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Growth and Antioxidant Responses of Lettuce (*Lactuca sativa* L.) to Arbuscular Mycorrhiza Inoculation and Seaweed Extract Foliar Application

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Abstract: Biofertilizers, such as arbuscular mycorrhiza fungi (AMF) and seaweed extract (SWE), have been effective in environmental and agricultural ecosystems. In this study, the effects of AMF, SWE, and their co-application were assayed on the growth and antioxidant potential of lettuce plants. The experiment was conducted as a factorial based on a completely randomized design with two factors and four replications under greenhouse conditions. The first factor was AMF (*Glomus mosseae*) at two levels consisting of AMF application (20 g pot^{-1}), and without using AMF; and the second factor was SWE foliar spraying (*Ascophyllum nodosum*) at 0.5, 1.5 and 3 g L^{-1} concentration. The results revealed that the highest root colonization (85%) belonged to AMF and SWE (3 g L^{-1}) × AMF; the lowest colonization rate (65%) was observed for AMF × SWE (0.5 g L^{-1}) treatment. The highest growth parameters (leaf number, shoot and root fresh weight, head diameter), biochemical traits (total soluble proteins, carbohydrates content) and TAA, total antioxidant activity by FRAP method and ascorbic acid, total phenolics, and flavonoids content were obtained with the co-applications. Therefore, the best results of the evaluated traits were achieved with AMF × SWE (3 g L^{-1}). The TAA value was increased three-fold compared to the control. Total phenolics and flavonoids content were 2.24 and 6.59 times higher than the control, respectively. On the other hand, leaf dry weight was decreased with the further growth of the plants. Overall, the co-application of AMF with SWE can be recommended to producers as an alternative and environment-friendly strategy to improve the qualitative and quantitative traits of the lettuce crop.

Keywords: biofertilizer; *Glomus mosseae*; colonization; biostimulant; FRAP

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1. Introduction

Lettuce (*Lactuca sativa* L.) belongs to the Asteraceae family and is rich in fiber, vitamins, minerals, and phenolic compounds [1]. In 2019, lettuce production reached 29,134,653 tons in the world from which about 547,590 tons were produced in Iran [2]. Considering the important and valuable nutritional role of lettuce due to its daily consumption and, on the other hand, the excessive application of chemical fertilizers with negative impacts on human health and the environment; there is an effort to find alternative methods for reducing the chemical fertilizers input in lettuce production areas.

The use of chemical fertilizers is widespread throughout the world, leading to soil degradation and environmental pollution. Therefore, the global approach to establishing a

sustainable agricultural system has changed with the employment of new management methods. Considering this, it is important to pay attention to the biological and integrated systems, especially biofertilizers, to meet the plant nutritional requirements and reduce the consumption of chemical fertilizers [3]. Bio-fertilizers (bio-stimulants) release their content to make it slowly available to the plants and simultaneously improve soil quality [4]. Therefore, the use of bio-fertilizers has advantages such as removing toxic substances and improving the physicochemical properties of soil [5].

Arbuscular mycorrhiza fungi (AMF) and seaweed extract (SWE) can be useful because they are organic, environmentally friend, cost-effective, and also a rich source of macro-and microelements, vitamins, pro-enzymes, and growth regulators and so play an important role in the sustainable soil fertility [6].

SWEs are a complex mixture of hormones, amino acids, proteins, sugars, lipids, vitamins, humic substances, and phenolic compounds. The organic features of SWEs and their physiological effects have led to their widespread use in the food and pharmaceutical industries [7]. Moreover, SWE contains carbohydrates, organic compounds, and high amounts of nitrogen, phosphorus, potassium, and other minerals that improve soil properties and are easily absorbed by the plants. Thereby, those extracts improve plant growth and the antioxidants pool by activating the respiratory cycle, photosynthesis, and delaying plant aging [8]. Di Mola et al. [9] reported that SWE increased the growth and yield in lettuce and significantly improved fresh and dry weight, stomatal conductance, potassium content, and total antioxidant activity [10]. Jung and Kim [11] reported increases in plant height, chlorophylls and carotenoids content, and total antioxidant activity in lettuce using SWE.

AMF is another natural fertilizer source used in organic farming systems [12]. AMF is one of the biological materials of arable soils that are related to the roots of 90% of plants and improve the effective root area and the ability of P uptake from immobile sources due to phosphatase activity and the insoluble phosphate solubilizing organic compounds release [13]. These microorganisms play an important role in plant nutrition, especially in the soils without humus and poor in P, N and other nutrients so that they can make unabsorbed and inaccessible P available to the plants in the growing medium [14]. In general, AMF symbiosis can play a key role in maintaining soil fertility and stabilizing soil structure when increasing plant water uptake, yield, and quality [15]. There are many reports on various effects of AMF and SWE on plants. Saia et al. [16] showed that AMF increased the content of various phenolic compounds and P, Mg, Fe, Mn, and Zn in lettuce. Tarraf et al. [17] stated that the symbiosis of AMF promoted growth, yield, and dry matter of maize and increased the concentration of P in the shoot. A wide range of studies has shown that AMF is effective in the biosynthesis and accumulation of plant secondary metabolites by modifying the polymorphisms and by stimulating the biosynthesis of polyphenols and can increase the activity of plant enzymes [18]. Moreover, AMF colony formation increases photosynthetic efficiency in plants [15]. Thus, bio-fertilizers can improve the quantity and quality of crops, especially vegetables.

Several studies reported the influence of AMF on lettuce and other vegetables' growth and antioxidant systems [19–21], and others focused on SWE to improve lettuce yield and quality [9]. However, there are no results in the literature on the application of AMF in combination with SWE to improve the growth, biochemical and antioxidant attributes of lettuce. In this context, the present research aimed to evaluate the effects of a SWE (*Aschophyllum nodosum*) and an AMF strain (*Glomus moseae*) and their interaction on the growth, yield, and antioxidant activity of lettuce under greenhouse conditions. The main question was: does the AMF inoculation combined with SWE improve the growth, yield, and antioxidant activity of lettuce?

2. Materials and Methods

This study was conducted in the research greenhouse of the Department of Horticultural Sciences, the University of Maragheh in East Azarbaijan province, Iran, with geographical coordinates of 37° and 23′ north latitude and 46° and 16′ east longitudes and

1485 m above sea level. The temperature regime for the night:day was 18:24 °C, and the relative humidity was around 65–75%. The experiment was arranged as factorial based on a completely randomized design with four replications.

Experimental treatments were: foliar application of SWE at four levels (0.5, 1.5 and 3 g L^{-1}, presented as SWE1, SWE2 and SWE3), AMF inoculation (20 g pot^{-1}), AMF inoculation plus the foliar application of SWE at three levels and control that consisted without AMF × 0 g L^{-1} SWE (control), AMF × 0.5 g L^{-1} SWE, AMF × 1.5 g L^{-1} SWE and AMF × 3 g L^{-1} SWE. Before sowing the seeds, the soil was sterilized to remove the soilborne fungi by autoclaving for 60 min at 121 °C under 1.2 atmospheric pressure. Lettuce seeds were planted in a culture tray containing coco-peat and perlite. The seedlings were transferred at two fully-developed leaves stage to 5-L pots. In AMF (inoculation with *Glomus mossae*) treatments 20 g of the autoclaved soil containing mycorrhiza fungal hyphae were added to each pot containing 5 kg soil at transplanting time. The soil was sandy clay loam with pH of 8.16, 1.23% organic carbon, 0.09% total N, 11.05, 570.85, 1.16 and 1.02 mg. kg^{-1} of available P, K, Zn and Fe, respectively. The plants were watered every 3 days and at the beginning of growth, 100 mL, and then 2 weeks later, 500 mL of tap water was given to each pot. Then, foliar application of different concentrations of SWE was started at the six-leaf stage and continued four times at weekly intervals, such that at the first stage 50 mL per plant of SWE solution was sprayed and at the other three stages 100 mL per plant was applied. Control plants were planted in the soil without AMF inoculation, sprayed with distilled water, and irrigated with tap water in the same manner until the harvest.

2.1. Morphological Traits

Plant morphological traits including head diameter, fresh weight of the head, dry weight of leaf, fresh and dry weight of root, and the number of leaves per head were recorded at harvest time. Head diameter was measured using a digital caliper. The weight of the head and root was measured separately by an analytical scale (A&D weighing Japan) (with an accuracy of 0.01 mg). Plant samples were dried in the oven at 75 °C for 48 h and the dry weight of root and leaves were recorded correspondingly.

2.2. Chlorophyll Index Determination

Chlorophyll content expressed as chlorophyll index (SPAD index) was determined in the fully expanded youngest leaves of lettuce using a portable chlorophyll meter (Instruments SPAD-502, Osaka, Japan).

2.3. Root Colonization

After head harvest, the fresh roots of lettuce were taken from the soil and rinsed with tap water to remove the residual soil particles. The root samples were divided into small segments (1 cm) and cleared in hot KOH solution (10%, v/v) for 10 min. The segments were washed with distilled water and then acidified with HCl (2%, v/v) at 25 °C for 20 min, and stained with trypan blue (0.05%) in lactic acid (80%, v/v) for 12 h [22,23]. Finally, the samples were washed with distilled water and stored in a solution containing water, glycerol, and lactic acid (1:1:1, $v/v/v$) [24]. The stained segments were identified and evaluated by an Olympus microscope (BH-2). The organs and hyphae of the fungus, which appeared blue, were recorded as high-quality photos (Figure 1). The percentage of colonization was calculated by the gridline intersection method based on Giovannetti and Mosse [25] so that, for each experimental treatment, the stained roots were cut into 1 cm pieces and randomly placed in a glass plate.

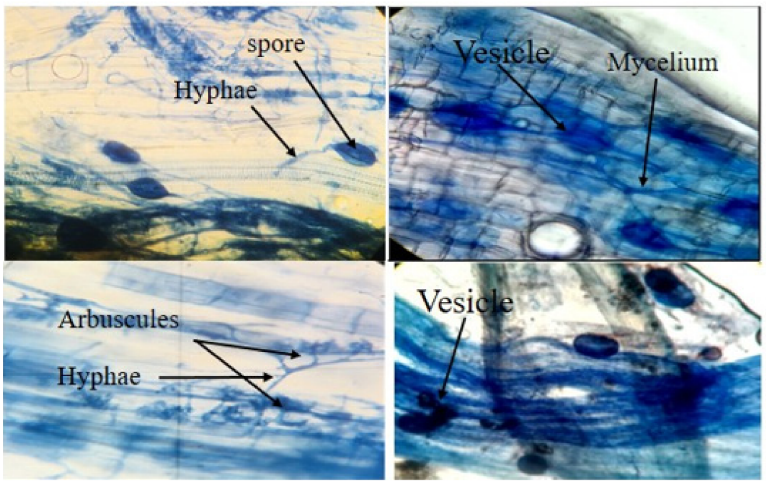

Figure 1. Microscopic images of the stained fragments of lettuce roots to detect the colonization of arbuscular mycorrhiza (*Glomus mosseae*).

2.4. Total Protein Content

Total protein content was recorded using the Bradford method [26]. A 5-fold Coomassie Brilliant Blue (CBB) stock solution was made as a Bradford reagent. We blended 50 mg of CBB with 25 mL of methanol and 50 mL of orthophosphoric acid in a dark bottle and stored this at −24 °C. Different concentrations (0.002−0.01 mg mL^{-1}) of bovine serum albumin were used as standard solutions. Fresh lettuce leaves (1 g) were powdered in 4 mL of 50 mM phosphate buffer solution and then centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was used for the total protein content assay. Protein extract (50 µL) was added to 1000 µL of Bradford reagent. The formation of blue color was assessed at the wavelength of 595 nm using the UV−VIS spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany). Total protein content was calculated as mg g^{-1} FW (fresh weight).

2.5. Total Carbohydrate Content

To measure total carbohydrate content [27], a sample of fresh lettuce leaves (0.2 g) was extracted with 10 mL of 95% ethanol for 1 h in a water bath at 80 °C, and then centrifuged at 12,000 rpm for 10 min. The supernatant was taken and then 1 mL of 0.5% phenol and 5 mL of 98% sulfuric acid were added. The absorption was detected at 483 nm with a spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany). Total carbohydrates content was recorded as mg. g^{-1} FW.

2.6. Total Antioxidant Activity (TAA)

Total antioxidant activity (TAA) was measured by ferric reducing power (FRAP) assay [28]. The reagents included acetate buffer (300 mM, pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine, 10 mM) solution in HCl (40 mM) and $FeC1_3.6H_2O$ (20 mM) solutions. The fresh working solution was prepared by mixing these reagents in the volume ratio of 10:1:1. methanolic extract of the sample (100 mL) was mixed with 3 mL of working FRAP reagent. Samples were then incubated at 37 °C in a water bath, and absorbance was recorded after 15 min at 593 nm with a UV−VIS spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany). L-ascorbic acid was used as a standard solution (100 mM–1000 mM). TAA was calculated as the inactivation of FRAP (%).

2.7. Ascorbic Acid Content

One gram of the leaf lettuce sample was completely digested with a 5% solution of metaphosphoric acid. The extracted sample was centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant was used to measure total ascorbic acid content [29]. To 4 mL of the centrifuged DCIP solution (2,6-dichlorophenolindophenol, 3 mM), 0.5 mL was added to

oxidize ascorbic acid to dehydroascorbic acid. The absorbance was measured using a UV-VIS spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany) at 520 nm. Ascorbic acid content was presented as mg. 100 g^{-1} FW.

2.8. Total Phenolics Content

The Folin–Ciocalteu method by Singleton et al. [30], with gallic acid as a standard was applied to measure total phenolics content. Plant extract (20 µL of 1% acidic methanol solution) and 100 µL of Folin–Ciocalteu reagent (10%) were mixed with 1.59 mL of distilled water and kept for 10 min in the dark. Sodium carbonate (7.5%, 2 mL) was then added into the mixture and placed for 2 h in the dark condition. The absorbance was measured at 765 nm with a UV−VIS spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany). The content was expressed, using the gallic acid calibration curve, as mg of gallic acid equivalents per 100 g of fresh weight (mg GAE 100 g^{-1} FW).

2.9. Total Flavonoids Content

The aluminum chloride colorimetric method by Chang et al. [31] was used for the measurement of total flavonoids content. A sample of the fresh leaf (1 g), crushed in liquid nitrogen was extracted with 4 mL 96% ethanol. To this extract (500 µL) was added potassium acetate (1 M, 100 µL) and aluminum chloride (10%, 0.1 mL). Then, 1.5 mL of methanol and 2.8 mL of distilled water were added and it was kept for 30 min at 25 °C. Finally, the absorbance was measured using a spectrophotometer (Spekol 1500, Analytik Jena, Jena, Germany) at 415 nm. Total flavonoid content was expressed using the quercetin calibration curve (0, 20, 40, 60, 80 and 100 µg ml^{-1}) as mg quercetin equivalent g^{-1} fresh weight (mg QE g^{-1} FW).

2.10. Statistical Analysis

ANOVA was performed using MSTAT-C ver. 2.1 software. The mean comparisons of the data were analyzed using the least significant difference (LSD) test at 5% probability level. Excel software was used to draw graphs. Pearson's correlation coefficients and a heat map were drawn using Rstudio ver. 14.2.1 software.

3. Results

3.1. Root Colonization Percentage

The results showed that root colonization percentage was significantly affected by the treatments (Figure 2). The highest colonization (85%) belonged to AMF × SWE (3 g L^{-1}) and the lowest percentage (65%) was observed in AMF × SWE 0.5 g L^{-1} (Figure 1).

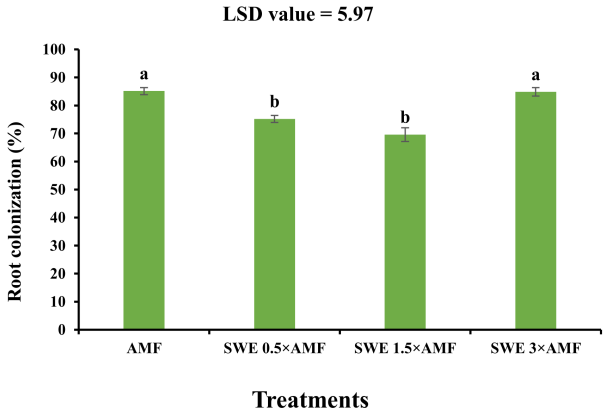

Figure 2. Mean root colonization percentage of lettuce plants affected by arbuscular mycorrhiza (AMF) inoculation × seaweed extract (SWE) foliar treatment. Different letters indicate significant differences at 5% level according to the least significant difference (LSD) test.

3.2. Morphological Traits

The number of leaves and head diameter were significantly affected by AMF × SWE interactions. The highest number of leaves and head diameter were obtained by applying AMF × SWE at concentrations of 1.5 and 3 g L^{-1}. The top number of leaves and head diameter were 44% and 3.6 times more than the control, respectively. The least number of leaves and head diameter were observed in the control (Table 1).

Table 1. The effects of arbuscular mycorrhiza (AMF) inoculation and seaweed (SWE) extract foliar spray on the growth-related traits of lettuce plants.

Fertilization	Number of Leaves	Head Diameter (cm)	Head Fresh Weight (g)	Root Fresh Weight (g)	Head Dry Weight (g)	Root Dry Weight (g)
control	25.5 ± 3.01d	20.75 ± 3.17g	72.5 ± 20.6f	22.38 ± 2.74g	1.42 ± 0.54e	13.88 ± 1.45g
SWE1	28.5 ± 2.96c	29.25 ± 2.39f	134.2 ± 18.90e	33.03 ± 1.26df	2.02 ± 0.035bc	20.9 ± 0.57f
SWE2	30.47 ± 2.75c	34.87 ± 2.57e	173 ± 27.1d	32.25 ± 1.572f	2.2 ± 0.035d	25.88 ± 0.32d
SWE3	34.75 ± 1.62bc	57.53 ± 3.54c	220.5 ± 14.4c	46.49 ± 2.275c	3.02 ± 0.022bc	30.01 ± 0.42c
AMF	31.5 ± 1.83bc	32.1 ± 2.39ef	180.2 ± 22.5d	35.6 ± 6.95ef	2.57 ± 0.041cd	23.4 ± 0.17e
AMF + SWE1	36 ± 2.61ab	43.72 ± 1.95d	199.1 ± 24.2de	42.22 ± 2.351cd	2.37 ± 0.056c	28.38 ± 0.43c
AMF + SWE2	37 ± 2.21a	66.48 ± 3.53b	245.7 ± 18.6b	52.11 ± 1.16b	3.4 ± 0.127b	32.86 ± 0.34b
AMF + SWE3	36.72 ± 3.83a	76.02 ± 3.13a	284.3 ± 17.01a	63.83 ± 3.31a	5.2 ± 0.437a	39.7 ± 2.08a
LSD at 0.05%	8.17	4.49	24.32	4.82	0.587	2.16
Significance						
AMF	ns	**	**	**	**	**
SWE	ns	**	**	**	**	**
AMF × SWE	**	**	*	*	**	**

Control (without arbuscular mycorrhiza fungi and seaweed extract); SWE 1, SWE 2 and SWE 3 (seaweed extract 0.5 g L^{-1}, 1.5 g L^{-1}, and 3 g L^{-1}, respectively), AMF (arbuscular mycorrhiza fungus), AMF × SWE 1, AMF × SWE 2 and AMF × SWE 3 (arbuscular mycorrhiza fungi × seaweed extract 0.5 g L^{-1}, 1.5 g L^{-1}, and 3 g L^{-1}, respectively). Different letters indicate significant differences according to LSD test at $p < 0.05$. ns, * and ** indicate no significant difference, significant at 5% probability level and significant at 1% probability level, respectively.

As shown in Table 1, the application of AMF × SWE treatment had a significant effect on the fresh weight of the lettuce head and roots. The highest dry weight of roots and head was recorded for AMF × SWE, 3 g L^{-1}, and the lowest values were observed in the control.

Moreover, the results showed that the dry weight of lettuce leaf and root was significantly affected by different concentrations of SWE. The highest dry weight of roots and leaves was obtained by applying AMF × SWE at 3 g L^{-1}, which showed an increase of 3.6 and 2.8 times compared to the control, respectively. The lowest dry weight of leaf and roots belonged to the control (Table 1).

3.3. Chlorophyll Index (SPAD)

The SPAD index was significantly increased by the co-application of SWE and AMF. The highest SPAD index (57.15) was obtained by using AMF × SWE, 3 g L^{-1}, while the lowest SPAD index (33.4) was recorded for the control. The top recorded data were 71.10% more than control (Figure 3a).

3.4. Total Protein Content

The total protein content of lettuce leaves was significantly affected by the co-application of AMF and SWE (Table 2). The highest leaf protein content (1.13 mg g^{-1} FW) was obtained for the treatment of AMF × SWE, 3 g L^{-1} which showed an increase of 4.21%, compared to the control. The lowest total protein content (0.268 mg g^{-1} FW) was recorded in the control samples (Figure 3b).

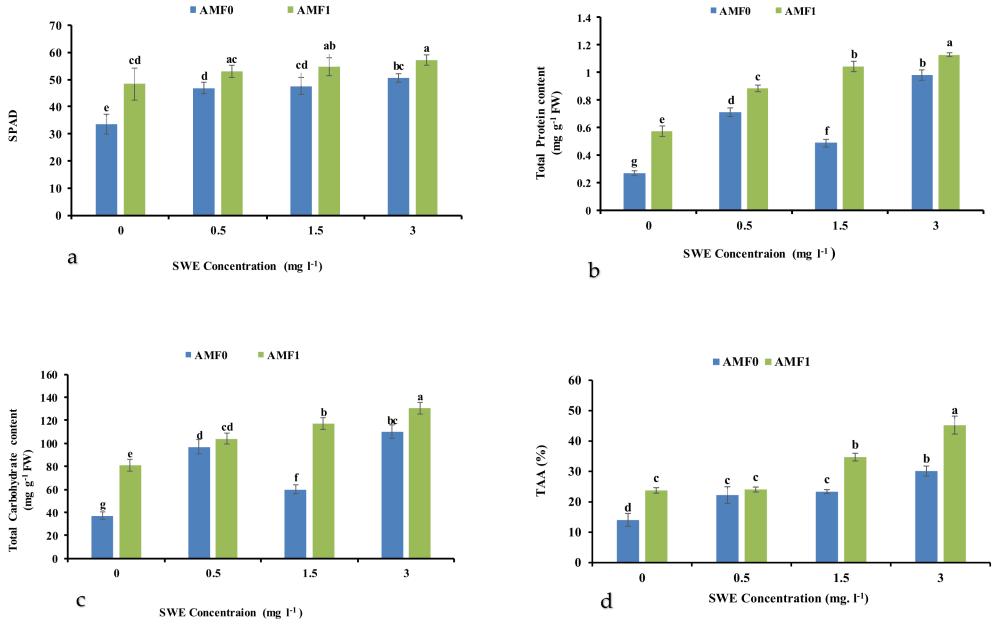

Figure 3. Effect of mycorrhiza (AMF) × seaweed extract (SWE) co-treatments on chlorophyll index (SPAD) (**a**), total proteins content (**b**), total carbohydrates content (**c**), and total antioxidant activity (TAA) (**d**) of lettuce plants. Different letters indicate significant differences according to LSD test at $p < 0.05$. AMF0 and AMF1 refer to without mycorrhiza and with mycorrhiza.

Table 2. Analysis of variance (ANOVA) for the effects of arbuscular mycorrhiza fungi (AMF) inoculation and seaweed extract (SWE) foliar spray on physiological traits of lettuce plant.

S.O.V.	df	Total Protein Content (mg g^{-1} FW)	Carbohydrate Content (mg g^{-1} FW)	TAA (%)	Ascorbic acid Content (mg $100g^{-1}$ FW)	Total Phenolics Content (mg $100g^{-1}$ FW)	Total Flavonoids Content (mg g^{-1} FW)
AMF	1	0.146 **	1708.3 **	392.91 **	262.43 **	7699.90 **	14,238.2 **
SWE	3	0.792 **	8346.9 **	663.45 **	961.32 **	19,875.95 **	48,877.1 **
AMF × SWE	3	0.007 **	103.2 *	37.60 *	125.95 **	3326.05 **	1733.1 **
Error	24	0.002	32.06	12.60	10.08	593.75	264.94
C.V.		5.51	6.15	13.05	5.92	12.55	8.82

AMF and SWE refer to arbuscular mycorrhiza fungi and seaweed extract, respectively. S.O.V. and df refer to the source of variation and degree of freedom. *, ** significant at the 5% and 1% probability levels, respectively.

3.5. Total Carbohydrate Content

The co-treatment of AMF and SWE significantly affected the total carbohydrate content of lettuce leaves (Table 2). The highest content of leaf carbohydrates (130.6 mg g^{-1} FW) was observed for AMF × SWE (3 g L^{-1}), which was 3.5 times more than the control (37.02 mg g^{-1} FW) (Figure 3c).

3.6. Total Antioxidant Activity (TAA)

According to Table 2, TAA values were significantly affected by the interaction of SWE and AMF. The highest antioxidant activity (42.24%) measured by the FRAP method was obtained by using AMF × SWE, 3 g L^{-1}. The activity was increased three times compared to the control while the lowest TAA value (14.06%) was recorded for the control sample (Figure 3d).

3.7. Ascorbic Acid Content

The interactions of AMF and SWE significantly affected the content of ascorbic acid as well (Table 2). The highest ascorbic acid content (67.66 mg 100g $^{-1}$ FW) was obtained for AMF × SWE, 3 g L^{-1}, and the least data (29.75 mg $100g^{-1}$ FW) belonged to the control (Figure 4a).

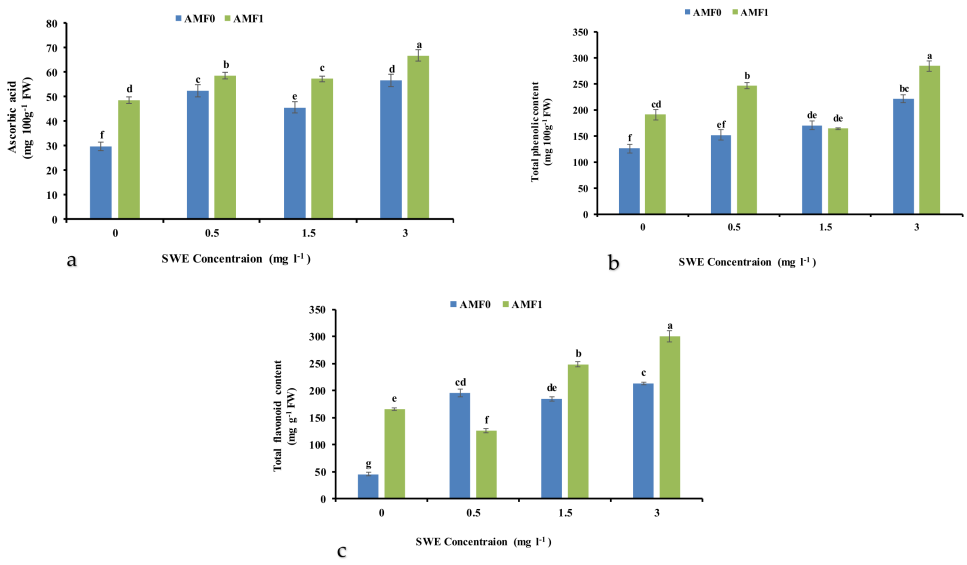

Figure 4. Effect of arbuscular mycorrhiza fungi (AMF) × seaweed extract (SWE) co-treatments on the ascorbic acid content (**a**) total phenolics content (**b**) and total flavonoids content (**c**) of lettuce plants. Different letters indicate significant differences according to the LSD test at $p < 0.05$. AMF0 and AMF1 refer to without and with arbuscular mycorrhiza fungi, respectively.

3.8. Total Phenolic and Flavonoid Content

Also, the content of phenolics and flavonoids in lettuce leaves was affected by the combined application of AMF and SWE (Table 2). The highest values of total phenolics (283.9 mg GAE 100 g^{-1} FW) and flavonoids (300 mg QE g^{-1} FW) were obtained by the co-application of AMF × SWE (3 g L^{-1}). It was 2.24 and 6.59 times higher than the control, respectively. The lowest content of total phenolics and flavonoid was 126.2 mg GAE 100 g^{-1} FW and 45.5 mg QE g^{-1} FW, respectively, which were observed for the control (Figure 4b,c).

3.9. Correlation Matrix and Relative Expressions

The Pearson's correlation of the morphological and biochemical traits is presented in Figure 4. The results revealed a positive significant correlation among phenolics, TAA, flavonoids, root FW, and ascorbic acid and protein content. Also, head diameter significantly correlated to SPAD and shoot FW. On the other hand, shoot DW significantly correlated with leaf number and shoot FW.

The heat map (Figure 5) showed that phenolics, TAA, flavonoids, root FW and DW, ascorbic acid, proteins content, leaf number, carbohydrates content, shoot FW, SPAD, and head diameter had positive compliance with the co-application of SWE and AMF.

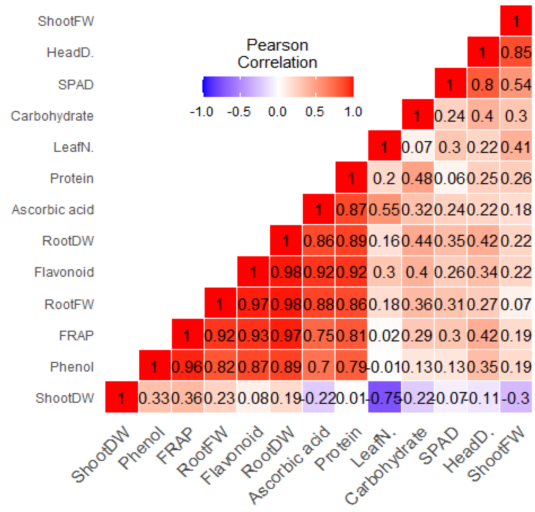

Figure 5. Heat map of Pearson's correlation analysis for the effect of arbuscular mycorrhiza (AMF) inoculation and seaweed extract (SWE) foliar spray on lettuce. Heat map representing head DW (dry weight), root FW (fresh weight), flavonoid (total flavonoids content), root DW, protein (total proteins content), TAA (total antioxidants activity by FRAP method), phenol (total phenolics content), carbohydrate content, head D (head diameter), head FW, SPAD (chlorophyll index), and leaf N (leaf number).

Cluster analysis and dendrograms in a heat map matrix (Figure 6) showed two main groups for the evaluated traits. Group 1 contained phenolics, TAA, flavonoids, root FW and DW, ascorbic acid content, protein content, leaf number, carbohydrates content, shoot FW, SPAD, and head diameter; and group 2 represented shoot DW that had a negative correlation with the higher growth of the plants. In general, cluster analysis of heat maps for treatments showed three main groups. Group 1 contained using AMF with SWE (3 g L^{-1}), group 2 contained, SWE (0.5 and 1.5 g L^{-1}) with and without AMF, and group 3 included the control.

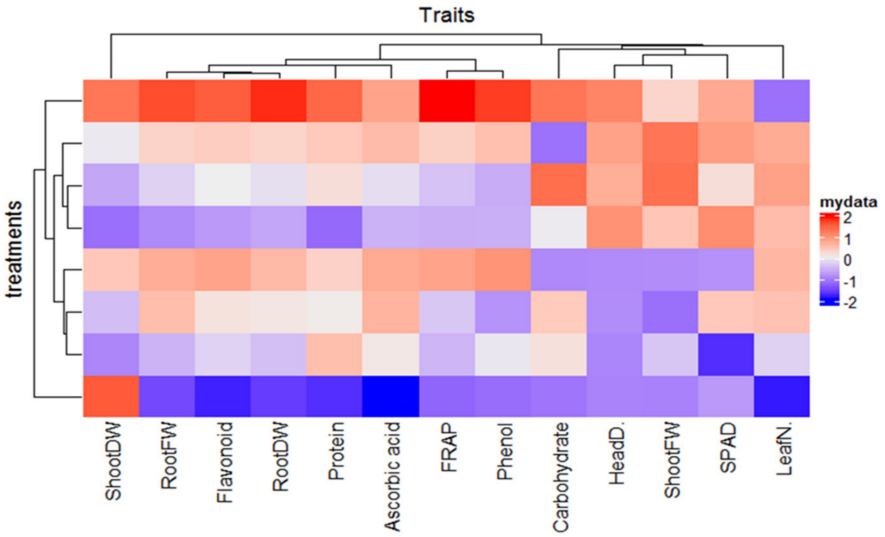

Figure 6. The morphological and biochemical changes in lettuce plants treated with arbuscular mycorrhiza (AMF) and seaweed extract (SWE). Heat map representing head DW (dry weight), root FW (fresh weight), flavonoid (total flavonoids content), root DW, protein (total proteins content), TAA (total antioxidants activity by FRAP method), phenol (total phenolics content), carbohydrates content, head D (head diameter), head FW, SPAD (chlorophyll index) and leaf N (leaf number).

4. Discussion

One of the crucial principles in planning the production of vegetables is to evaluate the effectiveness of diverse plant nutrition strategies. The organic production of vegetables along with a proper nutrition regime, while maintaining the environmental and health standards, will increase the yield and quality of those crops.

The results of this study showed that the highest average number of leaves, head diameter, head, and root fresh weight, and leaf and root dry weight was obtained by the combined application of biological stimulants (AMF and SWE). The increased plant height is a response to AMF, which helps in the appropriate absorption of essential nutrients. Furthermore, the inoculation of this fungus to plant roots causes the production of various hormones, such as auxin and gibberellins, and is effective in increasing the plant height and, consequently, the number of leaves [32]. It also improves water and nutrient uptake, photosynthesis potential, and carbohydrate production by altering the root morphology and thereby increasing shoot and root growth. The symbiosis of AMF plays a vital role in the carbon cycle and can increase the production of growth-promoting molecules [33]. In lettuce, the use of SWE increased plant growth probably due to the availability of significant amounts of growth hormones, amino acids, and macro-and microelements [34]. Following the present results, Dudaš et al. [35] in lettuce and Rouphael et al. [36] in zucchini reported an increase in plant height and number of leaves after the usage of SWE.

The results showed that the co-use of SWE and AMF positively affected the fresh and dry weight of the head and roots. In plants inoculated with AMF, some of the fungal hyphae enter the root system of the plant and reduce abscisic acid levels and increase cytokinins, which expand the root system and enhance water uptake, as well as the secretion of organic acids such as malic acid by extra-root hyphae increasing P uptake and thus improving root development and eventually growth of the aerial parts of the plant [37]. Biological properties of the soil [38] and this symbiosis in the root zone increase the uptake of minerals (P, K, Fe, Zn and Mn) and lead to an increase in dry weight biomass. SWE also improves photosynthetic efficiency by stimulating nutrients translocation and metabolism, phytohormones biosynthesis, proteins accumulation, and delaying the aging process, and can ultimately help to increase the fresh and dry weight of plants [39]. In this regard, Colla et al. [40] reported that foliar application of SWE increased the fresh and dry weight of lettuce.

In the present study, the application of AMF + SWE significantly increased the total proteins content in lettuce. In addition, the higher content of protein in AMF treatment can be attributed to the more nitrogen uptake in the symbiotic behavior of plants and fungi. Nitrogen in organic matter is usually present in the composition of peptides, proteins, and free amino acids. The AMF secretes peptidase and protease into the soil to absorb nitrogen-containing monomers, thus improving leaf proteins content [10]. The increase in proteins content due to the use of SWE can also be a result of the plant's ability to absorb a higher amount of other elements [41].

Consistent with the results of the current study, Sosnowski [42] reported that lettuce leaf carbohydrates content was increased by the combined application of AMF and SWE. Due to an increase in the stomatal conductance and P uptake, the symbiosis of mycorrhizae leads to the accumulation of secondary metabolites, vitamins, minerals, and photosynthetic pigments, and even raises the leaves' carbohydrates content [43]. In addition, AMF fortifies the plant sink for carbohydrates. An increase in carbohydrates content in tomatoes has been reported with the use of AMF [44]. Furthermore, the total carbohydrates content with the use of SWE can be attributed to the increase in chlorophyll index. The use of bio-stimulants raised the amount of soluble carbohydrates in *Vigna radiata* compared to the lack of foliar applications [45].

The ascorbic acid content in lettuce leaves was affected by the application of AMF × SWE. Lettuce is considered a good source of nutrients such as ascorbic acid and carotenoids. Photosynthesis and its products are directly related to the production of ascorbic acid in plants. Bio-fertilizer application improves the photosynthetic potential, N and P absorption,

and ultimately leads to an improvement in the ascorbic acid content [46]. Moreover, high amounts of vitamin C can be attributed to the improvements in chlorophyll content in response to the stimuli effects of SWE and AMF. Likewise, Subramanian et al. [47] reported that the highest ascorbic acid content was associated with AMF use in tomatoes. In another study on the spinach plant, Tiruvaimozhi et al. [48] reported an increase in ascorbic acid content using seaweed foliar treatments.

Total antioxidants activity and, phenolics and flavonoids contents in lettuce leaves were also affected by AMF and SWE in this study. The symbiosis of mycorrhiza fungi affects plant metabolism by stimulating the biosynthesis of secondary metabolites and potentially increasing the accumulation of antioxidant compounds in plants. This fungus causes changes in the concentration of phytohormones such as jasmonic acid, gibberellic acid, and cytokinins, which improve the absorption of elements and lead to the production of more antioxidant compounds [49]. Avio et al. [50] reported that mycorrhiza fungus application increased the antioxidant activity of lettuce. Gholinezhad et al. [51] also reported that fungi symbiosis improved antioxidant activity in soybeans. Fan et al. [52] observed a direct relationship between antioxidant capacity and the amount of phenolics in spinach.

Phenolics are a major component for plant cell protection against stressors. In artichoke plants inoculated with mycorrhiza fungus, Avio et al. [50] reported that the highest total phenolics content was obtained by using this fungus. Phenolic compounds appear to increase the symbiosis rate between plant and fungus and lead to the accumulation of secondary metabolites such as carotenoids and polyphenols in host plants by making significant changes in enzymatic activities and the physiological mechanisms involved [53]. Similarly, the increase in phenolics content in SWE treatment can be attributed to the stimulated biosynthesis of growth hormones and nutrient uptake in the roots and the improved polyphenol oxidase activity which increases the accumulation of phenolics and hence the antioxidant activity in the plant [54]. The results of Ashour et al. [55] on red hot pepper are consistent with these findings. Bio-stimulants improve the phenolics accumulation in plants by improving the growth-related traits and enhancing the nutrient uptake and even by enhancing the phenylalanine accumulation in roots.

Flavonoids are a major class of polyphenolic secondary metabolites in plants. De Assis et al. [56] concluded that the levels of phenolics and flavonoids in lemongrass increased with the use of mycorrhiza. In another study, an increase in phenolics, flavonoids, and antioxidant activity of the *Eruca vesicaria* L. plant was obtained by SWE treatment [57]. The results of Mahmoud et al. [58] on the red radish plant are consistent with our results as well.

Fungal hyphae can penetrate the very small pores that even the root hairs are not able to penetrate, increasing the absorption of water and nutrients and improve plant growth, and ultimately increasing crop yield [59]. The fungus provides the required carbon from the host roots and, in turn, increases the uptake of nutrients, especially phosphorus, by the host plant [60]. AMF coexistence can play an important role in maintaining soil fertility and stabilizing soil structure while increasing plant water uptake and product quality. In particular, enhancing the symbiotic activity with AMF is a way to improve food production at the lowest economic and environmental costs [13]. Mycorrhiza improves soil structure by coating a viscous glycoprotein called glomalin, which plays a key role in the formation of soil aggregates and large pores for better hyphae growth. These pores ease air and water penetration and also help to prevent progressive soil erosion [61]. Roots inoculated with AMF have richer secretions, and extra-root hyphae create an appropriate condition for the growth of some beneficial bacteria. Also, the extra-root hyphae of the AMF cause soil particles to stick together and the formation of soil aggregates that improve the airflow in the soil, which is essential for the growth and multiplication of soil bacteria [62]. Thus, in the rhizosphere of these plants, the population of plant growth-promoting rhizobacteria (PGPR), nitrogen-fixing bacteria, and several Gram-positive bacteria is higher which can inhibit the growth of pathogens. The enhancement in the population of the beneficial bacteria has been considered as a factor in helping to reduce the population of *Fusarium* or

Phytophthora pathogens [63]. Also, Atayese [64] showed that in peanut plants inoculated with *Glomus mosseae* the grain yield was increased up to 22% compared to non-symbiotic plants. Boomsma and Vyn [65] stated that inoculation with AMF caused extensive changes in root morphological characteristics, especially lateral root growth. Mycorrhiza fungus expands the root surface area up to 40 times and increases the population of beneficial soil bacteria [66].

Mycorrhizae regulate plant physiological functions such as leaf water potential, stomatal conductance, photosystem II efficiency, and carbon dioxide stabilization and this even occurs under stressful conditions [67]. It also improves nitrogen uptake by increasing the activity of nitrogen-absorbing enzymes such as glutamine synthase, and ultimately leads to an increase in proteins and amino acids content [10]. These fungi identify their host with the signals released from the plant root and coexist with it and, in the absence of the host root, they cannot form hypha and complete their life cycle [68,69]. Colony formation with AMF increases photosynthetic efficiency in plants as well [61].

5. Conclusions

The results showed that the co-application of SWE and AMF had a positive effect on the morphological and biochemical traits of lettuce. The sole application of AMF and SWE, as well as their combined application, significantly improved the percentage of colonization, number of leaves, head diameter, fresh and dry weight of roots, and dry weight of leaves compared to the control. Moreover, total proteins and carbohydrates content, antioxidant activity, ascorbic acid content, and phenolics and flavonoids content were responsive to the combined application of treatments. The application of AMF and SWE alone improved most of the evaluated traits, however, the co-application of AMF and SWE improved the traits more than their sole application. Therefore, we recommend the co-application of the tested biostimulants to improve the growth parameters and quality attributes of lettuce plants. Organic crop production may be one of the most important environmental challenges of sustainable agricultural systems. These bio-stimulants are eco-friendly alternative strategies to improve the quality and yield of vegetables that may pave the way for meeting the nutritional demands of the growing population of the world.

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