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Foliar Spray Application of *Chlorella vulgaris* Extract: Effect on the Growth of Lettuce Seedlings

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Abstract: Lettuce seedlings often require the use of fertilizers for their cultivation management to achieve appropriate yield. However, for eco-sustainable chemical-fertilizers-free agronomy, the implementation of totally organic farming often cannot support lettuce productivity, therefore new natural biostimulants able to increase lettuce yield could be considered of great interest. In this preliminary work, the foliar spray application of a *Chlorella vulgaris* extract in lettuce seedlings was investigated in order to achieve better yield performance. Its biostimulant effect was evaluated by monitoring the morphobiometric parameters, chlorophylls, carotenoids, total protein contents, and several enzymatic activities involved in primary and secondary metabolisms of the plant. The experimental trials were carried out by growing lettuce seedlings on inert substrate (pumice) with a 16 h photoperiod for 21 days. The treatment consisted of three consecutive applications by foliar spraying using a concentration of the *C. vulgaris* extract, corresponding to 1 mg C_{org} L^{-1}, which were performed one week apart. The results showed that the *C. vulgaris* extract positively influenced the growth of lettuce seedlings, by increasing the fresh and dry weights, chlorophylls, carotenoids, protein content, and ashes at shoot level. From a biochemical point of view, primary and secondary metabolisms of shoots, in particular nitrogen metabolism, were positively influenced. At the root level, the extract increased dry matter, proteins, and ash content.

Keywords: chlorophylls; citrate synthase; glutamine synthetase; glutamate synthase; malate dehydrogenase; phenylalanine ammonia-lyase

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1. Introduction

Among vegetable crops cultivated in the Mediterranean area, lettuce (*Lactuca sativa* L.) often requires for its cultivation the use of biostimulants and chemical fertilizers to reach a high degree of productivity and maximum growth, since it is a crop moderately sensitive to salt [1]. Unfortunately, the implementation of totally organic farming in some regions often cannot increase lettuce productivity [2]. In this respect, the application of natural biofertilizers and/or biostimulants to the agricultural field is becoming an attractive research topic. Researchers have discussed for a long time about the definition of biostimulants. Du Jardin [3] defined biostimulants as "substances or a mixture of molecules or microorganism which when applied to plants are able to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, independently of its nutrient content" [3,4]. Yaknin et al. [5] proposed to describe a biostimulant as "a formulated product of biological origin improving plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds." In Europe, the legislative framework on plant biostimulant is very complex, and it is regulated at a state level, since specific legislation or definitions are still not issued [4,6]. In this complex

context, du Jardin [4] also proposed to deem biofertilisers as a subcategory of biostimulants, which are able to increase nutrient use efficiency and allow new prospect for nutrients acquisition by plants.

Among these natural biostimulants, microalgae and their extracts showed to be good candidates, since it was shown that they may increase plant growth as well as improve the germination process, aiming to attain sustainable and environmentally friendly agricultural systems [7–15].

Regarding the microalgae effect on lettuce growth, Elhafiz et al. [8] successfully used *Chlorella vulgaris* and *Chlorella pyrenoidosa* living cells as biofertilizers for lettuce seedlings, providing them in the irrigation water of the culture, which strongly improved the dry weights and the chlorophyll content of cultivated lettuce. The same biofertilizer effect was also proven for other crops such as rice, cucumber, and eggplant [8]. Moreover, a formulation composed of *C. vulgaris* and plant growth-promoting bacteria (*Bacillus licheniformis*, *Bacillus megatherium*, *Azotobacter* sp., *Azospirillum* sp., and *Herbaspirillum* sp.) showed to positively affect the fresh weight, total antioxidant capacity, and total carotenoids content in lettuce cultivated for spring and summer crop [16]. More recently, an extract from *Scenedesmus quadricauda* showed a biostimulant effect on lettuce seedlings, increasing their growth at shoot level, and by influencing the activities of several enzymes involved in the primary and secondary plant metabolisms [14].

In recent years, several studies have been carried out on the biostimulant effect of microalgae and their extracts containing biologically active compounds on a great variety of vegetable crops [11,17]. Among these studies, the application of a mixture of microalgae (MaB-flocs and *Nannochloropsis* biomass) to the substrate showed to positively affect the growth of tomato seedlings [18]. The living cells of microalgae *C. vulgaris* and *S. quadricauda* showed to exert a biostimulant effect on tomato plants by increasing their growth parameters, both when tomato plants were grown in a microalgae co-cultivation system in Hoagland solution and when living cells were directly applied into the soil [12,13].

Moreover, the use of microalgal extracts applied by foliar spraying was proven to increase N-content in treated plants by improving nutrient uptake and by regulation of physiological plant metabolism [11,19]. Indeed, foliar spray application of microalgae-based products was recently considered as a promising and innovative agricultural technique, as it is safe to the environment, increases agricultural sustainability, and achieves high yield in crop production [11,19,20]. The application of 5% and 10% microalgal suspensions of *C. vulgaris* by spraying plants of Swiss chard and in soil, respectively, positively affected the initial growth of Swiss chard, and the content of photosynthetic pigments [21].

The aim of this work is to investigate, as a first approach, the biochemical response of lettuce seedlings treated by foliar spray application of an extract from *C. vulgaris*. The novelty of this study consists in the use of a little amount of the methanolic extract of *C. vulgaris* directly sprayed on the surface of the lettuce seedlings. In order to test the biostimulant effect of the treatment, both at leaf and root level, a set of morpho-physiological parameters, the protein contents, and ash contents of lettuce seedlings were investigated. Moreover, the chlorophyll and carotenoid contents of leaf tissues were also measured. Finally, the biochemical response at the shoot level was estimated by measuring the activities of glutamate synthase and glutamine synthetase (enzymes involved in nitrogen metabolism), citrate synthase and malate dehydrogenase (enzymes involved in carbon metabolism), phenylalanine ammonia-lyase (the key enzyme involved in secondary metabolism, leading to the synthesis of phenylpropanoids).

2. Materials and Methods

2.1. Microalgae Culture and Extract Preparation

Chlorella vulgaris (CCAP 211/11C) was obtained and maintained in the algal collection of the Department of Agriculture, Food and Environment (Di3A) (University of Catania, Catania, Italy). *C. vulgaris* was cultivated as detailed in Puglisi et al. [22]. Briefly, microalgae were grown in standard BG11 algae culture medium in a growth chamber, bubbled with air

using a pump at around 180 bubbles per minute through a plastic tube fitted to an air regulator, illuminated by a 3500-lux, average photon flux (PPF) 100 µmol m^{-2} s^{-1} light source (SON-T AGRO 400, PHILIPS, Eindhoven, the Netherlands), with a 12 h photoperiod (microphotography image is provided in Supplementary Figure S1). The microalgal biomass was collected when it reached the plateau growth phase and was centrifugated at 2500 rpm for 10 min at room temperature. The pellet was washed further with distilled water to reach a conductivity <200 µS cm^{-1} [23,24]. The final *C. vulgaris* biomass was treated with methanol (99.9% *v*/*v*) to lyse the cell walls and release the intracellular contents. Lysed cells were centrifugated 2500 rpm for 10 min at room temperature, and the organic solvent was evaporated, then the extract was collected with distilled water to obtain the microalgal extract stock solution. The complete characterization of the biomass of *C. vulgaris* and its extract was reported in Barone et al. [10], and the distribution of C intensity of ^{13}C NMR and element composition are summarized in Supplementary Tables S1 and S2.

2.2. Experimental Conditions

The experiments were carried out using pumice as an inert substrate in transparent containers (40 × 20 × 10 cm) as reported in Puglisi et al. [14]. The substrate was wetted with 1 L of Hoagland solution: $Ca(NO_3)_2 \cdot 4H_2O$, 1180 mg L^{-1}; KNO_3, 505 mg L^{-1}; KH_2PO_4, 68 mg L^{-1}; $MgSO_4 \cdot 7H_2O$, 493 mg L^{-1}; NH_4NO_3, 80 mg L^{-1}; H_3BO_3, 2.86 mg L^{-1}; $MnCl_2 \cdot 4H_2O$, 1.81 mg L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.22 mg L^{-1}; $CuSO_4 \cdot 5H_2O$, 0.051 mg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.12 mg L^{-1}; NaFeEDTA, 22.5 mg L^{-1} [25]. Lettuce seedlings (*Lactuca sativa* L.) at four true leaves, with a weight of around 4 g and height 8 cm, were provided by a local nursery in Catania. In a completely random design, 10 seedlings were transplanted in each container and were acclimatized by growing them for 6 days in a growth chamber at 25 ± 2 °C, with a 16 h photoperiod. Irrigation consisting of 100 mL distilled water was supplied every day, then 3 consecutive treatments one week apart were performed by spraying the seedlings with a solution of Hoagland (500 mL) containing *C. vulgaris* extract at the concentration of 1 mg of organic carbon per liter (C_{org} L^{-1}), whereas the control plants were sprayed with 500 mL of Hoagland solution. Experimental trials were composed of five replications for treatment and control, and each replicate was made of 10 seedlings. The seedlings were then grown for 21 days (from the first treatment) in a growth chamber at 25 ± 2 °C, with a 16 h photoperiod, being irrigated every day with 100 mL distilled water according to the experimental condition described in Puglisi et al. [14].

At the end of the experimental period, five plants for each treatment and replica were used for the morphobiometric parameters, whereas the remaining five plants were immediately frozen with liquid nitrogen and stored at −80 °C until further analysis.

2.3. Morpho-Biometric and Physiologic Parameters in Lettuce Seedlings

Lettuce seedlings (five plants for treatment and replica) were collected, separated into roots and shoots, and their lengths were measured by using a digital ruler to the nearest 0.5 mm, and the leaf number for each seedling was recorded. On the same seedlings, the fresh weights (FW) of leaves and roots were separately measured, and the dry weights (DW) were obtained by placing tissues in a drying oven at 105 °C until the constant weight was reached. Then, each sample was allowed to cool for 2 h inside a closed bell jar, then the dry weights of leaves and roots were separately measured.

Tissue ash contents were determined separately for shoots and roots by incineration of samples in a muffle furnace at 550 °C up to constant mass and were expressed as % respect to DW.

Relative growth rate (RGR) was determined, as reported in Gent [26], from the shoot weights harvested just before the treatment and at the end of the experimental period (21 days after the first treatment) using the following equation:

$$RGR = [\ln(weight_2) - \ln(weight_1)]/(day_2 - day_1) \quad (1)$$

where $weight_2$ represents the fresh weight at the end of the experimental period (21 days), $weight_1$ represents the fresh weight at the beginning of the experimental period, day_2 and day_1 represent the end and the beginning of the experimental period (21 and 0 days), respectively.

The pigment content in leaves (chlorophyll a, chlorophyll b, and carotenoids) were photometrically determined according to Vanni et al. [27] and Sumanta et al. [28]. Leaf tissues (0.5 g) were homogenized in 10 mL 80% acetone used as extraction solvent, then samples were centrifuged at 10,000 rpm for 15 min at 4 °C, and 0.5 mL of supernatant was mixed with 4.5 mL of the extraction solvent. Sample absorbance was then recorded at three different wavelengths: 470, 646.8, and 663.2 nm (Jasco V-530 UV-vis spectrophotometer) and the relative amount of Chlorophyll-a (Ch-a), Chlorophyll-b (Ch-b), and total carotenoids (C) were calculated as follows:

$$\text{Ch-a} = 12.25\ A_{663.2} - 279\ A_{646.8} \quad (2)$$

$$\text{Ch-b} = 21.5\ A_{646.8} - 5.1\ A_{663.2} \quad (3)$$

$$C = (1000\ A_{470} - 1.82\ \text{Ch-a} - 85.02\ \text{Ch-b})/198 \quad (4)$$

Pigment amounts were expressed as mg g^{-1} leaf dry weight (DW).

2.4. Total Protein Extraction from Lettuce Tissues

Extraction of total proteins and enzymes from leaves and roots of lettuce seedlings was performed as described in Puglisi et al. [29]. Briefly, samples of frozen leaves and roots of lettuce were ground with an extraction buffer made of 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, and 5 mM HEPES−KOH pH 7.5, in a 1:1.25 w/v ratio. The homogenate was then filtered with three layers of cheesecloth and centrifuged at 13,000 rpm for 30 min at 4 °C. The resulting supernatant was recovered, and the total proteins were precipitated with solid $(NH_4)_2SO_4$ at 55% of saturation. Total protein content, expressed as mg protein g^{-1} DW, was quantified according to the Bradford [30] method, using BSA as standard curve.

2.5. Enzyme Activities in Lettuce Leaves

Enzymatic activities were performed by using the total protein extract from lettuce leaves. Enzymatic aliquots (1 mL) were centrifuged at 13,000 rpm for 30 min at 4 °C, the supernatant was discarded, and the pellet was dissolved in the smallest volume possible with the appropriate buffer for each enzymatic activity. All enzymatic activities were performed as described in Puglisi et al. [14].

Glutamate synthase (GOGAT) activity was performed in an assay mixture containing 25 mM Hepes-NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2EDTA, and 100 µL of enzyme extract [31]. GOGAT activity was determined by a spectrophotometer (V-530 UV-vis spectrophotometer, Jasco, Japan), monitoring NADH oxidation at 340 nm by using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}, and was expressed as nmol NAD^+ min^{-1} mg^{-1} protein.

Glutamine synthetase (GS) was measured as transferase activity according to Canovas et al. [32]. The assay mixture (750 µL) contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine, and 100 µL of enzyme extract. The enzymatic reaction was incubated at 37 °C for 15 min, then 250 µL of a mixture (1:1:1) made of 10% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl was added. The γ-glutamyl hydroxamate produced during the reaction was spectrophotometrically quantified at 540 nm using a standard curve of γ-glutamyl hydroxamate, and activity was expressed as µmol γ-glutamyl hydroxamate mg^{-1} protein min^{-1}.

Citrate synthase (CS) activity was performed in an assay mixture of 3 mL, containing 50 µL of 0.17 mM oxalacetic acid, 50 µL of 0.2 mM acetyl coenzyme A (acetyl-CoA), and 100 µL of enzyme extract in 0.1 M Tris-HCl, pH 8.0 [33]. CS activity was spectrophotometrically determined by following the reduction of acetyl-CoA to CoA at 232 nm using a

molar extinction coefficient of 5400 L mol^{-1} cm^{-1} and was expressed as nmol CoA mg^{-1} protein min^{-1}.

Malate dehydrogenase (MDH) activity was carried out as described in Schiavon et al. [33]. The assay mixture (1 mL) was made of 94.6 mM phosphate buffer pH 6.7, 0.2 mM NADH, 0.5 mM oxalacetic acid, 1.67 mM $MgCl_2$, and 100 µL of enzyme extract. MDH activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1} and was expressed as nmol NAD^+ min^{-1}, mg^{-1} protein.

Phenylalanine ammonia-lyase (PAL) activity was performed as reported in Mori et al. [34]. The assay mixture (final volume of 1 mL) was made of 0.4 mL of 100 mM Tris–HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 200 µL of enzyme extract. The reaction was developed for 30 min at 37 °C, then stopped with 200 µL of 25% (*v*/*v*) TCA. Samples were then centrifugated at 10,000 rpm for 15 min at 4 °C, and the absorbance of the supernatant was registered at 280 nm. PAL activity was calculated by using a molar extinction coefficient of 16,890 L mol^{-1} cm^{-1} and was expressed as nmol cinnamic acid mg^{-1} protein min^{-1}.

All leaf enzymatic activities were performed as three separated extractions (on tissues sampled from five plants) for each replicate. Protein concentration in each aliquot used for the different enzymatic assays was measured by using the Bradford [30] method.

2.6. Statistical Analysis

Data were preliminarily checked for normality using the Shapiro–Wilk test. Data from the repeated experiments about growth performances, chlorophylls, carotenoids, proteins, and enzymatic activities of lettuce seedlings were analyzed using Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA) by one-way ANOVA ($p < 0.05$), followed by post hoc Tukey's test for multiple comparison procedures.

3. Results and Discussion

The foliar spray treatment with *C. vulgaris* extract (CV) showed to strongly affect the morphological traits of lettuce seedlings mainly at shoot level as shown in Figure 1. Therefore, these results suggest that foliar spray CV extract treatment seems to be a good strategy to obtain a greater yield of the edible portion of lettuce without the application of chemical fertilizers. *C. vulgaris* extract could be then considered a biostimulant, increasing lettuce growth according to the biostimulant definition provided by du Jardin [3] and Yakhin et al. [5].

Growth and the morphological traits of lettuce seedlings subjected to the foliar treatments were then measured, and the results are shown in Table 1. As confirmed by Figure 1, *C. vulgaris* extract positively affected all the morphological traits of lettuce seedlings at the shoot level (height, number of leaves, FW, and DW). On the contrary, at the root level, no significant differences were detected in length and FW, whereas DW of treated seedlings resulted significantly higher than in control plants (Table 1). These findings are in accordance with Puglisi et al. [14], who found that a biostimulant extract prepared from *Scenedesmus quadricauda* applied by root drenching on lettuce seedlings showed better effectiveness above all at shoot level. In particular, CV extract spray application resulted an increase of around 23% of leaf FW and around 20% of leaf DW as compared to those of control (Table 1). Similar values (around 22%) were also reported in lettuce seedlings treated with an *S. quadricauda* extract applied at the root level and grown for 14 days [14]. Data regarding root DW cannot be compared, as in lettuce treated with *S. quadricauda* extract at root level this parameter was not measured [14]. Even so, these results suggest that the spray treatment with *C. vulgaris* resulted to be as effective as those performed with *S. quadricauda* on the lettuce seedlings at the root level. Moreover, similar results were also obtained by Barone et al. [12] using the *C. vulgaris* extract at the concentration 1 mg C_{org} L^{-1} on tomato plants (the same concentration applied at leaf level in lettuce), grown in pots of soil for 18 days and treated by a singular soil application, which recorded an increase in their leaf

dry weights of around 33% concerning the control. On the contrary, with respect to the unaffected root length, which was observed in these experiments (Table 1), in the early stages of plant growth in sugar beet, the addition to Hoagland solution of the same amount used in the present study of *C. vulgaris* extract (1 mg C_{org} L^{-1}) significantly increased total root length of treated plants [35]. These different results taken together suggest that in the functioning of the CV extract great importance should be referred both to the application method and different variety of plant species.

Figure 1. Lettuce seedlings sprayed (CV) and not sprayed (Control) with *Chlorella vulgaris* extract after 21 days from the first treatment.

Table 1. Morphological traits of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) by the foliar application after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five plants for each replica. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; FW: fresh weight; DW: dry weight.

	Shoot Height (cm)	Leaves (N°)	Shoot FW (g)	Shoot DW (g)	Root Length (cm)	Root FW (g)	Root DW (g)
Ctr	19.37 ± 0.89 b	14.67 ± 1.15 b	13.66 ± 1.05 b	0.43 ± 0.04 b	11.72 ± 0.56 a	1.95 ± 0.20 a	0.099 ± 0.02 b
CV	23.53 ± 0.75 a	18 ± 1.15 a	16.85 ± 0.95 a	0.60 ± 0.05 a	11.33 ± 0.89 a	1.89 ± 0.16 a	0.154 ± 0.03 a

The FW root/shoot ratios confirmed that CV positively affected the plant weights mostly at the shoot level (Table 2), showing better growth performance. Indeed, it is well-known that, except for injury to the roots, the reduction in the root/shoot ratio is an index of more favorable growing conditions [36]. Moreover, the DW root/shoot ratios showed no significant difference among treated and untreated lettuce seedlings (Table 2). These results taken together suggest that the dry matter in the root system and epigeous part grow at the same rate, thus confirming that plants were not affected by stress conditions, simultaneously the lower FW root/shoot ratio in treated plants may be

attributed to the general wellness of plants, enhancing the growth of the epigeous part [36]. Interestingly, the lowest values of FW/DW ratios, calculated both for shoot and root, were observed in treated plants, suggesting that the treatment positively influenced the biomass accumulation in term of dry matter both at the shoot and root level (Table 2).

Table 2. Growth parameters of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) by foliar application after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five plants for each replica. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; FW: fresh weight; DW: dry weight; RGR: Relative Growth Rate.

	Root/Shoot FW Ratio	Root/Shoot DW Ratio	Shoot FW/DW	Root FW/DW	RGR
Ctr	0.14 ± 0.01 a	0.23 ± 0.01 a	31.77 ± 1.15 a	19.70 ± 1.05 a	0.035 ± 0.004 b
CV	0.11 ± 0.01 b	0.25 ± 0.02 a	28.08 ± 1.04 b	12.27 ± 1.25 b	0.042 ± 0.002 a

Finally, as reported in Table 2, the relative growth rate (RGR) calculated for treated seedlings resulted to be significantly higher than that estimated in control lettuce. Gent [26] showed that in lettuce the RGR, representing the relative increase in weight per day, slowly changes when plants grow in a constant environment, whereas environmental and nutritional alterations have effects on their growth. Therefore, being fixed in the experimental conditions, as it is in the present trial, the increase of RGR in treated lettuce was certainly linked to the biostimulant effect of CV extract.

As reported in Table 3, the pigment contents (chlorophylls a and b, and carotenoids) in treated lettuce seedlings showed values always significantly higher than the respective amounts in untreated plants. These data are in agreement with the results reported in other studies on a wide range of crops, including lettuce, in which an increase in chlorophyll contents was observed in plants treated with algae extracts [14,37,38]. Interestingly, the chlorophyll a and b ratio of treated plants resulted higher than the value calculated for control lettuce (Table 3). Indeed, this ratio being used as an indicator of N partitioning in leaves, it seems to be positively correlated with the ratio of PSII cores, supporting higher light captures by the chlorophyll-protein complex [39]. In accordance with previous results, Hajnal-Jafari et al. [21] found that treatments with 5% and 10% *C. vulgaris* suspensions applied on soil and Swiss card, respectively, positively affected the content of photosynthetic pigments, showing a correlation analysis between chlorophyll a content and leaf number, and chlorophyll b content and fresh leaf weight.

Table 3. Chlorophyll and carotenoid contents in leaves of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five replications. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; Ch-a: chlorophylls a; Ch-b: chlorophylls b; C: total carotenoids.

	Ch-a (mg g^{-1} DW)	Ch-b (mg g^{-1} DW)	C (mg g^{-1} DW)	Ch-a/Ch-b Ratio
Ctr	0.484 ± 0.042 b	0.239 ± 0.024 b	0.153 ± 0.010 b	2.02 ± 0.10 b
CV	0.699 ± 0.035 a	0.282 ± 0.023 a	0.282 ± 0.025 a	2.48 ± 0.11 a

Total protein contents extracted from the shoots and roots of lettuce seedlings are reported in Table 4. The foliar spray treatment strongly increased total protein contents (around 20% and 10% in shoot and root, respectively) compared to the control. An increase in protein contents was also observed in leaves of lettuce treated with *S. quadricauda* extract in Puglisi et al. [14], and it is probably related to the raised growth of plants subjected to the treatment. According to previous results of dry weights, ash content in shoots and roots also resulted higher in treated plants concerning the control (Table 4), showing that

CV treatment promoted an accumulation of mineral content both at the shoot and root level. These results suggest that the weight increase of the edible part of treated lettuce seedlings (Figure 1 and Table 1) probably was supported by the cumulative increase in pigments (Table 3), proteins, and ashes (Table 4). Meanwhile, the root apparatus supported epigeous growth by increasing the uptake of mineral by soil, since the photosynthates from leaves may be used either for new growth of the shoot itself or may be exported by phloem in root cells, which then increase their biomass [40]. This hypothesis is in agreement with Murchie et al. [41], who reported that an improvement in carbon fixation due to higher interception of solar radiation (chlorophyll content) is strictly related to an increase in yield and biomass in the most important crops. The effect on growth of lettuce seedlings is putatively linked to the action of one or more bioactive compounds present in *C.vulgaris* extract, and exerting their effect above all at shoot levels, determining the manifestation of the biostimulant effect in accordance with the definition of biostimulant proposed by du Jardin [3,4] and Yaknin [5].

Table 4. Total protein and ashes contents in leaves and roots of lettuce seedlings subjected to *Chlorella vulgaris* extract (CV) treatment after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five replications. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control.

	Shoot Protein Content (mg g^{-1} DW)	**Root Protein Content (mg g^{-1} DW)**	**Shoot Ashes (%)**	**Root Ashes (%)**
Ctr	92.10 ± 2.2 b	51.93 ± 2.0 b	18.55 ± 1.2 b	6.48 ± 1.0 b
CV	110.53 ± 2.3 a	56.91 ± 2.1 a	21.91 ± 1.5 a	11.28 ± 2.0 a

Finally, to deepen the effect of spray *C. vulgaris* extract on lettuce seedling metabolism, this preliminary study monitored the activities of GOGAT and GS as key enzymes involved in nitrogen primary metabolism, CS and MDH, involved in carbon primary metabolism, and PAL, as the key enzyme of the secondary metabolism (Figure 2). All the enzyme activities calculated in treated samples, except MDH, were always significantly higher than those measured in the controls (Figure 2).

GOGAT and GS isoenzymes play an important role in the primary nitrogen uptake through ammonium assimilation processes into organic form as glutamine and glutamate, representing the nitrogen donors in the biosynthesis of amino acids, nucleic acids, and other nitrogen compounds such as chlorophylls [42,43]. Our hypothesis is that greater nutrient absorption, in particular of nitrogen, may occur at the root level, involving an increase in biomass (Table 1), total proteins, and ashes (Table 4), thus contributing to enhancing the growth at the shoot level of the treated seedlings through the increase of nitrogen metabolism (Figure 2A,B). This hypothesis is in accordance with the results reported in several other studies and other crops. Among these studies, the ability of biostimulants to stimulate nitrogen metabolism was shown in lettuce [14], maize [33,44,45], and spinach [38]. Moreover, the application by foliar spray of microalgal extracts was proven to increase N content both in root and shoot tissues, by improving nutrient uptake and by a regulation of physiological plant mechanisms [11,19,20].

Regarding carbon metabolism, the treatment significantly increased CS activity when compared to that of untreated plants, whereas MDH activity was not significantly affected (Figure 2C,D). These results suggest that the increase of CS activity in the treated lettuce may be strictly related to the formation of α-ketoglutarate as a precursor in the GS-GOGAT pathway supporting N compounds synthesis. This hypothesis may also be confirmed by Hodges [46], who found that in N-starved tobacco plants after nitrate resupply, a coordinated expression level of CS and GS was measured.

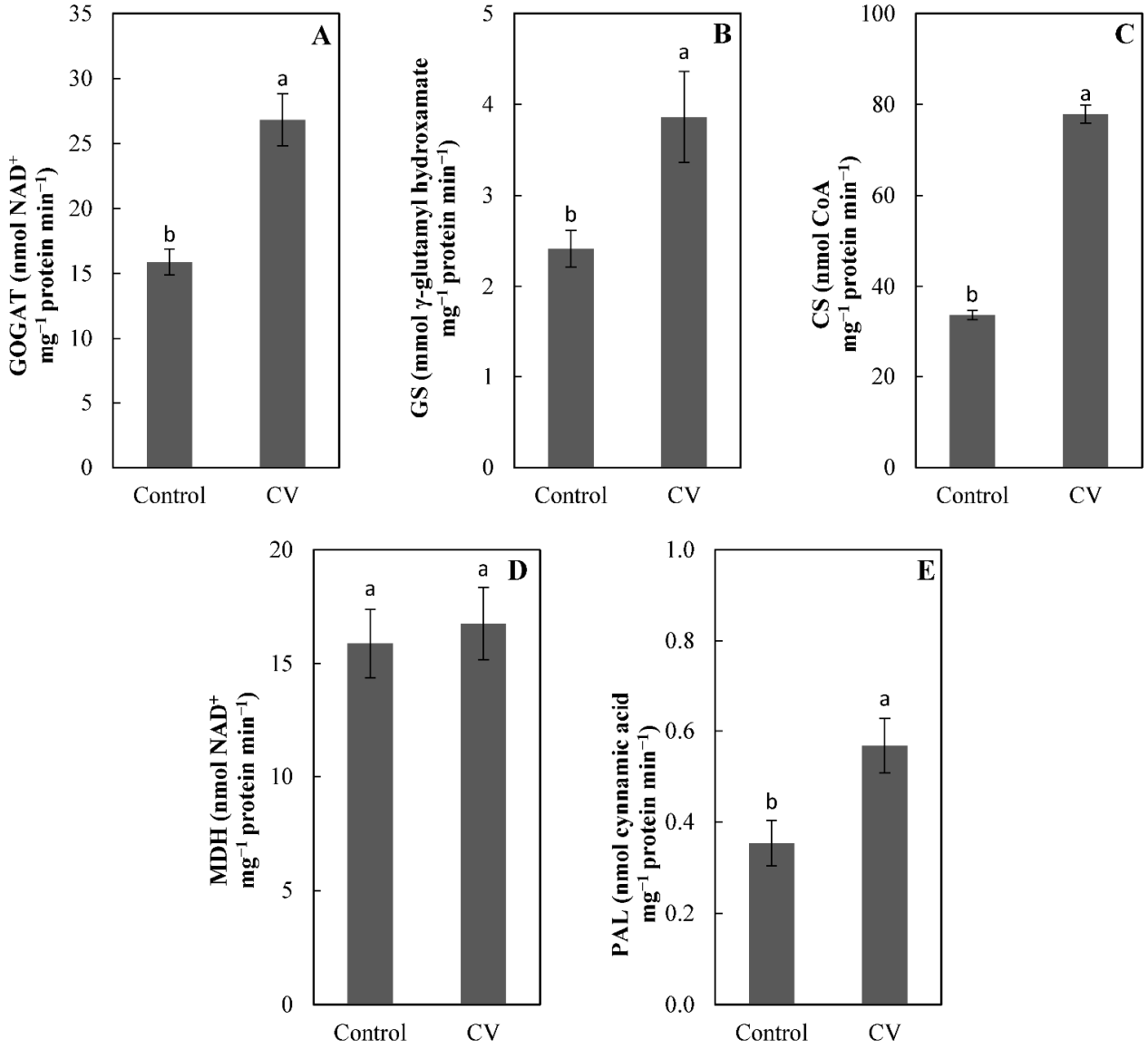

Figure 2. Glutamate synthase (GOGAT) activity (**A**), glutamine synthetase (GS) activity (**B**), citrate synthase (CS) activity (**C**), malate dehydrogenase (MDH) activity (**D**) and phenylalanine ammonia-lyase (PAL) activity (**E**) in leaves of lettuce seedlings. Error bars indicate standard deviation. The values are the means of data from five replications. Values followed by different letters are significantly different ($p < 0.05$).

Finally, to evaluate the effect of *C. vulgaris* extract on secondary metabolism, PAL activity was also evaluated (Figure 2E), resulting always significantly higher in treated plants respect to the control. Similarly, *S. quadricauda* extract applied at the root level of lettuce seedlings positively influenced PAL activity [14]. Indeed, it is well-known that treatments with algae-based extracts activate either primary metabolism or secondary metabolism by enhancing the biosynthetic pathway of plant defense compounds such as flavonoids and phenylpropanoid [47]. Given that in seaweeds and their extracts the major reason associated to biostimulation activity on crop plants has often been associated with hormonal effects [4], similarly, the increased growth performance observed in plants treated with microalgae extracts might be due to hormone-like substances, although other possible synergisms among different substances cannot be excluded.

4. Conclusions

The foliar spray application of microalgae-based biostimulant in agriculture practice is to be considered a promising and innovative agricultural technique, as it is safe to the environment, eases agricultural sustainability, and achieves high yield in crop production. Indeed, taking all the results together, *C. vulgaris* extract can be considered a biostimulant, being able to increase lettuce yield by enhancing crop growth and inducing plant metabolism. In this regard, these preliminary results represent the first study about a foliar spray application of *C. vulgaris* methanolic extract on lettuce seedlings, reporting a successful biostimulant effect on their growth and metabolism. For future studies it would be very interesting to investigate the comparison of different application strategies of the *C. vulgaris* extract and evaluate the best rate of dosage which allows to obtain the best biostimulant effect on lettuce. Although the application methods of *C. vulgaris* extract would deserve further investigation, the presented results are very promising, since the extract shows to act as a biostimulant on lettuce seedlings by increasing their growth and influencing plant physiology through coordinated induction of N and C metabolisms.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/2/308/s1. Figure S1: Optical microscope image of *Chlorella vulgaris* at 100X magnification. Table S1: Distri-bution of C intensity of 13C NMR of biomass of *Chlorella vulgaris* (CV) and its extract (CVextr). Table S2: Element composition (%) of biomass of *Chlorella vulgaris* (CV) and its extract (CVextr).

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