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Alleviation of salinity stress on maize plants using the extract of the halotolerant alga *Dunaliella bardawil*

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Abstract

Soil salinity has a great impact on decreasing yield potentials of the cultivated crops. The effect of presoaking *Zea mays* grains in the crude extract of the halotolerant alga *Dunaliella bardawil* on growth and some metabolic activities of salinized plants was investigated in this study. The Pre-soaking treatment demonstrated a highly significant enhancement in the percentage of seed germination. Moreover, the growth parameters of the pretreated plants were improved at hyposalinity stress conditions comparing with untreated ones. A huge augmentation in the soluble carbohydrates, polyols, free amino acids and proline contents was recorded with the pretreated plants at hypersaline stress conditions. GC-MS analysis identified some bioactive molecules in the algal extract such as nicotinamide, xanthine, dihydroxyphenylglycol and linalool that could potentially participate in the alleviation of the salinity stress.

INTRODUCTION

Plant salt stress is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death. Crop yields start declining when the salinity EC of the soil exceeds 4 dS m^{-1} [1]. Growth reduction of vascular plants under salinity stress may be attributed to the changes in photosynthesis, photorespiration, osmotic adjustment, amino acids and carbohydrate synthesis which could arise from adverse effects of sodium and chloride ions on metabolism [2]. It is well established that compatible solutes such as glycine, betaine, proline, glycerol and sorbitol play an important role in osmotic adjustment of the plants grow under osmotic stress [3].

Salinity stress lead to an enhanced generation of reactive oxygen species in plants due to disruption of cellular homeostasis [4]. The enhanced production of reactive oxygen species can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage of nucleic acids and enzyme inhibition [5]. Detoxification of excess the reactive oxygen species is achieved via an efficient antioxidative system comprising of the upregulation of the non-enzymatic endogenous antioxidants as well as the activation of the antioxidative enzymes [6].

Algae are classified according to the extent of their tolerance of salinity as halophytic and halotolerant. The first group of algae requires salt for optimum growth while the halotolerant algae have response mechanisms that permit their existence in saline medium. *Dunaliella bardawil* is halotolerant marine microalga that lacks a rigid cell wall and can store many economically important organic compounds [7]. The capability of this organism to tolerate hypertonic stresses was attributed to their ability to synthesize different intercellular metabolites such as β-carotene, glycerol and specific proteins that have a protective function against the inhibitory influence of salinity stress [7-8].

Several attempts had been done in order to overcome the depressive effect of salinity using some metabolites that are produced in salt tolerant plants such as proline, putrescine, sorbitol and glycerol [9]. In this study, it was intended to use the crude extract of the halotolerant alga *Dunaliella bardawil* which contains some bioactive metabolites to alleviate the negative impact of salinity on *Zea mays* plants.

MATERIALS AND METHODS

Algal strain and growth conditions

Dunaliella bardawil was isolated from salt marshes near El-Bardawil Lake, north part of Sinai Arish Governorate, Egypt. The alga was cultured in 250 mL Erlenmeyer flasks containing 100 mL of modified Johnson's medium [10] at 15% (w/v) NaCl. The algal culture was incubated in a shaking incubator at 25 ± 1ºC and light intensity 78 µmol $m^{-2}s^{-1}$ under 16/8 light/dark regime. The starting cultures were adjusted to contain 1.3 mg L^{-1} chlorophyll "a".

Algal extract preparation

After incubation period of 18 days under the previous conditions, the salinity level of the algal culture was raised to 25% (w/v) NaCl for an additional 5 days. Then algal cells were harvested by centrifugation at 4500g for 10 min. The pellet was freeze dried and stored at -20°C. Two grams of the freeze dried *Dunaliella* biomass was dissolved in 100 mL dH_2O. Then the algal suspension was subjected for homogenization using ultrasonic homogenizer model STH-750S (Sonictopia, Korea) for 2 min followed by centrifugation at 4500g for 10 min. The volume of the supernatant was completed to one liter by dH_2O. The *Dunaliella* extract was subjected to analysis of total chlorophylls and carotenoids, proteins, carbohydrates, glycerol and proline contents [11].

Antioxidant activity assay

About 200 mg of freeze dried *Dunaliella* biomass was extracted with 2 mL of hexane for 30 min at room temperature (25°C). The tube was centrifuged at 4500g for 10 min and the supernatant was recovered. The extraction was repeated with 2 mL of hexane and the two supernatants were combined. The residue was subsequently extracted twice with ethyl acetate (2 mL each time) for 30 min at room temperature and the supernatants were combined. Then, the residues were further extracted twice

with water (2 mL each time) for 30 min at 80°C and the supernatants were combined. Resultant extracts were filtered and solvent removed under reduced pressure to yield dry material. Extracts' weights were recorded. For the antioxidant activity assays a sample of each extract was prepared to give a final test concentration of 10 mg/mL. All samples were stored at 4°C until used.

Antioxidant activity of the algal extracts was determined using the ferric reducing antioxidant power (FRAP) assay according to Kelman *et al.* [12]. The working FRAP reagent was made by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio and heated to 37°C prior to use. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate with 16 mL glacial acetic acid and made to 1 L with ddH_2O. The TPTZ solution was prepared by mixing equal volumes of 10 mM TPTZ with 40 mM HCl. For the actual assays, 150 µL of FRAP reagent was added to each well of a 96-well microtiter plate. A blank reading was taken at 595 nm using a Bio-Rad (Hercules, CA, USA) microtiter plate reader. To each well 20 µL of sample in triplicate was then added, incubated for 8 min at room temperature and read at 595 nm. Triplicate standards of known Fe (II) concentrations were run simultaneously using concentrations between 50 and 1000 µM of $FeSO_4 \cdot 7H_2O$. A standard curve was plotted and the FRAP values (µM) was determined.

Maize grains and seed germination experiment

Grains of *Zea mays* L. double cross hybrid 215, pretreated with the fungicide β-fax, were used in all experiments of this work. Homogenous lots of seeds were selected for unity of size, shape and viability. Before germination, seeds were surface sterilized by soaking for 30 minutes in 2.5% sodium hypochlorite solution, rinsed several times with distilled water. Then the sterilized 20 seeds were presoaked in 100% the algal extract for 4, 8, 16, 20 and 24 hrs then they were transferred to sterile Petri dish (15 cm diameter) containing two sheets of Whatman No.1 filter paper, moisted with 10 mL of distilled water. The seeds were allowed to germinate in the darkness at 25°C. Petri dishes were daily watered with 2 mL of distilled water for 6 days.

Presoaking treatments

Lots of maize grains, each of 50 grain, were presoaked in 200 ml of the crude extract of *Dunaliella* cells for 4, 8, 12, 16, 20 and 24 hours. Thereafter the grains were allowed to drain for 1 hr on stainless steel screen, and then placed on filter paper for 24 hrs at normal room temperature and humidity to dry. The grains were then allowed to germinate and develop in sandy soil irrigated with Hoagland solution[13] supplemented with different concentrations of NaCl (0, 4, 8, 12 and 16 dS m^{-1}). Three replicates were done for each treatment.

Harvesting and analyses

Plants were harvested after 30 days where dry weight and the plant height were determined. Chlorophyll, soluble carbohydrates, soluble proteins, free amino acids, proline, total polyols were assayed according to Ali [14].

GC-MS analysis

The GC-MS analysis of the ethyl acetate extract was carried out in Faculty of Science (Fayoum University) on an Agilent 5973 single quadrupole mass spectrometer (Palo Alto, CA, USA), coupled with an Agilent 6890 gas chromatograph used with an Agilent DB-5.625 (30 mm × 0.25 mm inner diameter × 0.25 µm) analytical column (inlet temperature, 275°C; injection volume, 2 µL; J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas at a flow rate of 1.5 mL/min. The oven temperature was maintained at 150°C for 1 min and then increased to 250°C at a rate of 50°C/min (maintained for 1 min), followed by an increase to 300°C at a rate of 5°C/min (maintained for 1 min). Samples (1 µL) were injected into the GC, which was operating in the splitless mode with an injector port temperature of 280°C. We used electron impact (EI) as the ionization technique. Mass spectra data were acquired under selected ion monitoring (SIM) mode in which three to four ions per compound were scanned to enhance sensitivity.

Statistical methods

Results are presented as mean ± standard error of the mean. Data were compared for significant differences using Statistica Program (version 5) and Student's t-test. The levels of significance chosen were $p < 0.05$ and $p < 0.01$.

RESULT

Characterization of the *Dunaliella* extract

The data in Table (1) showed that the algal extract of *D. bardawil* contains total chlorophylls (19 mg L^{-1}), carotenoids (24.8 mg L^{-1}), total proteins (198.6 mg L^{-1}), total carbohydrates

Table 1. The analyses of *Dunaliella bardawil* extract.

***Dunaliella* extract analysis**	**Concentration** (mg L^{-1})
Chlorophyll "a" content	11.7 ± 2.4
Chlorophyll "b" content	7.3 ± 1.4
Carotenoid content	24.8 ± 2.6
Total protein content	198.7 ± 15.3
Total carbohydrates content	46.3 ± 3.2
Total lipids content	39.9 ± 4.1
Glycerol content	186.4 ± 13.9
Proline content	8.6 ± 1.7

The algal cell density was 7.8×10^8 cell mL^{-1}

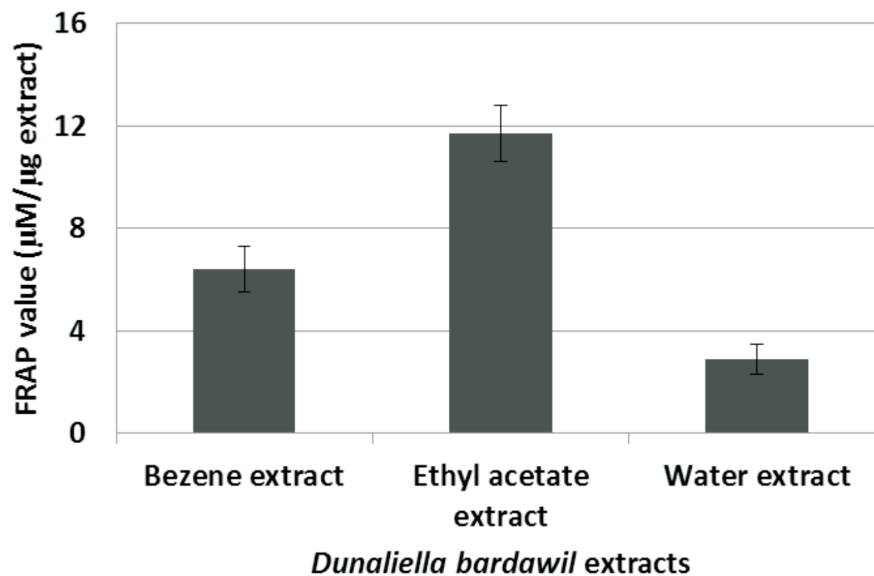

Fig. 1. Total antioxidant activity of the *Dunaliella bardawil* extracts presented as mean ferric reducing antioxidant power (FRAP) values in µM per µg extract. Error bars ± SD.

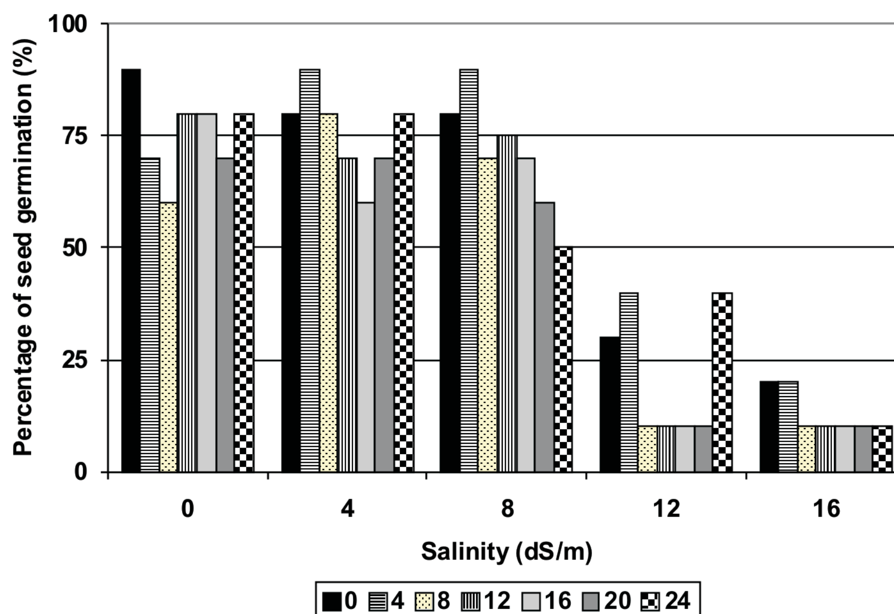

Fig. 2. Effect of pre-soaking of Maize grains in *Dunaliella bardawil* extract for 0, 4, 8, 12, 16, 20, 24 hrs on the percentage of seed germination under different salinity levels (0, 4, 8, 12 and 16 dS m^{-1}).

(46.3 mg L^{-1}), total lipids (39.9 mg L^{-1}), proline (8.6 mg L^{-1}) and glycerol (186.4 mg L^{-1}). *At the same time, the antioxidant potency of the D. bardawil* extracts (Figure 1) *demonstrated that the maximum activity was recorded with the ethyl acetate extract (11.7 µM/µg extract) while the benzene and water extracts demonstrated low antioxidant activities (6.4 and 2.9 µM/µg extract, respectively).*

Effect of presoaking of *Z. mays* in *D. bardawil* extract on seed germination

Data in Figure (2) clearly showed that the application of the crude extract of *Dunaliella* induced a highly significant positive effect on the percentage of seed germination of *Z. mays* at different salinity levels (up to 16 0, 4, 8, 12 and 16 dS m^{-1}) compared with the untreated seeds.

Effect of presoaking treatment on the Z. mays plants under salinity stress

The results presented in Table (2) clarified that the *Z. mays* plants cultivated in saline soil recorded significant decrease in their growth compared with the unstressed plants. Meanwhile, the presoaking *Z. mays* grains in *Dunaliella* extract for different periods of time showed a significant increase of the height and dry weight of the treated plants when cultivated at low soil salinity (4 dS m^{-1}) but non-significant effects were recorded at higher salinity levels (8, 12 &16 dS m^{-1}). The data concerning the biosynthesis of

Table 2. Effect of pre-soaking treatment on the height, dry weight, chlorophyll and protein contents of Maize plants treated with *Dunaliella bardawil* extract for 0, 4, 8, 12, 16, 20 & 24 hrs under different salinity levels.

Average plant height (cm)							
Salinity (dSm^{-1})	Soaking time (hrs)						
	0	4	8	12	16	20	24
0	27.5 ± 1.3	27.7 ± 2.1	27.6 ± 1.8	27.8 ± 2.0	27.6 ± 2.1	30.4 ± 1.7	28.4 ± 1.6
4	24.0 ± 1.9	27.2 ± 1.6	24.3 ± 1.3	23.3 ± 1.7	27.1 ± 1.4	24.8 ± 1.9	26.6 ± 1.5
8	19.6 ± 1.2	22.4 ± 1.5	20.1 ± 1.7	18.2 ± 1.3	17.5 ± 1.6	18.8 ± 1.4	19.8 ± 1.8
12	15.2 ± 1.4	12.6 ± 1.9	14.1 ± 2.1	12.4 ± 1.8	12.4 ± 1.7	10.8 ± 1.6	15.1 ± 1.3
16	12.4 ± 1.0	10.5 ± 1.1	11.2 ± 1.7	8.5 ± 1.4	9.8 ± 1.1	9.2 ± 1.2	9.2 ± 1.1
Average dry weight (mg/plant)							
Salinity (dSm^{-1})	Soaking time (hrs)						
	0	4	8	12	16	20	24
0	139 ± 14	156 ± 12	130 ± 16	144 ± 13	129 ± 15	160 ± 16	126 ± 11
4	123 ± 16	174 ± 14	126 ± 14	119 ± 17	180 ± 14	140 ± 13	173 ± 12
8	113 ± 15	127 ± 13	118 ± 17	92 ± 11	87 ± 16	97 ± 11	90 ± 14
12	60 ± 8	56 ± 9	97 ± 11	54 ± 7	56 ± 11	38 ± 8	74 ± 10
16	54 ± 6	42 ± 8	44 ± 9	30 ± 8	43 ± 7	36 ± 7	44 ± 9
Total chlorophyll content (mg/g fresh wt.)							
Salinity (dSm^{-1})	Soaking time (hrs)						
	0	4	8	12	16	20	24
0	1.2 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	1.3 ± 0.2	1.2 ± 0.4	1.2 ± 0.1	1.3 ± 0.1
4	1.2 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	1.2 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	1.2 ± 0.1
8	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.1 ± 0.3
12	1.4 ± 0.4	1.1 ± 0.3	1.1 ± 0.1	1.1 ± 0.3	1.4 ± 0.1	1.4 ± 0.2	1.3 ± 0.3
16	1.3 ± 0.2	1.2 ± 0.2	1.2 ± 0.3	1.2 ±0.3	1.0 ± 0.2	0.9 ± 0.3	1.0 ± 0.2
Total protein content (mg/g fresh wt.)							
Salinity (dSm^{-1})	Soaking time (hrs)						
	0	4	8	12	16	20	24
0	47 ± 4	29 ± 2	30 ± 3	35 ± 2	35 ± 7	44 ± 5	45 ± 3
4	41 ± 3	45 ± 4	42 ± 6	47 ± 3	42 ± 4	37 ± 8	37 ± 5
8	41 ± 6	43 ± 7	47 ± 8	42 ± 6	37 ± 3	41 ± 5	45 ± 4
12	41 ± 3	46 ± 8	43 ± 4	44 ± 4	45 ± 4	42 ± 8	39 ± 5
16	41 ± 7	41 ± 6	42 ± 3	43 ± 6	45 ± 5	45 ± 7	47 ± 4

chlorophylls and proteins of the pretreated *Z. mays* plants growing in soils containing different concentrations of NaCl remained more or less unchanged.

Figure (3) showed that the soluble carbohydrates content of *Z. mays* plants increased with increasing salinity level but in case of plants developed from the presoaking treatment, a sharp increase of the soluble carbohydrates content was recorded with increasing the salinity level especially under hypersalinity stress (12 &16 dS m^{-1}).

Results in Figure (3) indicated that the increment of salinity up to 8 dS m^{-1} led to a significant increase of polyols content in plant shoots, while high levels of salinity demonstrated a marked decrease in the content of these compounds. Presoaking of *Z. mays* grains in the algal extract have stimulated the accumulation of polyols in the plants under different salinity levels. It is worth to mention that the polyols content was significantly increased by the combination of presoaking grains for 8 hrs with soil salinity up to 8 dS m^{-1}.

The obtained results (Figure 3) recorded a general decrease in the free amino acids content which is correlated with an increase in the proline content of experimented plants against the increase of salinity level. The *Z. maize* plants developed from presoaking treatment recorded a sharp increase in the free amino acids and proline contents when grew under high salinity levels (12 & 16 dS m^{-1}).

GC-MS analysis of *Dunaliella bardawil* extract

GC-MS analysis of the ethyl acetate extract of *D. bardawil* (Figure 4) showed the presence of some bioactive molecules such as nicotinamide (122 g mol^{-1}), xanthine (152 g mol^{-1}), 3,4-dihydroxyphenylglycol (170 g mol^{-1}) and linalool (154 g mol^{-1}).

DISCUSSION

The ability of *Dunaliella* cells to grow at extremely high salinities was attributed to their capability to accumulate some

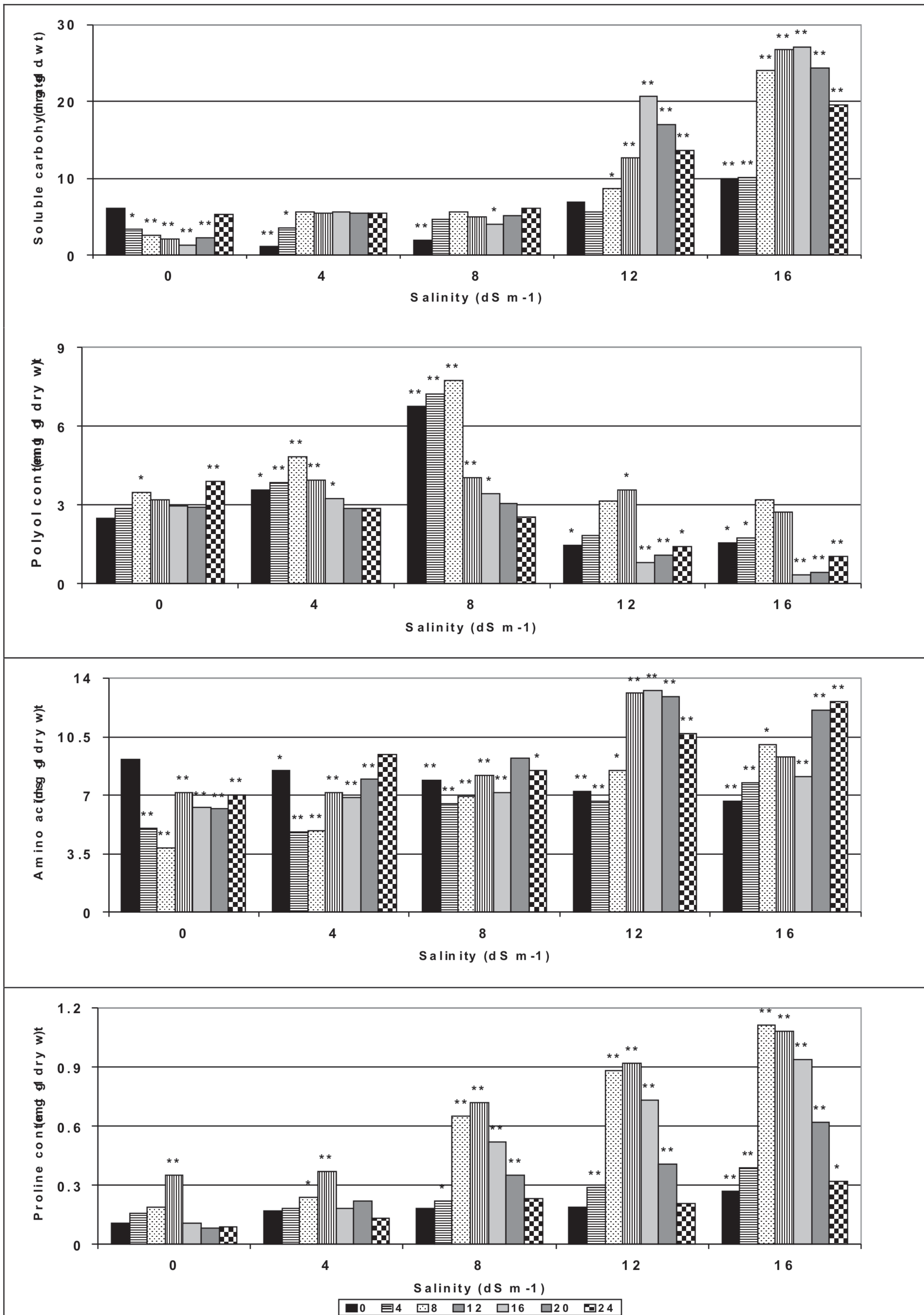

Fig. 3. Effect of pre-soaking of Maize grains in *Dunaliella bardawil* extract for 0, 4, 8, 12, 16, 20, 24 hrs on the soluble carbohydrate content, polyols content, amino acids content and proline content of plants cultivated under different salinity levels. (*) and (**) represent a significant and a highly significant differences, respectively.

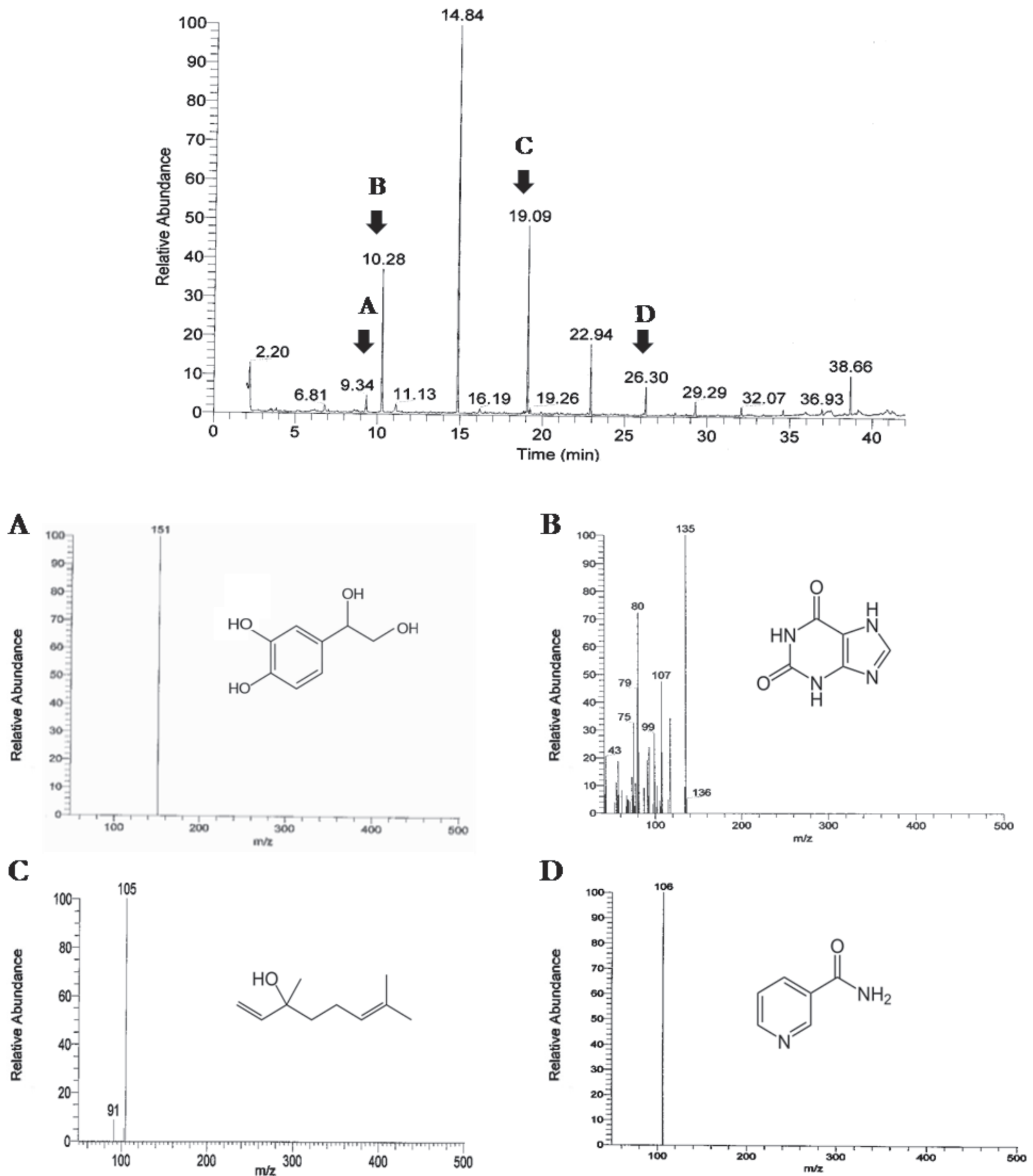

Fig. 4. Gas Chromatography mass spectral analysis of the *Dunaliella bardawil* extract. The GC spectrum shows peaks indicated with black arrows, the mass spectral analysis of which is identical to 3,4- dihydroxyphenyl (A), xanthine (B), linalool (C) and nicotinamide (D). Data are representative of at least two independent biological replicates.

osmoregulatory metabolites such as glycerol and proline that are linearly proportional to the salt concentration in which the algae are grown. Moreover, the accumulated carotenoids may play an important role in the protection of this alga against the oxidative damage [15-16].

The great antioxidant activity of *Dunaliella* was attributed to the accumulation of large quantities of antioxidant compounds as shown in Figure (4). Several bioactive compounds such as astaxanthin, zeaxanthin, glutathione, α-tocopherol and ascorbic acid have been identified in *Dunaliella* cells [17]. The antioxidant compounds of *Dunaliella* could play a crucial role as protective agents against the active oxygen species that are formed under oxidative stress.

Presoakink of *Z. mays* seeds in the extract of *Dunaliella* promoted the percentage of seed germination. These results agreed with Zhongqiang *et al*. [18] who recorded a positive correlation between seed germination of three species of *Vallisneria* and some freshwater microalgae, suggesting that these microalgae contain some compounds that may facilitate and enhance the germination process.

The negative impact of salinity on the growth of *Z. mays* plants could resulted from the oxidative damage of salinity that led to the generation of oxygen radicals such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals or energy expenditure during osmotic adjustment to salinity [19-20].

As a result of the exposure of *Z. mays* plants to salinity stress, the content of the soluble carbohydrates increased. These compounds could be used as osmoregulatory metabolites or as precursor for the biosynthesis of some osmoregulatory metabolites [21]. In case of the plants that were developed from the presoaking treatment, massive amounts of soluble carbohydrates were accumulated in the treated plants that might be resulted from the conversion of remarkable amounts of glycerol of the *Dunaliella* extract into carbohydrates inside the treated plants [22].

It is worth to mention that the polyols content of *Z. mays* plants was significantly increased by the combination of presoaking treatment with salinity. Polyols are compatible organic solutes accumulated in response to saline conditions in plants when subjected to increasing rates of sodium chloride. These compounds are suggested to act as an osmolyte, osmo-protectant or serve as storage carbohydrates under stress conditions [23]. It is important to note that there are several functions for polyols other than osmotic adjustment, such as translocation and storage of carbon, cryoprotection, prevention of activated oxygen species and energy delivery [24].

The *Z. maize* plants developed from presoaking treatment recorded a sharp increase in the free amino acids and proline contents when grew under salinity. The increment of proline and the free amino acids in the treated plants was attributed to the induction of these compounds via some bioactive molecules of the *Dunaliella* extract. The accumulated proline in plants cultivated under salinity can alleviate the inhibitory effect of salinity via acting; as a protective agent of enzymes, or as a free radical scavenger, or in the osmotic adjustment of stressed tissues [25].

Moreover, the accumulation of bioactive molecules such as nicotinamide, xanthine, 3,4-dihydroxyphenylglycol and linalool in addition to β-carotene might counteract the depressive effect of salinity. First, these bioactive molecules could enhance the enzymes regulating photosynthetic carbon reduction [26]. Second, they might promote the transcription of some defense genes [27]. Lastly, the antioxidant enzymes of the stressed plants might be activated by these active compounds which in turn decrease the build-up of active oxygen species [28-29].

CONCLUSION

It is clear from this study that presoaking *Z. maize* grains in the extract of *D. bardawil* had enhanced the percentage of seed germination and developed plants with high capability to grow under salinity stress. This effect was attributed to the presence of some substances such as glycerol which acts as osmoregulatory solute or as precursors for other osmoregulatory products. Moreover, the presence of high concentrations of carotenoids as well as other antioxidant compounds that have the ability to scavenge the highly reactive and potentially lethal oxygen species could be another benefit of the presoaking treatment. Although the presoaking treatment has mitigated the adverse effects of salinity on the treated plants, it appears to be complicated and needs further investigations.

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