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Improvement of Photosynthetic Pigment Characteristics, Mineral Content, and Antioxidant Activity of Lettuce (*Lactuca sativa* L.) by Arbuscular Mycorrhizal Fungus and Seaweed Extract Foliar Application

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Abstract: Beneficial plant–microbe interaction for enhancing crop yield and quality is a sustainable way to achieve eco-friendly, desirable agricultural productions. The main objective of this experiment was to evaluate the individual and combined effects of an arbuscular mycorrhizal fungus (AMF) strain (*Funneliformis mosseae*) and a seaweed extract (SWE) derived from *Ascophyllum nodosum*, on the growth and physiological responses of lettuce (*Lactuca sativa* L.). Lettuce plants were inoculated with commercial AMF inoculum (5 g kg^{-1} soil), and SWE foliar application was done at three levels (0.5, 1.5, and 3 g L^{-1}). The findings revealed that AMF along with SWE generated the greatest impact. In fact, co-application of AMF inoculation and 3 g L^{-1} SWE considerably enhanced root colonization, chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids, and mineral content in the shoots and roots (N, P, K, Ca, Fe, Zn, and Mn content) of lettuce plants. This combination improved initial fluorescence (F_0), photochemical efficiency of PSII (F_V/F_m) and Y(NO) and total antioxidant activity (TAA), whereas the maximum fluorescence, (F_m) and Y(II), showed the highest increase in lettuce plants treated with AMF and 1.5 g L^{-1} SWE. Furthermore, AMF inoculation along with SWE, at concentrations 1.5 and 3 g L^{-1}, considerably enhanced variable fluorescence (F_V) and the activity of water decomposition in electron donor photosystem II (F_V/F_0). As a result of these findings, it can be stated that the co-application of AMF and SWE positively improves the growth and development of lettuce plants.

Keywords: lettuce (*Lactuca sativa* L.); arbuscular mycorrhiza fungi; seaweed extracts; pigment parameters; chlorophyll fluorescence; macro and microminerals; antioxidant activity

1. Introduction

Sustainable agriculture, which protects environmental resources and human health, and improves the quality of crops, is the only possible way to meet the ever-increasing needs of the growing world population [1], and to address the major challenge presented by the environmental impact, on ecosystems, of the use of chemical fertilizers [2].

Biostimulants have been considered an effective way to achieve sustainable agricultural production, and to maintain soil health. These stimulants are mainly retrieved from plant extracts, for various applications in the production of crops, to increase the efficiency of the use of nutrients, and for growth improvement [3]. These compounds, as an alternative and, in most cases, as a supplement to chemical fertilizers, play important roles in the

sustainable production of agricultural systems. Furthermore, these fertilizers are composed of beneficial microorganisms, each of which is produced for a specific purpose, such as nitrogen fixation, the release of phosphate ions, potassium, iron, etc. [4]. In this regard, using growth stimulants as environmentally friendly compounds, improves flowering, fruit growth, crop yield, and nutrient efficiency [5].

Coexistence between plants and microorganisms is a common phenomenon in nature, and it can either be beneficial or detrimental to the host plants. The application of arbuscular mycorrhiza fungus (AMF) has been recognized as a valuable environmentally friendly method in organic farming systems [6]. Mycorrhiza plays a vital role in plant nutrition as a biofertilizer, especially in soils without humus, and which are poor in phosphorus, nitrogen, and other nutrients; hence, mycorrhiza establishes a symbiotic relationship with the plant roots, and improves the absorption of nutrients [7]. AMF forms a symbiotic relationship through the infiltration of plant roots and by fungal hyphae, improving the uptake of nutrients such as P and N and, in return, receiving organic carbon compounds from the plants [8]. Previous studies have shown that the coexistence of mycorrhizae and plants has been beneficial in various crops, including corn and wheat [9]. Among the AMF species, mycorrhiza (*Funneliformis mosseae*) is well-known for its symbiosis with the roots of many plant species: it promotes nutrient uptake through its mycelia which, by increasing root development and expansion through the outer membrane of the mycelia, attaches to the plant, thus facilitating the exchange of nutrients [10]. Baslam, et al. [11], in their study on lettuce, stated that AMF inoculation increased plant growth and nutrient uptake very significantly. Furthermore, in another research focused on lettuce [12], it was shown that AMF inoculation increased the phenolic compounds content and nutrients, including P, Mg, Fe, Mn, and Zn. On the other hand, in a study conducted by Papoui, et al. [13], it was found that ascorbic acid, phenolics and antioxidant potential were lower in lettuce and green onion plants grown organically after the application of two AMF (*Rhizophagus intraradices* and *Diversispora* spp.) than in control, while yield and color were unchanged. Tarraf, et al. [14], in their experiment, determined that AMF increased corn yield and dry matter, and the P content in shoots. In addition, Tchameni, et al. [15], found that AMF inoculation increased plant growth parameters and P content in cocoa plants. Elliott, et al. [16], reported that inoculation by arbuscular mycorrhizal fungi increased root colonization and phosphorus uptake in wheat cultivars by up to 30%.

Another new strategy to improve crop yields is the use of seaweed growth stimulants, which are used as cost-effective and environmentally friendly biofertilizers; this strategy has shown a significant increase in the yield of crops [17]. The importance of seaweed extracts as a biological stimulant is evident in many crops, and the possible ways of exploiting these stimuli in modern agriculture have been extensively studied in liquid, powder, or complete algal fertilizers [18]. The utilization of seaweed extract (SWE) as a biostimulant has increased worldwide [19]. SWE of *Ascophyllum nodosum* contains macronutrients and micronutrients, growth hormones, amino acids, vitamins, betaine, cytokinins, and sterols. These compounds promote early seed germination and improve root growth, leaf chlorophyll content, crop yield, and soil physicochemical and biological properties [20]. Moreover, SWE can combine with soil metal ions to form protective colloids [21]. SWE also has a wide and indirect effect on the structure and activity of soil microorganisms, and is one of the proposed solutions for improving soil fertility. This indirect effect on soil microorganisms improves and develops mutual coexistence between plants and microorganisms and, as a result, it leads to plant growth improvement [22]. In an experiment conducted by Abbas, et al. [19], it was observed that foliar application of SWE on onions caused a significant increase in crop yield, plant growth, nutrient uptake, and improved crop quality. In addition, some different species of SWE in rice positively affected crop yield [23]. Improved yield and quality of lettuce was achieved by Hoa, et al. [24], using foliar fertilizers of seaweed (*Vallisneria spiralis*) and water hyacinth.

Regarding the combined application of AMF and SWE, Rasouli, et al. [25], showed that the co-application of AMF and SWE positively affected some morphological and

biochemical characteristics of lettuce. Anli, et al. [26], identified the positive effect of AMF and SWE as an effective tool to enhance date palm growth and development, as total dry matter, leaf area, stomatal conductance, nitrogen, and phosphorus content increased. The combination of AMF with SWE application in common beans [27] improved their vegetative growth, yield, and quality, and this treatment was recommended for organic agricultural systems. This combination of biostimulants in tomato plants led to improvement in root growth, protein, and carbohydrate content [28]. Rashad, et al. [29], reported that AMF inoculation, along with SWE application at 3%, promoted the growth parameters of pea plants.

Lettuce (*Lactuca sativa* L.), which was also studied in this research, belongs to the Asteraceae family, and is one of the most popular leafy vegetables in the world. Lettuce is widely consumed in fresh form, as it is the source of plant nutrients, with health-promoting properties, such as polyphenols, flavonoids, vitamins A, C, and E, carotenoids, Fe, Ca, and some other minerals [30,31]. Vegetables have a short life cycle, and require more fertilizer during the growing season than other plant sources, such as cereal crops or fruit trees. Among vegetables, lettuce grows fast and produces a very shallow root system; it needs sufficient fertilizer levels during its growing season [32].

Considering the promising effects of AMF and SWE on the growth parameters of lettuce in previous research, and with minimum information in the literature about the effect of AMF and SWE—especially in regard to their combined application—on the total antioxidant activity and fluorescence parameters of lettuce, the aim of this study was to investigate the effect of AMF inoculation (*Funneliformis mosseae*) and SWE (*Ascophyllum nodosum*) foliar application on photosynthetic pigments, total antioxidant activity, chlorophyll fluorescence, and macromineral and micromineral content in lettuce shoots and roots. It would be advisable to plant producers to expand this type of production.

2. Materials and Methods

2.1. Experimental Treatments of Lettuce with AMF and SWE

The current experiment was performed in the research greenhouse of the Department of Horticultural Sciences, University of Maragheh, in East Azarbaijan province, Iran, with geographical coordinates of 37°23′ north latitude and 46°16′ east longitude, and 1485 m above sea level. The temperature regime for night and day was 17 and 23 °C, respectively, and the relative humidity was about 65–75%. The research was set up as a factorial experiment based on a completely randomized design (CRD), with four replications.

The experimental treatments included two factors; the first factor was arbuscular mycorrhiza fungus (AMF) (*Funneliformis mosseae*) inoculation (5 g kg^{-1} soil), which was added at transplanting time, and without AMF inoculation. AMF was purchased from Zist Fanavar Pishtaz Varian, Karaj, Iran, with 100 actives spores per g of soil. The second factor was seaweed extract (SWE) foliar spraying at 3 concentrations (0.5, 1.5, and 3 g L^{-1}) and control (0 g L^{-1}). SWE foliar application was done 20 days after transplanting, and replicated three times weekly. The treatment combinations of AMF inoculation and SWE foliar applications are given in Table 1.

Table 1. The treatment combinations—traits T1–T8.

Trait	SWE Concentration (g L^{-1})	AMF (g kg^{-1} soil)
T1	0	0
T2	0.5	
T3	1.5	
T4	3	
T5	0	5
T6	0.5	
T7	1.5	
T8	3	

Before planting the seeds of the lettuce plants (*Lactuca sativa* L.), the soil was autoclaved for 60 min at 121 °C under 1.2 atm, to eliminate the soilborne microorganisms. The lettuce seeds were planted in a container including coco-peat and perlite (2:1 ratio). The transplants were transferred at the two-fully-expanded-leaves stage to 5 L plastic pots. The lettuce plants were irrigated with tap water every 3 days.

In the AMF inoculation treatment, 20 g of *Funneliformis mosseae* was applied to each 5 L pot, including 4 kg soil at the transplanting time, based on our preliminary experiments. The soil texture was sandy clay loam with pH = 8.16, 1.23% organic carbon, 0.09% total N, and 11.05, 570.85, 1.16, and 1.02 mg kg^{-1} of available P, K, Zn, and Fe, respectively. The SWE foliar application was begun at the six-leaf stage, and repeated weekly, four times. The plants were foliar sprayed until the solution drops were run off the leaves. The control plants in the soil without AMF inoculation were sprayed with distilled water (100 mL).

2.2. Root Colonization

The lettuce roots were removed from the soil and washed with tap water, to eliminate the remaining soil particles. The fresh root samples were cut into smaller pieces (1 cm), and were put in a hot KOH solution (10% v/v, 10 min). The pieces were rinsed with de-ionized water, and then acidified with HCl (2%, v/v) at room temperature for 20 min, and dyed with trypan blue (0.05%) in lactic acid (80%, v/v) for 12 h [33,34]. Finally, the stained roots were rinsed with de-ionized water, and kept in a solution including water, glycerol, and lactic acid (1:1:1, $v/v/v$). The root mycorrhizal colonization was assessed using the grid line intersection method [35].

2.3. Photosynthetic Pigments

Chlorophylls (Chl *a* and Chl *b*) and carotenoids (Car) content were determined spectrophotometrically, using the Arnon method [36]. The leaf sample (0.5 g) was powdered with liquid nitrogen, and was homogenized in 10 mL of 80% acetone, after which 2 mL of the homogenate was removed. Finally, the absorbance (UV-1800, Shimadzu, Tokyo, Japan) was read at 663, 645, and 470 nm, to measure Chl *a*, Chl *b*, and Car content. The following equations (1; 2; 3) were used to calculate photosynthesis pigments content (mg g^{-1} fw), expressed as mg kg^{-1} fw (fresh weight):

$$\text{Chlorophyll } a \text{ (Chl } a\text{)} = [12.7\ (A_{663}) - 2.69\ (A_{645})] \quad (1)$$

$$\text{Chlorophyll } b \text{ (Chl } b\text{)} = [21.50\ (A_{645}) - 5.10\ (A_{663})] \quad (2)$$

$$\text{Carotenoids (Car)} = [1000\ (A_{470}) - 1.82\ \text{Chl } a - 85.02\ \text{Chl } b]/198 \quad (3)$$

2.4. Chlorophyll Fluorescence

Chlorophyll fluorescence parameters were measured on four completely developed young leaves, that were randomly chosen by means of pulse amplitude modulation fluorometer (PAM-2500, Heinz Walz, Effeltrich, Germany). The measurement was performed after 20 min, when the plants had adapted to the dark, and the data were analyzed by PamWin-3 software, as explained by Maxwell and Johnson [37]. The parameters with four replications were calculated by PamWin-3 software comprised of: minimum value for chlorophyll fluorescence (F_0); maximal possible fluorescence value (F_m); the difference between F_0 and F_m (F_V); the maximal quantum yield of PS II (F_V/F_m); and the non-photochemical quantum efficiency of PSII Y(NO).

2.5. Macro- and Microminerals

The wet digestion method was used for the measurement of macroelement and microelement concentration [38]. Dried and ground leaf samples (500 mg) were digested in a mixture of sulfuric acid (H_2SO_4) and nitric acid (HNO_3), at a ratio of 1:5 (v/v) at 60 °C for 24 h. In the next step, the homogenate mixture was exposed to nitric, and perchloric acid ($HNO_3/HClO_4$ with a ratio of 1:5; v/v). The Zn, Cu, Mn, and Fe contents were measured

by atomic absorption spectrophotometer (Model AA-6300, Shimadzu, Kyoto, Japan). A flame photometer (Model 410, Sherwood, UK) was used to determine K content.

2.6. Total Antioxidant Activity (TAA)

Using spectrophotometry, the TAA of the lettuce leaves was determined by the free radical scavenging power of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [39]. A fresh leaf sample (1 g) was extracted with 2 mL of 80% methanol, then centrifuged at 14,000 rpm for 15 min at 4 °C. The extract (20 µL) was added to 1 mL of 50 µM methanolic DPPH solution buffered with acetic acid buffer (pH 5.5). The mixture was incubated in the dark for 20 min. The reduction of DPPH absorption at 517 nm was measured using a spectrophotometer (AA-6300, Shimadzu, Japan).

Total Antioxidant Activity (TAA) was calculated as a % of inhibition, using the following Equation (4), where A_c was the absorbance of the control, and A_s was the absorbance of the sample:

$$TAA = [(A_c - A_s)/A_c] \times 100 \quad (4)$$

2.7. Statistical Analysis

Analysis of variance (ANOVA) was conducted using MSTAT-C version 2.1. In addition, the significant differences among means were compared with the Least Significance Difference test (LSD), at $p < 0.05$. Pearson correlation and cluster dendrogram heat maps were plotted in R software for statistical computing.

3. Results

3.1. Root Colonization

Microscopic images of lettuce root fragments stained to detect arbuscular mycorrhizal colonization, are shown in Figure 1. The AMF organs and hyphae, that appear in blue, were documented by high-quality photographs.

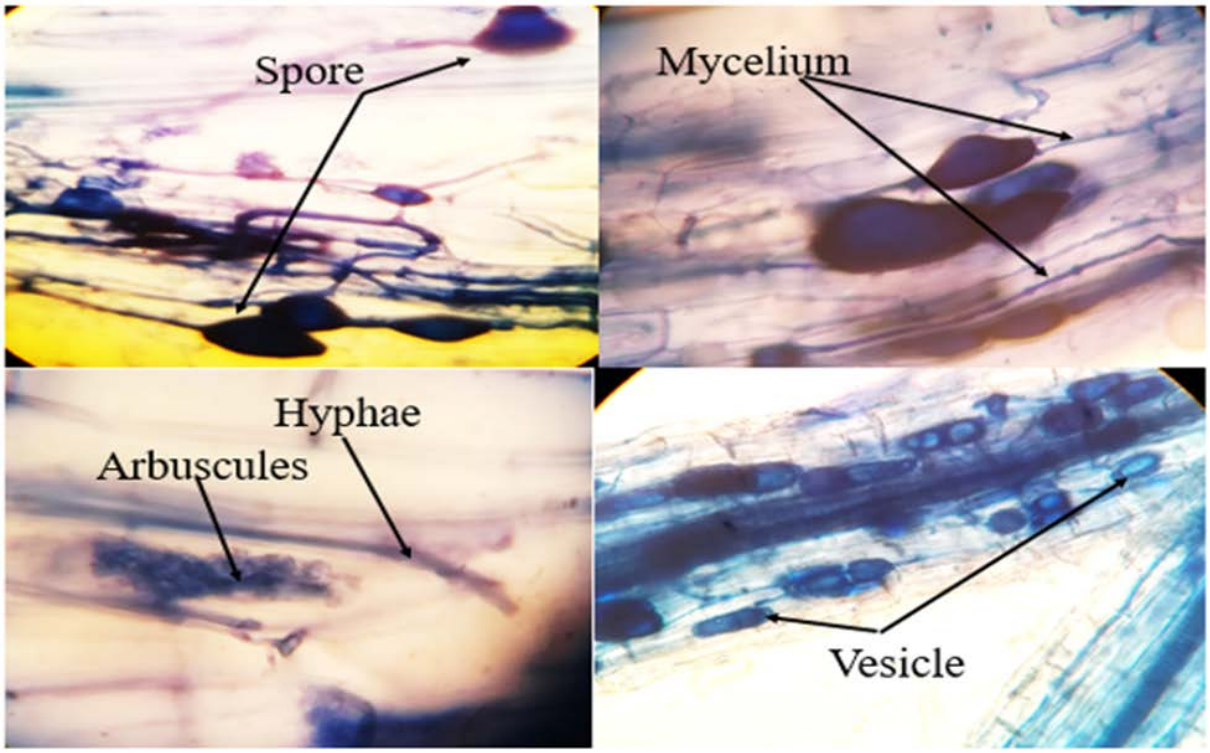

Figure 1. Microscopic images of stained lettuce root fragments for detection of arbuscular mycorrhizae (*Funneliformis mosseae*) colonization.

3.2. Photosynthetic Pigments

The results of photosynthetic pigments contents in lettuce plants, with/without AMF inoculation and SWE foliar application, are shown in Table 2.

Table 2. The effect of arbuscular mycorrhiza fungi (AMF) inoculation and seaweed extract (SWE) foliar application on the photosynthetic pigments contents of lettuce plants and their TAA.

SWE Concentration (g L^{-1})	AMF (g kg^{-1} soil)	Chl *a* (mg kg^{-1} fw)	Chl *b* (mg kg^{-1} fw)	Chl (*a* + *b*) (mg kg^{-1} fw)	Car (mg kg^{-1} fw)	TAA (%)
0	0	13.9 ± 1.4 ^{g}	6.49 ± 1.10 ^{g}	20.4 ± 2.16 ^{g}	3.73 ± 0.72 ^{h}	43.52 ± 3.35 ^{d}
	5	23.2 ± 0.53 ^{e,f}	15.8 ± 0.49 ^{e,f}	39.1 ± 1.02 ^{e,f}	10.5 ± 0.34 ^{f}	61.79 ± 0.77 ^{c}
0.5	0	30.3 ± 0.50 ^{d}	23.2 ± 0.75 ^{c,d}	53.5 ± 1.23 ^{c,d}	18.8 ± 0.61 ^{d}	59.69 ± 0.77 ^{c}
	5	26.6 ± 0.57 ^{d,e}	20.2 ± 0.68 ^{d,e}	46.8 ± 1.28 ^{d}	14.7 ± 0.69 ^{e}	61.70 ± 3.75 ^{c}
1.5	0	19.7 ± 0.59 ^{f}	11.8 ± 0.59 ^{f,g}	31.5 ± 1.16 ^{f}	7.01 ± 0.32 ^{g}	69.52 ± 0.39 ^{b}
	5	41.4 ± 0.61 ^{b}	33.6 ± 1.05 ^{b}	75.1 ± 1.61 ^{b}	28.1 ± 0.56 ^{b}	72.12 ± 0.12 ^{b}
3	0	34.9 ± 1.2 ^{c}	27.4 ± 0.63 ^{b,c}	62.3 ± 1.88 ^{c}	22.8 ± 0.36 ^{c}	70.00 ± 0.12 ^{b}
	5	53.6 ± 2.3 ^{a}	49.9 ± 5.36 ^{a}	103.6 ± 7.53 ^{a}	37.1 ± 0.68 ^{a}	81.85 ± 2.72 ^{a}
LSD at 0.05%		3.87	6.86		3.09	6.96
S.O.V						
AMF		336.8 **	453.1 **	1571.3 **	207.8 **	345.8 **
SWE		1370.40 **	1527.09 **	5790.07 **	1086.02 **	950.30 **
AMF + SWE		30.93 **	68.30 **	189.63 *	13.69 *	122.20 **
Error		7.062	22.120	48.770	4.485	22.700
C.V		8.71	19.9	14.52	11.86	7.34

Result ± SD (standard deviation); different letters in each column indicate a significant difference at $p \leq 0.05$; * and ** indicate significant difference at 5% probability level and at 1% probability level, respectively.

Table 2 shows an increasing of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids contents in plants grown under AMF treatment. The co-application of AMF and SWE significantly increased the content of chlorophyll *a*, chlorophyll *b*, and total chlorophyll (*a* + *b*). The highest content of chlorophyll *a* and *b* was observed in the plants treated with AMF inoculation × 3 g L^{-1} SWE, which were increased by 285, 787, and 405% compared to the control plant samples. The results also revealed that AMF × SWE application significantly increased the carotenoids content of lettuce leaves. The highest carotenoids content was related to the lettuce plants subjected to AMF × 3 g L^{-1} SWE, which increased by 894% compared to the control treatment.

3.3. Chlorophyll Fluorescence

The results of chlorophyll fluorescence parameters after AMF inoculation and seaweed extract (SWE) co-application on lettuce plants are given in Figure 2a–g. The F_0, F_V/F_m, and Y(NO) values of the lettuce leaves were significantly affected by AMF and SWE, as can be seen in Table 3. The uppermost improvements of the F_0, F_V/F_m, and Y(NO) of the leaves were obtained in the plants supplemented with AMF inoculation × 3 g L^{-1} SWE treatment, which were increased by 96.3%, 398%, and 105%, respectively, compared to the control. The lowest values of F_0, F_V/F_m, and Y(NO) were recorded in the control plants (Figure 2a–c). As shown in Figure 2, the amounts of F_m and Y(II) in the lettuce leaves were significantly affected by AMF inoculation × SWE treatment. AMF + SWE application at 1.5 g L^{-1} led to the maximum values of F_m and Y(II) for the lettuce leaves, with an enhancement of 97.3 and 202%, respectively, compared to the control plants (Figure 2d,f). Moreover, the F_V and F_V/F_0 parameters were significantly increased under the combined AMF and SWE treatments. The best values of F_V and F_V/F_0 were observed using 3 and 1.5 g L^{-1} SWE × AMF inoculation, and these treatments were up to 189% and 503% more than the control (Figure 2e,g).

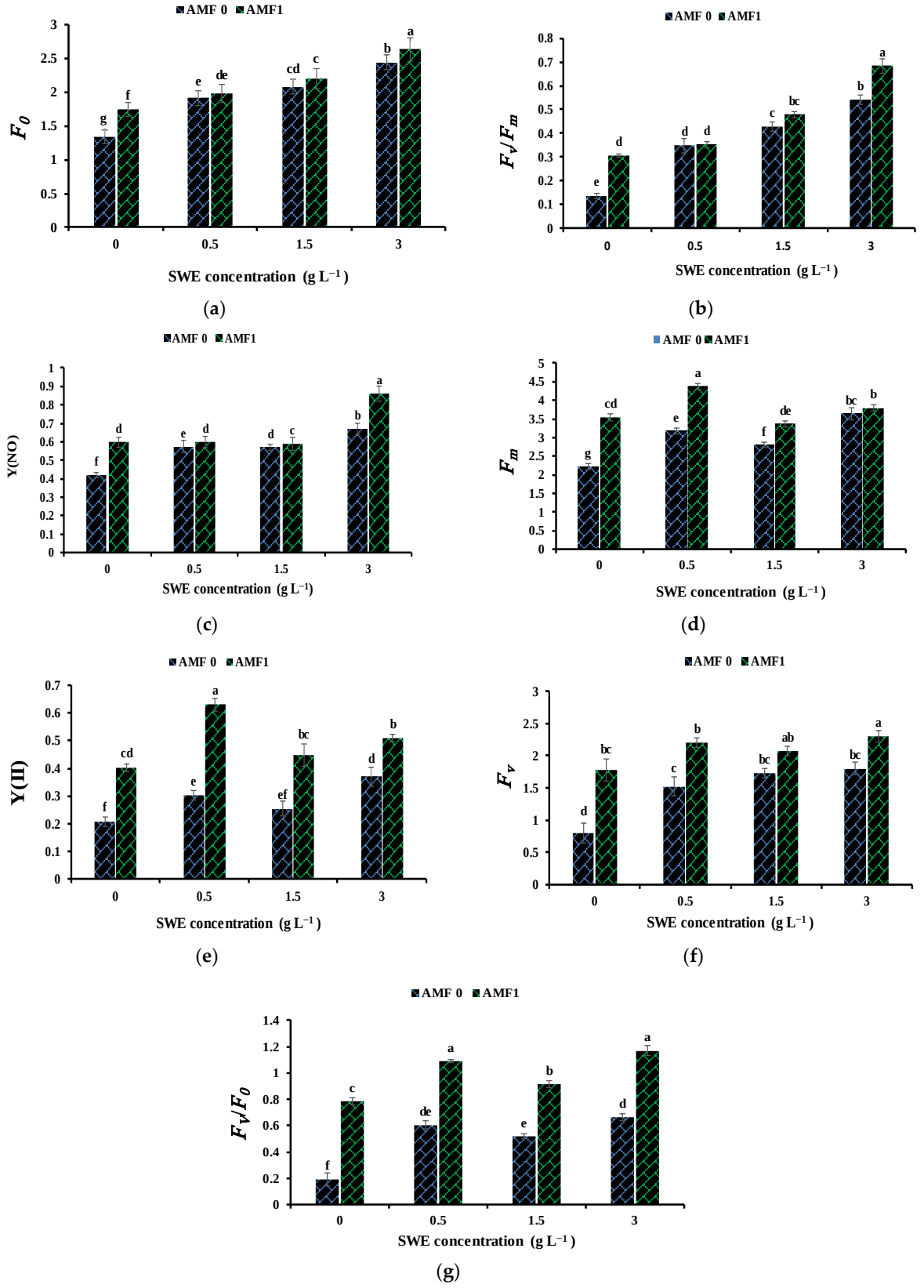

Figure 2. Effect of AMF inoculation (AMF 0 and AMF 1: without/with AMF, respectively) and seaweed extract (SWE) co-treatments on lettuce plants: (**a**) initial fluorescence (F_0); (**b**) photochemical efficiency of PSII (F_V/F_m); (**c**) Y(NO); (**d**) maximum fluorescence (F_m); (**e**) Y(II); (**f**) variable fluorescence (F_V); (**g**) electron donor photosystem II (F_V/F_0). Error bars represent standard deviations (SD). Different letters indicate significant differences according to the LSD test at $p < 0.05$.

Table 3. Analysis of variance (ANOVA) for the effects of arbuscular mycorrhiza fungi (AMF) inoculation and seaweed extract (SWE) foliar spray on some physiological traits of the lettuce plant.

S.O.V	df	F_0	F_m	F_V	F_V/F_m	F_V/F_0	Y(II)	Y(NO)
AMF	1	0.301 *	0.044 ^{ns}	1.242 **	0.068 **	0.173 **	0.001 ^{ns}	0.065 **
SWE	3	1.381 *	3.513 *	1.389 **	0.218 **	0.873 **	0.164 **	0.096 **
AMF + SWE	3	0.043 **	0.465 **	0.320 *	0.012 **	0.029 *	0.015 **	0.025 **
Error	24	0.013	0.025	0.059	0.002	0.005	0.002	0.005
C.V		5.63	4.73	13.66	11.02	9.66	10.37	11.44

^{ns} = no significant difference; * and ** indicate significant difference at 5% probability level and at 1% probability level, respectively.

3.4. Macrominerals and Microminerals

The contents of macrominerals and microminerals in the lettuce roots and shoots, after treatment with arbuscular mycorrhiza fungi and seaweed extract, are shown in Tables 4 and 5. Mineral concentrations were significantly affected by the application of AMF, and also by treatment combinations of AMF and SWE. In the inoculated plants, P and N concentrations were significantly enhanced (Table 4). Nevertheless, other elements were also influenced; meanwhile, the least content of minerals was detected in the control plants (without AMF and SWE foliar application) (Tables 4 and 5). With the application of a higher SWE concentration, a higher amount of most minerals in shoots and roots was observed.

3.5. Total Antioxidant Activity (TAA)

The results of the total antioxidant activity of lettuce plants treated with AMF and SWE are shown in Table 2. The TAA values were significantly affected by the combined AMF inoculation and SWE foliar application. Plants treated with the highest concentration of SWE presented the highest antioxidant activity. Thus, the highest TAA value of inhibition (81.9 %) was obtained in the combination of AMF × 3 g L^{-1} SWE, which increased by up to 88.1% compared to the control (Table 2). Without AMF inoculation, the lettuce plants increased their antioxidant effect by about 60.8% compared to the control.

Table 4. The macrominerals content in lettuce plants treated with arbuscular mycorrhiza fungi (AMF) and seaweed extract (SWE).

SWE Concentration (g L^{-1})	AMF (g kg^{-1} soil)	N Shoot (mg g^{-1} dw)	N Root (mg g^{-1} dw)	P Shoot (mg g^{-1} dw)	P Root (mg g^{-1} dw)	K Shoot (mg g^{-1} dw)	K Root (mg g^{-1} dw)	Ca Shoot (mg g^{-1} dw)	Ca Root (mg g^{-1} dw)
0	0	1.00 ± 0.02 ^{e}	1.07 ± 0.06 ^{d}	0.655 ± 0.04 ^{g}	1.32 ± 0.06 ^{f}	0.61 ± 0.03 ^{a}	0.71 ± 0.06 ^{f}	14.0 ± 0.3 ^{f}	15.7 ± 0.1 ^{g}
	5	1.38 ± 0.01 ^{d}	1.19 ± 0.02 ^{c,d}	1.16 ± 0.02 ^{e}	2.22 ± 0.01 ^{d}	0.82 ± 0.04 ^{a}	1.00 ± 0.01 ^{e}	21.2 ± 0.6 ^{d}	21.2 ± 0.3 ^{e}
0.5	0	1.46 ± 0.01 ^{c,d}	1.42 ± 0.01 ^{c,d}	1.30 ± 0.01 ^{d}	2.37 ± 0.01 ^{c}	1.36 ± 0.01 ^{a}	0.95 ± 0.01 ^{e}	18.2 ± 0.3 ^{e}	19.0 ± 0.2 ^{f}
	5	1.63 ± 0.05 ^{c}	1.64 ± 0.08 ^{c}	1.55 ± 0.01 ^{b}	2.59 ± 0.01 ^{b}	2.13 ± 0.04 ^{a}	1.08 ± 0.04 ^{e}	21.5 ± 0.5 ^{d}	22.5 ± 0.2 ^{d,e}
1.5	0	1.29 ± 0.03 ^{d}	1.12 ± 0.05 ^{d}	1.03 ± 0.01 ^{f}	2.07 ± 0.01 ^{e}	0.78 ± 0.07 ^{a}	1.40 ± 0.07 ^{d}	22.5 ± 0.4 ^{c,d}	23.5 ± 0.2 ^{c,d}
	5	2.69 ± 0.16 ^{a}	3.92 ± 0.37 ^{a}	1.22 ± 0.01 ^{e}	2.25 ± 0.01 ^{d}	0.91 ± 0.17 ^{a}	2.30 ± 0.06 ^{b}	27.0 ± 0.5 ^{b}	27.2 ± 0.2 ^{b}
3	0	2.14 ± 0.01 ^{b}	2.65 ± 0.05 ^{b}	1.41 ± 0.01 ^{c}	2.46 ± 0.02 ^{c}	1.63 ± 0.03 ^{a}	1.93 ± 0.02 ^{c}	23.7 ± 0.2 ^{c}	24.7 ± 0.2 ^{c}
	5	2.52 ± 0.02 ^{a}	3.63 ± 0.08 ^{a}	1.83 ± 0.02 ^{a}	2.71 ± 0.03 ^{a}	2.37 ± 0.04 ^{a}	2.54 ± 0.04 ^{a}	31.0 ± 0.3 ^{a}	31.5 ± 0.6 ^{a}
LSD at 0.05%		0.216	0.479	3.87	0.092	0.234	0.153	1.34	1.78
S.O.C									
AMF		0.342 **	0.575 **	0.552 **	1.26 **	0.267 **	0.591 **	47.53 **	50.0 **
SWE		0.994 **	1.442 **	3.27 **	11.85 **	3.87 **	4.12 **	225.3 **	199.1 **
AMF × SWE		0.046 **	0.303 **	0.07 *	0.363 *	0.013 ^{ns}	0.067 **	7.94 **	4.50 **
Error		0.002	0.004	0.022	0.108	0.026	0.011	0.844	1.50
CV		3.87	2.86	8.44	15.77	12.09	7.15	4.10	5.28

Result ± SD (standard deviation); different letters in each column indicate a significant difference at $p \leq 0.05$; ^{ns} = no significant difference; * and ** indicate significant difference at 5% probability level and at 1% probability level, respectively.

Table 5. The microminerals content in lettuce plants treated with arbuscular mycorrhiza fungi (AMF) and seaweed extract (SWE).

SWE Concentration (g L^{-1})	AMF (g kg^{-1} soil)	Fe Shoot (mg g^{-1} dw)	Fe Root (mg g^{-1} dw)	Zn Shoot (mg g^{-1} dw)	Zn Root (mg g^{-1} dw)	Mn Shoot (mg g^{-1} dw)	Mn Root (mg g^{-1} dw)
0	0	0.36 ± 0.04 ^{e}	0.65 ± 0.08 ^{g}	0.30 ± 0.02 ^{f}	0.58 ± 0.03 ^{e}	18.5 ± 0.5 ^{g}	16.5 ± 0.5 ^{g}
	5	0.90 ± 0.01 ^{e,f}	1.03 ± 0.03 ^{d}	0.98 ± 0.02 ^{d,e}	1.20 ± 0.03 ^{b,d}	29.5 ± 0.6 ^{e}	27.5 ± 0.3 ^{e,f}
0.5	0	0.99 ± 0.01 ^{d}	0.81 ± 0.05 ^{f,g}	0.62 ± 0.02 ^{e,f}	1.17 ± 0.04 ^{c,d}	26.7 ± 0.05 ^{f}	24.7 ± 0.04 ^{e,f}
	5	1.00 ± 0.01 ^{d}	1.24 ± 0.06 ^{c,d}	1.11 ± 0.01 ^{d}	1.23 ± 0.01 ^{b,c}	31.5 ± 0.07 ^{e}	29.5 ± 0.06 ^{e}
1.5	0	1.15 ± 0.01 ^{c}	1.05 ± 0.05 ^{d,e}	1.06 ± 0.03 ^{d}	1.29 ± 0.01 ^{d}	35.5 ± 0.05 ^{d}	31.5 ± 0.04 ^{c,d}
	5	1.43 ± 0.01 ^{b}	1.48 ± 0.02 ^{b}	1.44 ± 0.03 ^{a}	4.04 ± 0.10 ^{a}	41.7 ± 0.06 ^{b}	39.7 ± 0.03 ^{b}
3	0	1.27 ± 0.01 ^{c}	1.33 ± 0.01 ^{b,c}	1.28 ± 0.01 ^{a,c}	1.92 ± 0.02 ^{c}	38.2 ± 0.05 ^{c}	36.2 ± 0.03 ^{c}
	5	1.76 ± 0.04 ^{a}	1.84 ± 0.06 ^{a}	1.35 ± 0.02 ^{a,b}	2.89 ± 0.03 ^{b}	47.2 ± 0.08 ^{a}	45.2 ± 0.07 ^{a}
LSD at 0.05%		0.145	0.206	0.172	0.397	2.47	2.34
S.O.C							
AMF		0.581 **	0.001 ^{ns}	1.26 **	0.002 ^{ns}	171.12 **	171.1 **
SWE		1.199 **	1.295 **	11.85 **	13.56 **	692.25 **	692.25 **
AMF × SWE		0.153 **	0.127 **	0.363 *	1.234 **	16.20 **	16.20 *
Error		0.010	0.020	0.108	0.074	2.87 **	2.87 **
CV		8.81	12.28	16.59	8.44	5.04	5.36

Result ±SD (standard deviation); different letters in each column indicate a significant difference at $p \leq 0.05$; ^{ns} = no significant difference; * and ** indicate significant difference at 5% probability level and at 1% probability level, respectively.

3.6. Correlation Analysis

The Pearson's correlation of the photosynthesis pigments, fluorescence parameters, and mineral attributes is shown in Figure 3. The findings exposed a significantly positive correlation among the evaluated traits. However, a considerably positive correlation was observed among chlorophyll pigments Chl *a*, Chl *b*, Ch total (*a* + *b*), and carotenoids (Car), as well as among mineral K, Ca, Fe, Zn, and Mn concentrations of shoots and roots with one other. Also, F_0 positively correlated with phosphorus concentration in the shoots and roots, and with potassium in shoots.

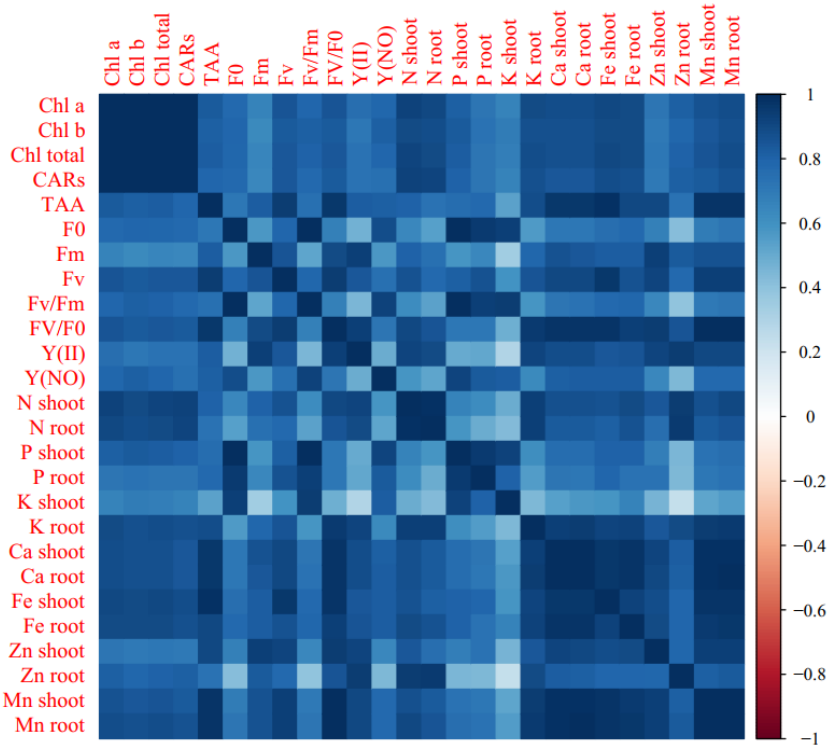

Figure 3. Heat map of Pearson's correlation analysis for the response of arbuscular mycorrhiza fungi

(AMF) inoculation along with seaweed extracts (SWE) foliar application to *Lactuca sativa* L. Heat map representing chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Chl total), carotenoids (CARs), total antioxidant activity (TAA), F_0, F_v, F_m, F_v/F_m, F_v/F_0, Y(NO), Y(II), and N, P, K, Ca, Fe, Zn, Ca concentrations in shoots and roots.

The heat map (Figure 4) shows that the application of AMF along with SWE had a positive effect on all evaluated traits. The highest positive compliance was observed for Y(NO) and photosynthesis pigments, and for some minerals, such as Ca in shoots and roots, Fe in roots, and P in shoots.

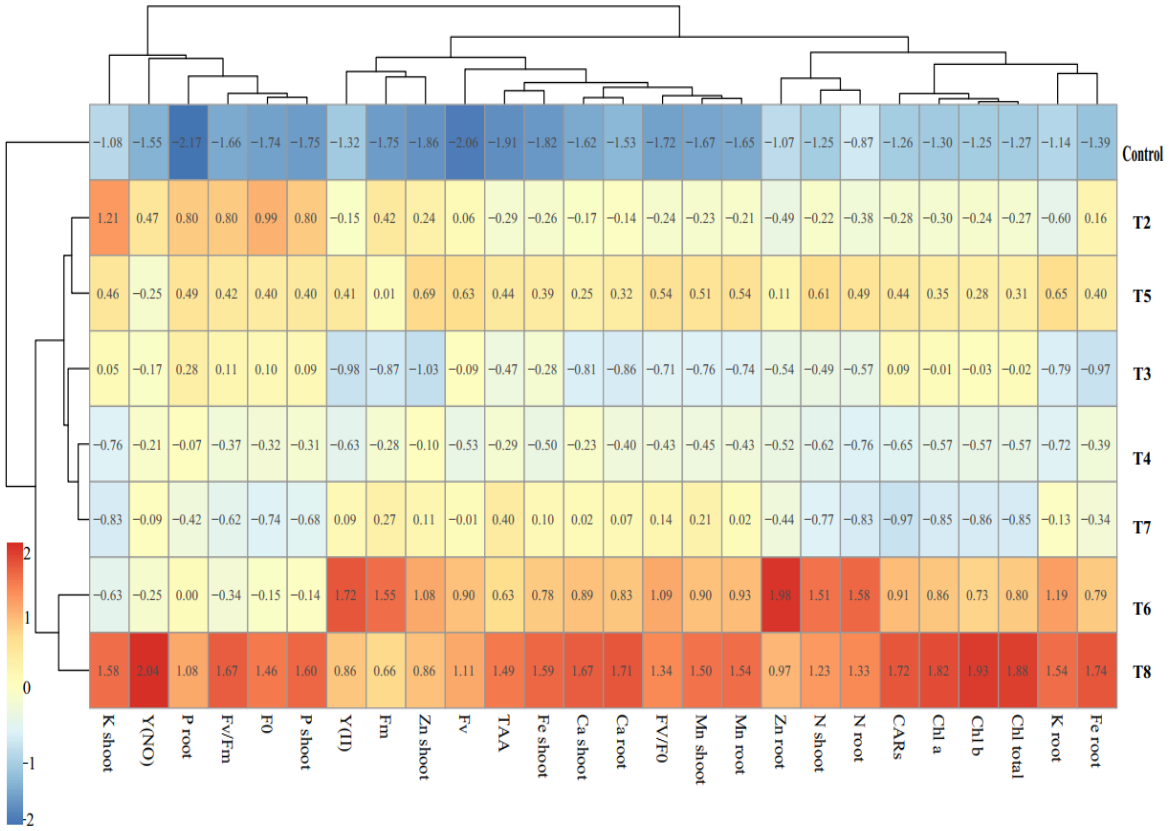

Figure 4. Cluster analysis heat map for the response of arbuscular mycorrhiza fungi (AMF) inoculation and seaweed extracts (SWE) foliar application to *Lactuca sativa* L. Photosynthesis pigments (chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Chl total), carotenoids (CARs), fluorescence chlorophyll parameters (F_0, F_v, F_m, F_v/F_m, F_v/F_0, Y(NO), Y(II),), mineral pool (N, P, K, Ca, Fe, Zn, Ca concentrations in shoots and roots), and total antioxidant activity (TAA), evaluated for the plants represented eight traits with different treatment combinations.

Cluster analysis and dendrograms in a heat map matrix (Figure 4) revealed three major clusters for the calculated features. Cluster 1 consisted of K in roots, Y(NO), P in shoots and roots, F_0, and F_V/F_m; cluster 2 contained Y(II), F_m, Zn in shoots, F_V, TAA, Fe in shoots, Ca in shoots and roots, F_V/F_0 and Mn in shoots and roots; and cluster 3 included Zn in roots, N in shoots and roots, photosynthesis pigments, K and Fe in roots. Overall, the heat map cluster analysis for the applied treatments exhibited three main groups, where cluster 1 contained only the control lettuce plants (trait 1); cluster 2 was the biggest group, comprising all samples without AMF inoculation × 0; 1.5, 3 g L^{-1} SWE, and samples with AMF and SWE at 0 and 1.5 g L^{-1} concentration; cluster 3 contained samples of lettuce plants inoculated with AMF along with SWE at levels of 0.5 and 3 g L^{-1}.

4. Discussion

The rhizosphere of plants is home to a wide variety of microbial species, many of which naturally interact with plant roots. Recently, there has been considerable interest in the use of beneficial microorganisms for sustainable crop production and soil health [40]. An extended mycelial network helps plant roots reach beyond the root depletion zone, allowing the plant to utilize more soil mineral nutrients and water [41,42]. AMF obtains photosynthetic carbohydrates/carbon from the roots of host plants, and AMF in turn provides nutrients and water from the soil to the host plants through its extended hyphal network [43]. Moreover, SWE supplies dozens of growth-essential nutrients, such as polysaccharides, amino acids, vitamins, and other active substances, which not only help the plant to build a robust root system, but also improve the absorption of nutrients and water [44]. Furthermore, El Chami, et al. [45], found that the application of SWE in pear production is very convenient, as it was possible to significantly reduce the total applied fertilization by 13%, and to improve the quantitative and qualitative characteristics (total yield, average fruit weight) of evaluated products. Overall, the application of SWE shows growth-stimulating activities in plants, and increased yields and quality in various horticultural crops [46], as was also observed in our findings, as discussed below.

The results of our research showed that photosynthetic pigments were significantly amplified by the use of AMF and SWE. The co-application of AMF and SWE significantly increased the content of chlorophyll *a*, chlorophyll *b*, and total chlorophyll (Table 2). Leaf chlorophyll content is an essential index for measuring plant photosynthesis and growth potential. A common response to the application of plant biostimulants is the increase of photosynthetic pigments such as chlorophyll *a*, chlorophyll *b*, and carotenoids, which play a significant role in chlorophyll biosynthesis [47,48]. The increase in the content of chlorophyll *a* and *b* in plants can be attributed to the high percentage of free amino acids, such as alanine, aspartate, asparagine, and glutamate in biostimulants [49]. The increase of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids contents in plants grown under AMF treatment could be due to the stimulation of root growth and the enhanced nutrient uptake. Our experimental results are consistent with the findings of previous studies. Spraying with SWE fertilizer can thus improve leaf chlorophyll content, and increase photosynthesis. Treatment with SWE can improve the net photosynthetic rate, stomatal conductance, and water use efficiency, as de Carvalho, et al. [50], found. On the other hand, SWE includes a large amount of plant-growth-regulating substances, such as auxins, gibberellins, betaine, polyamines, etc., which can effectively promote plant growth and improve leaf development, as well as enhancing photosynthetic pigments content [51]. In addition, seaweed extract contains a large amount of cytokinin, which has a protective role on chloroplasts, and promotes the biosynthesis of endogenous cytokinin in treated plants [52]. Similarly, the auxin and betaine in SWE fertilizers intensify the chlorophyll biosynthesis, and even promote the development of grana [53]. Furthermore, SWE contains a high mineral elements content (N, K, Mg, and Fe), of which magnesium is essential for chlorophyll biosynthesis, and potassium plays a positive role in enhancing photosynthesis, promoting meristem growth, and regulating the water status of the plants [54]. Our findings show that SWE and mycorrhiza co-application improves photosynthesis parameters and pigments content, and these results are consistent with those of Meng, et al. [55], Weisany, et al. [56], and Xu & Leskovar [57]. Overall, the obtained results may be due to the fact that the application of SWE tended to improve leaf total chlorophyll content, subsequently reflected in the capacity and efficiency of the photosynthetic process and, certainly, supportively reflected in plant vegetative growth. Our findings are in accordance with the report of Castellanos-Barriga, et al. [58], who stated that SWE application on mung bean led to an enhancement in leaf pigment contents compared to control plants, and that the total chlorophyll concentration of the seedlings was significantly higher. As Mahmoud, et al. [59], detected, the highest values of leaf photosynthesis pigments contents in red radish plants were obtained by spraying of brown SWE at 3 mL L^{-1} compared to the control, while the lowest values were achieved for the control sample. Similar trends were

noted in our study for lettuce plants supplemented with SWE foliar application at 3 g L^{-1}. The achieved results were in accordance with Mancuso, et al. [60], Shehata, et al. [61], Fan, et al. [62], and Kulkarni, et al. [63]. They confirmed that the application of SWE led to an increase in the leaf total chlorophyll content of treated plants, consequently reflected in the capacity and efficiency of the photosynthetic process.

Chlorophyll fluorescence parameters are commonly used to determine the potential and efficiency of photosynthesis. Evaluated fluorescence induction parameters include F_m (maximum fluorescence in photosystem II dark response), F_0 (minimum fluorescence or excitation energy loss index), F_V (variable fluorescence determined after adaptation to darkness), and fluorescence ratio F_V/F_m (photochemical capacity belonging to photosystem II). The F_0, F_V/F_m, and Y(NO) values in the current experiment were significantly improved by AMF inoculation and SWE foliar application (Table 3). These parameters, after combined treatment by AMF and SWE at 3 g L^{-1}, were the highest compared to the control (Figure 2a–g); the maximum value of F_m and Y(II) in the lettuce leaves was with AMF inoculation × SWE application at 1.5 g L^{-1}. This suggests that plants inoculated with AMF have a higher ability to protect their leaves from light damage. Moreover, the efficiency of the photochemical activity of photosystem II in plants inoculated with AMF was higher than in plants without AMF, and the reason for this could be related to the increase in photosynthetic efficiency due to the presence of AMF [64,65]. Furthermore, the symbiotic relationship of AMF fungi can increase the energy uptake efficiency of chloroplasts, and increase the photochemical capacity of photosystem II (PS II) in light-adapted leaves [66]. In general, AMF is involved in increasing F_V/F_m by improving plant nutritional status and activating several intermediate genes [67]. Considering that SWE will increase the amount of chlorophyll and photosynthesis potential, the increase in the intensity of chlorophyll fluorescence can be related to the improvements in the concentration of excited chlorophyll molecules [68]. Thus, SWE + AMF co-application can improve stomata conductance, increase photochemical quantum efficiency and CO_2 uptake, and ultimately increase chlorophyll fluorescence [69]. Moreover, AMF and SWE treatments significantly improved photochemical quantum efficiency (F_V/F_m) in date palms, compared to the control [26]. The conclusions of the above-mentioned reports are similar to the current findings.

The content of macrominerals and microminerals in lettuce roots and shoots was significantly affected by the application of AMF, and even more by treatment combinations of AMF and SWE (Tables 4 and 5). The plants inoculated with AMF absorbed more macromineral and micromineral nutrients than non-inoculated ones, therefore the colonization of plant roots with AMF led to extensive changes in the chemical and biochemical processes in the host plants. In the inoculated plants, P and N concentrations were significantly enhanced, and other elements were also influenced. This increment may have been attributable to the secretion of various types of siderophores and chelates, the stimulation of acid production by soil microorganisms, the release of cations from the soil particles, and improvement in the absorption potential of the roots. In general, AMF creates an extended hyphal network, stimulates root branching and expansion, and improves plants' ability to absorb water and nutrients [70]. The positive and beneficial effect of biostimulants, such as AMF, on enhancing the content of nutrients in plant tissues, shows a direct relationship with nutrient uptake by plant-living microorganisms and plant growth [71]. In agreement with our findings, Faria, et al. [72], have also shown that AMF inoculation increased the absorption of P, K, Zn, Fe, and Cu, compared to the control plants. In addition, AMF can indirectly increase the availability of nutrients in the soil, by producing phosphatase enzymes, and converting immobile phosphorus to a mobile form, increasing the uptake of the other nutrients and the degradation of organic matter [73].

It has been proven that SWE in microbial communities, such as mycorrhizal fungal populations, through low and high molecular weight organic diffusible compounds in the rhizosphere and root system structure, efficiently increases the absorption of macronutrient and micronutrient elements [74]. In addition, seaweed extracts include laminar polysaccharides, alginates, and carrageenan. Alginates from seaweed extracts promote the

growth of beneficial fungi, significantly increase hyphal growth, and increase the length of roots [75]. As with our findings, previous studies have documented the positive impact of AMF and SWE co-application on the N, K, P, and other micronutrients content of several plant species, including garlic (*Allium sativum*) [76], date palm (*Phoenix dactylifera*) [26], and *Pinus greggii* [77]. SWE fertilizer provides the crop with a complete range of macronutrients and micronutrients, such as polysaccharides, amino acids, vitamins, and other active substances, which help the plant to build a strong root system, but also improve the uptake of the nutrients, water and gases [78], as we showed also in our experiment. Natsir, et al. [79], stated that extract of Sargassum seaweed may accelerate growth, as it contains minerals such as N, P, K, Ca, Fe, S, Cu, and Zn. Those nutrients have a function as energy and cell development sources, as well as being important components in the formation of protein. Meng, et al. [55], revealed that lots of physiologically active ingredients contained in SWE contribute to the transport of organic and inorganic components in plants, and improve the absorption of nutrients.

The total antioxidant activity (TAA) of lettuce plants was also significantly affected by the combination of AMF inoculation and SWE foliar application, as seen in Table 2. As with our results, several previous experiments have recorded the positive effects of AMF and SWE on TAA in different plants. The coexistence of AMF induces the biosynthesis of the secondary metabolite, and may increase the antioxidant compound's accumulation and function. In an earlier study by Baslam, et al. [11], lettuce plants inoculated with AMF attained more antioxidant compounds. This fungus may interact with the host plant metabolism, and cause the accumulation of several phytochemicals and antioxidant molecules. As a result, it changes the plant's secondary metabolites pool and antioxidant potential [80]. In addition, the reason for the increase in antioxidant activity in lettuce can be attributed to the biostimulant effects of SWE, which increases the antioxidant activity in the host plant through the stimulation of antioxidant enzymes biosynthesis [81]. In an experiment with cowpea, Vasantharja, et al. [82], also observed that using SWE increased the antioxidant activity. In addition, in an investigation by Ashour, et al. [83], on the pepper plant, an enhancement in antioxidant activity was observed as a result of using the treatment of SWE as a biostimulant. Increase in phenolic contents in plant tissues can be linearly correlated with antioxidant capacity [84]. These results are underpinned by all the reports mentioned above.

5. Conclusions

In the current study, due to minimum information in the literature about the effects of AMF and SWE, and their combined application, on the fluorescence parameters and antioxidant activity of lettuce, these were studied more closely. The study found that their application, separately and in combination, positively affected the responses of the lettuce plants, especially in the co-application of AMF inoculation at 5 g kg^{-1} soil and 3 g L^{-1} of SWE derived from *Ascophyllum nodosum*. Photosynthetic pigments and chlorophyll fluorescence parameters, such as F_0, F_V/F_m, Y(NO), F_m, and Y(II), were significantly improved after the co-application of AMF and SWE, thereby leading to improvement of plant growth. AMF and SWE application greatly enhanced the mineral content of N, P, K, Fe, Mn, and Zn in shoots and roots, and also in the TAA of lettuce. Therefore, the treatment of the inoculation by AMF alone, and in combination with SWE foliar application, can be suggested to improve leafy vegetable production. Altogether, the combined application of AMF and SWE could contribute to a sustainable and viable biofertilization approach to improving lettuce growth. These biostimulants could be used as a management tool during conventional farming practices, to enhance crop growth and productivity; they also have the potential to improve crop production under nutrient-deficient systems, when they are applied individually or in combination. The findings of the present study show that the use of chemical fertilizers can be reduced or replaced by the application of AMF and SWE for leafy vegetables such as lettuce, wherever chemical fertilizers are widely used by growers.

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