The hemp substrate performed better than coconut, especially in mustard and radish, which could be related to the ability of hemp substrate to hold water better and provide a favorable environment for nutrient uptake. The efficiency of humic substances depends on environmental and substrate conditions, and substrates richer in nutrients or with higher water-holding capacity can amplify the biostimulatory effects of humic acids [63].

5. Conclusions

Following the analyses carried out in this work, a specific reaction of microgreens was highlighted both with the substrate used and with the Biohumussol biostimulator. High amounts of pigments were recorded in radishes and mustard on the hemp substrate without biostimulator, and spinach showed a positive reaction to humic substances, showing higher values in the treated variants.

In the case of polyphenols and flavonoids, a different behavior of microgreens was observed, the maximum values being observed in mustard and radish grown on hemp in the presence of the biostimulator Biohumussol. A similar effect is also observed in the case of the growth process.

The analyses carried out highlight the hemp fiber substrate as a good substrate for the growth of radish, mustard and spinach microgreens and also the role of the Biohumussol biostimulator on both antioxidant activity and vegetative growth. The interaction between the substrate and the biostimulator, as well as the specific behavior of the microgreens in the studied conditions, provide the premise for a larger study, with a larger number of microgreens grown in similar conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121238/s1, Table S1: Raw data for the determinations made on spinach (a–d), radish (e– h) and mustard (i–l) for the two substrate types tested under the influence biostimulant Biohumussol; Table S2: Results of t-tests performed for making heatmaps for spinach (a), radish (b) and mustard (c); Table S3: Height (cm) of mustard radish and spinach microgreens grown on hemp and coconut substrate under the action of Biohumussol biostimulator; Table S4: Weight (g) of mustard radish and spinach microgreens grown on hemp and coconut substrate under the action of Biohumussol biostimulator.

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