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Algae Extract Increases Seed Production of Soybean Plants and Alters Nitrogen Metabolism

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Abstract: Algae extract biostimulants increase nutrient uptake, stress tolerance, and productivity in several crops. However, there is still a gap in the knowledge of the mechanisms of action of algae extracts on nitrogen plant metabolism. This study aimed to evaluate the effect of a commercial *Ascophyllum nodosum* algae extract on nitrogen metabolism in nodulating soybean plants and their productivity. Two concentrations of algae extract (0.25% and 0.50%) were used, which were applied via seeds and leaf spray. Seeds were treated at sowing, and plants were sprayed twice at two vegetative phenological stages. Plants were harvested at the R5 phenological stage for leaf biochemical and enzyme activity analyses and leaf and root gene expression analyses. The experiment was carried out a second time to evaluate productivity. There was an increase in leaf and stem biomass, number of pods and seeds, weight of pods and seeds, and productivity in plants treated with both concentrations. Biochemical analysis showed increased amino acid content in leaves after extract application. No marked differences were found regarding the parameters related to nitrogen metabolism when the data were analysed individually. However, principal component analysis and gene expression heatmaps supported the conclusion that N metabolism was affected by algae extract application, leading to higher seed production.

Keywords: *Glycine max*; nitrogen metabolism; amino acids; growth; yield

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1. Introduction

Soybean (*Glycine max* (L.) Merrill) is among the most important commodities in the world. In Brazil, soybean cultivation occupied about 40 million hectares in the 2021–2022 season, averaging a productivity of 3478 kg ha^{-1} [1]. Nitrogen (N) is the most demanded nutrient by soybean plants, reaching 14 kg of N per kg of seeds [2], and its deficiency leads to severe yield reduction [3]. In Brazil, bacterial biological fixation is the main N source for soybean cultivation [4], representing an economy of $15 billion for soybean farmers, which do not use mineral N fertilizers [5].

In N biological fixation, N is assimilated by plants through the rhizobium root nodules and transformed into amino acids through its reduction to nitrite and then to ammonia (NH_4^+) by the enzymes nitrate reductase and nitrite reductase, respectively [6]. The incorporation of ammonia into amino acids is mediated by two sequentially acting enzymes, glutamine synthetase and glutamate synthase.

N may also be reassimilated from the ammonia generated in large amounts via photorespiration in photosynthetic tissues, from protein turnover during the senescence or protein reserves during seed germination [7]. Arginine is one of the main forms of N storage in several organs and in different plant species [8]. In mitochondria, arginine (Arg) is catabolized by arginase into ornithine and urea. Urea is hydrolyzed by urease into ammonia and carbon dioxide [9].

The absorption of nutrients by plants can be favored with the application of biostimulants, increasing nutrient use efficiency [10,11]. Several definitions have been given to biostimulants [12]. According to the European Biostimulant Industry Council (EBIC), biostimulants are products containing substance(s) and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to improve/benefit the absorption of nutrients, nutrient use efficiency, abiotic stress tolerance, and crop quality [13]. The ability to improve soil fertility, promote plant growth, and reduce the use of chemicals in crop management has made biostimulants an option to increase agriculture's sustainability [14–16].

Algae extracts (AE) are among the most commonly used biostimulants in agriculture [11,17]. Its composition is complex, primarily predominating polysaccharides such as laminarin, fucoidan, and alginate [18]. The use of AE improves seed germination, promotes plant growth and yield, and increases tolerance to abiotic (drought, heat, salinity, low soil fertility, and frost) and biotic (diseases, nematodes, and insects) stresses [19,20]. AE activates plant defenses against reactive oxygen species (ROS), which increases their formation during stresses [21].

Foliar application of AE from *A. nodosum* increased the water stress tolerance of soybean plants, which had less wilting and a greater capacity to recover turgor when irrigated [22]. AE from *A. nodosum* induces several genes in soybean, including genes related to primary and secondary metabolism, cell wall formation, photosynthesis, stress response, and hormone biosynthesis [23]. Although knowledge has accumulated over the years on the response of plants to biostimulants, still little is known about their mechanism of action, particularly their effect on N metabolism [24]. More importantly, considering how soybeans are cultivated in Brazil, to our knowledge, the impact of biostimulants on biological N fixation has not been investigated so far. Because biostimulants may improve plant biomass accumulation, the demand for N may increase, thus directly influencing the N fixation in the nodules of soybean roots. The increase in N fixation in soybean plants tends to increase productivity because the number of seeds depends on the availability of N during the flowering period [25].

Therefore, the lack of knowledge on the effects of biostimulants based on algae extracts on N fixation in soybean prompted us to evaluate the influence of an AE from the macroalgae *A. nodosum* on N metabolism in soybean plants and their productivity.

2. Material and Methods

2.1. Experimental Design and Treatments

The experiment was carried out in a greenhouse in Piracicaba, São Paulo (22°42′ S, 47°30′ W). Seeds of the soybean cv. Intacta RR2 Pro 57HO123 TP IPRO (HO-Genética) were sown in 10 L pots containing a mixture of soil, organic gardening substrate, and vermiculite (2:1:1, *v*/*v*/*v*). Seeds were placed at a depth of 2 cm and inoculated with Masterfix® L Soja (*Bradyrhizobium elkanii* and *Bradyrhizobium japonicum*) at a dose of 300 mL/ha. The inoculum was diluted in water (1/7, *v*/*v*) and distributed (1 mL) over the sown seeds. Once a week, the plants received 200 mL of a modified nutrient solution without N [26] containing 2 mM $MgSO_4.7H_2O$, 2.5 mM K_2SO_4, 2 mM $CaSO_4$, and 0.5 mM $Ca(H_2PO_4)_2$. Micronutrients were added at a normal rate. The plants were drip-watered daily.

AE from *Ascophyllum nodosum* (Acadian Plant Health™) was tested at 0.25% and 0.50%, applied via seed treatment and foliar spray. The AE composition was informed by the manufacturer as follows: N—8.12 g kg^{-1}; phosphorous (P)—6.82 g kg^{-1}; potassium (K)—12.00 g kg^{-1}; calcium (Ca)—1.60 g kg^{-1}; magnesium (Mg)—2.03 g kg^{-1}; sulfur (S)—8.16 g kg^{-1}; boron (B)—5.74 mg kg^{-1}; copper (Cu)—13.60 mg kg^{-1}; iron (Fe)—11.5 mg kg^{-1}; manganese (Mn)—0.04 mg kg^{-1}; zinc (Zn)—24.40 mg kg^{-1}; sodium (Na)—20 mg kg^{-1}; potassium hydroxide, with 61.48 g L^{-1} of water-soluble K_2O; 69.60 g L^{-1} of total organic carbon; and density of 1.16 g dm^{-3}. Seed treatment was made after *Bradyrhizobium* inoculation, using 1 mL per pot on the sown seeds. The foliar application was carried out with a CO_2-pressurized sprayer with a fan nozzle and a pressure of 2 bar on plants at

stages V3 and V5. Rizospray Extremo (Rizobacter) was used as an adjuvant at a concentration of 0.05% to break the water tension. Six replicates were made for each treatment. The experiment was carried out twice. Biochemical and gene expression analyses were carried out in the first experiment, and seed production was evaluated in the second experiment.

2.2. Samplings

The plants from the first-year experiment were collected at the R5 stage. The youngest, fully expanded leaves from several branches were collected and used for enzyme, biochemical, and gene expression analyses. Leaves used in the enzymatic analysis were frozen in liquid nitrogen and stored in a −20 °C freezer. Those for biochemical analysis were frozen in liquid nitrogen, freeze-dried, ground in an analytical mill, and kept at −20 °C in a freezer. The leaves used for gene expression were frozen in liquid nitrogen and kept at −80 °C in a freezer. After that, the plants were abundantly watered, and in the early morning of the next day, the stem was cut 5 cm from the soil surface to collect the xylem sap [27]. The dry masses of stem and remaining leaves were determined after drying at 70 °C for 72 h. After xylem sap collection, the roots were washed in running water, and a small part was reserved for gene expression. The rest of the roots were placed in internally moistened plastic bags and kept in a refrigerator to count the number of nodules. Then, the fresh and dry masses of the roots were determined at 70 °C after drying for 72 h.

2.3. Enzyme Activities

The activities of nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) were determined according to [28], [29], and [30], respectively. The concentrations of proteins in the enzyme extracts were obtained with a ready-to-use Bradford reagent from Bio-Rad [31]. Six replicates were made for each enzyme activity.

2.4. Nitrogen Compounds and Pigments

Nitrate (NO_3^-) was determined according to [32]. Free amino acids and ureides were determined in the leaves and in the xylem sap according to [33] and [34], respectively. Chlorophyll and carotenoid concentrations were determined after extraction in DMSO [35]. Six replicates were made for each analysis.

2.5. Nutrient Leaf Concentration

N, P, K, Ca, Mg, and S were determined in the leaves by plasma emission spectrometry (ICP-OES; JobinYvon, JY50P Longjumeau, France). Three replicates were made for this analysis.

2.6. Gene Expression by RT-qPCR

The expression of the following genes was analysed: NR, nitrite reductase (NiR), GS, GOGAT, nitrate transporter NTR1, asparagine synthetase, arginase, and urease (Table 1). The CYP2 and ACTII genes were used for gene expression normalization [36]. Total RNA was extracted from three replicates of leaves and roots using the Trizol reagent (Sigma, Kawasaki, Japan), following the manufacturer's instructions. The RNA was treated with "Turbo DNA-free" DNAse (Ambion, Inc., Austin, TX, USA) and quantified in a spectrophotometer at 260 nm. RNA integrity was checked by 1.5% agarose gel electrophoresis with ethidium bromide and UV light observation. Three µg of total RNA was used to synthesize the first cDNA strand with the SuperScript III First-Strand kit (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions.

Table 1. Primers used in the RT-qPCR reactions.

Gene	ID Phytozome	Primer F	Primer R
Nitrate reductase (NR)	Glyma.13G084000	GCGATTTTGAAGGACCCAGA	TTGCGATTTCCACCACGTAC
Nitrite reductase (NiR)	Glyma.02G132100	ACATTGGTTTCATGGGGTGC	ACAACAGGCACCAAGTCCTT
Glutamine synthetase (GS)	Glyma.02G127500	TGGGGAAGCAATGGAGAAGA	AACCAAGCCGAGTGACCAAT
Glutamate synthetase (GOGAT)	Glyma.14G162300	TTGCAGAGAAGTTGGGTGTG	CTTGCCGTCCCTCTGAAATC
10 Nitrate transporter 1 (NTR1)	Glyma.02G011600	AGGCTTGTATGAGGTTCCTGT	ACACTCCAAAAGCGACAGTT
Asparagine synthetase	Glyma.11g38130	TAGGCTCACTGTTCCTGGAG	ACCCTTGTTGTTCTGGTTTTCA
Arginase (ARGAH)	Glyma.17G097800	TGAGAGAACTCGCTGCAAAG	TGAGAGAACTCGCTGCAAAG
Urease (UreUU)	Glyma 2.0	ATCAAAGGTGGTGAGGTTGC	ATCAAAGGTGGTGAGGTTGC

The primers were designed using the Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0/, accessed on 1 April 2019) (Table 1) using gene sequences obtained from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html, accessed on 1 April 2019). The reverse transcription quantitative real-time PCR (RT-qPCR) reactions were prepared with a final volume of 10 µL, containing 3 µL of diluted cDNA, 1.6 µL of milliQ water, 0.2 µL sense primer, 0.2 µL antisense primer, and 5 µL of Sybr Green Master Mix (BioRad, Hercules, CA, USA). A StepOnePlus Real-Time PCR System (Thermo Fisher, Waltham, MA, USA) was used for gene amplification (95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s as a melting curve). Seven biological replicates were made for each treatment. The relative quantification of the expression was determined by comparing the transcriptional expression between the target genes and the reference genes by the $2^{-\Delta Ct}$ method [37].

2.7. Yield-Related Components

We repeated the first experiment using the same growth conditions and cultivated the soybean plants to full maturity to analyze the yield components. The number and mass of pods and seeds per plant, the number of seeds per pod, the mass per seed, the plant height, and the number of productive nodes were determined. These data were obtained from six replicates of each treatment.

2.8. Statistical Analyzes

The experimental design was in completely randomized blocks containing application mode (foliar and seed) X AE concentration (0, 0.25%, and 0.5%). Six replicates were used for each analysis, except for the gene expression and nutrient analyzes, for which seven and three replicates were used, respectively. The data were tested for normality using the Shapiro-Wilk test, and the Bartlett test was used to check the homogeneity of the variances. When the variables did not meet the assumptions of the tests, they were transformed into logs. Subsequently, the data were submitted to an analysis of variance (ANOVA), and the means were compared by Tukey's test at a 5% error probability. This analysis was performed using the "fac.2.dic()" function of the "ExpDes.pt" package [38]. All analyzes were performed using the R version 4.0.0 software (https://www.r-project.org/, accessed on 1 April 2019).

3. Results

3.1. Soybean Growth and Productivity

The parameters of growth and productivity of soybean plants treated with AE did not show statistically significant differences between the two application modes tested, i.e., foliar and seed application. Thus, only the effect of the biostimulant was analyzed. Plants treated with AE had higher leaf dry mass (0.25% and 0.50%) and stem mass (0.50%) (Table 2), were taller (0.50%), had a greater number of productive nodes (0.50%), a greater

number of pods (0.25% and 0.50%), a greater dry mass of pods (0.25% and 0.50%), a greater number of seeds (0.25% and 0.50%), a greater mass of seeds (0.25% and 0.50%), and a greater dry mass per seed (0.25% and 0.50%) (Table 3). The number of nodules in the roots and the number of seeds per pod were not altered.

Table 2. Growth features of soybean plants treated with *A. nodosun* algae extract (experiment 1).

	AE 0.25%	AE 0.50%	Control
Leaf dry weight (g)	23.14 a	24.46 a	21.24 b
Stem dry weight (g)	28.53 b	32.62 a	28.10 b
Root dry weight (g)	40.52 a	35.30 a	40.99 a
Total nodules dry weight (g)	3.91 a	3.91 a	3.68 a
Dry weight per nodule (mg)	4.84 a	4.82 a	4.87 a
Nodules per plant	811.38 a	845.13 a	758.88 a

Different small letters within the line indicate significant statistical differences among treatments (Tukey test, $p < 0.05$).

Table 3. Growth and productivity features of soybean plants treated with *A. nodosun* algae extract (experiment 2).

	AE 0.25%	AE 0.50%	Control
Plant height (cm)	73.33 ab	72.42 a	68.83 b
Number of nodes per plant	69.08 ab	70.5 a	64.83 b
Pods per plant	141.67 a	147.17 a	120.33 b
Pods dry weight per plant (g)	59.71 a	61.83 a	46.10 b
Seeds/plant	343.22 a	351.97 a	284.02 b
Seeds dry weight per plant (g)	39.54 a	39.91 a	28.7 b
Seeds per pod	2.42 a	2.40 a	2.37 a
Individual seed dry weight (g)	0.12 a	0.11 a	0.10 b

Different small letters within the line indicate significant statistical differences among treatments (Tukey test, $p < 0.05$).

In the second experiment, after seed maturation, the irrigation was suspended to induce shoot drying for the subsequent harvest of the pods. There was, phenotypically, a delay in senescence in the plants treated with the AE, keeping the leaves green for a longer time (Figure 1). All yield components determined, except the number of seeds per pod, had a significant increase in plants treated with AE. Plants treated with AE 0.50% showed higher seed productivity than untreated plants, increasing seed production per plant by 28% (Table 3).

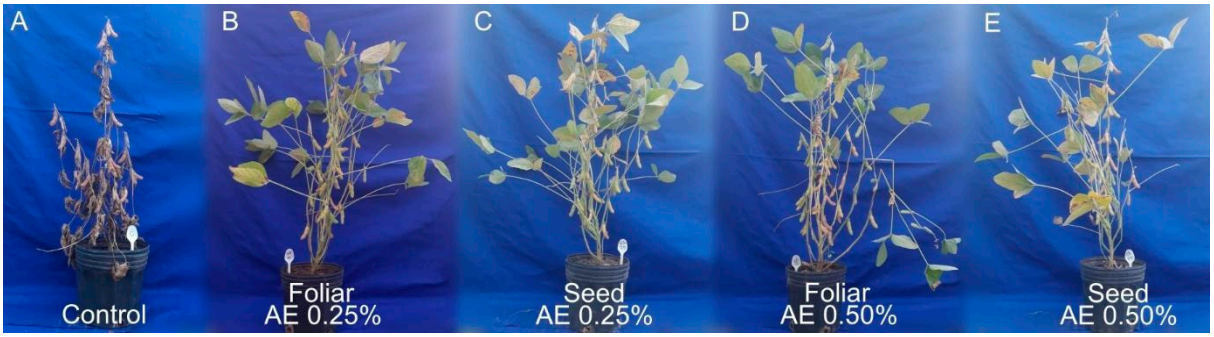

Figure 1. Soybean plants treated with *A. nodosun* algae extract at the seed maturation stage. (**A**) control plant, (**B**) 0.25% AE foliar application, (**C**) 0.25% AE seed treatment, (**D**) 0.50% AE foliar application, and (**E**) 0.50% AE seed treatment.

3.2. Enzyme Activities, Nitrogen Compounds and Pigments

Among the three enzymes analyzed, only NR showed statistical interaction between application modes and concentrations of biostimulants (Figure 2A). The AE seed treatment

did not show a statistical difference between the treatments, but there was a reduction in the NR activity in the foliar application in both AE concentrations. The highest GS activity was observed with AE 0.25% independently of the application mode; however, despite being 55% higher than the control, it did not differ statistically (Table 4). GOGAT activity did not differ among treatments (Table 4).

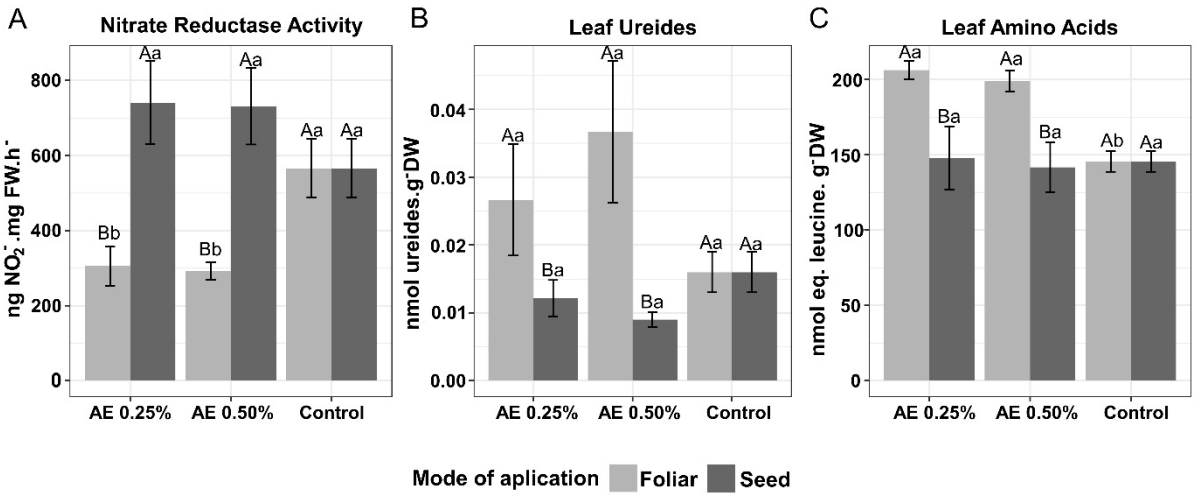

Figure 2. Effect of *A. nodosun* algae extracts application on nitrate reductase activity (**A**), ureide (**B**), and amino acid concentrations (**C**) in soybean leaves. Different lowercase letters indicate a statistical difference in the application mode, and different capital letters indicate a statistical difference in application mode in each treatment (Tukey test, $p < 0.05$). Bars indicate standard error.

Table 4. Enzyme activities, nitrogen compounds, and photosynthetic pigments in soybean plants treated with *A. nodosun* algae extract.

	AE 0.25%	AE 0.50%	Control
GS Activity (ΔAbsorbance.µg protein^{-1}·h^{-1})	1.32 a	0.63 b	0.85 ab
GOGAT Activity (ΔAbsorbance.µg protein^{-1}·h^{-1})	6.44 a	8.69 a	8.04 a
Leaf Nitrate (µg NO_3^-·g$^-$ fresh weight)	1.36 a	1.39 a	1.35 a
Xylem Sap Nitrate (µg NO_3^- µL)	0.61 a	0.58 a	0.64 a
Xylem Sap Ureides (nmol ureides·mL$^-$)	0.016 b	0.016 b	0.036 a
Xylem Sap Amino Acids (nmol eq. leucine·mL$^-$)	2.28 b	2.22 b	3.89 a
Chlorophyll a	0.39 a	0.38 a	0.39 a
Chlorophyll b	0.37 b	0.47 a	0.40 ab
Chlorophyll total	0.76 a	0.85 a	0.79 a
Carotenoids	0.083 a	0.042 b	0.073 ab

Different small letters within the line indicate significant differences among treatments (Tukey test, $p < 0.05$).

There was statistical interaction with the application mode only for ureides and amino acids in the leaves (Figure 2B,C and Table 4). The application mode did not differ for the nitrate levels in the leaf and xylem sap or the ureides and amino acids in the xylem sap. Compared to seed treatments, ureide concentrations in leaves were higher in foliar treatments at both AE concentrations. There was no statistical difference in the concentration of ureides in each algae treatment with the control, either in foliar spray or seed treatment (Figure 2B). The amino acid concentration followed a pattern similar to that of ureides, and in the foliar application, the AE treatments were higher than the control (Figure 2C). There was no difference between treatments regarding NO_3^- concentration in the leaves and xylem sap (Table 4). AE treatment reduced the concentration of ureides and amino acids in the xylem sap (Table 4). A few changes were observed in pigment concentration. Chlorophyll b concentration was lower in the treatment at AE 0.25%, and carotenoids were lower at AE 0.50% (Table 4).

3.3. Leaf Nutrient Content

N, P, and Ca concentrations did not differ statistically between application modes and did not differ among treatments (Table 5). The K concentration did not vary between the two algae concentrations via foliar application, but it was higher than via seed. Mg was higher in the AE 0.50% foliar treatment than in the seed treatment, and the same AE concentration also had the highest value compared to the foliar treatment. The concentration of S was higher in the AE 0.25% treatment when applied via foliar application and also the highest when considering only this type of application.

Table 5. Nutrient concentrations (g kg^{-1}) in the leaves of soybean plants treated with *A. nodosun* algae extract.

Treatments	N	P	Ca	K		Mg		S	
				Foliar	Seed	Foliar	Seed	Foliar	Seed
AE 0.25%	40.83 a	1.79 a	17.30 a	18.87 Aa	16.13 Bab	3.52 Ab	3.43 Aa	2.00 Aa	1.47 Ba
AE 0.50%	39.43 a	1.81 a	17.53 a	17.70 Aa	15.00 Bb	3.89 Aa	3.30 Ba	1.53 Ab	1.43 Aa
Control	41.42 a	1.81 a	17.45 a	17.15 Aa	17.15 Aa	3.37 Ab	3.37 Aa	1.58 Ab	1.58 Aa

Different small letters within the column indicate significant statistical differences among treatments, and capital letters within the line indicate significant statistical differences between application modes (Tukey test, $p < 0.05$).

3.4. Gene Expression Analysis

Although trends were observed for increased expression of several genes in AE-treated plants, most of the genes did not have statistically altered expression compared to the control (Figure 3). Only asparagine synthetase in the leaves of AE seed-treated plants (Figure 3B) showed a reduction in expression with AE of 0.50%. We did not detect the expression of the NO_3^- transporter in the roots, only in the leaves (Figure 3H).

Although we did not detect statistical differences in gene expression, we made a simplified scheme of the N fixation pathway with heatmaps representing the expression data (Figure 4). It is evident from the heatmaps that there is a continuous trend along the N pathway, indicating that AE 0.50% foliar treatment induced the expression of arginase, urease, NR, NiR, GOGAT, and asparagine synthetase in the roots (Figure 4A). Less evident but still consistent numerically with the results found in the roots, the expression values for the foliar AE 0.25% treatment were higher for GS, NR, NiR, GOGAT and asparagine synthetase. It is also evident that there is a trend in the gene expression in the roots of seed-treated plants, which is lower than that of control plants (Figure 4B).

To better understand how our data correlate and whether there are any distinctions between treatments, genes, and tissues studied, we also performed a principal component analysis (PCA). Figure 5 distinguishes between the control treatments and the two AE concentrations, applied via foliar (Figure 5A) or seed (Figure 5B), which occupy opposite axes of the quadrant. In both application modes, the AE concentrations were not separated.

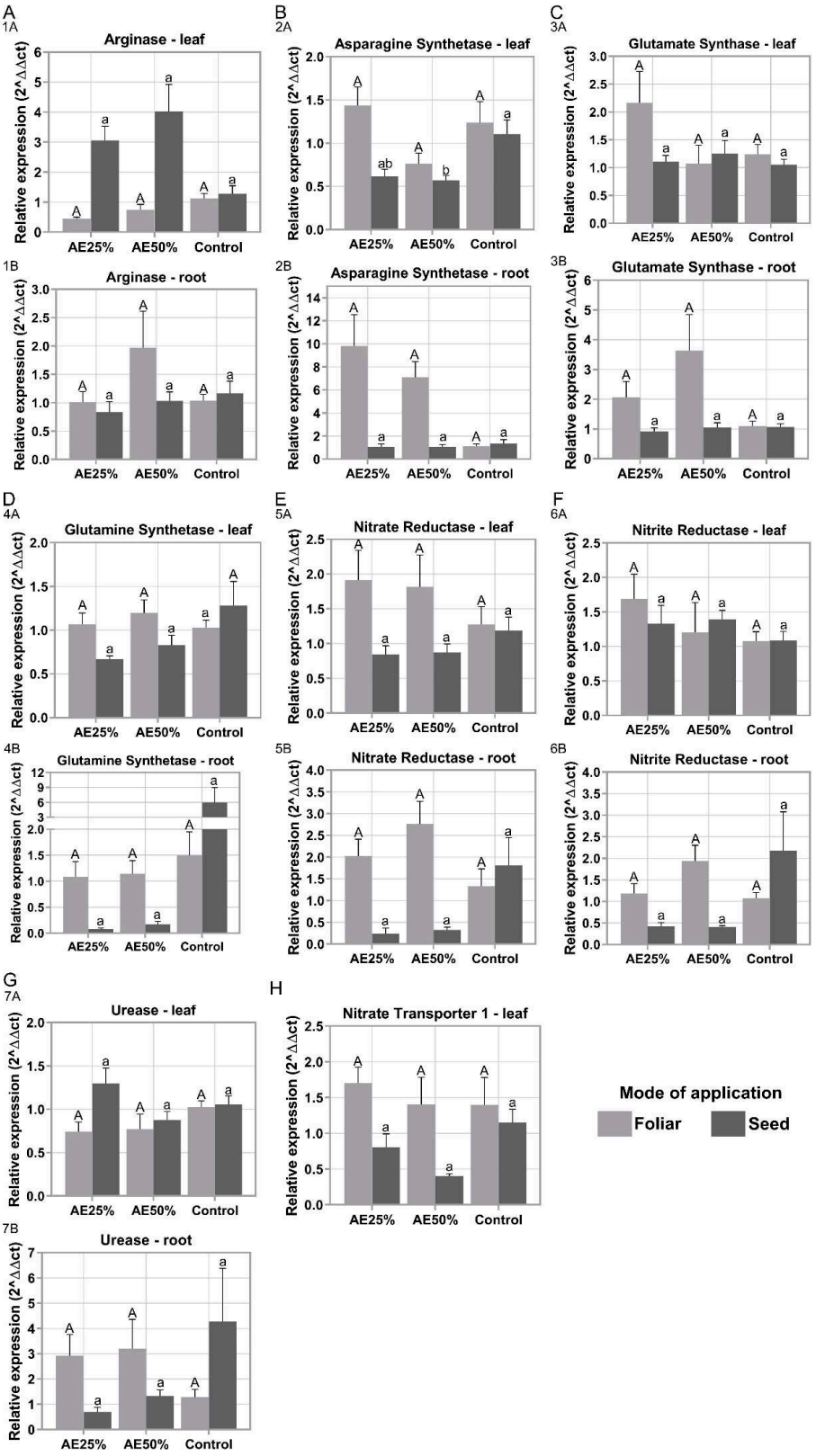

Figure 3. Relative expression of nitrogen metabolism genes in leaf and root of soybean plants treated with *A. nodosun* algae extract. (**A**) Arginase, (**B**) Asparagine synthetase, (**C**) GOGAT (Glutamate synthase), (**D**) Glutamine synthetase, (**E**) Nitrate reductase, (**F**) Nitrite reductase, (**G**) Urease and (**H**) Nitrate transporter 1. The figures were generated using relative expression data in relation to CYP2 and ACTII controls and calculated using the method described by [37]. Different lowercase letters indicate a statistical difference in the leaf treatment, and different capital letters indicate a statistical difference in the seed treatment (Tukey test, $p < 0.05$). Bars indicate standard error.

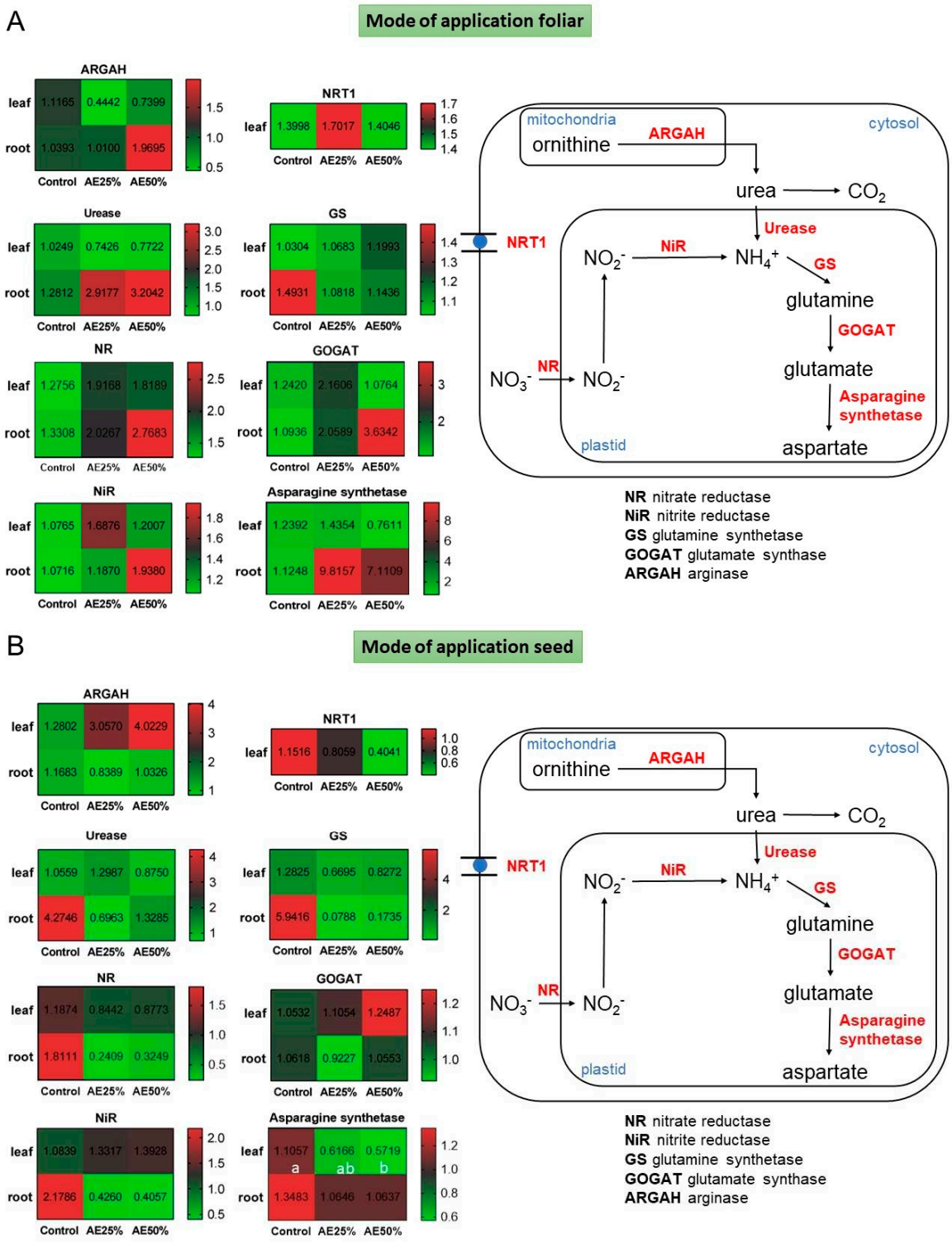

Figure 4. Simplified scheme of the nitrogen pathway in AE-foliar treated plants (**A**) and AE-root treated plants (**B**). The genes appear in red in the various cell compartments. The color scales in the heatmap charts show the relative expression values for each gene. White letters indicate the statistical differences found for asparagine synthetase in AE seed-treated plants.

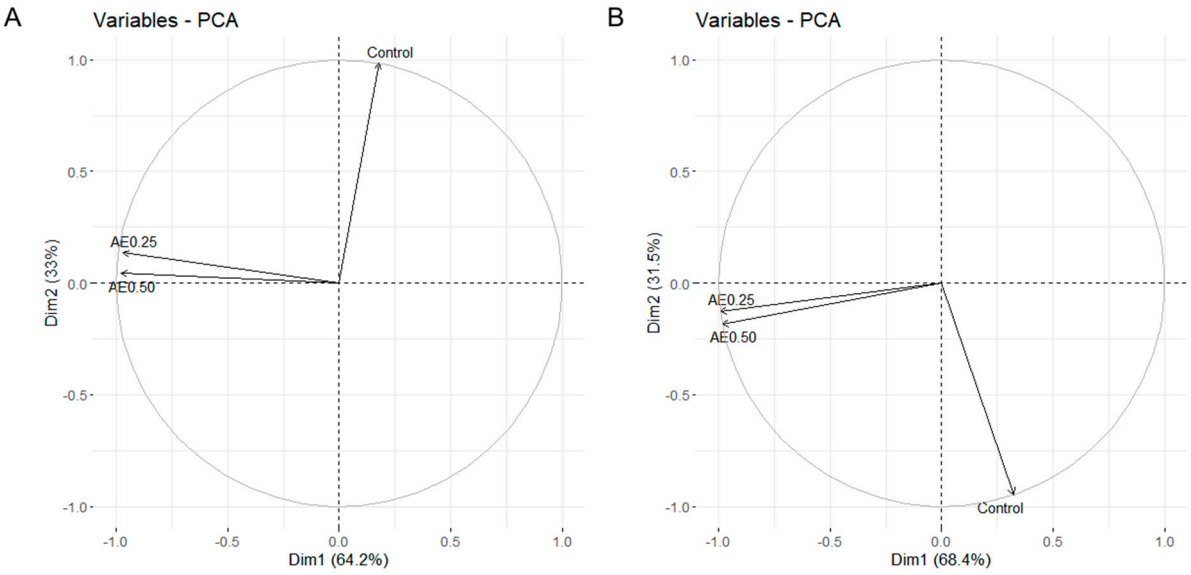

Figure 5. Principal component analysis of the gene expression data. (**A**) foliar AE application and (**B**) seed AE application. Algae extract 0.25% (AE0.25); algae extract 0.50% (AE0.50).

4. Discussion

The literature has described numerous benefits of biostimulants in various physiological parameters of plants, from productivity improvements to increased tolerance to various biotic and abiotic stresses [39]. Our results showed that the foliar and seed application of the AE of *A. nodosum* in soybean plants did not significantly alter most of the analyzed components of N metabolism, but it increased the seed production of the treated plants. Another study [40] reported that soybeans sprayed with AE from *Ecklonia maxima* had an increase in productivity, which was related to an increased number of pods and seeds and seed weight. We showed that the soybean plants treated with biostimulants based on AE 0.25% and 0.50% (leaf and seed application) induced more leaves and pods than the control. We also showed that AE-treated plants retained their green leaves for a longer period, which can provide greater biomass of pods and seeds, increasing the productivity of treated plants [41].

Algae-based biostimulants are complex mixtures and may have hormonal-like activities [18]. The delay in leaf senescence that we have observed in the AE-treated plants may be the result of cytokinin-like activity. It is known that cytokinins can delay senescence and chlorophyll degradation and induce chlorophyll biosynthesis [42,43]. Several studies have shown that chlorophyll concentrations were higher in plants treated with AE from different algae species [44]. Additionally, AE induces the expression of genes related to cytokinin biosynthesis [45], indicating that besides having hormone-like constituents, AE constituents induce the increase in the hormone in plants. However, we did not observe an increase in chlorophyll leaf concentration, suggesting that AE treatment directly influenced leaf senescence in the soybean plants.

The increase we found in plant biomass may be related to improved nutrient absorption, an effect already observed with plants treated with biostimulants [10,46]. We have observed an increase in the S, Mg, and K concentrations in soybean plants sprayed with AE leaves. Rathore et al. [47] showed that in soybean seeds of plants sprayed with *K. alvarezii* AE, the concentrations of N, P, K, and S increased by 36%, 61%, 49%, and 93%, respectively. Nelson and Staden [48] applied a commercial AE and observed an accumulation of 20% in the P concentration in the leaves. Turan and Köse [49] verified an increase in N, P, K, Ca, Mg, Fe, Zn, Mn, and Cu in grapes using AE from *A. nodosum*.

Amino acids and ureides tended to accumulate in the leaves of foliar AE-treated plants. Plants treated with AE showed numerically more nodules than control plants. We counted about 800 nodules per plant, including all sizes of nodules. However, amino

acids and ureides unexpectedly decreased in the xylem sap in both AE concentrations, irrespective of the AE application mode. It is important to comment that we sampled tissues for analysis at the R5 soybean developmental phase, when the seeds start to grow [50], but at this stage, leaves are still the main sinks for N in the plant, and they are far from being strong sinks compared to seeds. Later in the seed development, the leaf N will be remobilized to the seeds [50]. We also did not find a difference regarding N concentration in the leaves, but AE-treated plants had more leaf (and stem) dry weight than control plants, thus indicating more N per shoot. Therefore, the indication is that AE improved the N economy in AE-treated plants over time. Additionally, although we have not observed higher concentrations of chlorophyll, leaf dry mass and height were higher in AE-treated plants. We also observed more nodes, pods, and seeds per plant in these plants and higher individual seed weights.

The few statistical differences in gene expression among control and AE-treated plants are probably due to a large variation among replicates, although we have used seven biological replicates. Nevertheless, we could identify a logical pattern regarding the roots of plants sprayed with 0.5% AE using a heatmap analysis. Out of seven genes, only one did not have a higher value compared to the control plants. Numerically less evident, this same pattern was observed in the leaves of 0.25% AE-sprayed plants. In seed-treated plants, AE led to a different pattern in the roots, i.e., gene expression was numerically much lower than in the control plants. PCA analysis showed gene expression in the AE treatments grouped separately from the control plants, supporting the information on heatmaps.

5. Conclusions

Both applications of *A. nodosun* extract in the seeds and as a foliar spray increase soybean productivity. Although few statistically significant changes were observed regarding N-related parameters, PCA analysis separated all treatments, and heatmap charts showed an apparent effect of algae application on N metabolism, even though we have sampled tissues for analyses only at the R5 stage. Thus, the data strongly suggest that N metabolism was affected by algae extract application and induced higher seed production. New experiments with several samplings over the phenological cycle may definitively show AE's positive effect on soybean's N metabolism, leading to higher seed production.

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References

1. CONAB—Companhia Nacional de Abastecimento. *Acompanhamento da Safra Brasileira de Grãos*; Safra 2021/22, 9º Levantamento; Conab: Brasília, Brazil, 2022; p. 59.
2. Tamagno, S.; Balboa, G.R.; Assefa, Y.; Kovács, P.; Casteel, S.N.; Salvagiotti, F.; García, F.O.; Stewart, W.M.; Ciampitti, I.A. Nutrient partitioning and stoichiometry in soybean: A synthesis-analysis. *Field Crop. Res.* **2017**, *200*, 18–27. [CrossRef]

3. Cafaro La Menza, N.; Monzon, J.P.; Lindquist, J.L.; Arkebauer, T.J.; Knops, J.M.H.; Unkovich, M.; Specht, J.E.; Grassini, P. Insufficient nitrogen supply from symbiotic fixation reduces seasonal crop growth and nitrogen mobilization to seed in highly productive soybean crops. *Plant Cell Environ.* **2020**, *43*, 1958–1972. [CrossRef]
4. Hungria, M.; Nogueira, M.A. Fixação biológica do nitrogênio. In *Bioinsumos na Cultura da Soja*; Meyer, M.C., Ed.; Embrapa: Brasília, Brazil, 2022; p. 550; ISBN 978-65-87380-96-4.
5. Hungria, M.; Mendes, I.C. Nitrogen fixation with soybean: The perfect symbiosis? In *Biological Nitrogen Fixation*; Wiley Online Library: Vitural, 2015; pp. 1009–1024.
6. Krapp, A. Plant nitrogen assimilation and its regulation: A complex puzzle with missing pieces. *Curr. Opin. Plant Biol.* **2015**, *25*, 115–122. [CrossRef]
7. Xu, G.; Fan, X.; Miller, A.J. Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* **2012**, *63*, 153–182. [CrossRef] [PubMed]
8. Shargool, D.; Jain, J.C.; McKay, G. Ornithine biosynthesis, and arginine biosynthesis and degradation in plant cells. *Phytochemistry* **1988**, *27*, 1571–1574. [CrossRef]
9. Dixon, N.E.; Riddles, P.W.; Gazzola, C.; Blakeley, R.L.; Zerner, B. Jack bean urease (EC 3.5.1.5). V. On the mechanism of action of urease on urea, formamide, acetamide, N-methylurea, and related compounds. *Can. J. Biochem.* **1980**, *58*, 1335–1344. [CrossRef] [PubMed]
10. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 28–38. [CrossRef]
11. De Pascale, S.; Rouphael, Y.; Colla, G. Plant biostimulants: Innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* **2017**, *82*, 277–285. [CrossRef]
12. 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]
13. EBIC—European Biostimulant Industry Council. Available online: https://biostimulants.eu/ (accessed on 14 January 2022).
14. Halpern, M.; Bar-Tal, A.; Ofek, M.; Minz, D.; Muller, T.; Yermiyahu, U. The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* **2015**, *130*, 141–174. [CrossRef]
15. Van Oosten, M.J.; Pepe, O.; De Pascale, S.; Silletti, S.; Maggio, A. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* **2017**, *4*, 5. [CrossRef]
16. Win, T.T.; Barone, G.D.; Secundo, F.; Fu, P. Algal biofertilizers and plant growth stimulants for sustainable agriculture. *Ind. Biotechnol.* **2018**, *14*, 203–211. [CrossRef]
17. Battacharyya, D.; Babgohari, M.Z.; Rathor, P.; Prithiviraj, B. Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 39–48. [CrossRef]
18. 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*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2020; pp. 1–30. [CrossRef]
19. Khan, W.; Rayirath, U.P.; Subramanian, S.; Jithesh, M.N.; Rayorath, P.; Hodges, D.M.; Critchley, A.T.; Craigie, J.S.; Norrie, J.; Prithiviraj, B. Seaweed extracts as biostimulants of plant growth and development. *J. Plant Growth Regul.* **2009**, *28*, 386–399. [CrossRef]
20. Pohl, A.; Kalisz, A.; Sekara, A. Seaweed extracts' multifactorial action: Influence on physiological and biochemical status of Solanaceae plants. *Acta Agrobot.* **2019**, *72*, 1758. [CrossRef]
21. Omidbakhshfard, M.A.; Sujeeth, N.; Gupta, S.; Omranian, N.; Guinan, K.J.; Brotman, Y.; Nikoloski, Z.; Fernie, A.R.; Mueller-Roeber, B.; Gechev, T.S. A biostimulant obtained from the seaweed *Ascophyllum nodosum* protects *Arabidopsis thaliana* from severe oxidative stress. *Int. J. Mol. Sci.* **2020**, *21*, 474. [CrossRef]
22. Shukla, P.S.; Shotton, K.; Norman, E.; Neily, W.; Critchley, A.T.; Prithiviraj, B. Seaweed extract improve drought tolerance of soybean by regulating stress-response genes. *AoB Plants* **2018**, *10*, plx051. [CrossRef]
23. Goñi, O.; Fort, A.; Quille, P.; McKeown, P.C.; Spillane, C.; O'Connell, S. Comparative transcriptome analysis of two *Ascophyllum nodosum* extract biostimulants: Same seaweed but different. *J. Agric. Food Chem.* **2016**, *64*, 2980–2989. [CrossRef] [PubMed]
24. 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]
25. Thibodeau, P.S.; Jaworski, E.G. Patterns of nitrogen utilization in the soybean. *Planta* **1975**, *127*, 133–147. [CrossRef]
26. Hoagland, D.R.; Arnon, D.I. The water-culture method for growing plants without soil. *Circ. Calif. Agric. Exp. Stn.* **1950**, *347*, 29–31.
27. Souza, S.C.R.; Mazzafera, P.; Sodek, L. Flooding of the root system in soybean: Biochemical and molecular aspects of N metabolism in the nodule during stress and recovery. *Amino Acids* **2016**, *48*, 1285–1295. [CrossRef] [PubMed]
28. Foy, C.D.; Fleming, A.L. Aluminum tolerances of two wheat genotypes related to nitrate reductase activities. *J. Plant Nutr.* **1982**, *5*, 1313–1333. [CrossRef]
29. Rhodes, D.; Rendon, G.A.; Stewart, G.R. The control of glutamine synthetase level in *Lemna minor* L. *Planta* **1975**, *125*, 201–211. [CrossRef]
30. Dougall, D.K. Evidence for the presence of glutamate synthase in extracts of carrot cells cultures. *Biochem. Biophys. Res. Commun.* **1974**, *58*, 639–646. [CrossRef]

31. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
32. Cataldo, D.A.; Maroon, M.; Schrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80. [CrossRef]
33. Yemm, W.E.; Cocking, E.; Ricketts, E.R. The determination of amino acids with ninhydrin. *Analyst* **1955**, *80*, 209–214. [CrossRef]
34. Vogels, G.D.; Van Der Drift, C. Differential analyses of glyoxylate derivatives. *Anal. Biochem.* **1970**, *33*, 143–157. [CrossRef] [PubMed]
35. Hiscox, J.D.; Israelstam, G.F. A method for the extraction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.* **1979**, *57*, 1332–1334. [CrossRef]
36. de Souza, S.C.R.; Sodek, L.; Polacco, J.C.; Mazzafera, P. Urease deficiency alters nitrogen metabolism and gene expression in urease-null soybean without affecting growth or productivity under nitrate supply. *Acta Physiol. Plant.* **2020**, *42*, 34. [CrossRef]
37. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
38. Ferreira, E.; Cavalcanti, P.; Nogueira, D. ExpDes: An R Package for ANOVA and Experimental Designs. *Appl. Math.* **2014**, *5*, 2952–2958. [CrossRef]
39. Drobek, M.; Frąc, M.; Cybulska, J. Plant biostimulants: Importance of the quality and yield of horticultural crops and the improvement of plant tolerance to abiotic stress—A review. *Agronomy* **2019**, *9*, 335. [CrossRef]
40. Kocira, S.; Szparaga, A.; Kuboń, M.; Czerwińska, E.; Piskier, T. Morphological and biochemical responses of *Glycine max* (L.) Merr. to the use of seaweed extract. *Agronomy* **2019**, *9*, 93. [CrossRef]
41. Egli, D.B.; Bruening, W.P. Source-sink relationships, seed sucrose levels and seed growth rates in soybean. *Ann. Bot.* **2001**, *88*, 235–242. [CrossRef]
42. Misra, A.N.; Biswal, U.C. Effect of phytohormones on chlorophyll degradation during aging of chloroplasts in vivo and in vitro. *Protoplasma* **1980**, *105*, 1–8. [CrossRef]
43. Cortleven, A.; Schmülling, T. Regulation of chloroplast development and function by cytokinin. *J. Exp. Bot.* **2015**, *66*, 4999–5013. [CrossRef]
44. Ali, O.; Ramsubhag, A.; Jayaraman, J. Biostimulant properties of seaweed extracts in plants: Implications towards sustainable crop Production. *Plants* **2021**, *10*, 531. [CrossRef] [PubMed]
45. Trivedi, K.; Gopalakrishnan, V.A.K.; Kumar, R.; Ghosh, A. Transcriptional analysis of maize leaf tissue treated with seaweed extract under drought stress. *Front. Sustain. Food Syst.* **2021**, *5*, 774978. [CrossRef]
46. Jannin, L.; Arkoun, M.; Etienne, P.; Laîné, P.; Goux, D.; Garnica, M.; Fuentes, M.; Francisco, S.S.; Baigorri, R.; Cruz, F.; et al. Brassica napus growth is promoted by *Ascophyllum nodosum* (L.) Le Jol. seaweed extract: Microarray analysis and physiological characterization of N, C, and S metabolisms. *J. Plant Growth Regul.* **2013**, *32*, 31–52. [CrossRef]
47. Rathore, S.S.; Chaudhary, D.R.; Boricha, G.N.; Ghosh, A.; Bhatt, B.P.; Zodape, S.T.; Patolia, J.S. Effect of seaweed extract on the growth, yield and nutrient uptake of soybean (*Glycine max*) under rainfed conditions. *S. Afr. J. Bot.* **2009**, *75*, 351–355. [CrossRef]
48. Nelson, W.; Staden, J. The effect of seaweed concentrate on growth of nutrient-stressed greenhouse cucumbers. *HortScience* **1984**, *19*, 81–82. [CrossRef]
49. Turan, M.; Köse, C. Seaweed extracts improve copper uptake of grapevine. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2004**, *54*, 213–220. [CrossRef]
50. Ritchie, S.; Hanway, J.J.; Thompson, H.E.; Benson, G.O. *How a Soybean Plant Develops*; Iowa State University of Science and Technology Cooperative Extension Service: Ames, IA, USA, 1982; 20p.