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Reduced Effect of Commercial Leonardite and Seaweed Extract on Lettuce Growth under Mineral, Organic, and No Fertilization Regimes

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Abstract: In this study, two commercial products based on the main groups of contemporary biostimulants—a commercial leonardite and a seaweed extract—were tested with the objective of assessing the conditions under which they can enhance lettuce (*Lactuca sativa* L.) performance, particularly to determine if synergies with conventional fertilization methods can be observed. The experimental protocol was arranged as a factorial design with two factors: organic or mineral fertilization × plant biostimulant. The organic or mineral fertilization factor included five levels: two rates of a nitrogen (N) fertilizer (40 (Nmin40) and 80 (Nmin80) kg ha^{-1} of N), the same N rates applied as an organic amendment (Norg40 and Norg80), and an unfertilized control (N0). The plant biostimulants used were a commercial leonardite (leonardite) for soil application before planting, a commercial seaweed extract (algae) for foliar application during the growing season, and a control without plant biostimulant. Leonardite significantly increased lettuce dry matter yield (DMY) compared to the control only in the first growing cycle (11.5 and 13.5 g plant^{-1}) and showed no significant interaction with conventional fertilization. It also consistently increased phosphorus (P) levels in the plant tissues. The seaweed extract did not show any effect on the plant, nor did it have any interactions with conventional fertilization regarding DMY. In contrast, with mineral fertilization, lettuce DMY increased from 8.0 and 4.0 g plant^{-1} (N0) to 22.2 and 12.0 g plant^{-1} (Nmin80) in the first and second growing cycles, respectively. The response to organic fertilization was lower, yet DMY still increased from 4.0 to 8.1 g plant^{-1} in the second growing cycle. Generally, this type of plant biostimulant is tested under some form of environmental stress, where it often yields positive results. In this study, the optimal cultivation conditions maintained for the lettuce in the pots likely explain the limited response to the biostimulants. This study suggests that the product labels should more clearly indicate whether they are recommended for general cultivation conditions or specifically for situations where a particular environmental stress can be anticipated.

Keywords: plant biostimulant; *Lactuca sativa*; humic substances; sustainable agriculture

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Citation: Aguiar, P.; Corrêa, G.M.G.; Rodrigues, M.Â.; Arrobas, M. Reduced Effect of Commercial Leonardite and Seaweed Extract on Lettuce Growth under Mineral, Organic, and No Fertilization Regimes. *Agronomy* **2024**, *14*, 1939. https://doi.org/10.3390/agronomy14091939

Academic Editors: Gennaro Gennaro Brunetti, Francesco De Mastro and Małgorzata Szczepanek

Received: 13 July 2024
Revised: 17 August 2024
Accepted: 27 August 2024
Published: 28 August 2024

1. Introduction

Agricultural practices have intensified over the last few decades in the quest to produce more food for a world population that continues to grow rapidly and could surpass 10 billion inhabitants before the end of the 21st century [1]. The Green Revolution, which began after World War II, was based on several pillars, with the most significant being the development of improved crop varieties, the expansion of irrigation, and the widespread use of pesticides and fertilizers [2].

The intensification of agriculture, which has enabled the production of more food and various other products and services while maintaining accessible prices for populations, has diverse secondary effects. These effects include soil degradation, loss of biodiversity, contamination of water and the atmosphere, and disruptions to trophic chains, thereby posing a risk to human health [2,3]. Currently, scholars and politicians recognize the need for continued growth in food production to meet increasing demand. However, they also acknowledge that agricultural practices must undergo changes to achieve sustainable or ecological intensification [4].

Crop fertilization is a key agricultural practice in the sustainable intensification process. The use of mineral fertilizers, particularly N fertilizers, has been proven to enhance crop yields but can also result in negative side effects. The use of N fertilizers may contribute to the degradation of soil fertility, leading to acidification [5] and, under certain conditions, secondary salinization [6]. However, the most detrimental aspects associated with the excessive use of N fertilizers include the risk of water contamination due to nitrate leaching from agricultural fields [7] and the emission of N gases, such as N oxides, into the atmosphere [8].

The use of organic amendments, in turn, can enhance soil quality by increasing organic matter, thereby promoting important physical properties of the soil, such as aeration and water-holding capacity, and providing nutrients more gradually to plants and increasing the biological activity of the soil [9]. However, in many regions of the world, these resources are scarce due to agricultural specialization and the decline in livestock activity. It becomes necessary to turn to other organic amendments, such as composts from agro-industrial activities, municipal solid waste, or other sources [10].

In recent years, there has been a significant increase in the availability of products with a biostimulant effect on plants in the market. In the European Union, legislation has already been established to define and regulate the use of plant biostimulants in agriculture (Regulation (EU) 2019/1009). A plant biostimulant typically does not provide nutrients to plants, or, rather, that is not its primary function. Instead, its main purpose is to enhance the efficiency of processes in the soil or assist plants in coping with biotic and abiotic stresses [11–13]. While they do not substitute for organic amendments and fertilizers, they can enhance the efficiency of their utilization and contribute to a reduction in the required quantity. Among the different groups of plant biostimulants available on the market, commercial products containing humic and fulvic acids, as well as seaweed extracts, are among the most prevalent; the former is mainly designed for soil application, while the latter is primarily intended for use as foliar sprays.

Commercial leonardites are used in agriculture as an important source of humic and fulvic acids [14,15]. They can be used as a solid material for direct application to the soil or as raw material for preparing soluble products for fertigation. Humic substances play a significant role as soil conditioners, improving the soil's physical, chemical, and/or biological properties [13,16,17]. Humic substances based on leonardite have been recommended in agriculture for their ability to directly or indirectly enhance crop productivity, especially when crops are subjected to environmental stress [16–20]. However, in some studies, the effect on soil and crop productivity was not significant [21], and inhibitory effects of humic substances on plant growth have even been reported [22,23]. Furthermore, as these products are agricultural inputs of a high cost, it is necessary to make it even clearer under what specific conditions they can be an asset for producers to establish better guidelines for their use.

Seaweed extracts are obtained from various species, with the most common being *Ascophyllum nodosum* [24–27]. Seaweed extracts are currently agriculture's most widely used group of plant biostimulants. They appear in the market primarily as products for foliar application to crops but can also be applied to soils or in hydroponic solutions [11,28] and sometimes in combinations with other products that also have a biostimulant effect, such as humic substances, chitosan, and/or amino acids [12,27]. Due to their low nutrient content, they act as promoters of nutritional efficiency and help plants to better tolerate environmental stresses, such as salinity, extreme temperatures, nutrient deficiencies, and drought [24,26,29], or in controlling pathogenic biotic agents [12,27,30]. Although most studies have reported benefits in plant nutrition, productivity, and/or crop quality, some studies have not registered any significant effect or have found it to be minimal [31]. This justifies a continued effort in studying the optimization of conditions under which these production factors can be recommended to farmers.

For this study, a factorial design was outlined, where plants received mineral fertilization (two rates), organic fertilization (two rates), or no fertilization, combined with plants that were treated with leonardite, seaweed extract, or received no treatment. Lettuce was used as the test plant in two growing cycles, and oats (*Avena sativa*, cv. Boa Fé) were used at the end to assess the residual effect of fertilization. In the same study, mineral fertilization, organic fertilization, leonardite, and a commercial seaweed extract were integrated into a factorial experiment, aiming to observe potential synergistic effects on lettuce growth among these fertilizing materials—an aspect that has been relatively underexplored in previous research. Thus, we hypothesized that plant biostimulants (leonardite and/or algae extract) can have a measurable synergic effect with mineral fertilizer or organic amendment, increasing plant performance.

2. Materials and Methods

2.1. Characterization of Experimental Conditions

The pot experiment was conducted during May 2023 and February 2024 at Quinta de Santa Apolónia (41.797215, −6.761839; altitude 686 m) in Bragança, Northeastern Portugal. The region has a Mediterranean climate, Csb according to Köppen and Geiger classification, and is characterized by rainy winters and hot, dry summers [32]. The annual average temperature and precipitation are 12.3 °C and 758.3 mm, respectively [32]. The monthly values of average temperature and precipitation, both from the climatological normal and those recorded during the experimental period, are presented in Figure 1.

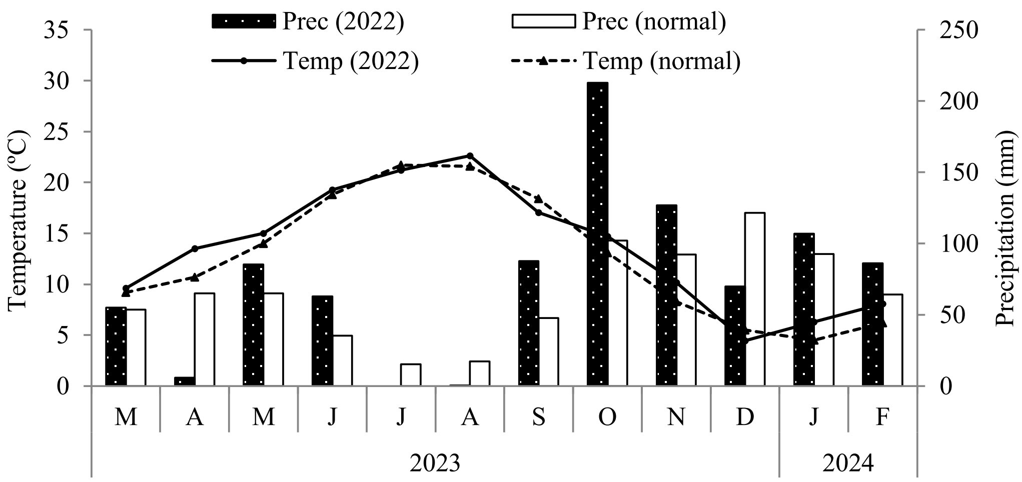

Figure 1. Monthly average temperature and precipitation from the climatological normal 1981–2010 [32] and values recorded at the Santa Apolónia farm meteorological station during the experimental period.

The soil used in this study was collected from the 0–0.20 m layer of a plot that had not been cultivated for the past three years. The soil is classified as eutric Regosol [33] and has a sandy clay loam texture (soil separates: 22.9%, 24.1%, and 53.0% of clay, silt, and sand, respectively). Other soil properties determined from this initial soil sampling are presented in Table 1.

Table 1. Selected soil properties (mean ± standard deviation, n = 3) from samples collected at a depth of 0–0.20 m immediately prior to the beginning of the trial.

Soil Properties		Soil Properties (Cont.)	
pH (H_2O)	6.5 ± 0.07	Boron (mg kg^{-1})	0.5 ± 0.09
Organic carbon (g kg^{-1})	12.0 ± 2.26	Exch. calcium (cmol$_c$ kg^{-1})	9.3 ± 0.48
Total nitrogen (g kg^{-1})	1.8 ± 0.21	Exch. magnesium (cmol$_c$ kg^{-1})	4.5 ± 0.62
Ammonium-N (mg kg^{-1})	6.8 ± 2.59	Exch. potassium (cmol$_c$ kg^{-1})	0.5 ± 0.23
Nitrate-N (mg kg^{-1})	18.8 ± 7.40	Exch. sodium (cmol$_c$ kg^{-1})	0.1 ± 0.01
Extr. phosphorus (P_2O_5) (mg kg^{-1})	150.1 ± 27.27	Exch. acidity (cmol$_c$ kg^{-1})	0.3 ± 0.00
Extr. potassium (K_2O) (mg kg^{-1})	175.3 ± 52.05	Cation exch. capacity (cmol$_c$ kg^{-1})	14.8 ± 1.25

2.2. Experimental Design and Fertilizing Materials

The experimental protocol was arranged as a factorial design with two factors: plant biostimulants × organic or mineral fertilization. The plant biostimulants used were a commercial leonardite for soil application before planting (leonardite), an algae extract for foliar spray application during the growing season (algae), and a control treatment without plant biostimulant application (control). The organic or mineral fertilization included a commercial compost used at rates corresponding to 40 (Norg40) and 80 (Norg80) kg ha^{-1} of N, along with a mineral-N fertilizer applied at rates of 40 (Nmin40) and 80 (Nmin80) kg ha^{-1} of N, and a control treatment without fertilization (N0). The experiment included three replicates per treatment. Thus, this resulted in a total of 45 pots (3 (plant biostimulant) × 5 (organic or mineral fertilization) × 3 (replicates) = 45). Pots with a mean diameter of 0.160 m and a height of 0.135 m were used, each filled with 3 kg of dry soil, sieved through a 2 mm mesh. Each pot received a fraction of 1/140,000 of the fertilizer amount corresponding to the hectare application rate previously mentioned, based on a typical planting density for this crop of 140,000 plants per hectare.

Leonardite (Humitec®, Mirandela, Portugal) has been manufactured from humified forest masses for around 40 million–60 million years. It contains 8% moisture, 21.3% organic carbon (C), 27.6% humic acids, and 9.2% fulvic acids. It was applied at the manufacturer's recommended rate (500 kg ha^{-1}). The rate to be applied to each pot was estimated considering the typical lettuce planting density of 140,000 plants ha^{-1}, with each pot receiving the corresponding fraction for each lettuce (3.57 g pot^{-1}). Algae extract (Fitoalgas Green®, Lisboa Portugal) is a pure extract of *Ascophyllum nodosum* obtained through a cold extraction process. It contains small quantities of macro- and micronutrients, as well as organic compounds such as polyphenols, alginates, and mannitol. It was applied under the conditions specified by the manufacturer (3 kg ha^{-1}), with three applications made during the lettuce's growing cycle, and the doses (21.5 mL lettuce^{-1}) were estimated in the same manner as for the leonardite.

The organic and mineral fertilizers were applied at rates equivalent to 40 and 80 kg ha^{-1} of N. Each pot (lettuce) received a N rate corresponding to a fraction of 140,000 (the number of lettuces per hectare), namely 0.29 and 0.57 g pot^{-1} of N. The organic amendment is derived from composting forestry, agro-industrial, and organic domestic waste and is authorized for organic farming in Portugal. The applied rates of compost were calculated by considering its moisture content and N concentration (Table 2). The doses of compost used were 15.16 and 30.32 g pot^{-1} in the Norg40 and Norg80 treatments, respectively. Ammonium nitrate 27% (50% NH_4^+ and 50% NO_3^-) was used as the mineral-N fertilizer. The fertilizer rates were estimated considering its N concentration (1.06 and 2.12 g in the Nmin40 and Nmin80 treatments, respectively). The organic compost was applied before planting, while the mineral fertilizer was split, with half applied at planting and the other half as a side dressing. The procedures were applied to the two lettuce growing cycles.

Table 2. Composition of the organic compost (mean ± standard deviation, n = 3) immediately before the start of the experiment.

Compost Properties		Compost Properties (Cont.)	
pH (H_2O)	8.2 ± 0.20	Calcium (g kg^{-1})	86.6 ± 18.69
Moisture (%)	9.3 ± 1.70	Magnesium (g kg^{-1})	3.5 ± 0.57
Carbon (g kg^{-1})	345.4 ± 16.96	Boron (mg kg^{-1})	8.3 ± 0.65
Nitrogen (g kg^{-1})	25.0 ± 1.02	Iron (mg kg^{-1})	5116.9 ± 247.32
Carbon/nitrogen ratio	13.8 ± 0.51	Manganese (mg kg^{-1})	130.8 ± 5.42
Phosphorus (g kg^{-1})	5.4 ± 0.99	Zinc (mg kg^{-1})	100.5 ± 5.88
Potassium (g kg^{-1})	16.5 ± 1.36	Copper (mg kg^{-1})	38.7 ± 3.75

2.3. Experimental Setup and Implementation

The study involved two growing cycles of lettuce (cv. Sumner Wonder) and one cycle of oats a period considered sufficient to consolidate the effects of the treatments. Lettuces in each growing cycle were fertilized according to the previously mentioned conditions. Oats were left unfertilized and were included in the study to assess the residual effect of lettuce fertilization. Oats serve as a biological indicator of soil fertility, as plants utilize only a portion of the nutrients provided by fertilizers during the main crop's growth cycle. Fertilizing materials applied at preplant (leonardite, organic compost, and half the rate of mineral fertilizer) were mixed into 3 kg of dry soil (the soil mass used in each pot) before planting in each lettuce growing cycle. The lettuce cycles took place between 5 May and 26 June 2023 and between 25 August and 9 October 2023. The oat cycle occurred from 16 October 2023 to 19 February 2024. Topdress fertilizations were carried out on June 2 and September 25 for the first and second lettuce cycles, respectively. The topdressed fertilizers were not incorporated into the soil. After soil application, irrigation was performed. The algae extract was applied on 26 May and 2 and 9 June for the first lettuce cycle, and on 18 and 25 September and 2 October for the second lettuce cycle. A household sprayer was used for algae extract application.

For lettuce cultivation, seedlings at phenological stage 13 (3rd true leaf unfolded) were used [34]. Harvesting of lettuce was carried out at phenological stage 49 (typical size, form, and firmness of heads reached) [34]. For oat sowing, 15 seeds per pot were used, with the final population adjusted to 10 plants per pot by removing surplus plants. Oats were harvested when plants exhibited stunted growth due to clear nutrient shortage, at phenological stage 31 (first node at least 1 cm above tillering node) [34].

The pots were kept outdoors. Wooden protections were placed on the sides of the pots to prevent those on the south side (Northern Hemisphere) from receiving direct radiation on the pot body causing overheating. Throughout the growing cycle, the pots were randomly rearranged weekly from their original positions to ensure equal exposure to radiation for all. After lettuce planting, weed emergence was monitored, and weeds were manually removed immediately after germination. The pots were watered as needed. Considering that evapotranspiration depends on the size of the lettuce, which varies between treatments, and on environmental conditions (especially temperature and radiation), the pots were watered by daily observing soil moisture and the water status of each lettuce. The aim was to apply water quantities that ensured adequate growth of all lettuces.

2.4. Measurements during the Growing Season

During the growing season, leaf greenness was measured using the portable SPAD (Soil and Plant Analysis Development)-502 Plus chlorophyll meter (Spectrum Technologies, Inc., Aurora, IL, USA), and Chlorophyll a fluorescence was assessed with the OS-30p+ fluorometer (Opti-sciences, Inc., Hudson, NH, USA). The SPAD-502 provides dimensionless readings, proportional to the chlorophyll content of the leaves, by measuring the transmittance of light through the leaves at 650 nm (red light, absorbed by chlorophyll) and 940 nm (infrared light, not absorbed by chlorophyll) [35]. Each mean value was obtained from six individual readings taken from fully expanded young leaves. Chlorophyll a fluorescence was determined using the dark adaptation protocol F_V/F_M with the OS-30p+ fluorometer [36]. F_M and F_V represent maximum and variable fluorescence, respectively, from dark-adapted leaves. Measurements were taken from the youngest fully expanded leaves after a dark adaptation period of more than 35 min. Both measurements were conducted a few days after the last application of the algae extract, on 12 June and 4 October, respectively.

2.5. Harvesting of Plants and Laboratory Determinations

The lettuces were cut at ground level using a knife. Using a sharp-edged round puncher, slices with a diameter of 0.03 m were cut from young fully expanded leaves and were then placed in a Falcon tube that was hermetically sealed and placed in a thermal bag. In the laboratory, fresh and dry weights (at 70 °C) of the lettuce slices and Falcon tubes were obtained to determine the dry mass of the sample. The main lettuce sample was also oven-dried at 70 °C until constant weight. This allowed for the determination of the dry matter of the lettuce and its total leaf area, calculated from the area of the slice and the dry mass of both the slice and the entire lettuce. The oats were also cut at ground level and oven-dried at 70 °C until constant weight was reached.

The dried lettuce samples were used for elemental composition determination. Only the N concentration was determined in the dried oat samples to assess the residual effect of lettuce fertilization, as the oats themselves were not fertilized. N was determined by the Kjeldahl method, while boron (B) and P were determined by colorimetry, and potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) were determined by atomic absorption spectrophotometry [37] after digesting the samples with nitric acid in a microwave oven. Nitrates in lettuce were determined by placing 1 g of tissue in 50 mL of distilled water, followed by 1-h agitation and filtration with Whatman No. 42 filter paper. Nitrate concentrations in the extracts were analyzed by UV–Vis spectrophotometry [38].

After the second lettuce growing cycle, a soil sample of approximately 50 g per pot was collected following thorough homogenization of all soil within the pot. This sample was oven-dried at 40 °C and used to assess the effect of treatments on soil mineral N and various other properties. The remaining soil was then placed back into the pots to initiate the oat growing cycle.

The soil samples were analyzed for pH (H_2O) (soil/solution, 1:2.5), organic C (dichromate oxidation), total N (Kjeldahl method), extractable P and K (ammonium lactate solution at pH 3.7), cation-exchange capacity (ammonium acetate at pH 7.0), and extractable B (hot water and azomethine-H) [39]. The availability of cationic metals (Cu, Fe, Zn, and Mn) in the soil was determined by atomic absorption spectrometry after extraction with diethylenetriaminepentaacetic acid (DTPA) buffered at pH 7.3, following the standard procedure of FAO [40]. Nitrate and ammonium concentrations in soil solution were determined in a solution prepared from 20 g of soil and 40 mL of 2 M KCl, following 1-h agitation and filtration through Whatman No. 42 filter paper. The ions in solution were analyzed using a UV–Vis spectrophotometer [38]. Soil separates were determined in initial soil samples using the Robinson pipette method [39].

2.6. Data Analysis

The data analysis was conducted using the statistical software SPSS Statistics (version 25, IBM SPSS, Armonk, NY, USA). Normality and homogeneity of variances for the data were initially assessed using the Shapiro–Wilk test and Levene's test, respectively. The effects of the treatments were compared using a two-way ANOVA. When significant differences were found ($p < 0.05$), means were separated using the Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1. Dry Matter Yield of Lettuce and Leaf Surface Area

In the first lettuce growing cycle, significant differences were observed in DMY among the plant biostimulant treatments, with pots receiving leonardite displaying higher values compared to the control treatment (Figure 2). However, this result was not replicated in the second year, as no significant differences were recorded between treatments. Mineral fertilization markedly influenced the results, with significant differences observed between Nmin40 (17.2 and 9.7 g pot^{-1} in the first and second growing cycles, respectively) and N0 (8.0, 4.0 g pot^{-1}) and between Nmin80 (22.2, 12.0 g pot^{-1}) and Nmin40. The effect of organic amendments was less pronounced. In the first growing cycle, no significant differences were observed between Norg40 or Norg80 treatments and the N0 treatment. However, in the second cycle, DMY was significantly higher in Norg80 (8.1 g pot^{-1}), followed by Norg40 (5.8 g pot^{-1}) and, finally, N0 (4.0 g pot^{-1}).

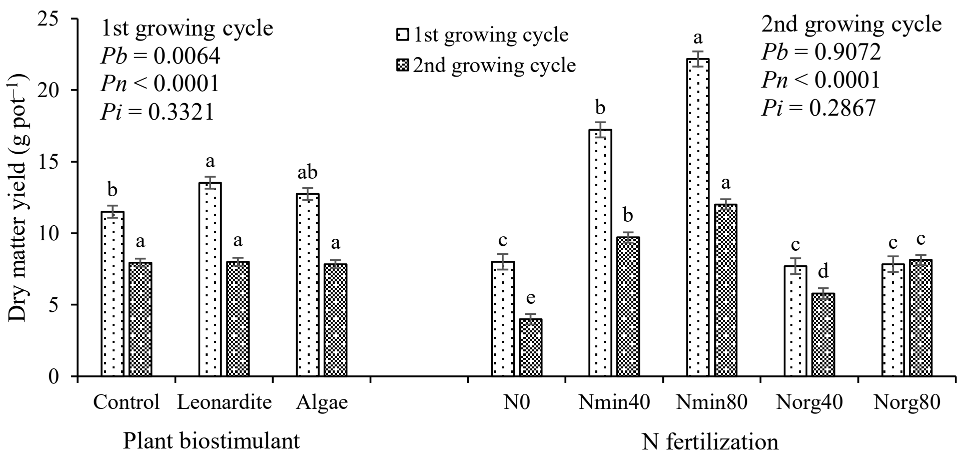

Figure 2. Dry matter yield in the first and second cycles of lettuce as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively. Within plant biostimulant, N fertilization, and growing cycle, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05). Error bars indicate standard errors.

The results for leaf area mirrored, to some extent, the results for DMY (Figure 3). Significant differences were observed among plant biostimulants in the first growing cycle, with the leonardite treatment resulting in significantly higher values compared to the control treatment. Regarding the application of mineral and organic N, the mean values followed a similar pattern, with significant differences in both cycles for the mineral fertilizer rates and only in the second cycle for the organic amendment rates.

3.2. SPAD-Readings, Chlorophyll Fluorescence Variables, and Nitrogen Nutritional Status of Lettuce Plants

The SPAD-readings differed significantly among plant biostimulants only in the first growing cycle, with the algae treatment showing lower values than the control treatment (Table 3). Comparing different rates of mineral fertilizer, significant differences were observed between Nmin40 and Nmin80 and the N0 treatment in both growing cycles. Additionally, significant differences were found between Nmin80 and Nmin40 in the first growing cycle. The values observed in the organic amendment treatments did not differ significantly from the values observed in the N0 treatment in either growing cycle. Within the plant biostimulant factor, mean values ranged from 26.7 to 28.2 and from 27.3 to 28.1 in the first and second growing cycles, respectively. Within fertilization treatments, mean values ranged from 23.5 to 35.5 and from 22.5 to 25.3 in the first and second growing cycles, respectively.

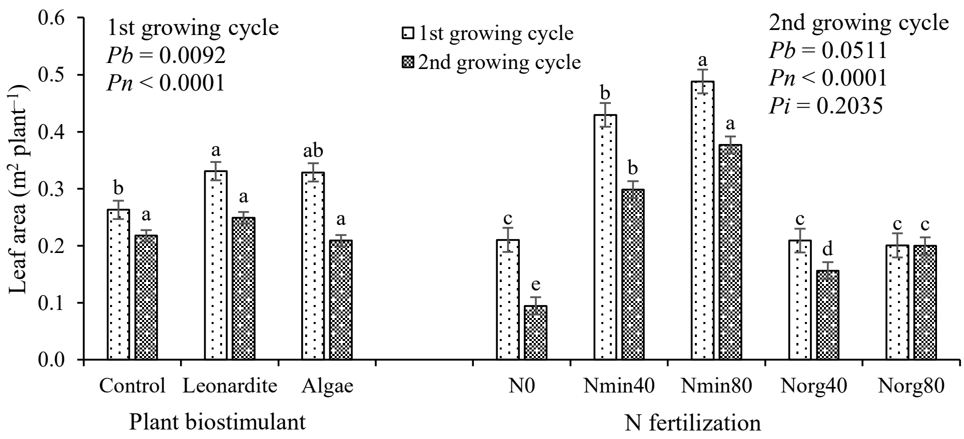

Figure 3. Total leaf area in the first and second cycles of lettuce as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively. Within plant biostimulant, N fertilization, and growing cycle, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05). Error bars indicate standard errors.

Table 3. SPAD-readings and F_V/F_M (ratio of variable fluorescence/maximum fluorescence) in the first and second growing cycles (GCs) of lettuce as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	SPAD Readings		**F_V/F_M**	
	1st GC	**2nd GC**	**1st GC**	**2nd GC**
Control	28.2 a	28.1 a	0.815 a	0.799 a
Leonardite	27.2 ab	27.3 a	0.818 a	0.801 a
Algae	26.7 b	28.1 a	0.812 a	0.803 a
N0	23.8 c	22.7 b	0.809 a	0.772 b
Nmin40	29.8 b	34.1 a	0.818 a	0.816 a
Nmin80	35.5 a	35.3 a	0.823 a	0.820 a
Norg40	24.1 c	22.5 b	0.812 a	0.796 ab
Norg80	23.5 c	24.6 b	0.814 a	0.802 ab
Pb	0.0353	0.4076	0.5121	0.9283
Pn	<0.0001	<0.0001	0.1954	0.0135
Pi	0.1607	0.3436	0.0700	0.5845

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

In the first growing cycle, F_V/F_M did not vary significantly with the effect of plant biostimulants or fertilization treatments (Table 1). Average values varied from 0.809 and 0.823. In the second growing cycle, significant differences occurred only between the mineral fertilizer treatments and the control. In the N0 treatment, the average value was 0.772, and in the mineral fertilizer treatments, the average values were 0.816 and 0.820 in the Nmin40 and Nmin80 treatments, respectively.

The application of leonardite and algae extract did not significantly influence the N concentration in lettuce tissues in either the first or second growing cycles (Table 4). The average values in the first growing cycle varied between 13.7 and 14.3 g kg^{-1}. In the second growing cycle, the average values were higher, ranging from 17.6 to 18.8 g kg^{-1}.

In contrast, mineral fertilization significantly influenced N concentration in the tissues in both cycles. The mean values for the Nmin80 treatment (18.4 and 27.5 g kg^{-1} in the first and second cycles, respectively) were significantly higher than those for the Nmin40 treatment (15.1 and 23.9 g kg^{-1}), and these were significantly higher than those for the N0 (11.3 and 12.7 g kg^{-1}). Organic amendment treatments resulted in significant differences in N concentration in the tissues compared to the N0 treatment only in the first growing cycle.

Table 4. Tissue N concentration, N recovery, and tissue nitrate concentration in the first and second growing cycles (GCs) of lettuce as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	Tissue N (g kg^{-1})		**N Recovery (g pot^{-1})**		**Tissue Nitrate (g kg^{-1})**	
	1st GC	**2nd GC**	**1st GC**	**2nd GC**	**1st GC**	**2nd GC**
Control	13.7 a	18.8 a	171.6 b	165.9 a	11.4 b	16.2 ab
Leonardite	13.9 a	17.6 a	204.2 a	155.2 a	11.1 b	14.5 b
Algae	14.3 a	18.4 a	194.5 a	158.1 a	12.5 a	17.1 a
N0	11.3 d	12.7 c	90.5 c	50.3 d	10.4 bc	7.5 c
Nmin40	15.1 b	23.9 b	259.7 b	230.5 b	11.2 b	19.9 b
Nmin80	18.4 a	27.5 a	406.2 a	328.4 a	16.2 a	34.2 a
Norg40	12.4 c	13.2 c	94.8 c	76.3 cd	10.1 c	9.0 c
Norg80	12.7 c	13.9 c	99.2 c	113.2 c	10.4 bc	9.2 c
Pb	0.0560	0.1317	0.0001	0.5438	<0.0001	0.0352
Pn	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Pi	0.0511	0.0004	0.0262	0.9193	<0.0001	0.0155

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

The N recovered in the tissues was significantly lower in the control treatment (171.6 g pot^{-1}) compared to the leonardite (204.2 g pot^{-1}) and algae (194.5 g pot^{-1}) treatments in the first growing cycle, but no significant differences among treatments were observed in the second cycle (values ranging from 155.2 and 165.9 g pot^{-1}) (Table 4). The treatments with mineral fertilization maintained a strong response pattern, proportional to the fertilizer rate applied, in both the first and second growing cycles. The application of organic amendment had a modest effect, with significant differences observed only in the second growing cycle between the Norg80 treatment and the N0 treatment.

The nitrate concentration in the lettuce tissues showed a tendency for higher values in the algae treatment for the plant biostimulant factor (Table 4). The response to mineral and organic fertilizers followed the trend of N concentration in the tissues and N recovery, with a strong response to mineral fertilizer and a lesser response to organic amendment.

3.3. Concentration of Other Macro- and Micronutrients in Plant Tissues

In the first growing cycle, the P concentration in the tissues was significantly higher in lettuce plants that received leonardite compared to those in the control treatment (Table 5). Treatments with mineral fertilizer showed lower P levels in the tissues compared to organic amendments and the N0 treatment. The K levels in the tissues did not vary among treatments of the plant biostimulant factor and were significantly lower in the treatments with mineral N compared to the other treatments. The Ca levels in lettuce tissues were significantly lower in the leonardite treatment compared to the algae treatment. Within the fertilization factor, values were higher in the N0 treatment and lower in the treatments that received mineral fertilizer. The M levels in the tissues did not vary significantly with the plant biostimulant and were significantly lower in the Norg80 treatment compared to the other fertilization treatments. The B levels in the tissues were significantly higher in the algae treatment compared to the control and significantly lower in the treatments with mineral N compared to organic amendments and the N0 treatment. Tissue concentrations of

Fe, Zn, and Cu tended to be higher in the leonardite treatment compared to the control and algae treatments. The Fe levels in the tissues tended to be higher in the organic amendment treatments, while Mn and Zn levels tended to be higher in the mineral N treatments.

Table 5. The concentration of macro- and micronutrients in lettuce tissues in the first growing cycle as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
	g kg^{-1}				mg kg^{-1}				
Control	2.0 b	33.9 a	6.3 ab	2.4 a	27.7 b	419.7 b	50.6 ab	29.8 a	6.6 b
Leonardite	2.3 a	35.8 a	6.0 b	2.2 a	28.8 ab	698.4 a	55.3 a	32.8 a	9.0 a
Algae	2.2 ab	35.3 a	6.7 a	2.3 a	30.8 a	443.6 b	50.1 b	31.8 a	7.4 b
N0	2.4 a	39.0 a	7.1 a	2.4 a	30.6 a	506.3 b	47.6 c	31.0 bc	7.2 a
Nmin40	1.8 b	32.0 b	5.8 c	2.3 a	26.4 b	441.4 b	61.4 b	34.2 b	7.9 a
Nmin80	1.8 b	25.9 c	5.4 c	2.4 a	23.5 b	300.3 c	72.7 a	44.8 a	7.6 a
Norg40	2.3 a	37.9 a	6.9 ab	2.3 a	32.2 a	816.7 a	42.3 cd	26.8 cd	7.9 a
Norg80	2.5 a	40.2 a	6.5 b	2.0 b	32.8 a	538.1 b	35.8 d	20.5 d	7.6 a
Pb	0.0159	0.1943	0.0004	0.2597	0.0033	<0.0001	0.0339	0.2424	<0.0001
Pn	<0.0001	<0.0001	<0.0001	0.0015	<0.0001	<0.0001	<0.0001	<0.0001	0.6593
Pi	0.0716	0.0006	0.1326	0.4935	0.2580	<0.0001	0.1531	0.1575	0.0008

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

In the second growing cycle, the P concentrations in the tissues remained consistent with those of the first cycle, showing elevated values in the leonardite treatment for the plant biostimulant factor and in the organic amendment treatments for the fertilization factor (Table 6). The K and Ca levels also showed consistency with those of the first cycle for the fertilization factor, with values tending to be lower in the treatments receiving mineral N. The B levels in the tissues were also significantly lower in the Nmin40 and Nmin80 treatments compared to the others, consistent with the results from the first growing cycle. The Fe levels in the tissues were higher in the leonardite treatment compared to the control and algae treatments, consistent with the first growing cycle results. There were no significant differences among fertilization treatments regarding the Fe levels. The Mn and Zn levels tended to be higher in the mineral fertilization treatments, consistent with the first growing cycle results. For some variables, significant interactions were observed, but these interactions mostly did not align with what was observed in the first growing cycle.

3.4. Residual Effect of Lettuce Fertilization Treatments on Oat Growth and Soil Properties

The application of leonardite to the pots and foliar sprays with algae extract on the plants did not significantly influence oat DMY, N concentration in the tissues, and N recovery (Table 7). Mineral fertilization significantly influenced DMY and N recovery, depending on the applied rate, but did not affect N concentration in the tissues. Organic amendment, applied at any of the rates, resulted in significantly higher oat DMY, N concentration in the tissues, and N recovery compared to the N0 treatment. In terms of N recovery, the Norg80 treatment exhibited a significantly higher value compared to the Norg40 treatment.

The total inorganic N in the soil at the end of the lettuce growing cycle did not vary significantly among treatments of the plant biostimulant factor, although significant differences were observed for each of the inorganic N forms (nitrate and ammonium) in the soil (Figure 4). The application of mineral N, especially at the higher rate (Nmin80), significantly increased the nitrate, ammonium, and total inorganic N content in the soil, whereas organic amendment application did not result in significantly different values compared to the N0 treatment. Soil mineral N levels showed significant interaction between treatments of the plant biostimulant factor and the N fertilization factor.

Table 6. The concentration of macro- and micronutrients in lettuce tissues in the second growing cycle as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
		g kg^{-1}					mg kg^{-1}		
Control	2.2 ab	35.7 a	6.1 a	2.8 a	25.5 a	449.6 b	55.5 a	52.5 a	9.1 a
Leonardite	2.3 a	37.7 a	6.2 a	2.8 a	26.0 a	801.5 a	53.6 a	54.1 a	10.1 a
Algae	2.1 b	37.8 a	6.1 a	2.5 a	26.2 a	476.6 ab	54.1 a	46.4 a	8.8 a
N0	2.1 b	36.8 bc	7.1 a	2.7 a	29.2 a	575.8 a	48.5 b	43.0 b	8.2 a
Nmin40	1.9 b	32.9 c	5.7 b	2.8 a	22.3 b	358.2 a	54.3 b	50.8 ab	10.2 a
Nmin80	1.9 b	34.4 c	5.7 b	2.8 a	23.5 b	382.5 a	67.4 a	69.6 a	10.3 a
Norg40	2.6 a	40.5 ab	6.4 ab	2.6 a	28.0 a	801.3 a	53.8 b	48.1 b	9.4 a
Norg80	2.6 a	40.7 a	5.8 b	2.6 a	26.5 a	761.7 a	48.1 b	43.4 b	8.5 a
Pb	0.0062	0.0882	0.9221	0.0677	0.6231	0.0298	0.8164	0.3399	0.1675
Pn	<0.0001	<0.0001	0.0001	0.2701	<0.0001	0.0537	0.0002	0.0034	0.1102
Pi	<0.0001	0.0221	0.3447	0.2553	0.2602	0.4767	0.1620	0.1157	0.4823

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

Table 7. Dry matter yield (DMY), tissue nitrogen (N), and N recovery in oats as a function of the factorial: plant biostimulant (control, leonardite, and algae extract); and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	DMY	Tissue N	N Recovery
	g pot^{-1}	g kg^{-1}	mg pot^{-1}
Control	2.2 a	15.1 a	33.8 a
Leonardite	2.2 a	15.5 a	34.0 a
Algae	2.2 a	15.6 a	33.5 a
N0	1.3 c	13.4 b	16.8 c
Nmin40	2.2 b	14.4 b	31.1 b
Nmin80	3.3 a	14.7 b	48.7 a
Norg40	1.8 b	16.6 a	30.5 b
Norg80	2.3 b	18.0 a	41.9 a
Pb	0.9509	0.5596	0.9767
Pn	<0.0001	<0.0001	<0.0001
Pi	0.0255	0.1262	0.0237

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

The two-way ANOVA applied to soil properties often showed significant interaction between the plant biostimulant and N fertilization factors (Table 8). Additionally, some soil properties were significantly influenced by the plant biostimulant factor, notably pH, which was significantly lower in the leonardite treatment compared to the others, and extractable P, which was lower in the algae treatment. Conversely, soil B levels were significantly higher in the leonardite treatment compared to the other treatments. The application of organic amendment tended to increase several soil properties, including organic C, pH, extractable P, exchangeable Mg, K, sodium (Na), and extractable B, often with significant differences observed compared to mineral N and the N0 treatments, especially when the organic amendment was applied at the higher rate (Norg80).

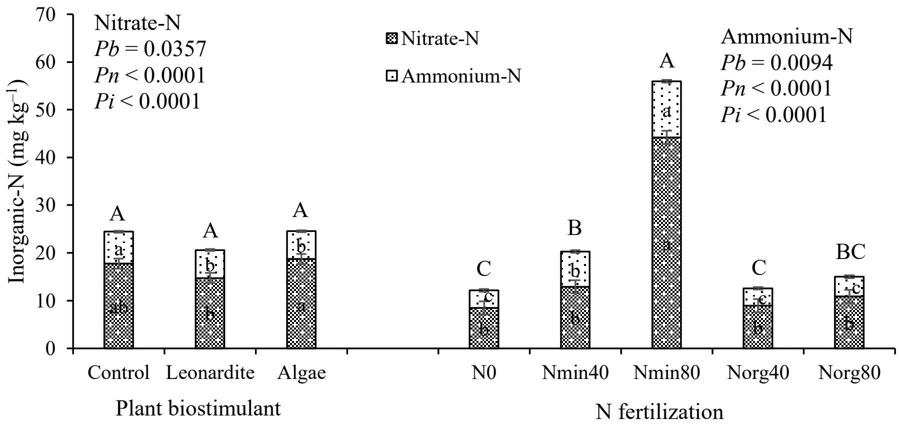

Figure 4. Nitrate and ammonium ions in the soil at the end of the second growing cycle of lettuce as a function of the factorial: plant biostimulant (control, leonardite, and algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40 and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively. Within plant biostimulant and N fertilization and separated into nitrate-N and ammonium-N (lowercase) and total (uppercase), means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05). Error bars indicate standard errors.

Table 8. Organic carbon (OC), pH(H_2O), extractable phosphorus (P), exchangeable calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), cation-exchange capacity (CEC), and extractable boron (B) in the soil as a function of the factorial: plant biostimulant (control, leonardite, algae extract) and N fertilization (mineral (min) and organic (org) applied at 0, 40, and 80 kg ha^{-1} of N). *Pb*, *Pn*, and *Pi* represent the probability of the two-way ANOVA associated with plant biostimulant, N fertilization, and interaction, respectively.

	OC		**P (P_2O_5)**	**Ca**	**Mg**	**K**	**Na**	**CTC**	**B**
	g kg^{-1}	**pH**	**mg kg^{-1}**			**$cmol_c$ kg^{-1}**			**mg kg^{-1}**
Control	8.82 a	6.60 a	157.7 ab	14.00 a	4.56 a	0.41 a	0.20 a	19.17 a	0.36 b
Leonardite	9.21 a	6.39 b	146.5 b	13.37 a	4.64 a	0.45 a	0.18 a	18.65 a	0.53 a
Algae	9.00 a	6.54 a	166.2 a	14.14 a	4.51 a	0.46 a	0.20 a	19.30 a	0.39 b
N0	8.52 bc	6.50 bc	110.4 c	14.19 a	4.49 b	0.32 c	0.17 c	19.17 a	0.26 c
Nmin40	8.06 c	6.45 c	83.0 d	13.73 a	4.39 b	0.25 c	0.15 c	18.54 a	0.28 c
Nmin80	8.91 bc	6.11 d	91.8 cd	13.97 a	4.43 b	0.23 c	0.14 c	18.78 a	0.36 c
Norg40	9.50 ab	6.63 b	199.4 b	13.36 a	4.63 ab	0.54 b	0.22 b	18.78 a	0.49 b
Norg80	10.06 a	6.88 a	299.4 a	13.91 a	4.91 a	0.85 a	0.27 a	19.93 a	0.75 a
Pb	0.4557	<0.0001	0.0180	0.0731	0.2971	0.1986	0.3527	0.4101	<0.0001
Pn	0.0002	<0.0001	<0.0001	0.4390	0.0003	<0.0001	<0.0001	0.1068	<0.0001
Pi	0.0083	0.0018	0.0176	0.6042	0.0298	0.4605	0.0611	0.3973	0.0007

In the columns, separated by the factors plant biostimulant and N fertilization, means followed by the same letter are not significantly different according to the Tukey HSD test (α = 0.05).

4. Discussion

4.1. Soil Applied Leonardite

The application of leonardite significantly increased DMY and total leaf area compared to the control during the first growing cycle of lettuce. Although leonardite application did not enhance N concentration in the tissues, it did increase N recovery in lettuce due to the boosted DMY. However, these effects were inconsistent in the second cycle, as the leonardite did not exhibit significant differences compared to the control treatment. Leonardite is advocated in agriculture primarily for its richness in humic and fulvic acids [14]. Due to its relatively low cost, leonardite has become an important raw material in the production of many commercial products enriched with humic substances [14]. Humic substances are considered a vital group within the category of plant biostimulants [11,14]. Their use is generally expected to enhance plants' ability to cope with various environmental stresses and improve nutrient use efficiency. In recent years, numerous studies have reported positive effects on crop yield from the application of humic substances [15,17,41–43]. However, some studies have also reported no significant positive effects [21] or even negative effects on plant productivity following the application of humic substances [22,23]. One of the central issues in using humic substances is their interaction with native organic matter, microorganisms, and plant roots, as such an interaction influences soil properties and nutrient cycling and ultimately determines plant performance in an unpredictable manner [11,18,19]. Furthermore, humic substances are complex mixtures of biomolecules containing sugars, fatty acids, polypeptides, aliphatic chains, and aromatic rings, thus making their mode of action challenging to elucidate [44,45]. Consequently, while various modes of action have been proposed to interpret their effects, such as involvement in metabolic and signaling pathways in plant development [17,46], increased activity of plant development-related enzymes [42,47], or upregulation of genes related to plant hormonal responses [41,48], it is generally agreed that the mechanisms through which humic substances exert biostimulatory effects on plants remain largely unknown [11,44,49].

In this study, the application of leonardite reduced the soil pH but consistently increased the P concentration in plant tissues. P tends to be less available to plants in acid soils, where it precipitates as $AlPO_4$ and $FePO_4$ [2]. However, the positive effect of using humic substances on crop growth and yield can also be attributed to improvements in nutrient use efficiency [11,18,19], and sometimes to enhanced P nutrition [18,19]. The positive effect on P nutrition may be due to a stimulation of microbial activity that is occasionally observed [13,15], or because humic substances can compete with P for soil adsorption sites, thereby releasing the nutrient for easier absorption by plants [18,19].

Applying leonardite tended to increase the concentration of micronutrient cations such as Fe and Mn in plant tissues. Few studies have reported soil pH variation due to leonardite application, possibly because of the typically low quantities applied. Regarding Fe and Mn, previous studies have shown increased levels of available Fe and Mn in the soil [50], but also contradictory effects to those presented here, reporting immobilization of trace metals due to leonardites' oxidized functional groups that bind metal ions [16,17]. In this study, the pH reduction may be attributed to enhanced biological activity, which increases the mineralization of organic substrates and can lead to soil acidification, as nitrification is generally an acidifying process [2]. This hypothesis is supported by the increased N recovery in plant tissues during the first lettuce growing cycle, potentially resulting from enhanced mineralization of native soil organic matter. The elevated levels of Fe and Mn may be attributed to soil acidification, which increases the solubility of these metals [2,51], possibly overshadowing the effects of the oxidized functional groups binding metal ions.

4.2. Foliar Spray of Algae Extract

The seaweed extract did not demonstrate a measurable positive effect on DMY, or the mineral composition of lettuce tissues compared to the control. Although there was a trend towards higher nitrate levels in the tissues, no other significant effects on plant growth or soil properties were observed. Seaweed extracts have become prominent commercial products used as plant biostimulants [11,24,25]. Their popularity stems from a long tradition dating back to ancient times when seaweeds were utilized in coastal agriculture [24,52]. The introduction of seaweed extracts to the market has facilitated their widespread use without territorial restrictions. Studies employing these biostimulants frequently report positive effects on crop productivity and/or the quality of agricultural products [30,53]. These beneficial outcomes are often attributed to the protective effects they provide plants against biotic [12,27] or abiotic [26,31] stresses.

Seaweed extracts are complex products containing macro- and micronutrients, albeit in small quantities, as well as phytohormones such as cytokinins, auxins, and abscisic acid; polysaccharides; fatty acids; phenolics; and a diverse array of other compounds [54,55]. Many of these compounds are known to have positive effects on plants and often form the basis for observed beneficial outcomes. However, the complexity of seaweed extract composition poses challenges in establishing clear cause-and-effect relationships or fully understanding their mode of action [11,25,55]. While positive results from seaweed extracts are frequently reported, numerous studies, particularly from field trials, have not observed such benefits [31,56].

Positive outcomes are more commonly observed under environmental stresses such as salinity, drought, or heat [12,27,57]. Conversely, the absence of significant environmental stress has been used to explain situations where no evident benefit to plants from seaweed extract application was observed [31]. In this study, where lettuce was grown in carefully managed pots under controlled conditions, this may also explain the lack of response to the plant biostimulant application. This result indicates that further research is needed to more precisely establish the conditions for the use of plant biostimulants, ensuring that their application consistently yields regular and beneficial outcomes for farmers.

4.3. Inorganic Nitrogen Fertilization

The N applied to the soil had a pronounced effect on DMY and the total leaf area of the lettuce. The average values for the N80 treatment were significantly higher than those for the N40 treatment, and the values for the N40 treatment were higher than those for the N0 treatment. Additionally, N concentration, N recovery, and nitrate levels in the tissues also increased significantly with higher N rates. N is often the most limiting nutrient in agricultural soils [2,58]. This limitation, combined with its essential role in numerous organic structures within cells, including proteins, nucleic acids, chlorophylls, and secondary metabolites, typically leads to robust plant responses when applied in sufficient quantities [59]. In pot experiments, these responses are often more pronounced due to the limited soil volume available for root expansion [10,21]. The consistent relationship observed between the DMY and N concentration in the tissues underscores N as the primary determinant of productivity in this study.

The SPAD readings and F_V/F_M ratio, while showing a trend towards higher values with increased N rates, exhibited lower sensitivity to nutrient application compared to DMY and the plant nutritional status indices evaluated through N and nitrate concentration in the tissues. SPAD-502 measures the greenness of leaves, providing an indirect estimate of chlorophyll content, which correlates with N concentration in leaves [2,60]. Despite being a popular index of plant nutritional status due to its simplicity and low cost [60,61], its sensitivity was lower than conventional laboratory analyses. The F_V/F_M ratio provides an indication of photoinhibition or other injuries affecting the photosystem II (PSII) complexes [62], with values dropping below 0.78 under environmental stress impacting its function [36,63]. While inadequate plant nutritional status can affect PSII function [36,62], the maximum quantum efficiency of PSII values observed in this study suggested a weak response of the photochemical reactions of photosynthesis to the experimental treatments.

The application of N to the soil has a residual effect on oat yield and its N nutritional status. Despite N applied in the form of ammonium nitrate being theoretically fully available to plants, nutrient use efficiency, as assessed by the percentage of N recovered, tends to be low [2,64]. This is primarily due to the high risk of losses through denitrification [8] since leaching was prevented in this study by trays at the bottom of the pots. Additionally, N applied to the soil can undergo temporary biological immobilization due to competition for resources by soil microorganisms and NH_4^+ fixation in clay minerals, where the cation contributes to charge neutrality through isomorphic substitution of silicon with aluminum, providing structural stability to clay minerals [2]. However, these forms of N immobilization are temporary, and the nutrient can later be utilized by plants [2]. These dynamics in N cycling within the soil may explain the positive residual effect observed in oats from N previously applied to lettuce in the two preceding cropping cycles.

The application of mineral N to the soil tended to decrease the concentration of other nutrients in plant tissues, consistently reducing P, K, Ca, and B levels. While increased availability of a specific nutrient in the soil can interact with other nutrients in various ways, such as through synergistic or antagonistic effects on absorption [2], in this study, the primary cause appears to be a concentration/dilution effect. That is, the increased availability of N in the soil led to increased biomass production, which resulted in a dilution effect on other nutrients because their absorption cannot increase as much as N absorption since they were not applied. This phenomenon of concentration/dilution is well-documented in the literature [58] and occurs when an environmental factor influences biomass production without affecting the availability of a specific nutrient in the medium. This effect has been observed in recent studies, particularly in pot experiments [21,64], accentuated by the restricted root expansion typical of pot cultivation.

The decrease in pH was the most significant effect of mineral N application on soil properties. N fertilizers containing ammonium ions can induce reactions that lead to pH reduction, as documented in the literature [2,5]. Additionally, Mn levels were increased in lettuce leaves, a result that contrasts with the dilution effect observed for other nutrients. It is plausible that soil acidification increased Mn solubility [49], thereby enhancing its availability in the soil and its concentration in lettuce tissue.

4.4. Organic Amendment Application

The application of organic amendment did not significantly influence DMY or total leaf area in the first lettuce growing cycle. Similarly, other variables associated with plant photosynthetic performance, such as F_V/F_M and N nutritional indices, did not show significant differences compared to the control during this growing cycle. However, significant differences in DMY emerged in the second lettuce growing cycle for the control treatment, which became more pronounced during oat cultivation. Organic amendments release their nutrients gradually as they undergo mineralization by soil heterotrophic microorganisms [2,65]. The rate at which the mineralization/biological immobilization process occurs depends on the material's decomposition nature and particularly its N concentration or C/N ratio [65]. The composted material used in this study exhibited low reactivity, despite its relatively low C/N ratio (C/N ratio of 13.8). Typically, net mineralization tends to occur when the C/N ratio is below 20:1 [2,65]. However, the short growing season of lettuce also helps explain the lack of response to organic amendment in the first growing cycle. As typical of the mineralization/biological immobilization process, nutrient availability increased over time, becoming more pronounced during the oat growing cycle.

Some important nutrients, such as P and K, tended to increase in plant tissues. This result can be attributed to the organic amendment supplying these nutrients without significant dilution effect, as it had a limited impact on DMY. Additionally, there was a notable trend towards increased soil organic matter content, pH, P, and K levels, directly influenced by the application of the amendment, which contains these nutrients and has a higher pH compared to the soil. These findings align with those of a previous study using similar organic amendments [10].

5. Conclusions

The application of leonardite significantly influenced lettuce DMY in only one of the growing cycles. The most consistent result appeared to be an increase in the P concentration in the tissues. However, this outcome seems underwhelming given that leonardite is a commercial product expected to provide economic returns to farmers upon application.

The seaweed extract did not influence lettuce growth compared to the control treatment. This outcome can be justified by the optimal growth conditions maintained for the lettuce plants, as positive effects of seaweed extract application are more commonly observed under environmental stress conditions.

Inorganic N fertilization had a striking effect on lettuce DMY, highlighting the importance of N as a limiting ecological factor in crop productivity.

The organic amendment exhibited typical slow-release behavior, showing a significant effect on lettuce DMY only in the second growing cycle, with its residual value becoming more pronounced during oat cultivation.

This study was designed to investigate potential synergies between mineral and organic fertilization and two types of commercial biostimulants, leonardite and a seaweed extract, in lettuce growth. However, the primary finding may be the clear indication that both companies and academics need to make a greater effort to establish the conditions under which plant biostimulants should be applied, specifically whether their use can be generalized regardless of the cultivated species and growing conditions, or if their application should be tailored to help plants respond to environmental stresses.

Author Contributions: P.A., investigation, data curation, and writing—original draft preparation; G.M.G.C., investigation and writing—review and editing; M.A., funding acquisition, methodology, supervision, review, and editing; M.Â.R., conceptualization, funding acquisition, project administration, data curation, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support from national funds FCT/MCTES (PIDDAC), to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2020), and for the Doctoral scholarship PRT/BD/154361/2023 to Peltier Rossi Lino de Aguiar.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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

References

1. United Nations. Population. 2023. Available online: https://www.un.org/en/global-issues/population (accessed on 6 May 2024).
2. Weil, R.R.; Brady, N.C. *Nature and Properties of Soils*, 15th ed.; Pearson: London, UK, 2017.
3. Hakeem, K.R.; Dar, G.H.; Mehmood, M.A.; Bhat, R.A. *Microbiota and Biofertilizers: A Sustainable Continuum for Plant and Soil Health*, 1st ed.; Springer International Publishing: Berlin/Heidelberg, Germany, 2021.
4. Ajibade, S.; Simon, B.; Gulyas, M.; Balint, C. Sustainable intensification of agriculture as a tool to promote food security: A bibliometric analysis. *Front. Sustain. Food Syst.* **2023**, *7*, 1101528. [CrossRef]
5. Sparks, D.L.; Singh, B.; Siebecker, M.G. The chemistry of soil acidity. In *Environmental Soil Chemistry*, 3rd ed.; Sparks, D.L., Singh, B., Siebecker, M.G., Eds.; Academic Press: Cambridge, MA, USA, 2024; pp. 381–410. [CrossRef]
6. Han, J.; Shi, J.; Zeng, L.; Xu, J.; Wu, L. Effects of nitrogen fertilization on the acidity and salinity of greenhouse soils. *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 2976–2986. [CrossRef] [PubMed]
7. Wang, Y.C.; Ying, H.; Yin, Y.L.; Zheng, H.F.; Cui, Z.L. Estimating soil nitrate leaching of nitrogen fertilizer from global meta-analysis. *Sci. Total Environ.* **2019**, *657*, 96–102. [CrossRef] [PubMed]
8. McDonald, M.D.; Lewis, K.L.; DeLaune, P.B.; Hux, B.A.; Boutton, T.W.; Gentry, T.J. Nitrogen fertilizer driven nitrous and nitric oxide production is decoupled from microbial genetic potential in low carbon semi-arid soil. *Front. Soil Sci.* **2023**, *2*, 1050779. [CrossRef]
9. Bhanwaria, R.; Singh, B.; Musarella, C.M. Effect of organic manure and moisture regimes on soil physicochemical properties, microbial biomass Cmic: Nmic: Pmic turnover and yield of mustard grains in arid climate. *Plants* **2022**, *11*, 722. [CrossRef]
10. Rodrigues, M.A.; Sawimbo, A.; da Silva, J.M.; Correia, C.M.; Arrobas, M. Sewage Sludge Increased Lettuce Yields by Releasing Valuable Nutrients While Keeping Heavy Metals in Soil and Plants at Levels Well Below International Legislative Limits. *Horticulturae* **2024**, *10*, 706. [CrossRef]
11. Du Jardin, P.; Xu, L.; Geelen, D. Agricultural functions and action mechanisms of plant biostimulants (PBs): An introduction. In *The Chemical Biology of Plant Biostimulants*; Geelen, D., Xu, L., Eds.; Wiley: Hoboken, NJ, USA, 2020; pp. 3–29.
12. Patel, J.S.; Selvaraj, V.; Gunupuru, L.R.; Rathor, P.K.; Prithiviraj, B. Combined application of *Ascophyllum nodosum* extract and chitosan synergistically activates host-defence of peas against powdery mildew. *BMC Plant Biol.* **2020**, *20*, 113. [CrossRef]
13. Sun, Q.; Liu, J.; Huo, L.; Li, Y.C.; Li, X.; Xia, L.; Zhou, Z.; Zhang, M.; Li, B. Humic acids derived from Leonardite to improve enzymatic activities and bioavailability of nutrients in a calcareous soil. *Int. J. Agric. Biol. Eng.* **2020**, *13*, 200–205. [CrossRef]
14. Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Jones, D.L.; Nebbioso, A.; Mazzei, P.; Piccolo, A. Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 15–27. [CrossRef]
15. Litardo, R.C.M.; Bendezú, S.J.G.; Zenteno, M.D.C.; Pérez-Almeida, I.B.; Parismoreno, L.L.; García, E.D.L. Effect of mineral and organic amendments on rice growth and yield in saline soils. *J. Saudi Soc. Agric. Sci.* **2022**, *21*, 29–37. [CrossRef]

16. Cieschi, M.T.; Lucena, J.L. Leonardite iron humate and synthetic iron chelate mixtures in Glycine max nutrition. *J. Sci. Food Agric.* **2021**, *101*, 4207–4219. [CrossRef] [PubMed]
17. Sugier, D.; Kołodziej, B.; Bielińska, E. The effect of leonardite application on *Arnica montana* L. yielding and chosen chemical properties and enzymatic activity of the soil. *J. Geochem. Explor.* **2013**, *129*, 76–82. [CrossRef]
18. Kaya, C.; Senbayram, M.; Akram, N.A.; Ashraf, M.; Alyemeni, M.N.; Ahmad, P. Sulfur-enriched leonardite and humic acid soil amendments enhance tolerance to drought and phosphorus deficiency stress in maize (*Zea mays* L.). *Sci. Rep.* **2020**, *10*, 6432. [CrossRef]
19. Purwanto, B.H.; Wulandari, P.; Sulistyaningsih, E.; Utami, S.N.H.; Handayani, S. Improved corn yields when humic acid extracted from composted manure is applied to acid soils with phosphorus fertilizer. *Appl. Environ. Soil Sci.* **2021**, *2021*, 8838420. [CrossRef]
20. Sesveren, S.; Taş, B. Response of *Lactuca sativa* var. Crispa to deficit irrigation and leonardite treatments. *All Life* **2022**, *15*, 105–117. [CrossRef]
21. Arrobas, M.; de Almeida, S.F.; Raimundo, S.; da Silva Domingues, L.; Rodrigues, M.Â. Leonardites rich in humic and fulvic acids had little effect on tissue elemental composition and dry matter yield in pot-grown olive cuttings. *Soil Syst.* **2022**, *6*, 7. [CrossRef]
22. Atiyeh, R.M.; Lee, S.; Edwards, C.A.; Arancon, N.Q.; Metzger, J.D. The influence of humic acids derived from earthworm processed organic wastes on plant growth. *Bioresour. Technol.* **2002**, *84*, 7–14. [CrossRef] [PubMed]
23. Lee, J.G.; Yoon, H.Y.; Cha, J.-Y.; Kim, E.-K.; Kim, P.J.; Jeon, J.-R. Artificial humification of lignin architecture: Top-down and bottom-up approaches. *Biotechnol. Adv.* **2019**, *37*, 107416. [CrossRef] [PubMed]
24. Battacharyya, D.; Babgohari, M.Z.; Rathor, P.; Prithiviraj, B. Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 39–48. [CrossRef]
25. De Saeger, J.; Van Praet, S.; Vereecke, D.; Park, J.; Jacques, S.; Han, T.; Depuydt, S. Toward the molecular understanding of the action mechanism of *Ascophyllum nodosum* extracts on plants. *J. Appl. Phycol.* **2020**, *32*, 573–597. [CrossRef]
26. Carmody, N.; Goñi, O.; Łangowski, Ł.; O'Connell, S. *Ascophyllum nodosum* extract biostimulant processing and its impact on enhancing heat stress tolerance during tomato fruit set. *Front. Plant Sci.* **2020**, *11*, 807. [CrossRef] [PubMed]
27. Gunupuru, L.R.; Patel, J.S.; Sumarah, M.W.; Renaud, J.B.; Mantin, E.G.; Prithiviraj, B. A plant biostimulant made from the marine brown algae *Ascophyllum nodosum* and chitosan reduce Fusarium head blight and mycotoxin contamination in wheat. *PLoS ONE* **2019**, *14*, e0220562. [CrossRef] [PubMed]
28. Frioni, T.; VanderWeide, J.; Palliotti, A.; Tombesi, S.; Poni, S.; Sabbatini, P. Foliar vs. soil application of *Ascophyllum nodosum* extracts to improve grapevine water stress tolerance. *Sci. Hortic.* **2021**, *277*, 109807. [CrossRef]
29. Engel, D.C.H.; Feltrim, D.; Rodrigues, M.; Baptistella, J.L.C.; Mazzafera, P. Algae extract increases seed production of soybean plants and alters nitrogen metabolism. *Agriculture* **2023**, *13*, 1296. [CrossRef]
30. Viencz, T.; Oliari, I.C.R.; Ayub, R.A.; Faria, C.M.D.R.; Botelho, R.V. Postharvest quality and brown rot incidence in plums treated with *Ascophyllum nodosum* extract. *Cienc. Agrar.* **2020**, *41*, 753–766. [CrossRef]
31. Afonso, S.; Dias, M.I.; Ferreira, I.C.F.R.; Arrobas, M.; Cunha, M.; Barros, L.; Rodrigues, M.Â. The phenolic composition of hops (*Humulus lupulus* L.) was highly influenced by cultivar and year and little by soil liming or foliar spray rich in nutrients or algae. *Horticulturae* **2022**, *8*, 385. [CrossRef]
32. IPMA. Normais Climatológicas. Instituto Português do Mar e da Atmosfera. Available online: http://www.ipma.pt/pt/oclima/normais.clima/ (accessed on 6 July 2024).
33. WRB. World Reference Base for Soil Resources 2014 Update 2015. In *International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*; World Soil Resources Reports No. 106; FAO: Rome, Italy, 2015.
34. Meier, U. *Growth Stages of Mono and Dicotyledonous Plants*; Federal Biological Research Centre for Agriculture and Forestry: Berlin, Germany, 2018.
35. Konica Minolta Optics, Inc. *Chlorophyll Meter SPAD-502Plus*; A lightweight handled meter for measuring the chlorophyll content of leaves without causing damage to plants; Konica Minolta Optics, Inc.: Osaka, Japan, 2009.
36. Opti-Sciences. *Desktop Plant Stress Guide*; Edition 3.0; Opti-Sciences: Hudson, WI, USA, 2014; Available online: www.optisci.com (accessed on 15 April 2022).
37. Temminghoff, E.E.; Houba, V.J. (Eds.) *Plant Analysis Procedures*, 2nd ed.; Kluwer Academic Publishers: London, UK, 2004. [CrossRef]
38. Baird, R.B.; Eaton, A.D.; Rice, E.W. Nitrate by ultraviolet spectrophotometric method. In *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association, American Water Works Association, Water Environment Federation: Washington, DC, USA, 2017.
39. Van Reeuwijk, L. *Procedures for Soil Analysis*; Technical Paper 9; International Soil Reference and Information Centre: Wageningen, The Netherlands, 2002.
40. FAO. *Standard Operating Procedure for Oil Available Micronutrients (Cu, Fe, Mn, Zn) and Heavy Metals (Ni, Pb, Cd) DTPA Extraction Method*; FAO: Rome, Italy, 2022; Available online: https://www.fao.org/3/cc0048en/cc0048en.pdf (accessed on 9 July 2024).
41. Barone, V.; Bertoldo, G.; Magro, F.; Broccanello, C.; Puglisi, I.; Baglieri, A.; Cagnin, M.; Concheri, G.; Squartini, A.; Pizzeghello, D.; et al. Molecular and morphological changes induced by leonardite-based biostimulant in *Beta vulgaris* L. *Plants* **2019**, *68*, 181. [CrossRef]

42. Ertani, A.; Nardi, S.; Francioso, O.; Pizzeghello, D.; Tinti, A.; Schiavon, M. Metabolite-targeted analysis and physiological traits of *Zea mays* L. in response to application of a leonardite-humate and lignosulfonate-based products for their evaluation as potential biostimulants. *Agronomy* **2019**, *89*, 445. [CrossRef]
43. Atero-Calvo, S.; Magro, F.; Masetti, G.; Navarro-León, E.; Rios, J.J.; Blasco, B.; Ruiz, J.M. Comparative effects of root and foliar leonardite-suspension concentrate application on plant growth and photosynthetic efficiency of lettuce plants (*Lactuca sativa* L.). *J. Plant Growth Regul.* **2024**. [CrossRef]
44. Lamar, R.T. Possible role for electron shuttling capacity in elicitation of PB activity of humic substances on plant growth enhancement. In *The Chemical Biology of Plant Biostimulants*; Geelen, D., Xu, L., Eds.; Wiley: Hoboken, NJ, USA, 2020; pp. 97–121.
45. Gupta, S.; Kulkarni, M.G.; White, J.F.; Stirk, W.A.; Papenfus, H.B.; Doležal, K.; Ördög, V.; Norrie, J.; Critchley, A.T.; Van Staden, J. Categories of various plant biostimulants—Mode of application and shelf-life. In *Biostimulants for Crops from Seed Germination to Plant Development*; Gupta, S., Van Staden, J., Eds.; Academic Press: Cambridge, MA, USA, 2021; pp. 1–60. [CrossRef]
46. Rathor, P.; Gorim, L.Y.; Thilakarathna, M.S. Plant physiological and molecular responses triggered by humic based biostimulants A way forward to sustainable agriculture. *Plant Soil* **2023**, *492*, 31–60. [CrossRef]
47. Pizzeghello, D.; Schiavon, M.; Francioso, O.; Dalla Vecchia, F.; Ertani, A.; Nardi, S. Bioactivity of size fractionated and unfractionated humic substances from two forest soils and comparative effects on N and S metabolism, nutrition and root anatomy of *Allium sativum* L. *Front. Plant Sci.* **2020**, *11*, 1203. [CrossRef] [PubMed]
48. Arslan, E.; Agar, G.; Aydin, M. Humic acid as a biostimulant in improving drought tolerance in wheat: The expression patterns of drought-related genes. *Plant Mol. Biol. Rep.* **2021**, *39*, 432–451. [CrossRef]
49. Rathor, P.; Upadhyay, P.; Ullah, A.; Gorim, L.Y.; Thilakarathna, M.S. Humic acid improves wheat growth by modulating auxin and cytokinin biosynthesis pathways. *AoB Plants* **2024**, *16*, plae018. [CrossRef] [PubMed]
50. Sonmez, S.; Filiz Asri, Ö. Effects of leonardite on soil properties and the nutrients concentrations of tomato plants. *Commun. Soil Sci. Plant Anal.* **2024**, *55*, 1463–1475. [CrossRef]
51. Rengel, Z. Plant responses to soil-borne ion toxicities. In *Marschner's Mineral Nutrition of Higher Plants*, 4th ed.; Rengel, Z., Cakmak, I., White, P.J., Eds.; Elsevier Ltd.: Chennai, India, 2023; pp. 665–722. [CrossRef]
52. Pereira, L.; Cotas, J. Historical use of seaweed as an agricultural fertilizer in the European Atlantic area. In *Seaweeds as Plant Fertilizers, and Agricultural Biostimulants and Animal Fooder*; Pereira, L., Bahcevandziev, K., Joshi, N.H., Eds.; CRC Press: Boca Raton, FL, USA, 2020; pp. 1–22.
53. Procházka, P.; Štranc, K.; Pazderů, J.; Vostřel, J.; Řehoř, J. Use of biologically active substances in hops. *Plant Soil Environ.* **2018**, *64*, 626–632. [CrossRef]
54. Stirk, W.A.; Rengasamy, K.R.R.; Kulkarni, M.G.; van Staden, J. Plant biostimulants from seaweed. In *The Chemical Biology of Plant Biostimulants*; Geelen, D., Xu, L., Eds.; John Wiley & Sons: Hoboken, NJ, USA, 2020; pp. 31–55. [CrossRef]
55. Prisa, D.; Spagnuolo, D. Plant Production with Microalgal Biostimulants. *Horticulturae* **2023**, *9*, 829. [CrossRef]
56. Amiri, M.E.; Fallahi, E.; Golchin, A. Influence of foliar and ground fertilization on yield, fruit quality, and soil, leaf, and fruit mineral nutrients in apple. *J. Plant Nutr.* **2008**, *31*, 515–525. [CrossRef]
57. Di Stasio, E.; Van Oosten, M.J.; Silletti, S.; Raimondi, G.; Dell'Aversana, E.; Carillo, P.; Maggio, A. *Ascophyllum nodosum*-based algal extracts act as enhancers of growth, fruit quality, and adaptation to stress in salinized tomato plants. *J. Appl. Phycol.* **2018**, *30*, 2675–2686. [CrossRef]
58. Bell, R. Diagnosis and prediction of deficiency and toxicity of nutrients. In *Marschner's Mineral Nutrition of Plants*, 4th ed.; Rengel, Z., Cakmak, I., White, P.J., Eds.; Elsevier, Ltd.: Chennai, India, 2023; pp. 477–495. [CrossRef]
59. Hawkesford, M.J.; Cakmak, I.; Coskun, D.; De Kok, L.J.; Lambers, H.; Schjoerring, J.K.; White, P.J. Functions of macronutrients. In *Marschner's Mineral Nutrition of Plants*, 4th ed.; Rengel, Z., Cakmak, I., White, P.J., Eds.; Elsevier, Ltd.: Chennai, India, 2023; pp. 201–281. [CrossRef]
60. Wicharuck, S.; Suang, S.; Chaichana, C.; Chromkaew, Y.; Mawan, N.; Soilueang, P.; Khongdee, N. The implementation of the SPAD-502 chlorophyll meter for the quantification of nitrogen content in Arabica coffee leaves. *MethodsX* **2024**, *12*, 102566. [CrossRef]
61. Afonso, S.; Arrobas, M.; Ferreira, I.Q.; Rodrigues, M.A. Assessing the potential use of two portable chlorophyll meters in diagnosing the nutritional status of plants. *J. Plant Nutr.* **2018**, *41*, 261–271. [CrossRef]
62. Maxwell, K.; Johnson, G.N. Chlorophyll fluorescence—A practical guide. *J. Exp. Bot.* **2000**, *51*, 659–668. [CrossRef] [PubMed]
63. Arrobas, M.; Afonso, S.; Ferreira, I.Q.; Moutinho-Pereira, J.; Correia, C.M.; Rodrigues, M. Liming and application of nitrogen, phosphorus, potassium, and boron on a young plantation of chestnut. *Turk. J. Agric. For.* **2017**, *41*, 441–451. [CrossRef]
64. Arrobas, M.; Correia, C.M.; Rodrigues, M.Â. *Methylobacterium symbioticum* applied as a foliar inoculant was little effective in enhancing nitrogen fixation and lettuce dry matter yield. *Sustainability* **2024**, *16*, 4512. [CrossRef]
65. Beegle, B.; Kelling, A.; Schmitt, A. Nitrogen from animal manures. In *Nitrogen in Agricultural Systems*; Schepers, J., Raun, W.R., Eds.; Agronomy Monograph n.° 49; ASA, CSSA, SSSA: Madison, WI, USA, 2008; pp. 823–881.