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Use of *Chlorella vulgaris* and *Ulva lactuca* as Biostimulant on Lettuce

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Abstract: The important purpose of this work is to evaluate the biostimulant activity of the algae *Chlorella vulgaris* and *Ulva lactuca* extracts on the crop plants *Lactuca sativa*, to compare the effect of these two green algae on plant growth and development as a part of a sustainable plant production method and show that these extracts can be a promissory source for replacing chemical fertilization. The study faces all the phases of plant growth, from the germination of the seeds to the greenhouse plant growth and treatment, matched with the chemical characterization of both the green algae used and the lettuce plants. This work is meant to define a tool to be improved by more experiments and studies in order to suggest a sustainable method that could ensure an adequate use of organic fertilizer. After the identification of the best concentration of the respective extracts of *C. vulgaris* and *U. lactuca*, the experiment affirmed that a low concentration (15% *C. vulgaris* and 25% *U. lactuca*) of the extracts contributed to the production of plants with a satisfying nutritional profile, while a high concentration (75% *U. lactuca*) is conducive to lettuce production with some parameters not suitable for human diet and health.

Keywords: *Lactuca sativa* L.; bioactive compounds; biostimulant activity; green algal extracts; sustainable agriculture

1. Introduction

Today, food production must be increased to ensure the wellness of the growing population around the world, but, at the same time, it is important to reduce the use of synthetic chemical fertilizers that are dangerous for human and environmental health. To prevent this issue, it is essential to take the direction towards more sustainable agricultural practices that can assure an increased production with the minimum negative impact on the environment [1,2].

One of the strategies to face this goal is the use of biostimulants, the definition of which has evolved over time. Biostimulants are defined as a biological formulation that enhance plant health and productivity as a result of an induced action led by the complex mixture's compounds, rather than just the presence of mineral content and/or the presence of plant growth regulators [3]. There are several kinds of biostimulants involved in agriculture, such as humic substances (humic acid, fulvic acids, and humins), fungi, bacteria, and algae extracts [4]. The market for these products is growing steadily for different reasons: (a) unexpected climatic changes that adversely affect crop yields, (b) the need to promote efficient use of organic materials, and (c) the increasing availability of new commercial formulates [5].

Sustainable development is a central issue in XXI century, considering that the United Nation Agenda 2030 plan promotes, among other things, sustainable food production in its goal 12: "Ensure sustainable consumption and production patterns" [6]. Therefore, interest in biostimulants used in crop production is increasing, and studies demonstrate that algae contain components used to perform this task on crop plants [4].

Chlorella vulgaris and *Ulva lactuca* are both green algae; the first is a unicellular microalga which can grow in both fresh and brackish sweet water and in varied environmental conditions, while the second is a macroalga (seaweed) which grows in brackish and seawater. Both have several applications, from biofuel production to human nutrition, from animal feed to pharmaceutical use and, not least, in agrochemical applications to increase the growth rate and physiological responses of many crops. When applied to soil or leaves, seaweed extract demonstrates a variety of biostimulating properties in agricultural output. Biostimulants are metabolic enhancers that promote plant production due to the unique or emergent features of a complex of ingredients employed in modest amounts. Plants benefit from biostimulants in a variety of ways, including enhanced stress tolerance to nutrients, water, and salt, germination and early seed establishment, improved crop performance and production, maximal resilience to abiotic and biotic stress, and a longer shelf life of the fruits after harvesting, which are all favorable impacts on plants. To increase agricultural yield, liquid seaweed extracts are administered as a foliar spray to crops. These seaweed extracts include cytokinins, auxins, betaines, gibberellins, carbohydrates, vitamins, polysaccharides, alginates, amino acids, and trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni) that operate in the control and development of plants. The methods of action of these seaweed chemicals are complicated, and it is unknown why they are effective; nonetheless, it is probable that these substances operate synergistically when combined [7–10].

Lactuca sativa L. is one of the most valuable vegetable crops around the world used in a human diet for its nutritional properties rich in vitamins, fibers, and polyphenols. Furthermore, studies confirm that lettuce has important effects in the prevention of cardiovascular diseases [11]. The consumption of this species is increasingly becoming one of the leading vegetable crops in the United States [12]. Moreover, lettuce is an important crop in different countries including Italy, Spain, Japan, and Mexico [11]. In Europe, the major producers of lettuce are Spain, with 850 million tons of production, followed by Italy (490 million tons) and France (315 million tons) [13].

This study gives an overview of a possible sustainable system where algae are used as a foliar spray organic fertilizer treatment, a valuable practice in lettuce cultivation.

2. Materials and Methods

2.1. Algal Biomass

The green microalgae *C. vulgaris* dry biomass was supplied by the company Allmicroalgae (Leiria, Portugal), while the green seaweed *U. lactuca* was provided by the start-up Lusalgae (Figueira da Foz, Portugal) and was collected in Lusalgae's seaweed aquaculture (now UMIMARE) (40.12832234344434, −8.820083137469934) located in Ilha da Morraceira (Figueira da Foz, Portugal), Mondego River estuary.

Following that, seaweeds were carried to the laboratory in plastic bags in a cold box and frozen at −20 °C for later use. After removing the seaweeds from the freezer, they were washed with filtered saltwater (collected at the sample sites) to remove the sand, epiphytes, and other debris. Following that, the biomass was rinsed with distilled water to eliminate the salt content of seawater, deposited on plastic trays, and dried for 48 h at 60 °C in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain). Following this, the biological samples were milled (1 cm diameter) with a commercial grinder (Taurus aromatic, Oliana, Spain), and the algal biomass was kept in sterile flasks at room temperature in a dark and dry environment.

2.2. Algal Extract Preparation

The aqueous extracts of *C. vulgaris* and *U. lactuca* were prepared with dried algal biomass and distilled water up to a concentration of 12 g/L and placed in a Moulinex LM811D11 blender (SEB, Selongey, France). The liquification was 2500 rpm for 2 min. After the algae liquification, the crude extracts were filtered through a cotton crude cloth with a pore of 0.5 mm, with a Buchner filter (Linex, Spain) under vacuum. The crude extract solution was diluted with the addition of distilled water, in ratios, to obtain different concentrations for the experiment: 15%, 50%, 75%, and 100% of *C. vulgaris*; 25%, 50%, 75%, and 100% of *U. lactuca*. This concentration solution was based on previous extract analysis and preliminary studies to define the best concentrations of each extract.

The pH and electric conductivity of the crude extract solution were then determined using a pH meter (3310 Jenway, Staffordshire, UK) and an electric conductivity meter (Portable conductivity meter ProfiLine Cond 3310 WTW, Oberbayern, Germany).

2.3. Seed Germination Assay

Lettuce (*Lactuca sativa* var Grand Rapids; Flora Lusitana Lda; Cantanhede; Portugal) and bean (*Phaseulus vulgaris* var. Catarino; Flora Lusitana Lda; Cantanhede; Portugal) seeds were disinfected through emersion for 1 min in a solution of sodium hypochlorite (José Manuel Gomes dos Santos, Portugal) (NaClO) 2% and rinsed for 3 min in a volume of 250 mL of distilled water [14]. The seed germination assay was also performed on beans to investigate if the treatments behaved differently on various species. Sterilized Petri plates (12 cm of the diameter) were previously prepared with cotton and filter paper. Following that, 70 mL of each extract was added. The control was accomplished by adding the same volume of distilled water. Then, 25 disinfected lettuce seeds and 10 disinfected bean seeds were sowed in each Petri dish and incubated for 14 days at 23 ± 1 °C in darkness (Heraeus B5090E Incubator, Thermo Scientific, Osterode, Germany). There were two Petri dish repetitions for each treatment (*Chlorella* dilutions: 15%, 50%, 75%, 100%; *Ulva* dilutions: 25%, 50%, 75%, 100%) and two repetitions for the control. Three observations of the seeds in the Petri dishes were done, respectively, after 3, 7, and 10 days from the incubation.

The plant growth parameters assessed were: the germination percentage (GP), calculated by the equation described by Hernández-Herrera et al. [15], GP = (number of germinated seeds/total number of seeds) $\times$ 100 and radicular length, using a ruler.

2.4. Greenhouse Assay

Lettuce seedlings with 5 leaves were planted in the soil inside a greenhouse belonging to the Coimbra School of Agriculture, Polytechnic Institute of Coimbra in rows, randomly distributed in group of 5 plants for each repetition, 4 repetitions per each treatment were performed. From each treatment, 12 mL of extract dilution was used per repetition, for a total of 60 mL of extract per each dilution. The treatments used were the ones that performed the best results from the germination assay (15% *C. vulgaris*; 25% and 75% *U. lactuca*).

The seedlings were irrigated everyday with water and, one week after planting, three consecutive treatments distributed one per week were applied, consisting of a foliar spray with a solution composed of distilled water and algal extract (12 mL per repetition) according to the different dilutions used. With control plants, no treatment was done.

After 21 days from the first treatment, plants were harvested and the growth parameters evaluated: root length and aerial part diameter with a ruler and their weight with a digital weight scale.

The biomass was dried at 60 °C overnight and after that reweighted to measure the water content by subtraction of the dry matter weight and the fresh matter weight. Then, the dried biomass was used to perform the chemical characterization of lettuce.

2.5. Algal Biomass and Lettuce Chemical Characterization

2.5.1. Moisture and Ashes Content

This was performed according to the international standard method 930.04 (Official Methods of Analysis of AOAC International) [16].

2.5.2. Crude Lipids

The total lipids content was gravimetrically quantified following a continuous extraction process with diethyl ether in a Soxhlet apparatus (Behr Labor-Technik GmbH, Germany), following the international standard AOAC method 930.09 [16].

2.5.3. Total Nitrogen/Protein

The total nitrogen/protein content was determined using the Kjeldahl method (AOAC method 978.04) [16]; whilst 5 was used as a protein conversion factor for the algal samples [17], 6.25 was used for lettuce samples.

2.5.4. Crude Fiber

This was performed according to the standard method 930.10 of AOAC [16].

2.5.5. Total Carbohydrates/Nitrogen-Free Extractives

Nitrogen-free extractives are the difference of 100 from the remaining constituents (moisture, lipids, protein, crude fiber, and ash), while the total carbohydrates correspond approximately to the difference between 100 and the sum of the moisture, ash, lipids, and protein [18].

2.5.6. Mineral and Trace Element Characterization

With the ashes obtained (Section 2.5.1), the mineral content was analyzed through dry mineralization and assessed using flame atomic absorption spectrometry (PerkinElmer PinAAcle 900 T, EUA) [19]. Apart from phosphorus analysis that was performed by spectrophotometry (PG instruments T80+ UV/VIS spectrophotometer, UK) [20].

An acid digestion with nitric acid 65% (m/v) was performed in a water bath at 100 °C for 30 min for this analysis. Finally, the samples were filtered into a volumetric flask and the volume was adjusted to 100 mL using distilled water. Following the requisite dilutions (1:10, 1:100, and 1:500), the analysis was performed on an atomic absorption spectrophotometer outfitted with the cathode corresponding to each element.

2.5.7. Caloric Value

The caloric value, energy value or nuclear energy of food is the amount of energy available after the digestion of a food. Food energy value data are expressed in kilocalories or kilojoules. In a food, energy is present in macro nutrients: fat, protein, and carbohydrates. To calculate the caloric value, the Atwater factors are used, which are 9 Kcal for each gram of fat, 4 Kcal for each gram of protein, and 4 Kcal for each gram of carbohydrate.

The caloric value of the food is given by FAO [21]:

$$\text{caloric value (Kcal)} = (\text{fat} \times 9) + (\text{protein} \times 4) + (\text{carbohydrate} \times 4)$$

To obtain the values in kilojoules from kilocalories, the following conversion was used:

$$1 \text{ Kcal} = 4.1868 \text{ KJ}$$

2.6. Statistical Analysis

The statistical analysis was carried out using the program Sigma Plot v.14. An ANOVA analysis was used to analyze statistical differences in the germination assay assessment parameters, as well as in the chemical characterization of algae and lettuces, across the different treatments. Following the rejection of the ANOVA null hypothesis, the Bonferroni

multiple comparison *t*-test was utilized to distinguish the differences between radicular and aerial component length and weight, as well as the statistical differences between both algal biomass characterization. However, after rejecting the ANOVA null hypothesis, an all pairwise comparison *t*-test (Tukey test) was used to determine significant differences in lettuce chemical characterization between treatments. A statistical analysis was performed comparing the different treatments, being considered statistically different when *p*-value < 0.05.

3. Results and Discussion

3.1. Algal Extract Dilutions Treatment on Seeds

The measured EC values of the crude extracts from *U. lactuca* and *C. vulgaris* (Table 1) were below 2 dS/m (above that, it was considered that the extract was too conductive for plants, which can drive plants cells into osmotic stress and have a negative impact on the plants itself), only for the dilutions of 15% and 50% of *C. vulgaris* and 25% of *U. lactuca*. pH (Table 1) was approximately between 5.7 and 5.9 for *C. vulgaris* dilutions and in the range of 6.7 and 7.0 for *U. lactuca* dilutions; since this parameter was not very different between the extract dilutions, it cannot be considered as a selection standard for the choice of the best treatment.

Table 1. pH and electrical conductivity (EC) of the algal extract dilutions.

Algal Extract Dilutions		pH	EC (mS)
Chlorella	15%	5.69	0.54
	50%	5.91	1.67
	75%	5.88	2.19
	100%	5.90	2.33
Ulva	25%	6.79	1.27
	50%	7.04	2.70
	75%	6.67	2.86
	100%	6.97	3.23

The physical-chemical characterization of extracts is critical for successful outcomes. As a result, the electrical conductivity of the tested extracts was revealed to be not so suitable for lettuce growth, because previous research has found that using nutrient solutions with an electric conductivity greater than 1600 S/cm to cultivate lettuce may cause nutrient imbalance, resulting in decreased leaf number, area, and weight. Furthermore, high electrical conductivity values in the nutrient solution (>1700 S/cm) might cause early bolting as well as chlorotic and necrotic patches on lettuce's lower leaves. Another important aspect in plant growth is pH; thus, nutrient solutions with pH values around 6 are advised for lettuce development [7–9].

These results had consequences on the germination rate (GR) performed by the lettuce seeds during the germination assay seven days after the start of the experiment in Petri dishes (Figure 1). After seven days, no changes were observed in the assay. The GR of lettuce seeds treated with 50% *C. vulgaris* was more than 50% lower than the GR of 15%, that is much higher than 75% and 100%. For the beans treated with *C. vulgaris*, there were no statistical differences between the dilutions. Regarding the *U. lactuca* treatments in beans, the difference is irrelevant with a lower value of 50%. In lettuce, the best results were obtained with 25% and 75% *U. lactuca*, however, these results were not statistically significant when compared with the control.
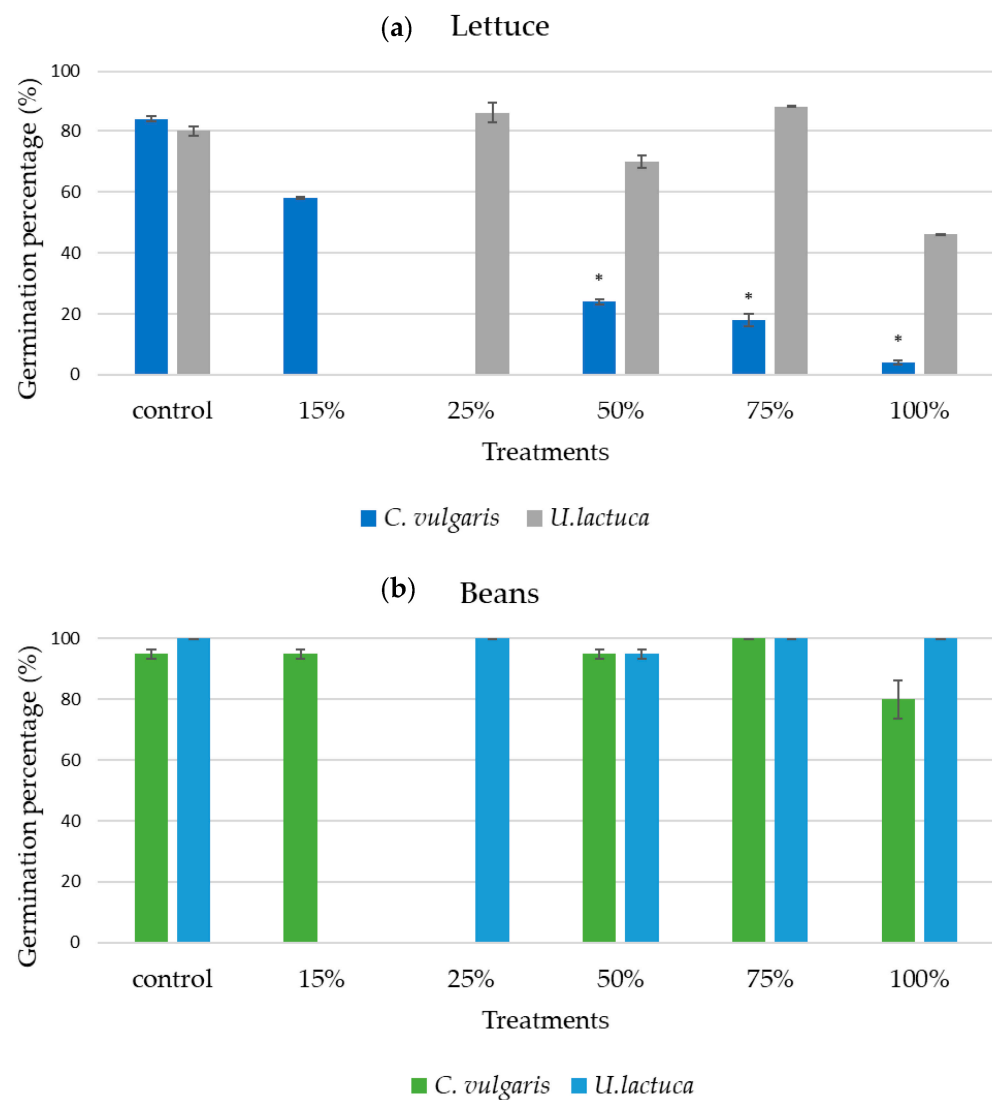
(a) Lettuce

(b) Beans

Figure 1. Germination rate (%) seven days after the beginning of the seed (**a**) lettuce and (**b**) beans treatments in Petri dishes. The results are expressed by mean ± standard error. In comparison with the control, statistically significant differences are expressed by the symbol * (*p*-value ≤ 0.05).

The results in lettuce demonstrated that the physico-chemical parameters in *C. vulgaris* add a direct impact in the seed germination, where pH and EC were out of limits. Unlike the *C. vulgaris*, the *U. latuca* extracts demonstrated that the pH can interact with the negative impact of EC where, at 100%, there is lower germination without being significantly different.

According to Hassan and Ghareib [22], whose experiment was performed on lettuce and tomatoes with the acetone extract of *U. lactuca*, a low concentration of extract had a positive effect on the GR. The only exception is presented with the 75% *U. lactuca* dilution that appeared to be a very efficient dilution for beans. The above-cited study, as an answer to this effect, explains that these results can be connected to the action of free phenolic compounds able to stimulate the germination of different plants.

3.2. Greenhouse Treatment

Considering the results from the germination assay, the best concentrations of the algal extract were 15% with *C. vulgaris* and 25% and 75% with *U. lactuca*. Relevant effects of treating lettuce plants with these solutions were observed in roots (Table 2 and Figure 2).

Table 2. Lettuce morphological analysis after the three treatments. The results are expressed by mean ± standard error. In comparison with the control, statistically significant differences are expressed by different letters [a,b].

Treatment	Root Length mm	se	Root Weight g	se	Aerial Part Weight se	se	Aerial Part Diameter mm	se	FW/DW Ratio (Aerial Part)
control	14.38 [a]	0.88	22.84 [b]	1.37	776.00 [a]	39.09	20.88 [a]	0.84	24.98
C. vulgaris 15%	14.25 [a]	0.63	36.18 [a]	4.61	738.74 [a]	30.56	21.25 [a]	1.00	25.29
U. lactuca 25%	15.63 [a]	1.31	24.86 [a,b]	2.15	624.93 [a]	40.97	19.31 [a]	0.28	24.14
U. lactuca 75%	15.38 [a]	0.92	27.16 [a,b]	1.45	615.45 [a]	33.03	18.50 [a]	0.25	23.61

Figure 2. Different root lengths observed in the control and treated plants.

No significant statistical differences were detected between treatments (Figure 1 and Table 2). This indicates that the tested extracts do not affect the lettuce root system. The highest root weight was performed with 15% *C. vulgaris*, while the least was with the non-treated plants (control) with low weight roots (Table 2).

Concerning the plant aerial part (Table 2), there were no statistically significant differences between the treated and the control plants. It is interesting to notice that the fresh weight (FW)/Dry weight (DW) ratio (Table 2) shows a lower value for the treatments with *U. lactuca*. This result suggests that *U. lactuca* extracts improve the production of leaf biomass in lettuce compared to non-treated plants and *C. vulgaris* extract. It leads to a lower water content in leaves of plants treated with *U. lactuca*.

The root weight results are in accordance with Hajnal-Jafari et al. [23], who confirmed that the green algae treatment with *Chlorella vulgaris* on lettuce positively influenced root growth, especially the weight. Indeed, in our work, the root weight value of the plants treated with *C. vulgaris* (15%) appeared 36.9% higher than in the control plants (Table 2).

3.3. Chemical Composition

3.3.1. Algal Chemical Composition

The results of the chemical analysis of *C. vulgaris* and *U. lactuca* (Table 3) show some differences between the two green algae. Firstly, the ash contents were visibly different; *U. lactuca* had lower water content compared to *C. vulgaris*, this means that the first one is characterized by a higher biomass matter. *C. vulgaris* fiber content is about 14.5% of the *U. lactuca* fiber value; the opposite situation was true for the protein content values where *U. lactuca* produced 37% less than *C. vulgaris*.

Table 3. Moisture value (g/100 g) and the chemical composition of *Chlorella vulgaris* and *Ulva lactuca* dry matter. The results are expressed by mean ± standard deviation. Statistically significant differences in the same element content among the species are expressed by different letters.

	Dry Matter	
	C. vulgaris	*U. lactuca*
MOISTURE (g/100 g)	4.94 [a]	11.16 [a]
ASHES (g/100 g)	9.33 ± 0.04 [a]	26.25 ± 0.08 [b]
FATS (g/100 g)	1.36 ± 0.05 [a]	0.99 ± 0.02 [a]
FIBER (g/100 g)	1.09 ± 0.03 [a]	7.51 ± 0.08 [b]
PROTEINS (g/100 g)	37.11 ± 0.06 [a]	13.58 ± 0.06 [b]
NON-NITROGEN EXTRACTIVES (g/100 g)	51.11 ± 0.00 [a]	51.67 ± 0.08 [a]
ENERGY (Kcal/100 g)	365 ± 0.20 [a]	270 ± 0.07 [b]
ENERGY (KJ/100 g)	1529 ± 0.85 [a]	1130 ± 0.31 [b]
N (%)	5.94 ± 0.01 [a]	2.17 ± 0.15 [b]
P (%)	1.78 ± 0.03 [a]	0.26 ± 0.01 [b]
Ca (%)	1.13 ± 0.06 [a]	0.64 ± 0.06 [b]
Mg (%)	0.21 ± 0.01 [a]	0.82 ± 0.10 [b]
K (%)	0.75 ± 0.05 [a]	1.57 ± 0.04 [b]
Na (%)	0.13 ± 0.05 [a]	0.20 ± 0.04 [b]
Cu (mg/Kg)	25.75 ± 1.24 [a]	12.75 ± 2.28 [b]
Zn (mg/Kg)	30.51 ± 1.90 [a]	23.64 ± 1.53 [b]
Fe (mg/Kg)	38.24 ± 5.60 [a]	210.70 ± 10.84 [b]
Mn (mg/Kg)	93.21 ± 2.82 [a]	33.53 ± 1.91 [b]

Other relevant differences concerned: phosphorus (P) in *U. lactuca*, which was nearly 85% less than in *C. vulgaris*; nitrogen (N) in *U. lactuca*, which was about 63.5% less than in *C. vulgaris;* copper (Cu) in *U. lactuca*, which was about 50% less the other algae; and the fact that around 63% of *C. vulgaris* manganese (Mn) content was more than in *U. lactuca*. On the other hand, iron (Fe) and magnesium (Mg) in *C. vulgaris*, were, respectively, about 82% and 74% less than in the *U. lactuca* total content. The difference in calcium (Ca) content between the two algae was about 57% more in *C. vulgaris*; potassium (K) content differed by approximately 48% more in *U. lactuca*. Regarding the other chemical compounds, there were not many differences in values; non-nitrogen extractives content was almost the same in the two kinds of algae. *C. vulgaris* contains 30% more fats and zinc (Zn) compared to *U. lactuca*. The opposite situation was true for sodium (Na), where the seaweed (*U. lactuca*) contains 30% more Na than the microalgae (*C. vulgaris*).

Regarding energy, the analysis showed that *C. vulgaris* had a 26% higher content than *U. lactuca.*

In *C. vulgaris*, the protein content (37.11%) was lower than most of the commercial extracts produced with *Chlorella* spp. and *Auxenochlorella* spp. (Chlorophyta) (extract brands: Biotona, Piura, Purasana, Soleil Vie, Alver) as analyzed in Canelli et al. [24], where the range of protein content was around 60–66% and also lower than the values of crude protein reported by Tokuşoglu and Ünal [25] (47.82%).

For the carbohydrate content, the non-nitrogen extractive reported in this study (51.11%) was between that shown by Canelli et al. [24], where the medium range for most of the commercial extracts made with the different *Chlorella* spp. considered (extract brands: Biotina, Piura, Purasana, Soleil Vie) was around 9.9–14.4%, apart from Alver (extract from *Auxenochlorella protothecoides*) (20.2%) and LG-Chlorella (65.0%), which contained

more carbohydrates. Moisture (4.49%) was higher than in the research of Tokuşoglu and Ünal [25], while fat content (1.36%) was much lower than the 13.32% of the same study.

According to Rasyid [26], who analyzed dried *Ulva lactuca* for its nutritional composition, this is considered a good source of carbohydrates, since it has 58.1% of non-nitrogen extractives, while, in this study, we found 51.67%. Protein presented a percentage of 13.6 in this study, which is the same as in Rasyid [26], but higher than in Yaich et al. [27]. The iron (Fe) and sodium (Na) content results were similar to Rasyid [26]. In the present study, moisture was 11.16% (Table 4), which is lower than the considered study where the same parameter is 16.9%, as shown by Khairyet al. [28]. The same was true for fiber (7.51% in this study against 28.4% in Rasyid [26]) and calcium (Ca) (0.64% while in Rasyid [26] it was 1.83%). Other parameters were higher in the present study than in Rasyid [26]. The main differences concern ashes: 26.25% in this study and 11.2% in the cited work. This parameter seems to be unstable in the different studies in which it is analyzed, for example, in Yaich et al. [27] it was 19.59%. Fats and potassium (K), as ashes, were higher in the characterized *U. lactuca* compared to Rasyid [26].

Table 4. Chemical composition of lettuce plants treated with three algal extracts (15% *C. vulgaris*; 25% and 75% *U. lactuca*) compared with non-treated plants (control). Moisture value in g/100 g are: 96.33 (control); 96.40 (15% *C. vulgaris*); 96.22 (25% *U. lactuca*); 96.14 (75% *U. lactuca*). Statistically significant differences in the same element content among the treatments are expressed by different letters.

	Dry Matter			
	Control	**15% *C. vulgaris***	**25% *U. lactuca***	**75% *U. lactuca***
ASHES (g/100 g)	23.20 ± 0.26 [a]	22.90 ± 0.09 [a]	21.35 ± 0.73 [a]	22.59 ± 0.09 [a]
FATS (g/100 g)	3.37 ± 0.15 [a]	2.98 ± 0.06 [a,b]	2.75 ± 0.03 [b]	3.13 ± 0.06 [a]
FIBER (g/100 g)	14.42 ± 0.15 [a,b]	14.58 ± 0.13 [a]	14.18 ± 0.04 [a,b]	14.03 ± 0.10 [b]
PROTEINS (g/100 g)	24.07 ± 0.17 [a]	20.51 ± 0.19 [b]	21.10 ± 0.04 [a,b]	21.71 ± 0.04 [a,b]
NON-NITROGEN EXTRACTIVES (g/100 g)	34.94 ± 0.09 [b]	39.02 ± 0.09 [a,b]	40.62 ± 0.76 [a]	38.54 ± 0.09 [a,b]
ENERGY (Kcal/100 g)	266 ± 0.34 [a,b]	265 ± 0.57 [b]	272 ± 2.94 [a]	269 ± 1.04 [a,b]
ENERGY (KJ/100 g)	1115 ± 1.40 [a,b]	1109 ± 2.37 [b]	1137 ± 12.31 [a]	1127 ± 4.37 [a,b]
N (%)	4.24 ± 0.06 [a]	3.61 ± 0.03 [a]	3.63 ± 0.06 [a]	3.82 ± 0.01 [a]
P (%)	0.68 ± 0.01 [a]	0.62 ± 0.01 [a]	0.64 ± 0.01 [a]	0.66 ± 0.02 [a]
Ca (%)	0.74 ± 0.03 [a]	0.62 ± 0.01 [a]	0.67 ± 0.01 [a]	0.67 ± 0.01 [a]
Mg (%)	0.21 ± 0.01 [a]	0.19 ± 0.01 [a]	0.19 ± 0.01 [a]	0.20 ± 0.01 [a]
K (%)	7.39 ± 0.78 [a]	7.13 ± 0.14 [a]	7.20 ± 0.35 [a]	8.01 ± 0.05 [a]
Na (%)	0.17 ± 0.01 [a]	0.16 ± 0.01 [a]	0.12 ± 0.01 [b]	0.15 ± 0.01 [a]
Cu (mg/Kg)	14.94 ± 0.95 [a]	13.14 ± 0.68 [a]	13.54 ± 0.53 [a]	13.32 ± 1.09 [a]
Zn (mg/Kg)	42.06 ± 1.75 [a]	31.23 ± 0.18 [a]	40.06 ± 0.81 [a]	531.80 ± 24.31 [b]
Fe (mg/Kg)	20.28 ± 0.35 [b]	29.44 ± 1.42 [a]	25.30 ± 1.82 [a,b]	23.21 ± 1.02 [a,b]
Mn (mg/Kg)	25.23 ± 0.30 [a]	22.00 ± 0.27 [a]	22.05 ± 0.96 [a]	22.19 ± 1.30 [a]

3.3.2. Lettuce Chemical Composition

The lettuce plant dry matter content was analyzed after the treatments with algal extracts (Table 4).

The ash values were mostly similar between the different lettuce treated samples, but it is interesting to note that the lettuce treated with 25% extract dilution of *U. lactuca* had the lowest ash value. More visible differences were observed in the iron (Fe) content, which was higher in lettuce treated with 15% *C. vulgaris* compared to the control and other

treatments. The extract of this green algae had less Fe but influenced its production in the lettuce leaves.

A relevant result observed was the zinc (Zn) content which, in plants treated with 75% *U. lactuca*, was impressively high, about 93% more than with the other extracts (Table 3). The 75% *U. lactuca* had more impact on the lettuce fiber and in non-nitrogen extractives yield, where the control had more protein and fats in comparison to the algae extracts.

The control added higher protein and fats and lowered iron content when compared to the other samples. The algae extracts appear to not be morphological biostimulating, where only the 15% *C. vulgaris* extract demonstrated to produce some difference in the root weigh and lettuce aerial diameter (not statistically different). Where U. latuca extracts appear to produce more leaf biomass, the extract appears to stimulate a different nutritional profile that needs to be considered. The application of 15% *C. vulgaris* extract is the most acceptable for lettuce plant production under a controlled environment; these extracts produced the heathiest plants in terms of mineral composition and fiber yield, where 25% *U. latuca* was more significant in terms of lettuce production based on low fat and high carbohydrate content, thus, producing high energetic vegetables (1137 Kj/ 100 g).

According to Kim et al. [29], Na, K, Ca, P, and Mg fit in the range of values that generally characterizes lettuce crops. This is also suggested in a study by Geraldson and Tyler [30], where the common nutrient range of minerals in crops are reported and, for lettuce, the average values described are in accordance with the measured values detailed in the present study regarding P, K, N, Ca, and Mg in each treated lettuce and control. Zn is included in the range of 30–50 mg/Kg—the reported range in the mentioned study—for the control, 15% *C. vulgaris* and 25% *U. lactuca*, but this was not true of 75% *U. lactuca*, where zinc content was highly above this range. A quantity of zinc above 300 mg/Kg in plants is usually toxic, so 531.80 mg/Kg makes the lettuce treated with 75% dilution of *U. lactuca* extract non-advisable for human diets [31].

Based on a study by Kim et al. [29], the iron content in our lettuce leaves (treated and non-treated) was lower than the average presented value range from 20.28 mg/Kg to 29.44 mg/Kg (dry weight), while it should be approximately 60 mg/Kg to 200 mg/Kg. This deficiency being common to every treated and non-treated plant, this could be attributed to soil condition or other issues that do not concern the type of treatment applied.

4. Conclusions

This study shows that the use of algae as a biostimulant and bio fertilizer in agriculture is a potentially innovative method that could contribute to the evolution of the world agricultural system that is moving more and more towards sustainability in the practices adopted in every process of the agriculture chain. The obtained results reveal that it is possible to think about an improvement in the production using natural sustainable sources that do not affect the environment.

The present work gives an overview of the production of lettuce without the use of chemical fertilizers and leads to promising results in the cultivation of this vegetable which is used in human diets.

This study can be a part of the sustainable development process, which, in the prospective view of innovation and improvement in this research field, can lead to new approaches and to a new way of developing sustainable agriculture worldwide. However, there is still some necessary assays and new method—mostly to analyze the field trials, such as in abiotic monitoring—because we cannot forget that plants can have direct impacts due to abiotic factors and soil conditions. Another measure that can explain the results is the microbiome and soil changing due to the extracts' properties, although the assay demonstrated that the pH of an extract can be key in stimulating the seed germination in highly conductive soil and liquids. Thus, high pH solutions, like those with *U. latuca*, can be used to enhance seed germination in highly conductive soils.

The research on algal extracts and their use in agriculture will surely be implemented in the near future, with more studies also in the poorest and most disadvantaged areas of

the world; here, increasing production with no negative effect on the environment, and with low-tech practices, could improve the social wellness of societies. The future road in this field is to try to understand how algae extracts affect plant metabolism and interfere in the production of secondary metabolites.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Colla, G.; Hoagland, L.; Ruzzi, M.; Cardarelli, M.; Bonini, P.; Canaguier, R.; Rouphael, Y. Biostimulant action of protein hydrolysates: Unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* **2017**, *8*, 2202. [CrossRef]
2. Savci, S. Investigation of effect of chemical fertilizers on environment. *Apcbee Procedia* **2012**, *1*, 287–292. [CrossRef]
3. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in plant science: A global perspective. *Front. Plant Sci.* **2017**, *7*, 2049. [CrossRef]
4. Kapoore, R.V.; Wood, E.E.; Llewellyn, C.A. Algae biostimulants: A critical look at microalgal biostimulants for sustainable agricultural practices. *Biotechnol. Adv.* **2021**, *49*, 107754. [CrossRef] [PubMed]
5. Rouphael, Y.; Lucini, L.; Miras-Moreno, B.; Colla, G.; Bonini, P.; Cardarelli, M. Metabolomic responses of maize shoots and roots elicited by combinatorial seed treatments with microbial and non-microbial biostimulants. *Front. Microbiol.* **2020**, *11*, 664. [CrossRef] [PubMed]
6. Agenda 2030. Available online: https://sdgs.un.org/2030agenda (accessed on 24 January 2023).
7. Dominguez, H.; Loret, E.P. *Ulva lactuca*, a source of troubles and potential riches. *Mar. Drugs* **2019**, *17*, 357. [CrossRef] [PubMed]
8. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278. [CrossRef]
9. Pacheco, D. *Seaweeds as Plant Health Promoters*; University of Coimbra: Coimbra, Portugal, 2022.
10. Sousa, T.; Nunes, J.P.; Lopes, J.; Cotas, J.; Gonçalves, A.M.M.; Bahcevandziev, K.; Pereira, L. *Seaweed as Plant Biostimulants*; Apple Academic Press: Palm Bay, FL, USA, 2022; pp. 183–200.
11. Shatilov, M.; Razin, A.; Ivanova, M. Analysis of the world lettuce market. In Proceedings of the IOP Conference Series: Earth and Environmental Science, Moscow, Russia, 27 May–6 June 2019; p. 012053.
12. Boriss, H.; Brunke, H. *Commodity Profile: Lettuce*; University of California: San Diego, CA, USA, 2005.
13. Available online: https://www.zipmec.com/la-produzione-di-insalata.html#:~:text=La%20Spagna%20possiede%20il%20primato,315%20milioni%20di%20tonnellate%20prodotte (accessed on 24 January 2023).
14. Rayorath, P.; Jithesh, M.N.; Farid, A.; Khan, W.; Palanisamy, R.; Hankins, S.D.; Critchley, A.T.; Prithiviraj, B. Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, *Arabidopsis thaliana* (L.) Heynh. *J. Appl. Phycol.* **2007**, *20*, 423–429. [CrossRef]
15. Hernández-Herrera, R.M.; Santacruz-Ruvalcaba, F.; Ruiz-López, M.A.; Norrie, J.; Hernández-Carmona, G. Effect of liquid seaweed extracts on growth of tomato seedlings (*Solanum lycopersicum* L.). *J. Appl. Phycol.* **2014**, *26*, 619–628. [CrossRef]
16. Cunniff, P.; Washington, D. Official methods of analysis of aoac international. *J. AOAC Int.* **1997**, *80*, 127A.
17. Angell, A.R.; Mata, L.; de Nys, R.; Paul, N.A. The protein content of seaweeds: A universal nitrogen-to-protein conversion factor of five. *J. Appl. Phycol.* **2016**, *28*, 511–524. [CrossRef]
18. FAO. *Food Energy—Methods of Analysis and Conversion Factors*; FAO: Rome, Italy, 2002.
19. Lucas, M.; Sequeira, E. Determinação do Cu, Zn, Mn, Fe, Ca, Mg, K, e Na totais das plantas por espectrofotometria de absorção atómica e fotometria de chama. *Pedologia* **1976**, *11*, 163–169.
20. Ribas, M.; Veiga, M.; Curto, A.; Oliveira, E.; Barbeitos, M.; Ferreira, M.; Pacheco, C.; Peralta, M.; Duarte, M. Métodos de análise de material vegetal e terras. *Pedologia* **1988**, *11*, 163–169.
21. FAO. Available online: https://www.fao.org/3/y5022e/y5022e04.htm (accessed on 24 January 2023).

22. Hassan, S.; Ghareib, H. Bioactivity of *Ulva lactuca* L. acetone extract on germination and growth of lettuce and tomato plants. *Afr. J. Biotechnol.* **2009**, *8*, 3832–3838.

23. Hajnal-Jafari, T.I.; Đurić, S.S.; Stamenov, D.R. Influence of green algae *Chlorella vulgaris* on initial growth of different agricultural crops. *Zbornik Matice Srpske za Prirodne Nauke* **2016**, *2016*, 29–33. [CrossRef]

24. Canelli, G.; Tarnutzer, C.; Carpine, R.; Neutsch, L.; Bolten, C.J.; Dionisi, F.; Mathys, A. Biochemical and nutritional evaluation of Chlorella and Auxenochlorella biomasses relevant for food application. *Front. Nutr.* **2020**, *7*, 565996. [CrossRef] [PubMed]

25. Tokuşoglu, Ö.; Üunal, M. Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. *J. Food Sci.* **2003**, *68*, 1144–1148. [CrossRef]

26. Rasyid, A. Evaluation of nutritional composition of the dried seaweed *Ulva lactuca* from Pameungpeuk waters, Indonesia. *Trop. Life Sci. Res.* **2017**, *28*, 119. [CrossRef]

27. Yaich, H.; Garna, H.; Besbes, S.; Paquot, M.; Blecker, C.; Attia, H. Chemical composition and functional properties of *Ulva lactuca* seaweed collected in Tunisia. *Food Chem.* **2011**, *128*, 895–901. [CrossRef]

28. Khairy, H.M.; El-Shafay, S.M. Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt. *Oceanologia* **2013**, *55*, 435–452. [CrossRef]

29. Kim, M.J.; Moon, Y.; Tou, J.C.; Mou, B.; Waterland, N.L. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *J. Food Compos. Anal.* **2016**, *49*, 19–34. [CrossRef]

30. Geraldson, C.M.; Tyler, K.B. Plant Analysis as an Aid in Fertilizing Vegetable Crops. In *Soil Testing and Plant Analysis*, 3rd ed.; Westerman, R.L., Ed.; American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America: Hoboken, NJ, USA, 1990; Volume 3.

31. Noulas, C.; Tziouvalekas, M.; Karyotis, T. Zinc in soils, water and food crops. *J. Trace Elem. Med. Biol.* **2018**, *49*, 252–260. [CrossRef] [PubMed]