Foliar Application of *Ulva rigida* Water Extracts Improves Salinity Tolerance in Wheat (*Triticum durum* L.)

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Abstract: This study investigated the effect of seaweed extract (SWE) sprays obtained from *Ulva rigida* on wheat plants (*Triticum durum* L., variety Karim) grown under salt stress conditions for six weeks. Three levels of NaCl (0, 34.22, 68.44 mM) and four different concentrations (0, 12.5, 25 and 50%) of *Ulva rigida* as a water extract were applied. The obtained results indicated that seaweed treated plants showed higher ability to tolerate salt stress (34.22 or 68.44 mM of NaCl) by a significant (*p* < 0.05) increase of plant growth and the photosynthetic pigment contents, compared to those of control (non-treated plants). Furthermore, there was a significant improvement in antioxidant enzyme activity, such as superoxide dismutase (SOD), isocitrate dehydrogenase (ICDH), glutathione peroxidase (GPx), glutathione reductase (GR), and peroxidase (GP) activities in the stressed plants, especially in those treated with 12.5% of SWE. Overall, our results suggest that the application of the *Ulva rigida* water extract could be used as a promising plant growth biostimulant for treating wheat plants under salinity stress.

Keywords: *Triticum durum*; seaweed extracts; antioxidant enzymes; NaCl; biostimulant; abiotic stress

1. Introduction

Salinity is one of the major abiotic stresses which limit the yield of major crops [1–3]. It was estimated that up to 20% of irrigated lands in the world are affected by different levels of salinity and sodium content [4]. Salt stress like many abiotic stresses induces oxidative damage to plant cell catalyzed by reactive oxygen species (ROS). The Reactive Oxygen Species (ROS), comprise both free radical (O\(^{-}\)-, superoxide radicals; OH\(^{-}\), hydroxyl radical; HO\(_2\)\(^{-}\), perhydroxyl radical and alkoxy radicals RO\(^{-}\)), and non-radical forms (H\(_2\)O\(_2\), hydrogen peroxide; and O\(_2\), singlet oxygen). These species are responsible for damage to membranes and other essential macromolecules such as photosynthetic pigment, protein, DNA and lipids [5]. Nevertheless, plants have the high ability to scavenge ROS radicals by producing two types of antioxidants including: enzymatic and non-enzymatic systems [6,7]. Enzymatic antioxidants comprise Superoxide Dismutase (SOD), Isocitrate Dehydrogenase (ICDH), Glutathione Reductase (GR), Phosphoenolpyruvate Carboxylase (PEPC), Glutamate Dehydrogenase (GDH), Glutathione Peroxidase (GPx), and Glutathione-S-Transferase (GST). Whereas, non-enzymatic antioxidants include (low molecular weight): glutathione (GSH), ascorbate (AsA), carotenoids, and phenolic compounds [8,9]. In order...
to minimize the harmful consequence of salinity in crops, previous studies have shown that exogenous protectants such as osmoregulators (proline, trehalose, etc.), plant hormone (gibberellic acids, salicylic acid, etc.), antioxidants (ascorbic acid, glutathione, tocopherol, etc.), trace elements (selenium, silicon, etc.) have been found effective in mitigating the salt induced damage in plant [10,11]. These metabolites showed the capacity to enhance the plant’s growth, yield as well as stress tolerance under salinity. Moreover, various research investigations have revealed the significant application of liquid seaweed extracts as foliar sprays in numerous crops, to advance their growth under favorable or unfavorable conditions [12]. Seaweed Extract (SWE) concentrates are known to cause many beneficial effects on plants, as they contain many growth promoting hormones such as Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Cytokinins, trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni), vitamins, and amino acids [13–15]. Furthermore, seaweed extracts have been reported to stimulate the growth and yield of plants, enhance tolerance to environmental stress, and improve nutrients availability and nutrients uptake from the soil [15–18]. Many research studies have shown the beneficial effect of seaweed extracts in stimulating the growth of plants [19,20]. Aziz et al. [17] showed that the application of Ascophyllum nodosum extracts to Amaranthus tricolor has enhanced flowering and its chemical constituents under high salinity conditions. Additionally, the utilization of seaweed extracts to stimulate the germination and growth of tomato (Solanum lycopersicum) seedlings under salt stress was successful [21].

Latique et al. [22], reported that macroalgae could enhance plant salt tolerance through the increase of production of non-antioxidant compounds, and elevate the activities of antioxidant enzyme system. Therefore, the beneficial effect of seaweed extract application is as a result of many components that work synergistically at different concentrations.

The main objective of this paper is to gain insight into the mechanisms by which seaweed extract application contributes to protection of plant against salt stress. In our study, we evaluate the impact of the foliar applications of seaweed extract obtained from a macroalgae species Ulva rigida on salt stress tolerance in wheat plants (Triticum durum L.), one of the major cereals in the world. Accordingly, some physiological and biochemical parameter such as, growth parameters, chlorophyll content, and antioxidant enzymes activities (SOD, ICDH, GR, PEPC, GDH, GPx, GST) of wheat plants (Triticum durum L.) were studied under salt condition (0, 34.22, 68.44 mM of NaCl). Our goal was to gain insight into the mechanisms by which seaweeds extract application contributes to protection of plant against salt stress.

2. Materials and Methods

2.1. Algae Material and Seaweed Liquid Preparation

In this study, green seaweed Ulva rigida was used. Ulva species (Ulvophyceae), are widely distributed across the globe, being one of the most heavily traded edible seaweeds [23]. Ulva rigida, also known as sea lettuce, is a cosmopolite species [24]. This algae’s tallus size varies from 1–2 cm up to 30 cm, and clings to the substrate with a short stem [25]. Ulva rigida was collected from a coastal area called Akhfenir near the city of Laâyoune in Morocco, in October 2019.

Seaweeds were hand-picked and thoroughly washed with seawater to remove all the unwanted impurities, adhering sand particles, and epiphytes. Morphologically, distinct thalli were placed separately in new polythene bags, kept in an ice box containing slush ice, and transported to the laboratory. Samples were washed thoroughly, under ambient temperature, using tap water to remove the surface salt, and then blotted to remove excess water. Fresh material was cut into small pieces and preserved at −20 °C until use.

1 kg of the fresh crushed algal material was mixed with 1 L of distilled water, and boiled separately for one hour, then the mixture was filtered to remove debris [26]. This filtrate represented the 100% crude extract. The 12.5% 25% and 50% concentrations were subsequently prepared by adding distilled water.
2.2. 

2.2.1. Color, pH and Minerals Determination

Color, pH, calcium, sodium, chloride, magnesium, and potassium contents were analyzed by the method described by the American Public Health Association [27]. The powdered algae samples were ignited and incinerated in a muffle furnace (Karl Kolb Scientific, Technical Supplies, D-6072) at 550 °C for 4 h. The mineral constituents (Ca, K, Na, Mg and Cl) were dissolved in HNO3 [27] and the mineral were determined using an atomic absorption spectrophotometer (Analytik Jena AAS Zeenit 700).

2.2.2. Nitrogen Content

Total nitrogen was determined based on Kjeldahl’s method [28]. The sample is digested in sulfuric acid, using Copper sulphate (CuSO4) and Titanium dioxide (TiO2) as catalysts, converting N to NH3, which is distilled and titrated.

Calculate nitrogen:

The amount of nitrogen was calculated according to the following equation:

\[
\% \text{N} = \left( \frac{(TS - TB) \times \text{Strength of HCL} \times 0.014}{\text{Weight of the sample (g)}} \right) \times 100
\]

where TS = Titer value of the sample in ml; TB = Titer value of the blank; Strength of HCL acid = 0.1 N.

2.2.3. Protein Content

The soluble protein content of algal extract was calculated according to the method of Lowry et al. [29]. Protein content was calculated by converting the nitrogen content, determined by micro-Kjeldahl method (6.25 × N).

2.2.4. Lipid Content

The lipid content of algal extract was estimated by taking a sample weighing 3 g in filter paper sacks, and placing it into the Soxhlet system AOAC [30]. The extraction was continuous over 6 h using petroleum ether as a solvent.

2.2.5. Ascorbic Acid Content

Ascorbic acid is oxidized by copper to form dehydroascorbate, which reacts with 2, 4-dinitrophenyl hydrazine to form a colored substance with a maximum absorption at 520 nm. This was measured to estimate the ascorbic acid (Vitamin C) content; using it as a standard by following the method of Omaye et al. [31].

2.2.6. Determination of Glycinebetaine (GB)

Analysis of GB was carried out according to the method of Gorham [32]. Leaf extract was prepared in 20 mL test tubes by chopping 0.5 g leaves in 5 mL of toluene-water mixture (0.05% toluene). All the tubes were mechanically shaken for 24 h at 25 °C. After filtration, 0.5 mL of extract was mixed with 1 mL of 2 N HCl solution and 0.1 mL of potassium tri-iodide solution (containing 7.5 g iodine and 10 g potassium iodide in 100 mL of 1 N HCl) was added and shaken in an ice-cold water bath for 90 min and then 2 mL of ice-cooled water was added after gentle shaking 10 mL of dichloromethane (Chilled at −10 °C) was poured in it. By passing a continuous stream of air for 1–2 min, two layers were separated, the upper aqueous layer was discarded, and optical density of the organic layer was recorded at 365 nm. The concentration was estimated by using a standard curve developed with different concentration of GB.
2.2.7. Estimating Concentration of Phenolic Compounds

0.5 g leaves were homogenized with 1 mL 80% methanol at 4 °C. The homogenate was centrifuged at 19,000 rpm for 20 min and the supernatant was used for phenol content analysis. Phenolic content of the extract was estimated by the method of Sauvestry, Page, and Huot [33]. One hundred microliters of sample supernatant was mixed with 2 mL of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature.

After incubation, 100 µL of 50% Folin-Ciocalteau’s phenol reagent was added and then the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using a UV-visible spectrophotometer.

2.2.8. Amino Acids Analysis

Amino acids content was determined by high-performance liquid chromatography (HPLC) according to the OJEC standard method [34]. A mass sample of algae powder samples were hydrolysed with 6 N hydrochloric acid, in an ampoule containing 0.1% phenol (for protection of tyrosine) for 24 h at 110 °C. After acid hydrolysis, 30 mL of citrate buffer (pH 2.2) were added, and the pH was adjusted. The obtained sample was diluted to 100 mL with citrate buffer after adding 1 mL of a norleucine solution 50 at l mol (as an internal standard). The sample was filtered through a 0.2 µm nylon filter before being analysed by HPLC. The contents of the different recovered amino acids were presented as mg g⁻¹ protein.

2.3. Soil Characterization

Concerning the chemical characterization of the soil, total N content was calculated following the Kjeldahl method [28]. Olsen’s method [35] was used to measure the available phosphorus (P) in soil by colorimetry. Potassium content was measured by atomic absorption spectrophotometry following the method described by Mountier et al. [36].

2.4. Plant Material and Growth Conditions

Wheat plants (variety Karim) were grown under controlled conditions; under 16 h light regimes at 25 °C, and 8 h dark regime at 18 °C. After disinfection with sodium hypochlorite (6%) for 3 min and rinsing with distilled water, the seeds of durum wheat were germinated in Petri dishes [37]. The pre-germinated seed, the elderly a week, have been planted into plastic pots containing soil. The different pots were watered every three days. A week after transplanting and from the 4-leaf stage (Tillering stage), plants under study were treated with salt at various concentrations (0, 34.22, 68.44 mM of NaCl) corresponding respectively to the growth conditions: without stress (WS), moderate salt stress (MSS), severe salt stress (SSS). The lot of plants treated with saline solution were considered as control as they did not receive extract of Ulva rigida. Another lot of plants were treated with salt at various concentrations (0, 34.22, 68.44 mM of NaCl) and for each concentration we sprayed with seaweed extracts (SWE) of Ulva rigida separately at four different concentrations (0, 12.5, 25, 50% (v/v)) of SWE in water. The selected gradients concentrations were based on previous studies [18,22,26].

The treatment was applied until beginning of the heading stage (index 50 according to the Zadoks scale), and the plants were aged 8 weeks, of which the last six weeks were under saline stress and sprays of seaweed liquid extracts alternatively during the whole week.

All the plants were harvested 8 weeks after initiating seaweed treatments, and the morphological characteristics such as shoot length and fresh weight were measured. All experiments and enzyme preparations were performed with freshly harvested plants.
2.5. Determination of Chlorophyll Content

300 mg of leaf samples were homogenized in 6 mL of 80% acetone in the dark. The chlorophyll content was determined in three independent replicates. The methods of Arnon [38] were used to estimate the chlorophyll a and chlorophyll b contents:

\[
\text{Chlorophyll a mg L}^{-1} = 12.7 \times \text{OD.663 nm} - 2.69 \times \text{OD.645 nm}
\]

\[
\text{Chlorophyll b mg L}^{-1} = 22.9 \times \text{OD.645 nm} - 4.68 \times \text{OD.663 nm}
\]

2.6. Preparation of Plant Extracts for Enzyme Activity Assays

Using a pre-cooled mortar and pestle, leaves were homogenized in 50 mM ice-cold phosphate buffer (pH 7.6) containing 14 mM \(\beta\)-mercaptoethanol, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 9.4 \(\mu\)M leupeptin, and 10% (w/v) glycerol. The homogenates were centrifuged at 12,000 g for 20 min, and the supernatants were used for determination of enzyme activities. All procedures were performed at 0–4 °C.

For PEPC and NADP-ICDH, the supernatant was saturated (60%) with solid ammonium sulfate for 30 min. The saturated supernatant was centrifuged again in the same conditions, and the resulting pellet was re-suspended in the extraction buffer and used for enzyme assays.

Enzyme Activity Assays

The activity of SOD (EC 1.15.1.1) was assayed according to the method described by Beauchamp and Fridovich [39]. The reaction mixture was composed of 0.05 M phosphate buffer, pH 7.5, 10 mM methionine, 0.1 \(\mu\)M EDTA, 2 \(\mu\)M riboflavin, 75 \(\mu\)M nitroblue tetrazolium (NBT) and the enzyme extract. The SOD activity was measured at 560 nm. One unit of SOD activity was defined as the quantity of SOD required to obtain a 50% inhibition of the reduction of NBT. The activity was expressed as units per mg of protein content.

The GR activity was determined by the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM\(^{-1}\) cm\(^{-1}\)), as described by Rao [40]. The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 \(mM\) EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH at 30 °C.

The activity of GST was estimated by the method of Habig [41] with a slight modification. The assay mixture contained enzyme extract, 5 mM GSH, 2.5 mM 1-chloro-2, 4-dinitrobenzene (CDNB), and 0.1 M phosphate buffer (pH 5.5). The reaction was monitored spectrophotometrically at 340 nm at 30 °C. The product concentrations were calculated using a molar extinction coefficient of 9.6 mM\(^{-1}\) cm\(^{-1}\).

The NADP-ICDH activity was measured according to Magalhães and Huber [40] with some modifications as reported by Ben Mrid et al. [42]. The ICDH activity was measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm for 5 min. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM MnCl\(_2\), 1 mM NADP\(^+\), and 4 mM isocitrate.

The PEPC activity was determined according to the method described by Ben Mrid et al. [43]. The activity was assayed by coupling to NAD-malic dehydrogenase (MDH), and monitoring NADH oxidation at 340 nm spectrophotometrically in a 1 mL assay mixture containing 100 mM Hepes—KOH (pH 7.3), 5 mM MgCl\(_2\), 0.2 mM NADH, 5 U of MDH, 2.5 mM PEP (for roots 1 mM), 5 mM NaHCO\(_3\) and leaves or roots extract. One unit of PEPC is the amount of enzyme extract which catalyzes the transformation of 1 \(\mu\)mol substrate per minute at 30 °C.

The GDH activity was measured in the aminating direction, as described by Ben Mrid et al. [44]. The activity was performed in the amination direction at 30 °C in reaction buffer containing 100 mM Tris-HCl (pH 8), 1 mM CaCl\(_2\), 13 mM a-ketoglutarate, 50 mM (NH\(_4\))\(_2\)SO\(_4\) and 0.25 mM NADH. Kinetic activity was determined spectrophotometrically by monitoring NADH at 340 nm. The activity of NAD+ malate dehydrogenase was assayed by monitoring NADH at 340 nm. The reaction buffer contained 50 mM
potassium phosphate buffer (pH 7.5), 1 mM oxaloacetic acid, 0.25 mM NADH and the enzyme solution.

The GPx was carried out according to the method described by Bouchmaa et al. [45] with some modifications. The reaction mixture contained 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, GR (10 µg/mL), 0.25 mM NADPH and enzyme extract. The mixture was incubated at 25 °C for 3 min and completed by adding 0.25 mM of H₂O₂. The rate of NADPH oxidation was monitored at 340 nm for 5 min. GPx activity was calculated and expressed as µmol of NADPH oxidized/min/mg protein by using the extinction coefficient of 6.2 mM⁻¹ cm⁻¹.

The total soluble protein content of the enzyme extracts was determined following the method of Bradford [46], using Bovine Serum Albumin (BSA) as a protein standard.

2.7. Statistical Analysis

SPSS 10.0.5 package for Windows, version 10.0.1 was used for all statistical analyses. Two-way ANOVA (factor 1, salt stress level; factor 2, SWE treatment), followed by the Student Newman—Keuls post hoc test, were used to compare differences in the means (p < 0.05). Different letters indicate significant differences. Principal component analysis (PCA) was performed, with KMO and Bartlett’s test and Oblimin with Kaiser Normalization in Rotation Method, to draw a biplot of the studied parameters using IBM SPSS version 26.

3. Results

3.1. Physicochemical Properties and Biochemical Analysis of Seaweed Ulva rigida Extract

Results obtained from the nutrient analysis (Table 1) showed the presence of the macro-elements K, Mg, Na, Ca, and Cl in all samples. Ulva rigida liquid extract is high in potassium (12.33 mg/g) and magnesium (35.10 mg/g) contents. The pH value of the green liquid SWE was slightly acidic (6.58).

Table 1. Physical, biochemical, and mineral constituents of seaweed (Ulva rigida) extract.

| Algal Species | Physical Property | Minerals Content (mg/g) | Biochemicals Content (mg/g) | Total Nitrogen (%) |
|--------------|------------------|-------------------------|----------------------------|------------------|
| Ulva rigida  | Green            | 6.58 ± 0.1             | 12.33 ± 0.49               | 35.10 ± 0.68     | 0.68 ± 0.031     | 0.42 ± 0.021     | 0.15 ± 0.012     | 9.2 ± 0.3       | 2.8 ± 0.1       | 35 ± 1         | 0.99 ± 0.006   | 8.03 ± 0.093   | 1.28           |

Values are average ± standard error (n = 5).

3.2. Biochemical Analysis of Ulva rigida Extract (SWE)

The algal biomass contains 0.99 mg/g of phenolic compounds, 9.2 mg/g of protein, and 2.8 mg/g of lipid. In addition, SWE contains 35 mg/g of ascorbic acid. Furthermore, the SWE analysis revealed that Ulva rigida contains betain, which is an osmoregulatory compound accumulated in plants allowing them to resist to salt and cold stresses [47]; it had a concentration value of 8.03 mg/g (Table 1).

The amino acids contents of Ulva rigida are illustrated in Figure 1. The essential amino acids (EAA) included are methionine, leucine, isoleucine, lysine, phenylalanine, tyrosine, arginine, threonine, valine, and tryptophan. The levels of the different essential amino acids ranged from 0.646 to 15.83 g 100 g⁻¹ protein. Ulva rigida contains a large amount of leucine (15.8 g 100 g⁻¹ protein), and less content of lysine (1.93 g 100 g⁻¹ protein) and methionine (0.64 g 100 g⁻¹ protein), respectively. The Non-essential amino acids (NEAA), namely histidine, aspartic acid, glutamic acid, serine, proline, glycine, and alanine, ranged from 3.7 to 19.6 g 100 g⁻¹ protein. This marine macroalgae contained a large amount of aspartic (19.6 g 100 g⁻¹ protein) and glutamic acids (9.2 g 100 g⁻¹ protein), followed by alanine (10.44 g 100 g⁻¹ protein), glycine (9.19 g 100 g⁻¹ protein), serine (3.98 g 100 g⁻¹ protein),
and proline (6.67 g 100 g\(^{-1}\) protein). The lowest quantity of these non-EAA was histidine (3.7 g 100 g\(^{-1}\) protein). Similar results have been obtained in previous studies [48,49].

![Amino Acids contents of Ulva rigida](image)

**Figure 1.** Amino acids contents of *Ulva rigida* from Akhfenir coastal. Results are means ± S.D. Different letters in a single line show statistically significant differences for \(p<0.05\) level. Abbreviations are as follows: Met: Methionine, Trp: Tryptophan, Lys: Lysine, Tyr: Tyrosine, Phe: Phenylalanine, Ile: Isoleucine, His: Histidine, Ser: Serine, Thr: Threonine, Val: Valine, Pro: proline, Gly: Glycine, Ala: Alanine, Leu: Leucine, Asp: Aspartic Acid.

All macroalgae species contain low concentrations of basic amino acids, and show a general prevalence of the same compounds (aspartic and glutamic acids, which often make up 50% or more of the total, alanine, glycine, and serine) [50].

### 3.3. Soil Analysis

In the current study, soil analysis was conducted under controlled conditions. However, as substrate for the plant growth, we used a soil that has never received any fertilizer. Moreover, no fertilizers have been added during this experiment. The exploration of the soil was realized, and the results of the main macro-elements (N, P, and K) are presented in Table 2.

| Chemical Compound | \(\text{P}_2\text{O}_5\) (ppm) | \(\text{K}_2\text{O}\) (ppm) | N (%)       |
|-------------------|-----------------|-----------------|------------|
| Concentration     | 13.16 ± 0.056   | 147.575 ± 0.134 | 0.146 ± 0.004 |
| Soil characteristics | Moderately poor \(^1\) | Poor \(^2\) | Moderately poor \(^3\) |

\(^1\) according to Olsen et al. [35]; \(^2\) according to Mountier et al. [36]; \(^3\) according to Kjeldahl [28].

### 3.4. Effect of Algal Extracts on Shoot Length and Fresh Weight of Wheat Plants Cultivated under Salt Stress

The importance of different concentrations of *Ulva rigida* water extract on growth of wheat plants was investigated under different growth conditions: without stress (WS), moderate salt stress (MSS), and severe salt stress (SSS).
Data in Table 3 reveal that shoot length was reduced by 17% and 20% under MSS and SSS respectively, compared to control plants; moreover, the fresh weight was decreased by 25% and 48% under the same conditions. However, this decrease was less pronounced in wheat plants treated with *U. rigida* extract (SWE). All the obtained results were statistically insignificant. From the present results, it can be seen that the application of SWE improved notably the shoot length plants in all salt treatments (Table 3). Our findings are in accordance with previous studies carried out on soybean [16], where there was an increase in vegetative growth by the application of seaweed extract.

**Table 3.** The effect of Salt stress on shoot length (SL) and fresh weight (FW) of *Triticum durum* L. sprayed with SWE. Results are means ± SD (*n* = 5). Different letters for each mean show statistically significant differences at *p* < 0.05. Abbreviations are as follows: WS: Without stress (0 mM of NaCl), MSS: Moderate salt stress (34.22 mM of NaCl), SSS: Severe salt stress (68.44 mM of NaCl), %: concentration of *Ulva rigida* extract in the solution of treatment.

| Treatment            | Growth Conditions |
|----------------------|-------------------|
|                      | WS         | MSS         | SSS         |
| **Shoot length (cm)**|            |             |             |
| Control              | 41.3 ± 5.91 a   | 34.1 ± 2.83 b | 33.14 ± 1.56 b |
| *Ulva rigida* extract| 39 ± 5.65 ab    | 38.9 ± 2.3 ab | 38.7 ± 4.08 ab |
| 12.5%                | 38 ± 1.27 ab    | 37.4 ± 1.14 ab | 35.8 ± 1.79 ab |
| 25%                  | 38.8 ± 0.76 ab  | 37.5 ± 4.47 ab | 37.5 ± 1.87 ab |
| 50%                  | 38.8 ± 0.76 ab  | 37.5 ± 4.47 ab | 37.5 ± 1.87 ab |
| **Fresh weight (g plant⁻¹)**|          |             |             |
| Control              | 2.01 ± 0.45 a   | 1.50 ± 0.25 ab | 1.05 ± 0.03 b |
| *Ulva rigida* extract| 2.09 ± 0.33 a   | 1.96 ± 0.14 a  | 1.51 ± 0.27 ab |
| 12.5%                | 1.98 ± 0.01 a   | 1.89 ± 0.35 a  | 1.89 ± 0.47 a |
| 25%                  | 1.97 ± 0.22 a   | 1.62 ± 0.32 ab | 1.42 ± 0.16 ab |
| 50%                  | 1.97 ± 0.22 a   | 1.62 ± 0.32 ab | 1.42 ± 0.16 ab |

3.5. Effect of Algal Extracts on Photosynthetic Pigment Contents of Wheat Plants Cultivated under Salt Stress

Our results show that the chlorophyll a and b content decreased significantly in leaves of wheat plants under salt stress (Figure 2). The chlorophyll a was reduced by 23.78% and 35.8% under MSS (34.22 mM of NaCl) and SSS (68.44 mM of NaCl) respectively compared to the control plant, while the chlorophyll b was reduced by 15.32% and 28.62% under the same conditions (Figure 2). The treatment of stressed plants with seaweed extract reduced significantly the stress effect on pigment content.

Under moderate salt stress (MSS), the application of seaweed extracts significantly increased the chlorophyll a content from 73.61 µg g⁻¹ FW (untreated plant) to 218.83 µg g⁻¹ FW and 208.76 µg g⁻¹ FW in plant sprayed with 12.5% and 25% of *Ulva rigida* extract, respectively. However, the maximum values of chlorophyll a were found in stressed plants by 68.44 mM of NaCl that were sprayed with 25% of *Ulva rigida* extract (230 µg g⁻¹ FW).
The chlorophyll b content was increased under severe salt stress (SSS) in plants sprayed with 50% of Ulva rigida extract (66.52 µg g⁻¹ FW) compared to untreated and stressed plant (40.48 µg g⁻¹ FW). The highest values of chlorophyll b were recorded with unstressed plants sprayed with 50% of Ulva rigida extract (79 µg g⁻¹ FW).

The results showed that plants treated with seaweed extract had increased leaf chlorophyll content in both conditions (without stress and under stress conditions).

3.6. Effect of Algal Extracts on Antioxidant Enzymes of Wheat Plants Cultivated under Salt Stress

The effect of the seaweed extracts, Ulva rigida, and the oxidative stress was also evaluated on the antioxidant system through the determination of enzyme activities (Figure 3).

Figure 2. The effect of Salt stress on chlorophyll content in plant leaves of Triticum durum sprayed with SWE. Results are means ±SD. Different letters for each mean show statistically significant differences at p < 0.05. Abbreviations are as follows: WS: Without stress, MSS: Moderate salt stress, SSS: Severe salt stress, %: concentration of Ulva rigida extract in the solution of treatment, Chl a: Chlorophyll a, Chl b: Chlorophyll b, Chl T: Chlorophyll total, FW: Fresh weight.

Figure 3. Cont.
Figure 3. The effect of salt stress on antioxidant enzymes ((A): ICDH, (B): GDH, (C): SOD, (D): GPx, (E): GR, (F): GST, (G): PEPC. Abbreviations are as follows: WS: Without stress, MSS: Moderate salt stress, SSS: Severe salt stress, %: concentration of Ulva rigida extract in the solution of treatment, Superoxide Dismutase (SOD), Glutathione Reductase (GR), Phosphoenolpyruvate Carboxylase (PEPC), Glutamate Dehydrogenase (GDH), Glutathione Peroxidase (GPx), Glutathione-S-Transferase (GST), and Isocitrate Dehydrogenase (ICDH).

3.6.1. Isocitrate Dehydrogenase (ICDH)

Our results show that salinity treatments increased NADP⁺-ICDH activity (Figure 3A), which was similar to the studies in Mesembryanthemum crystallinum [51]. In the present study, the activity of NADP⁺-ICDH has been increased in all conditions of plant treatment with the seaweed extracts either under salt stress conditions compared to the control plant, and the increase was dose-dependent (Figure 3A).
The highest ICDH activity was observed for the plants sprayed with 12.5% of *Ulva rigida* under severe salt stress treatment. The increase in the NADP+ - ICDH activity could be responsible for the provision of NADPH, used to counteract oxidative stress that could be generated in the culture conditions of our experiments.

### 3.6.2. Glutamate Dehydrogenase (GDH)

Our data reveal that in leaves of wheat, the GDH activity was reduced by 25.75% and 39.71%, under salt conditions 34.22 and 68.44 mM of NaCl respectively compared to the control plant (Figure 3B). However, this enzymatic activity increased when the stressed plants were treated with SWE.

The beneficial effect of algal treatment varied with *Ulva rigida* extract concentrations. Thus, the highest levels of GDH activity were detected in plants treated with 50% of *Ulva rigida* extract (17.96 and 17.23 μmol min⁻¹ mg⁻¹ protein, respectively, under MSS and SSS). Therefore, the SWE treatment by 12.5% and 50% were statistically insignificant under SSS on the GDH activity.

The algal treatment increased GDH activity in plants without stress, and the maximum was attained with 50% treatment (22.13 μmol min⁻¹ mg⁻¹ protein), compared to untreated control plants (Figure 3B).

### 3.6.3. Superoxide Dismutase (SOD)

Superoxide dismutase is considered the first enzyme which behaves as a barrier against ROS [52], and which is responsible for the dismutation of superoxide anion radicals (O₂⁻) to hydrogen peroxide (H₂O₂), and O₂.

In the present study, the result showed (Figure 3C) that plants treated with 50% of *Ulva rigida* had a similar enzymatic activity under MSS and SSS compared to control (untreated and unstressed) plants.

Therefore, when the stressed plants were sprayed with different concentrations of seaweed extract, the enzymatic activity increased considerably.

The highest SOD activity was detected in plants treated with 12.5% of *Ulva rigida* extract (31.01 and 31.4 U mg⁻¹ protein, respectively, under MSS and SSS).

### 3.6.4. Glutathione Peroxidase (GPx)

The results of our study reveal that in the leaves of wheat, the GPx activity was enhanced at a concentration of 12.5% of SWE, under severe salt stress (68.44 mM of NaCl), and reached 137.7 μmol min⁻¹ mg⁻¹ protein compared to the control (104.5 μmol min⁻¹ mg⁻¹ protein) (Figure 3D). This result confirmed that the application of seaweed extracts enhances the glutathione peroxidase activity, and plays a protective role against ROS that are formed during salt stress.

### 3.6.5. Glutathione Reductase (GR)

Our results indicated that GR activity increased in all the studied conditions. The highest GR activity was observed for the plants sprayed with 12.5% of SWE, especially under moderate salt stress (34.22 mM of NaCl) (Figure 3E).

The high activity of GR enzyme, responsible for reducing oxidized glutathione (GSSG) to its reduced form (GSH), could be a response to the high demand for GSH, needed both for detoxifying plant cells from H₂O₂, and also for its role in different metabolisms required for plant growth and development [43]. In fact, GR is implicated in maintaining high content of the reduced glutathione in plant cells. The GSH is used in several redox reactions, among them, those responsible for the amino acid transport, and protein and DNA synthesis [53,54]. High ratio of GSH/GSSG was also reported to be required for accelerating the H₂O₂ scavenging pathway, especially when the plants are facing stressful conditions [55,56]. The increase in plant height and fresh weight at the 12.5% concentration of seaweed could explain the high level of GR enzyme at this condition, to allow synthesis of
high levels of GSH required for the metabolic pathways implicated in biomass production, and also to cope with the eventual stressful conditions faced by wheat plants.

3.6.6. Glutathione S-Transferase (GST)

Our data indicated also an increase of GST activity in all treatments. The main enhancement of GST activity was observed for the plants sprayed with 25% of SWE, especially under moderate salt stress (34.22 Mm of NaCl) (Figure 3F). It is known that GST may help reducing a wide range of organic hydroperoxides in the presence of GSH [56]. It has been shown that GST enzyme can protect plants exposed to salt stress by removing its effect against lipid peroxidation [53]. In another study, Iannelli [57] reported that GST is implicated in the detoxification pathway to reduce the impact of high levels of cadmium in Phragmites australis.

3.6.7. Phosphoenolpyruvate Carboxylase (PECP)

The activity of phosphoenolpyruvate carboxylase (PECP) in leaf extracts of wheat plants subjected to different concentrated NaCl is shown in Figure 3G. The wheat enzyme activity was inhibited 17%, and 20% respectively by 34.22, and 68.44 Mm of NaCl. However, the application of SWE improved notably the PECP activity in all salt treatments. PEPC is a cytosolic enzyme widely distributed in most plant tissues. This enzyme catalyzes the β-carboxylation of phosphoenolpyruvate by HCO$_3^-$ in the presence of a divalent cation to yield Pi and oxaloacetate (OAA), which is readily converted to malate by NAD(P)-malate dehydrogenase [58,59].

3.7. Principle Component Analysis (PCA) Analysis

The PCA was performed to find the association of the different groups of treatments and biochemical traits of wheat plants variety Karim (Figure 4).

![Figure 4. PCA to understand treatment-variable relationships for antioxidant enzyme activity of wheat plants. Abbreviations are as follows: Superoxide dismutase (SOD), Isocitrate dehydrogenase (ICDH), Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferase (GST), Glutamate dehydrogenase (GDH), Phosphoenolpyruvate carboxylase (PECP), Algal extract (EA), Sodium chloride (NaCl).](image-url)

The two components of PCA in Figure 4 (Component 1 and 2) collectively explained 73.57% of the total variation. Based on the pattern matrix with rotation method: Oblimin with Kaiser normalization, it was observed that SOD, ICDH, GPx and GR were correlated to component 1, however GDH, PEPC, NaCl, GST and EA (Algal extract) were correlated to component 2. Globally, our results confirm that the salinity stress has a negative effect on chlorophyll content, shoot length and fresh weight, but a positive effect on the activation of the following enzymes, for example, ICDH and SOD.
Nevertheless, the seaweed liquid extract treatment shows a significant effect on plants grown under salt stress by enhancing shoot length, fresh weight, chlorophyll a, total chlorophyll contents and specially the activity of the following enzymes: phosphoenolpyruvate carboxylase (PEPC) and glutamate dehydrogenase (GDH). This means that the seaweed liquid extract could be used as a promising plant growth biostimulant for treating wheat plants (variety Karim) irrigated with saline solution.

4. Discussion

Our results revealed that *Ulva rigida* provide an excellent source of bioactive compounds such as amino acids (Figure 1), minerals (Table 1), and growth promoting substances. Therefore, these compounds in *U. rigida* extract could potentially participate in the alleviation of salinity stress [60].

According to the results, wheat shoot fresh weights decreased in all NaCl treatments and this decline was also less evident in wheat plants treated with seaweed liquid extract (Table 3). This constructive effect might be due to the macronutrient content in SWE, such as potassium, magnesium, and phosphorous (Table 1), and due to the auxin content in *Ulva rigida* extracts which has a great role in cell division and enlargement, and may lead to an increase in plant biomass growth [22,61,62].

The application of algal *Ulva rigida* extract to wheat plants irrigated by saline water also led to a significant increase in the concentrations of T-Chl, Chl-a, and chl-b as compared with the values of control plants. Thus, the level of photosynthetic pigment was found to be restored in treated wheat plants irrigated by saline water due to application of algal extracts. The concentration of SWE improves the pigment content of stressed and unstressed plants. This increase in chlorophyll content could be a result of reduction in chlorophyll degradation, which might be caused in part by betaines in the seaweed extract [63].

We showed that *Ulva rigida* extract was rich of glycinebetaine (Table 1). This component delays the loss of photosynthetic activity by inhibiting chlorophyll degradation during storage conditions in isolated chloroplasts [64]. Furthermore, these results revealed that algal extract contained high amounts of antioxidant constituents, which were positively correlated with an increase of photosynthetic pigments, restored in wheat plants cultivated under salt conditions. These chlorophylls in photosynthetic membranes could be protected by the photosynthetic apparatus from excessive ROS by a quenching of singlet oxygen and other radicals.

NaCl stress has a strong influence on nitrogen (N) and carbon (C) metabolism in plants and this is reflected in a number of changes occurring in a range of physiological and biochemical processes [65,66]. Carbon metabolism could play a significant role to discern the behaviours of plants in response to salinity, via enzymatic and/or metabolic processes. Among the key enzymes of carbon metabolism, dehydrogenases are considered important in generating reducing powers which are utilized in various metabolic activities including reductive biosynthesis of amino acids.

Isocitrate dehydrogenase (ICDH) catalyses the reversible conversion of isocitrate to 2-oxoglutarate (2-OG), as proposed by Chen and Gadal [67] and links C and N metabolism [68]. The carbon generated through this reaction may be used for the process of biosynthesis of amino acids [43].

Phosphoenolpyruvate carboxylase (PEPC) also play an important role within carbon metabolism and it has a major anaplerotic function of replenishing the tricarboxylic acid (TCA) cycle with intermediates to meet the demand of carbon skeletons for synthesis of organic acids and amino acids by glutamate dehydrogenase (GDH), which plays an important role in ammonium. Additionally, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) in conjugation with glutathione reductase (GR) form another pathway to protect the organism from reactive oxygen species (ROS).

The application of *Ulva rigida* extract (SWE) in wheat plants leads to the increase in SOD activity. This rise may be due to the presence of some biochemical compounds in *Ulva rigida* extract, such as soluble sugar, polyphenol, and proteins which might be necessary
for the stimulation of antioxidant enzymes [22,69]. Our obtained result is in line with the works conducted on the effect of seaweed extracts as biostimulants and their ability to improve nutrient uptake [14]. The SWE applied as foliar spray increased the antioxidant potential in stressed plants when compared to untreated control plants. Our result can be explained by the effect of seaweed extract on the reduction of cells damage caused by ROS [37,70]. The application of seaweed extract increased the tolerance to oxidative stress, by the activation of some antioxidant enzyme like superoxide dismutase (SOD), which scavenges superoxide [37,71].

Antioxidant enzymes are essential for decreasing ROS levels in salt stressed plants [72]. The application of *Ulva rigida* extract in wheat seedlings leads to the increase in the activities of antioxidant enzymes. This rise may be due to the presence of some biochemical compounds in *Ulva rigida* extract, such as ascorbic acid, polyphenol, and proteins (Table 2), which might be necessary for the stimulation of antioxidant enzymes [69].

5. Conclusions

In the present study, we observed that 12.5% and 25% concentrations of *Ulva rigida* water extracts applied as foliar spray at wheat plants showed better vegetative growth, and increased stress tolerance.

This extract enhanced the antioxidant potential of plants by activation of antioxidant enzymatic system of SOD, GPx, GST and GR and consequently reduced the salt stress. This rise may be due to the presence of some biochemical compounds in *Ulva rigida* extract, such as soluble sugar, polyphenol and proteins, which might be necessary for the stimulation of antioxidant enzymes [69].

Alternatively, SWE represents an important source of plant biostimulants allowing treated crops to tolerate soil salinity as one of the abiotic stress types. In addition, the application of SWE contributes to the protection of plants against peroxidation imposed by salt stress. Therefore, the present findings encourage the application of such seaweed as a natural fertilizer in the agricultural sector. As a perspective, it would be beneficial to carry out more research including the study of antioxidant and active substances in seaweed extracts that stimulate the enzymatic system, and lead to salt stress tolerance in wheat plants.

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