Halophilic microalgae *Dunaliella salina* extracts improve seed germination and seedling growth of *Triticum aestivum* L. under salt stress

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Abstract

Salinity is among the major limiting factors of crop productivity worldwide. Salt stress affects the plant growth at all developmental stages. The present study investigates the potential of halotolerant microalgae *Dunaliella salina* extracts to suppress the inhibition of seeds germination and seedlings growth caused by salt stress on wheat (*Triticum aestivum*). Germination percentage and height of coleoptiles and root system were measured as a response of *D. salina* extracts treatment. Biomass hydrolyzate of *D. salina* cultured in different concentrations of NaCl (1, 2 and 4 M of NaCl) improved germination and seedling growth of wheat under salt stress. The effect of molecules accumulated in *D. salina* which are proline, carotenoids and exopolysaccharides on germination and seedling growth was investigated. Our results showed that exopolysaccharides extracted from the microalgae had the major stimulating effect on germination and seedling growth in wheat. Exopolysaccharides extracts from *D. salina* improved the germination percentage of wheat seeds under salt stress of 3 and 6 g L^{-1} NaCl by 96 and 83%, respectively, compared to control, while the height of root system was stimulated with 133 and 444% and the coleoptiles height was increased by 105 and 750% in response to 3 and 6 g L^{-1} NaCl, respectively, compared to control stressed untreated. Our study demonstrates that extracts of halotolerant microalgae especially exopolysaccharides could help plants to tolerate salt stress.

Keywords: *Microalgae*, exopolysaccharides, plant, salt stress, germination

INTRODUCTION

Plants are subjected to a wide range of environmental stresses both biotic and abiotic, which may drastically reduce their growth, development and yield (Monteiro et al., 2011). Soil salinity is among the most harmful stresses for plants which leads to serious metabolic and physiological alterations of plant. More than 900 million ha of land worldwide which represent 20% of the total agricultural and, are affected by salinity. NaCl is the predominant salt causing salinity (Munns, 2002). Wheat (*Triticum* spp.) is the world's major cereal crop and is affected by this agro-economical problem (Yildiz, 2007). Soil salinity is the main constraint to wheat seed germination and seedling establishment particularly in the arid and semi-arid regions of the world. Germination is the first stage of the plant cycle and the improvement of tolerance to high salinity at this stage can save plant growth and improve its productivity. Seeds and seedlings are particularly vulnerable to salinity increase at this stage, even in tolerant species, since plants have not yet developed their physiological mechanisms to tolerate rising salinity (Adam, 1990). Hence, the improvement of germination potential and seeds germination in saline conditions appears as a good alternative.

Salinity reduces the ability of plants to take up water (Munns, 2002). This kind of

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adaptation involves the accumulation of osmolytes, such as proline, glycine-betaine, and sugar alcohols, or late-embryo-abundant (Lea) proteins. This is why some researchers have attempted to improve salt tolerance in plants by adding some osmolytes (Kasuga et al., 1999).

D. salina is a motile unicellular halotolerant green microalgae belonging to the class *Chlorophyta* and the family *Polyblepharidaceae,* frequently found naturally in habitats like salt marshes (García et al., 2007). This alga can adapt to hypersaline environments and is considered a model photosynthetic organism for salinity tolerance (Takagi et al., 2006). Due to their similarity to plants and because of close evolutionary linkages between plants and *Chlorophyceae*, it has been demonstrated that salt stress applied to the green algae *D. salina* causes an increase of osmolytes content such as amino acids; serine and glycine, and induces modulation in the activities of ROS scavenging enzymes (Tammam et al., 2011). Many research studies have shown the beneficial effect of algae extracts in stimulating seeds germination and growth of plants under stresses (Ghareib, 2010; Blunden, 1991).

The aim of this work is to study the beneficial effects of the salt-tolerant microalgae extracts in wheat seeds germination and seedlings growth under salt stress condition.

MATERIALS AND METHODS

Microalgae and culture conditions

D. salina strain used in the current study belongs to MAScIR's Collection of Moroccan Microalgae and was isolated from Oualidia lagoon in the Atlantic Moroccan Coast.

Microalga was grown in sterile seawater enriched with F/2 medium nutrients, with different NaCl concentration (0.5, 1, 2 and 4 M). Batch cultures were carried out at 25°C, under continuous illumination of 150 µmol m^{-2} s^{-1}. The biomass was harvested at 14th day by centrifugation at 4000 rpm for 10 min and biomass were washed twice with distilled water and freeze-dried and stored frozen at -20°C until extraction.

Extracts preparation

Three extracts were prepared from *D. salina* biomass*:*

- Hydrolyzates extracts (HE): 0.5 g of *D. salina* dry biomass was suspended in 50 mL of distilled water with 3% sulfuric acid (v:v), and autoclaved at 121°C for 30 min. After hydrolysis, solutions were neutralized and filtered with 0.45-µm filter.
- Exopolysaccharides extract (EPS): 1 L of *D. salina* in culture medium was incubated at 90°C under continuous agitation at 400 rpm for 2 h. After cooling, supernatant was recuperated by centrifugation and pellet refluxed twice to increase extraction efficiency. Extracts were concentrated to 1/4 by evaporation at 50°C in a rotavapor and cooled at 4°C. Three volumes of ethanol were added and the final mixture was centrifuged and pellet (total polysaccharides extract) was lyophilized and stored at -80°C until use.
- Carotenoids extract (CE): Two grams of algal biomass were crushed in a mortar with a mixture of hexane and acetone (1:1 v:v). 5 mL of acetone was added slowly at regular intervals. The solvents were recuperated separately and the process was repeated twice. The solvents containing carotenoids were filtered through a filter paper and transferred into a separating funnel. 50 mL of water solution with 10% NaCl was added. The mixture was shaken vigorously and kept aside for the layers to separate. The upper layer containing carotenoids was collected separately and lyophilized.

Seed germination and seedling growth

Wheat seeds (*Triticum aestivum* 'Amal') were used in this study. Seeds were sterilized with 1.0% sodium hypochloride (v:v) and then washed with distilled water. Seeds were cultured under two concentrations of salt 3, 6 g L^{-1} NaCl and water as a control in sterile petri dishes each hydrolyzate extracts (0.01% DW) of *D. salina* cultured in different concentrations of NaCl (0.5, 1, 2 and 4 M) and microalgae polysaccharides extract (2 mg L^{-1})

were tested in this study. Petri dishes were incubated at 25°C. In the 2nd day, the number of germinated seeds was counted; in 3th, 4th, 5th, and 7th day lengths of shoots and roots were measured.

***D. salina* biomass characterization**

1. Chlorophylls and carotenoids content measurement.

In this study, we used the Lichtenthaler equations (Lichtenthaler and Buschmann, 2001) to determine pigments content when ethanol is used as solvent extraction. Pigment was measured by spectrophotometer (ULTROSPEC 3100 PRO, Thermo-Scientific) for chlorophyll a, chlorophyll b and total carotenoids. Equations for pigment determination in ethanol extract previously reported (Miazek and Ledakowicz, 2013), were used for carotenoids content determination:

$$Chla=13{,}36\times A_{664}- 5{,}19\ A_{648}$$

$$Chlb=27{,}43\times A_{648} - 8{,}12\ A_{664}$$

$$Carotenoids_{totaux}= (1000\times A_{470} - 1{,}63\times chla- 104{,}96\times chlb)/221$$

2. Proline determination.

Proline colorimetric determination was proceeded according to Bates et al. (1973) based on proline's reaction with ninhydrin. For proline colorimetric determinations in dried algal biomass, a 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100°C for 1 h. The reaction was stopped in an iced bath and the cromophore was extracted with 4 mL of toluene and its absorbance at 520 nm was determined.

Wheat seedling analysis

1. Proline determination in wheat seedling.

Similarly to algal biomass, proline determination in wheat seedlings was carried out according to Bates et al. (1973) based on proline's reaction with ninhydrin.

2. ROS scavenging enzymes activities analysis.

For the two enzymes activities analysis, leaf protein was extracted from frozen leaflet samples ground in liquid nitrogen using ice cold mortar and pestle. Protein was extracted in 3 mL of extraction buffer containing 100 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 14 mM 2-mercaptoethanol, and 0.1% (v/v) Triton X-100 for SOD (EC 1.15.1.1) activity. The homogenate was than centrifuged at 15000 ×g for 15 min at 4°C. The supernatant was used for total protein and enzymatic assays.

3. Peroxidase (POD) activity.

POD activity was measured by the method of Maehly and Chance (1954). The assay mixture contained 50 mM sodium acetate buffer pH 5.6 (adjusted by 1 N HCl), 5.4 mM guaiacol, 15 mM H_2O_2, and the enzyme extract approximately 5 µg mL^{-1} protein extract. The increase in absorbance due to the oxidation of guaiacol to tetraguaiacol (e=26.6 mM^{-1} cm^{-1}) was monitored at 470 nm. The enzyme activity was expressed in units, each representing 1 µmole of tetraguaiacol formed min^{-1} mg^{-1} protein. The temperature reaction was 24°C. Reaction was stated at 290 nm after by H_2O_2. The reaction time was 3 min and results were taken every 15 s.

4. Superoxide dismutase (SOD) activity.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) (Jiang and Zhang, 2002). The assay mixture consists of 50 mM sodium phosphate buffer (pH 7.8) 13 mM methionine, 75 µM

NBT, 2 µM riboflavin, 0.1 mM EDTA, approximately 20 µg mL^{-1} of enzyme extract. The reaction mixture was filled in ELISA plate and illuminated for 15 min at a light intensity of 5000 lux. Reaction started by adding riboflavin and illumination and ended by switching-off the light and placing the plate in the dark. A non-irradiated reaction mixture which was run in parallel and which should not develop color served as blank. The absorbance was read at 560 nm by a spectrophotometer. One enzyme unit (U) of SOD activity is defined as the amount that inhibits the NBT photoreduction by 50% min^{-1}.

RESULT AND DISCUSSION

Effect of salt stress on *D. salina*

1. *D. salina* growth under salt stress.

Figure 1 shows that the optimum growth of *D. salina* occurred at 0.5 M NaCl concentration in the culture medium, while reduced growth occurred starting from 4 M NaCl medium. The reduced growth in 1 M NaCl is not obvious over 0.5 M, which may be due to halophilic character of the genera *Dunaliella.*

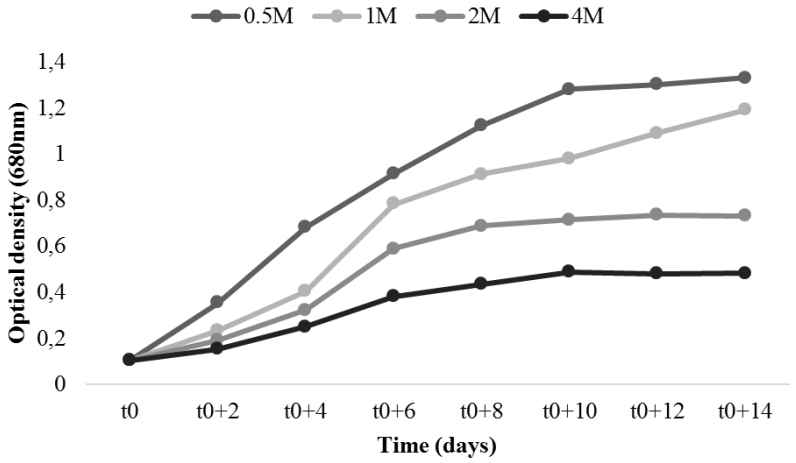

Figure 1. *D. salina* growth cultured in Walne medium under salt stress with different concentrations of NaCl (0.5, 1, 2 and 4 M) at 25°C, agitation 140 rpm and continuous illumination: 5000 lux.

D. salina is the only eukaryotic and photosynthetic organism that can grow in salt concentrations ranging from 0.05 M to saturation (5.5 M) (García et al., 2007; Yu et al., 2015). *Dunaliella* cope with salt stress by massive accumulation of several organic compounds such as glycerol, β-carotene (Ghetti et al., 1999), and it seems to serve as an osmotic regulator (Hadi et al., 2008). *D. salina and D. tertiolecta* showed a remarkable tolerance to hyper saline conditions and survived in nearly all tested concentrations (0.05 to 4.0 M NaCl) (Tammam et al., 2011).

2. Biochemical indicators of *D. salina* tolerance to salt stress

Chlorophylls and carotenoids.

The effect of salinity on the chlorophylls and carotenoid content in *D. salina* was studied. Figure 2 shows a proportional inversely decrease of chlorophyll as a result of increasing salinity in the culture medium of *D. salina*. Chlorophyll a decreased with 45, 66 and 90% in cells cultured in 1, 2 and 4 M NaCl, respectively. Similarly, chlorophyll b declined 75, 77 and 89.6% in cells cultured in 1, 2 and 4 M NaCl, respectively. On the other hand, carotenoids increased compared to control with 43, 328 and 700% in 1, 2 and 4 M NaCl, respectively.

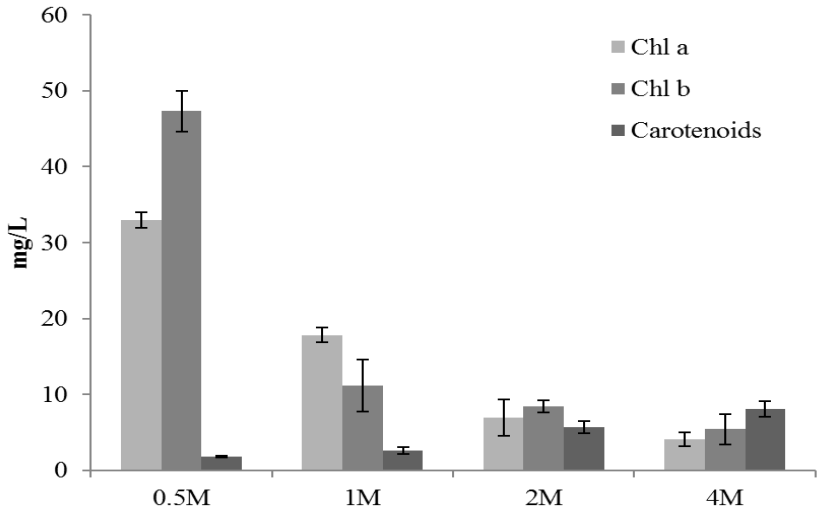

Figure 2. Chlorophylls and carotenoids in *D. salina* cultured in Walne medium with different concentrations of NaCl (0.5, 1, 2, 4 M). All tests were realized in triplicate and data are mean ± standard deviation of the mean of 9 samples.

The highest carotenoids content in *D. salina* was achieved under high salinity (Borowitzka and Borowitzka, 1990; Cray et al., 2013; Fachet et al., 2016). Ratio of carotenoids/chlorophylls was increased as 0.09, 0.37, and 0.84 in 1, 2 and 4 M compared to control (0.023). Araújo et al. (2009) have reported similar results where carotenoids/chlorophylls ratio increased by salinity in the culture medium of *D. salina*.

Exopolysaccharides content in D. salina *under salt stress.*

The exopolysaccharides produced by *D. salina* under different NaCl concentrations was isolated at the 15th day of culture which corresponds to the middle of stationary phase. It was found that exopolysaccharide content increased concomitantly with salt concentration. It began from 5 mg L^{-1} when algae was grown in 1 M NaCl and reached the maximum of 10.5 mg L^{-1} when 4 M NaCl was added to the culture medium (Figure 3).

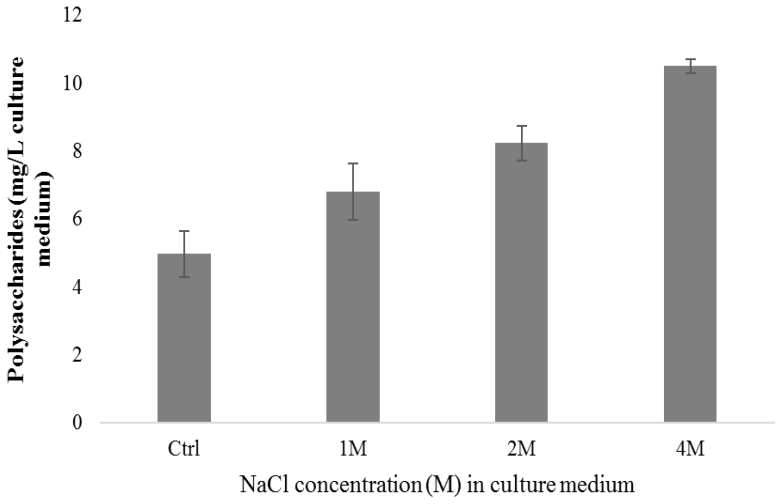

Figure 3. Exopolysaccharides content liberate by *D. salina* cultured in Walne medium with different concentrations of NaCl (0.5, 1, 2, 4 M). All tests were realized in triplicate and data expressed by the mean ± standard deviation of the mean of 9 samples.

The improvement in the production of polysaccharides by increasing salinity in several marine algae was established in several studies (Liu et al., 200; Taraldsvik and Myklestad, 2000). Priester et al. (2006) reported increase the production of extracellular carbohydrates under various environmental stresses. The EPS production from three isolates microalgae cultured in 0.4 M of NaCl was considerably higher than the control and a good correlation was observed between NaCl exposure and EPS production in each isolates (Ozturk and Aslim, 2010).

EPS from *D. salina* have recently been characterized. FT-IR spectrum confirms the presence of primary amine-group, aromatic-compound, halide-group, aliphatic alkyl-group and polysaccharides (Mishra et al., 2011). Monosaccharides detected in EPSs of *D. salina* can be categorized as aldohexoses (glucose and galactose), ketohexose (fructose) and pentose (xylose) sugars (Mishra et al., 2011). The same study showed that variation in the salinity level in medium culture has no effect on the composition of EPSs of *D*. *salina* (Mishra et al., 2011).

Proline content in D. salina *under salt stress.*

The accumulation of proline in *D. salina* grown under different NaCl concentrations (0.5, 1, 2 and 4 M) was determined after 15 days of culture. Proline content in *D. salina* biomass increased in 1 M NaCl compared to the control (0.5 M) and reached 127 µmol g^{-1}. Proline content dropped to 86 and 21 µmol g^{-1} in 2 and 4 M NaCl treatment, respectively (Figure 4).

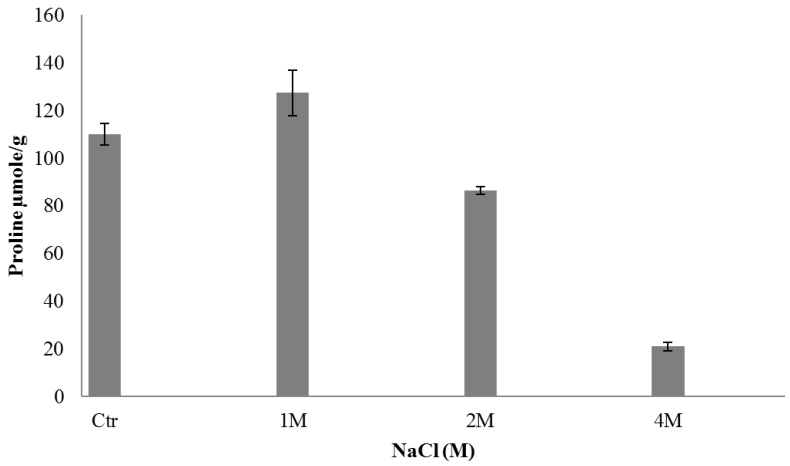

Figure 4. Proline concentration of *D. salina* cultured in 1 L of Walne medium with different concentrations of NaCl (0.5, 1, 2, 4 M) for 14 days. All tests were realized in triplicate and data are mean ± standard deviation of the mean of 9 samples.

Proline plays an important role in ameliorating tolerance to environmental stresses in plants and microorganisms (Siripornadulsil et al., 2002). According to Hong-Qi et al. (1985) proline is one of the metabolites that increase resistance to oxidative stress after their accumulation. In microalgae and other plants, proline is an osmoprotector which acts as a free radical scavenging and increases salt tolerance in microorganisms (Hiremath and Mathad, 2010).

Effect of *D. salina* extracts on germination of wheat seeds and seedlings growth under salt stress

1. Effect of *D. salina* hydrolyzate extracts (HE) on seed germination and seedling growth.

Hydrolyzate extracts (HE) from *D. salina* cultured in different salinity levels (0.5, 1, 2, 4 M) were applied on wheat and their impact on germination and seedlings growth are

investigated and results are presented in (Figure 5). 0.1% of HE of *D. salina* cultured at different concentration of NaCl increased germination of wheat seeds at both 3 and 6 g L^{-1} NaCl. HE from *D. salina* cultured in 1 and 2 M NaCl raised germination rate from 45 to 50 and 75%, respectively, for wheat seeds cultured in 3 g L^{-1} NaCl (Figure 5a). Wheat seeds cultured in 6 g L^{-1} NaCl were also stimulated from 37 to 40 and 70% when treated by 0.1% of HE of *D. salina* cultured in 1 and 2 M, NaCl, respectively, (Figure 5b).Whereas extracts of *D. salina* cultured in 4 M NaCl had no effect on seed germination in salt stress (3 and 6 g L^{-1} NaCl). Moreover, the HE from *D. salina* grown at different salinity levels stimulated root growth and coleoptile size of wheat seedling cultured in the two concentrations of at 3 and 6 g L^{-1} NaCl (Figure 5c).

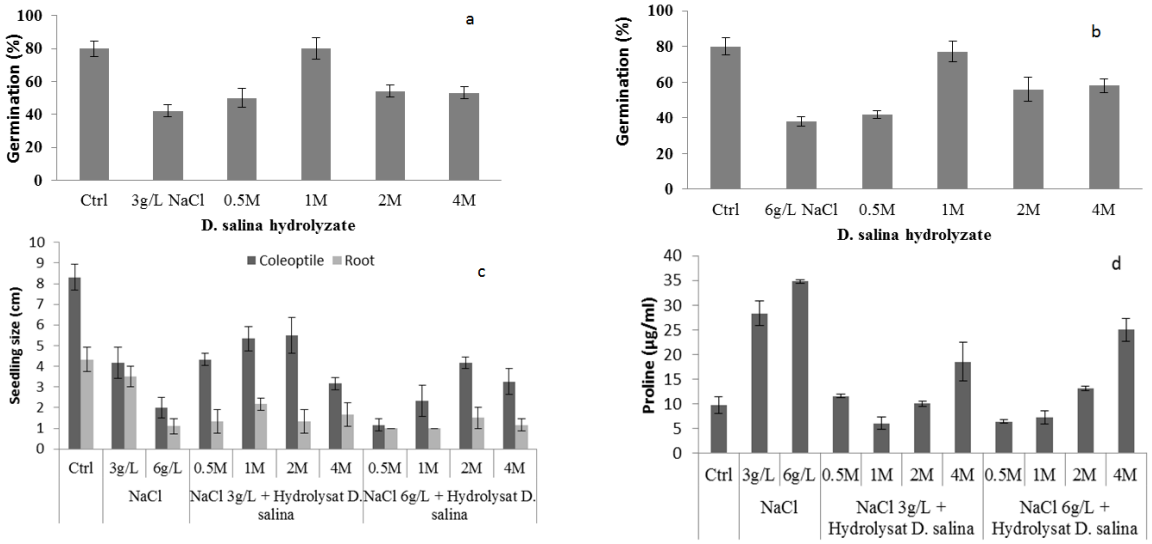

Figure 5. Hydrolyzates extracts effect (0 and 1%) of biomass *D. salina* (washed twice by distilled water) cultured at different concentrations of salinity (0.5, 1, 2, 4 M of NaCl) on germination of wheat and seedling growth under salt stress (3 and 6 g L^{-1} NaCl) at the 4 day of incubation. a) Effect on germination of seeds wheat cultured in 3 g L^{-1} NaCl; b) effect on germination of seeds wheat cultured in 6 g L^{-1} NaCl; c) effect on seedling growth; and d) proline content in wheat seedling under salt stress. All tests were realized in triplicate and data are mean ± standard deviation of the mean of 4 petri dishes (25 seeds $petri^{-1}$).

Several studies have shown the positive effect of seaweeds extracts on plant seeds germination. Ghareib (2010) showed that methanolic extract of *Sargassum virgatum* stimulated the seeds germination and seedling growth of lettuce, tomato and barely crops. *Ascophyllum nodosum* extract also has a positive effect on the stimulation of germination and seedling growth of tomato (Basher et al., 2012). As well, the extracts of *Ulva rigida* improve antioxidative mechanisms under salt stress and consequently enhance wheat growth under salt stress condition (Chernane et al., 2015).

We also found an increase in proline accumulation in wheat seedlings under salt stress (3 and 6 g L^{-1} NaCl). Figure 5d shows the accumulation of proline which increased remarkably under both salt stress levels, whereas after seeds treatment with HE from *D. salina* grown at different concentrations of NaCl, proline content decreased gradually. The accumulation of proline after microalgae hydrolyzate treatment was decreased compared to salt stress conditions (3 and 6 g L^{-1}) and was inversely proportional to proline content of extracts prepared from *D. salina* grown at different concentrations of NaCl. Accumulation of solutes, especially proline, glycine-betaine and sugars is a common observation under stress condition (Ashraf et al., 1994; Naqvi et al., 1994). Ashraf et al. (1994) reported that proline is an important osmolyte to adjust the plant osmoregulation under drought/saline conditions.

The results support the idea that proline accumulation occurs in many plants under saline conditions (Ashraf, 2004).

2. Effect of bioactive molecules extracted from *D. salina* on wheat germination and seedling growth.

The effect of three bioactive molecules- polysaccharides, carotenoids and proline which are the major constituents of HE were studied on wheat germination and seedling growth under salt stress conditions.

3. Effect of exopolysaccharides (EPS) on germination and seedling growth of wheat under salt stress.

We investigated the effect of total polysaccharides extract from *D. salina* cultured under salt stress at different concentrations of NaCl (0.5, 1, 2, 4 M) on the germination and seedlings growth under salt stress conditions.

Figures 6a and 7a, show that exopolysaccharides (EPS) extracted from *D. salina* induced germination under salt stress which reached 96% under 3 g L^{-1} NaCl and 83% under 6 g L^{-1} NaCl after 4 days of culture compared to controls in which the germination rate was 60% under 3 g L^{-1} and 55% under 6 g L^{-1} NaCl.

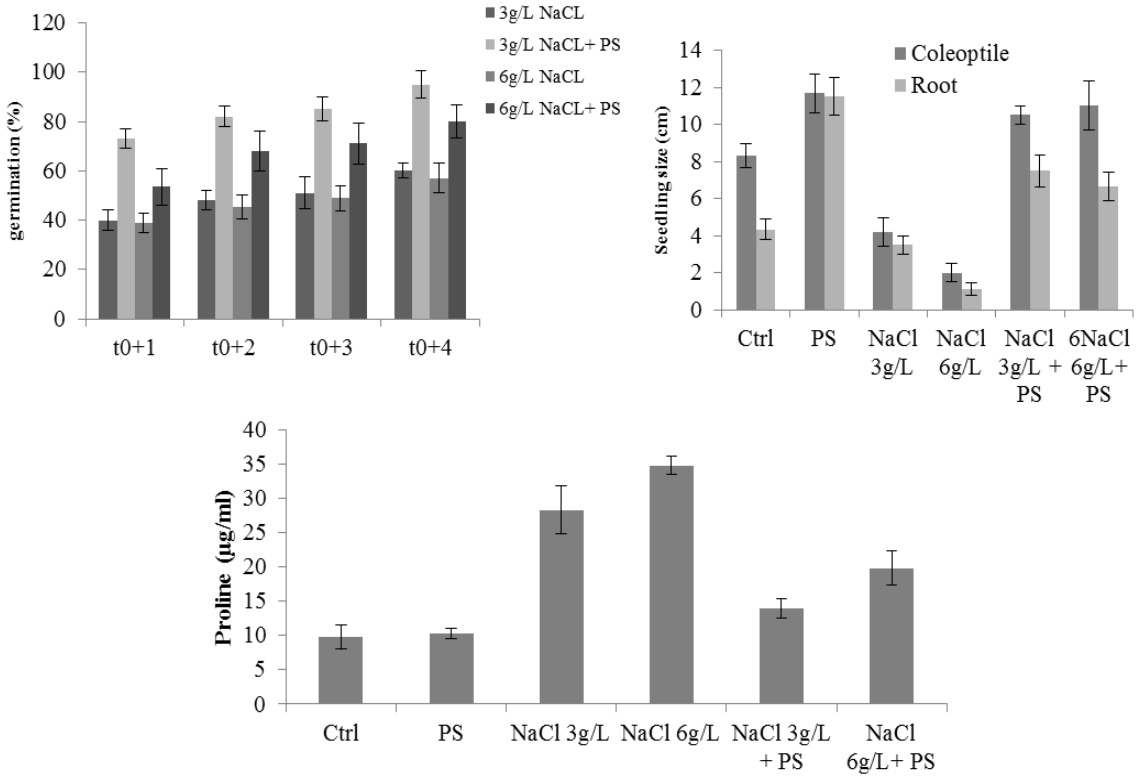

Figure 6. Effect of polysaccharides (2 mg L^{-1}) extracted from *D. salina* cultured at different salinity level (0.5, 1, 2, 4 M of NaCl) on germination of wheat and seedling growth under salt stress (3 and 6 g L^{-1} NaCl) at 4 days of incubation. Top left – effect on germination of seeds wheat cultured in 3 and 6 g L^{-1} NaCl; top right – effect of PS on seedling growth; and bottom – PS effect on proline content in wheat seedling cultured under salt stress condition. All tests were realized in triplicate and data are mean ± standard deviation of the mean of 4 petri dishes (25 seeds $petri^{-1}$).

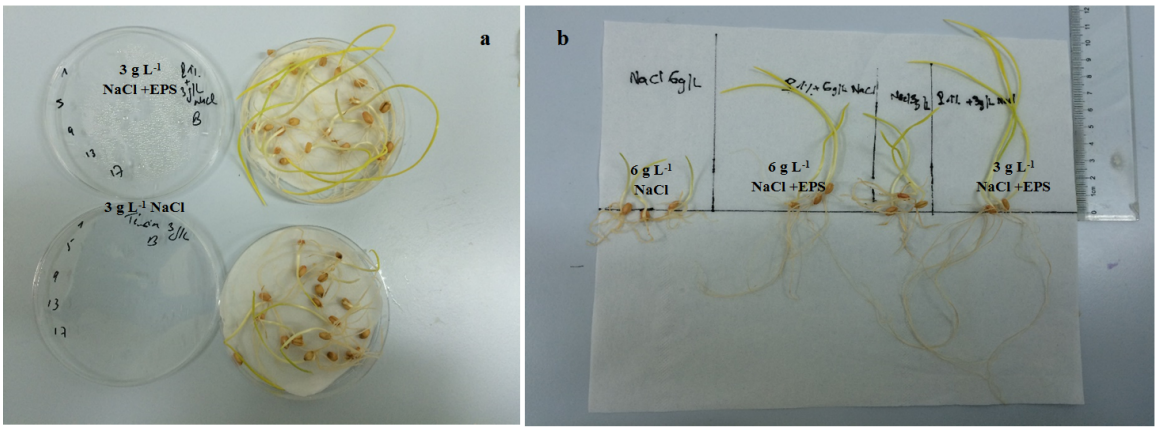

Figure 7. Effect of EPS treatment on wheat seeds germination and seedling growth. a) Wheat seeds cultured in salt stress conditions and treated with 2 mg mL^{-1} of EPS; b) Seedling wheat under salt stress and treated with EPS.

Polysaccharides treatment significantly improved seedlings growth at 2 mg L^{-1} polysaccharides (Figures 6b and 7b). This treatment stimulated root and coleoptiles with 120 and 50%, respectively, compared to the control. Seedling growth was inhibited by salinity, root and coleoptile systems were decreased by 50 and 25%, respectively, at 3 g L^{-1} NaCl and 75 and 73%, respectively, at 6 g L^{-1} NaCl. Polysaccharide treatment improved size of the roots and coleoptiles system 133 and 105%, respectively, at 3 g L^{-1} NaCl compared to control (3 g L^{-1} NaCl untreated), and 444 and 750% at 6 g L^{-1} NaCl, respectively, compared to control (6 g L^{-1} NaCl, untreated). Thus, PS acts as a biological protector against salinity. Previous works generally reported the use of whole microalgae biomass on fertilization and growth stimulation of plants (Aly and Esawy, 2008; Bhowmik et al., 2010). It was demonstrated that polysaccharides extracted from other organisms especially, seaweeds *Sargassum vulgare*, stimulated germination rate with 2-5% and enhanced seedlings growth of *Phaseolus vulgaris* cultured under salt stress (4 g L^{-1} NaCl) (Mansori et al., 2015). Arora et al. (2010) showed that cyanobacterial exopolysaccharides stimulated seeds germination from 13 to 30% in wheat, maize and rice under salt stress. Another study show that *Ulva lactica* polysaccharides improved germination percentage and seedling growth of wheat under salt stress (Saqib et al., 2013).

Proline is another plant stress indicator studied in this work. Figure 6c shows that accumulation of proline increased remarkably under salt stress, whereas after treatment of seeds with EPS extracted from *D. salina*, proline content decreased gradually. Accumulation of compatible solutes especially proline is a common observation under stress condition (Ashraf et al., 1998).

4. Effect of exogenous proline on germination of wheat seeds under salt stress.

We investigated the effect of exogenous application of proline extracted from *D. salina* on salt tolerance of wheat seeds (Figure 8). Proline application improved germination percentage under salt stress at 3 and 6 g L^{-1} NaCl compared to untreated control.

As shown in Figure 4, proline increased when salinity of microalgae *D. salina* rose from 0.5 to 1 M and then decreased at 2 and 4 M. The same tendency was observed when microalgae HE was tested on stressed seeds (Figure 5a, b). This suggests that the proline was an essential component of HE for germination and it is involved in the enhancement of tolerance to stress seeds. This was confirmed by the analysis of the effect of exogenous proline on germination (Figure 8). Although salt stress reduced the germination percentage, plant biomass and chlorophyll contents of wheat and the addition of proline (40 and 50 mM) showed lighter improvement of germination percentage under salt stress (Ismail, 2014; Talat et al., 2013).

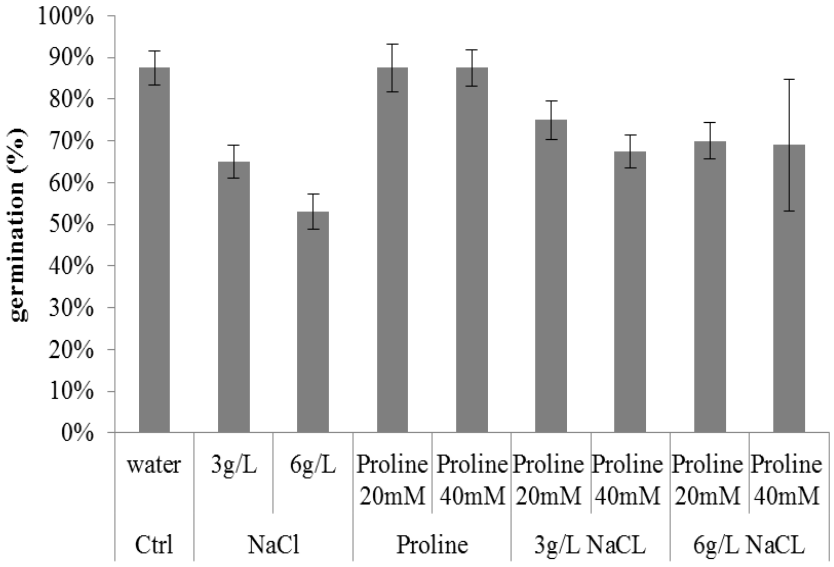

Figure 8. Proline effect on germination of wheat under salt stress. Wheat seeds cultured in salt stress (3 and 6 g L^{-1} NaCl) and treated with two concentration of proline (20 and 40 mM). All tests were realized in 4 replicate and data are mean ± standard deviation of the mean of 4 petri dishes (25 seeds $Boite^{-1}$).

5. Effect of carotenoids extract from *D.salina* on germination of wheat seeds under salt stress.

Carotenoids are structurally and functionally a very diverse group of natural pigments of the polyenetype. Carotenoids are important constituents of photosynthetic organelles of all higher plants, mosses, ferns andalgae. Carotenoids are pigments which play a major role in the protection of plants against photooxidative processes.

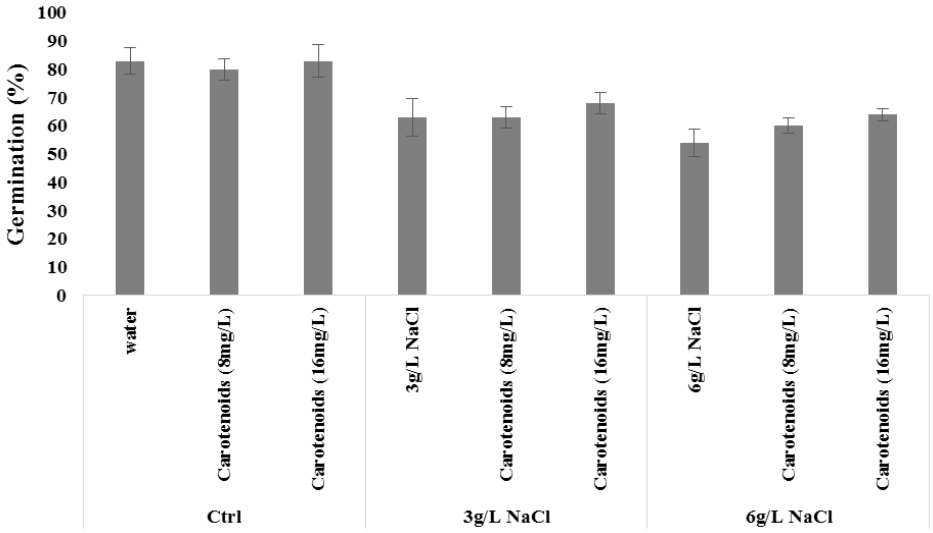

Figure 9. Effect of carotenoids extract from *D. salina* on germination of wheat under salt stress. Wheat seeds cultured in salt stress (3 and 6 g L^{-1} NaCl) and treated with two concentrations of carotenoids extract (8 and 16 mg L^{-1}). All tests were realized in 4 replicates and data are mean ± standard deviation of the mean of 4 petri dishes (25 seeds $Boite^{-1}$).

Carotenoids extract has no effect on germination at standard conditions (Figure 9). Carotenoids treated seeds showed no significant improvement on germination under salt stress for both salinity level compared to untreated control. They are efficient antioxidants scavenging singlet molecular oxygen and peroxyl radicals (Paiva and Russell, 1999). Carotenoids are known to be very efficient physical and chemical quenchers of singlet oxygen (1 O_2), as well as potent scavengers of other reactive oxygen species (ROS) (Cvetkovic et al., 2013; Fiedor et al., 2005).

6. Effect of *D. salina* exopolysaccharides on the ROS scavenging enzymes activities.

The effect of microalgae exopolysaccharides on ROS scavenging enzymes activities in wheat seedling under salt stress was investigated. Results showed an increase in POD and SOD activities in stressed seeds by adding 3 or 6 g of NaCl (Figure 10).

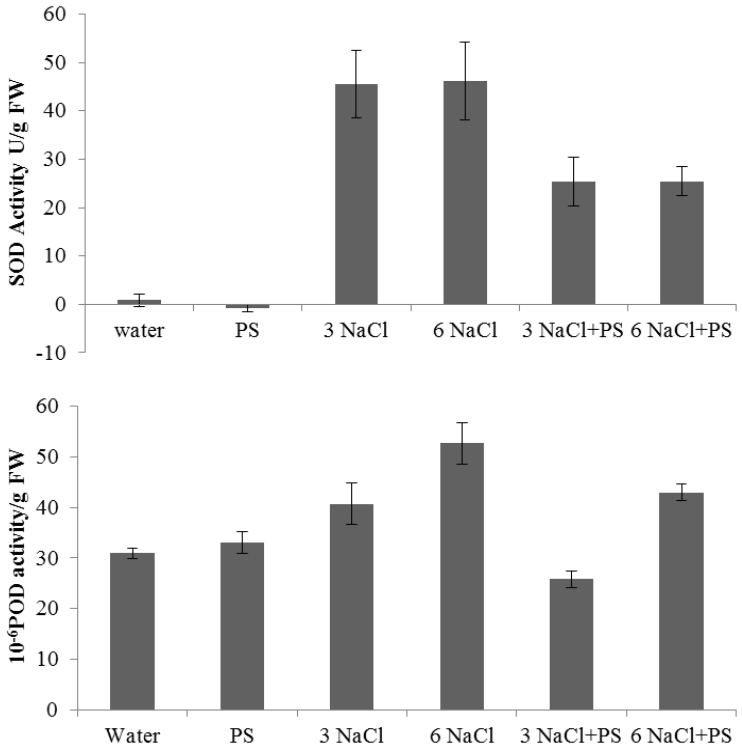

Figure 10. ROS scavenging enzymes activities (POD and SOD) under salt stress on wheat seedlings treated with 2 g L^{-1} of *D. salina* exopolysaccharides.

The increase was more pronounced in SOD activity, from less than 1 to 46 U g^{-1} FW as compared to POD activity which increased by 32 and 70% in 3 and 6 g L^{-1} treated seeds, respectively. This gives an indication on cellular ROS rate which is directly proportional to these activities.

POD and SOD activities of seeds dropped under salt treatment by adding exopolysaccharides. POD activity decreased by 51 and 18% under 3 and 6 g L^{-1} NaCl, respectively, while SOD activity decreased by 46% in both treatments (3 and 6 g L^{-1}). The result indicates that exopolysaccharides could contribute to ROS reduction caused by salt stress under control. On the other hand, for seeds cultivated under normal conditions, SOD and POD activities remain steady when treated by polysaccharides. It has been reported that salt stress provoke the increase of ROS scavenging enzymes under salt stress in *Triticum aestivum* (Shafi et al., 2009).

The study of the mechanisms by which exopolysaccharides positively intervene to alleviate salt stress is important. No such study has been reported, but we know at least, that halophyte plant secrete mucilage and polysaccharides under salt stress. Presence of

mucopolysaccharides has been observed in xylem vessels and it was suggested that the pectin polysaccharides could be involved in Na + fixation and therefore in salt tolerance mechanisms of halophyte plants (Ghanem et al., 2010).

CONCLUSIONS

D. salina tolerated salt stress by accumulating several osmoprotectants such as proline, carotenoids and exopolysaccharides. *D. salina* hydrolysates enhanced seeds germination and seedling growth and helped plant to tolerate salt stress. Exopolysaccharides showed the major effect of *D. salina* extracts on wheat seeds germination and seedling growth. Further studies should be undertaken to understand mechanisms involved in the improvement of plants tolerance by exopolysaccharides.

Literature cited

Adam, P. (1990). Salt Marsh Ecology (Cambridge, UK: Cambridge University Press).

Almansouri, M., Kinet, J.-M., and Lutts, S. (2001). Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). Plant Soil *231* (*2*), 243–254 http://dx.doi.org/10.1023/A:1010378409663.

Aly, M.S., and Esawy, M.A. (2008). Evaluation of *Spirulina platensis* as biostimulator for organic farming systems. Journal of Genetic Engineering and Biotechnology *6* (*2*), 1–7.

Araújo, O.Q.F., Gobbi, C.N., Chaloub, R.M., and Coelho, M.A.Z. (2009). Assessment of the impact of salinity and irradiance on the combined carbon dioxide sequestration and carotenoids production by *Dunaliella salina*: A mathematical model. Biotechnol. Bioeng. *102* (*2*), 425–435. PubMed http://dx.doi.org/10.1002/bit.22079

Arora, M., Kaushik, A., Rani, N., and Kaushik, C.P. (2010). Effect of cyanobacterial exopolysaccharides on salt stress alleviation and seed germination. J Environ Biol *31* (*5*), 701–704 PubMed.

Ashraf, M. (2004). Some important physiological selection criteria for salt tolerance in plants. Flora Review *199* (*5*), 361–376 http://dx.doi.org/10.1078/0367-2530-00165.

Ashraf, M.Y., Azmi, A.R., Khan, A.H., and Ala, S.A. (1994). Effect of water stress on total phenol, peroxidase activity and chlorophyll contents in wheat (*Triticum aestivum* L.). Acta Physiol. Plant. *16*, 185–191.

Ashraf, M.Y., Ali, Y., and Qureshi, T.M. (1998). Effect of salinity on photosynthetic efficiency and yield of rice genotypes. Pak. J. Biol. Sci. *1* (*2*), 72–74 http://dx.doi.org/10.3923/pjbs.1998.72.74.

Basher, A.A., Mohammed, A.J., and Teeb, A.I.H. (2012). Effect of seaweed and drainage water on germination and seedling growth of tomato (*Lycopersicon* spp.). Euphrates J Ag Sci *4*, 24–39.

Bates, L., Waldren, R.P., and Teare, I.D. (1973). Rapid determination of free proline for water-stress studies. Plant Soil *39* (*1*), 205–207 http://dx.doi.org/10.1007/BF00018060.

Bhowmik, D., Dubey, J., and Mehra, S. (2010). Evaluating potential of *Spirulina* as inoculant for pulses. Academic Journal of Plant Sciences *3* (*4*), 161–164.

Blunden, G. (1991). Agricultural uses of seaweeds and seaweed extracts. In Seaweed Resources in Europe: Uses and Potential, M.D. Guiry, and G. Blunden (Chichester, UK: John Wiley & Sons Ltd.), p.65–81.

Borowitzka, L.J., and Borowitzka, M.A. (1990). Commercial production of B-carotene by *D. salina* in open ponds. Bull. Mar. Sci. *47* (*1*), 244–252.

Chernane, H., Latique, S., Mansori, M., and El Kaoua, M. (2015). Salt stress tolerance and antioxidative mechanisms in wheat plants (*Triticum durum* L.) by seaweed extracts application. J of Agri and Veter Sci. *8* (*1*), 36–44.

Cray, J.A., Bell, A.N., Bhaganna, P., Mswaka, A.Y., Timson, D.J., and Hallsworth, J.E. (2013). The biology of habitat dominance; can microbes behave as weeds? Microb Biotechnol *6* (*5*), 453–492. PubMed http://dx.doi.org/10.1111/1751-7915.12027

Cvetkovic, D., Fiedor, L., Fiedor, J., Wiśniewska-Becker, A., and Markovic, D. (2013). Molecular base for carotenoids antioxidant activity in model and biological systems: the health-related effects. In Carotenoids: Food Sources, Production and Health Benefits, M. Yamaguchi, ed. (Atlanta, GA, USA), p.93–126.

Fachet, M., Hermsdorf, D., Rihko-Struckmann, L., and Sundmacher, K. (2016). Flow cytometry enables dynamic tracking of algal stress response:aA case study using carotenogenesis in *Dunaliella salina.* Algal Research *13*, 227–234 http://dx.doi.org/10.1016/j.algal.2015.11.014.

Fiedor, J., Fiedor, L., Haessner, R., and Scheer, H. (2005). Cyclic endoperoxides of β-carotene, potential pro-oxidants, as products of chemical quenching of singlet oxygen. Biochim. Biophys. Acta *1709* (*1*), 1–4. PubMed

http://dx.doi.org/10.1016/j.bbabio.2005.05.008

García, F., Freile-Pelegrín, Y., and Robledo, D. (2007). Physiological characterization of *Dunaliella* sp. (*Chlorophyta, Volvocales*) from Yucatan, Mexico. Bioresour. Technol. *98* (*7*), 1359–1365. PubMed http://dx.doi.org/10.1016/j.biortech.2006.05.051

Ghanem, M.E., Han, R.M., Classen, B., Quetin-Leclerq, J., Mahy, G., Ruan, C.J., Qin, P., Pérez-Alfocea, F., and Lutts, S. (2010). Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. J. Plant Physiol. *167* (*5*), 382–392. PubMed http://dx.doi.org/10.1016/j.jplph.2009.10.012

Ghareib, H.R. (2010). Effect of methanol extract of *Sargassum virgatum* AG (MERT.) – a marine brown macroalga on seed germination and seedling growth of some agriculture crops. An Inter J of Mar Sci *26* (*1*), 13–21.

Ghetti, F., Herrmann, H., Häder, D.P., and Seidlitz, H.K. (1999). Spectral dependence of the inhibition of photosynthesis under simulated global radiation in the unicellular green alga *Dunaliellasalina.* J. Photochem. Photobiol. Bol. Biol. *48*, 166–173.

Hadi, M.R., Shariati, M., and Afsharzadeh, A. (2008). Microalgal biotechnology: carotenoid and glycerol production by the green algae *Dunaliella* isolated from the Gave-Khooni salt marsh, Iran. Biotechnol. and Bioprocess Engin *13* (*5*), 540–544 http://dx.doi.org/10.1007/s12257-007-0185-7.

Hiremath, S., and Mathad, P. (2010). Impact of salinity on the physiological and biochemical traits of *Chlorella vulgaris* Beijerinck. J. Algal Biomass Utln *1* (*2*), 51–59.

Hong-Qi, Z., Croes, A.F., and Linskens, H.F. (1985). Proline accumulation during another development in *Petunia hybrida.* Acta Bot. Neerl. *34* (*2*), 213–222 http://dx.doi.org/10.1111/j.1438-8677.1985.tb01880.x.

Ismail, M.A. (2014). Exogenous proline induced changes in SDS-PAGE protein profile for salt tolerance in wheat (*Triticum aestivum* L.) seedlings. Res. J. of Pharma. Biol. and Chem. Sci. *5* (*4*), 748–755.

Jiang, M., and Zhang, J. (2002). Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. J. Exp. Bot. *53* (*379*), 2401–2410. PubMed http://dx.doi.org/10.1093/jxb/erf090

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. *17* (*3*), 287–291. PubMed http://dx.doi.org/10.1038/7036

Lichtenthaler, H.K., and Buschmann, C. (2001). Chlorophylls and carotenoids: measurement and characterization by UV-VIS Spectroscopy. Curr. Prot. in Food Analyt. Chem., F4.3.1-F4.3.8.

Liu, H., and Buskey, E.J. (2000). Hyper salinity enhances the production of extracellular polymeric substance (EPS) in the Texas browntide alga, *Aureoumbra lagunensis* (*Pelagophyceae*). J. Phycol. *36* (*1*), 71–77 http://dx.doi.org/10.1046/j.1529-8817.2000.99076.x.

Maehly, A.C., and Chance, B. (1954). The assay of catalases and peroxidases. Methods Biochem Anal *1*, 357–424. PubMed http://dx.doi.org/10.1002/9780470110171.ch14

Mansori, M., Chernane, H., Latique, S., Benaliat, A., Hsissou, D., and El Kaoua, M. (2015). Seaweed extract effect on water deficit and antioxidative mechanisms in bean plants (*Phaseolus vulgaris* L.). J. Appl. Phycol. *27* (*4*), 1689–1698 http://dx.doi.org/10.1007/s10811-014-0455-7.

Miazek, K., and Ledakowicz, S. (2013). Chlorphyll from leaves, needles and microalgae: a kinetic approach. Int J Agric and Biol. Eng. *6* (*2*), 107–115.

Mishra, A., Kavita, K., and Jha, B. (2011). Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina.* Carbohydr. Polym. *83* (*2*), 852–857 http://dx.doi.org/10.1016/j.carbpol.2010.08.067.

Monteiro, C.C., Carvalho, R.F., Gratão, P.L., Carvalho, G., Tezotto, T., Medici, L.O., Peres, L.E.P., and Azevedo, R.A. (2011). Biochemical responses of the ethylene-insensitive never ripe tomato mutant subjected to cadmium and sodium stresses. Environ. Exp. Bot. *71* (*2*), 306–320 http://dx.doi.org/10.1016/j.envexpbot.2010.12.020.

Munns, R. (2002). Comparative physiology of salt and water stress. Plant Cell Environ. *25* (*2*), 239–250. PubMed http://dx.doi.org/10.1046/j.0016-8025.2001.00808.x

Naqvi, S.S.M., Mumtaz, S., Ali, S.A., Shereen, A., Khan, A.H., Ashraf, M.Y., and Khan, M.A. (1994). Proline accumulation under salinity stress. Is abscisic acid involved? Acta Physiol. Plant. *16* (*2*), 117–122.

Ozturk, S., and Aslim, B. (2010). Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress. Environ Sci Pollut Res Int *17* (*3*), 595–602. PubMed http://dx.doi.org/10.1007/s11356-009-0233-2

Paiva, S.A., and Russell, R.M. (1999). Beta-carotene and other carotenoids as antioxidants. J Am Coll Nutr *18* (*5*),

426–433. PubMed http://dx.doi.org/10.1080/07315724.1999.10718880

Priester, J.H., Olson, S.G., Webb, S.M., Neu, M.P., Hersman, L.E., and Holden, P.A. (2006). Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. Appl. Environ. Microbiol. *72* (*3*), 1988–1996. PubMed http://dx.doi.org/10.1128/AEM.72.3.1988-1996.2006

Saqib, A., Tabbssum, M.R., Rashid, U., Ibrahim, M., Gill, S.S., and Mehmood, M.A. (2013). Marine macroalgae Ulva: a potential feed-stock for bioethanol and biogas production. Asian J. Agri. Biol *1*, 155–163.

Shafi, M., Bakht, J., Hassan, M.J., Raziuddin, M., and Zhang, G. (2009). Effect of cadmium and salinity stresses on growth and antioxidant enzyme activities of wheat (*Triticum aestivum* L.). Bull Environ Contam Toxicol *82* (*6*), 772–776. PubMed http://dx.doi.org/10.1007/s00128-009-9707-7

Siripornadulsil, S., Traina, S., Verma, D.P., and Sayre, R.T. (2002). Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell *14* (*11*), 2837–2847. PubMed http://dx.doi.org/10.1105/tpc.004853

Takagi, M., Karseno, and Yoshida, T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng. *101* (*3*), 223–226. PubMed http://dx.doi.org/10.1263/jbb.101.223

Talat, A., Nawaz, K., Hussian, K., Bhatti, K.H., Siddiqi, E.H., Aneela, K., Sehrish, A., and Sharif, M.U. (2013). Foliar application of proline for salt tolerance of two wheat (*Triticum aestivum* L.) cultivars. W. Appli. Sci. J. *22* (*4*), 547–554.

Tammam, A.A., Fakhry, E.M., and El-Sheekh, M. (2011). Effect of salt stress on antioxidant system and the metabolism of the reactive oxygen species in *Dunaliella salina* and *Dunaliella tertiolecta.* Afr. J. Biotechnol. *10* (*19*), 3795–3808.

Taraldsvik, M., and Myklestad, S.M. (2000). The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonem acostatum.* J. Phycol. *35* (*2*), 189–194 http://dx.doi.org/10.1080/09670260010001735781.

Yildiz, M. (2007). Two-dimensional electrophoretic analysis of soluble leaf proteins of a salt-sensitive (*Triticum aestivum*) and a salt-tolerant (*Triticum durum*) cultivar in response to NaCl stress. J. Integr. Plant Biol. *49* (*7*), 975–981 http://dx.doi.org/10.1111/j.1672-9072.2007.00465.x.

Yu, X., Chen, L., and Zhang, W. (2015). Chemicals to enhance microalgal growth and accumulation of high-value bioproducts. Front Microbiol *6*, 56. PubMed http://dx.doi.org/10.3389/fmicb.2015.00056