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Full Length Research Paper

Effect of salt stress on antioxidant system and the metabolism of the reactive oxygen species in *Dunaliella salina* and *Dunaliella tertiolecta*

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The physiological and biochemical adaptations of two chlorophytes *Dunaliella salina and Dunaliella tertiolecta* developed to extreme saline environment were assessed in the present study. Both *Dunaliella* cultures were treated with a range of NaCl concentrations ranging from 0.05 to 4 M NaCl and the influence of salinity on growth and antioxidant parameters were determined. Biomass yields, chlorophyll and carbohydrate contents were reduced at salinity extremes. Protein contents were elevated under low salinities. No evidence or for large change was found in soluble amino acids during salinity stress. Osmoregulation is mediated by glycerol as compatible solute in both *Dunaliella* species. The maximum glycerol production was observed at high growth salinities. Under hyposaline conditions, a low content of β-carotene was noticed, whereas hypersaline conditions induced an increase in this product, about 1.4 and 1.1-fold more than its value at optimum salinities for *D. salina* and *D. tertiolecta,* respectively. An exposure to 0.5, 0.1, and 4 M NaCl increased H_2O_2 contents were positively correlated with the level of thiobarbituric acid reactive substances. The levels of six antioxidant enzymes (superoxide dismutase, catalase, peroxidase, ascorbate peroidase, glutathione reductase and glutathione S-transferase) and two antioxidant substrates (glutathione and ascorbic acid) were quantified. The data revealed a differential response between *D. salina* and *D. tertiolecta* in response to different salinity levels. The involvement of oxidative stress at various salinity levels is implied by the alterations in antioxidant enzymes and substrates, but the specific changes are very different between hypo and hypersaline stress conditions.

Key words: Antioxidant system, β-carotene*, Dunaliella*, glycerol, reactive oxygen species, salt stress.

INTRODUCTION

Salt stress is one of the most significant abiotic stresses and affects every aspect of plant physiology and metabolism. The physiological and biochemical responses are extensively studied to understand how algae respond and adapt to salinity changes (Kirst, 1990). However, information on the effects of salinity stress on the induction of oxidative stress is limited in algae (Jahnke and White, 2003; Liu et al*.,* 2007). Algae adapt differently to salinity and are classified according to the extent of their tolerance as halophytic (salt requiring for optimum growth) and halo tolerant (having response mechanisms that permit their existence in saline medium (Rao et al., 2007). *Dunaliella* is a unicellular photoautotroph halotolerant green alga that lacks a rigid cell wall and can store many economically important organic compounds such as glycerol, β-carotene and others, (Ghetti et al*.,* 1999). *Dunaliella* responds to salt stress by massive accumulation of glycerol, enhanced elimination of Na^+ ions and accumulation of specific proteins (Pick, 2002). However, the physiological role and function of glycerol may be different in each *Dunaliella* species and it seems to serve as an osmotic regulator (Hadi et al., 2008). As wall-less cells, they must maintain iso-osmotic conditions with their environment. With changing external salinity,

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the major biochemical response is seen in the internal glycerol level, which changes in direct proportion to the external salinity (Ben-Amotz 1975, Chitlaru and Pick, 1991).

Algae contain a number of vitamins such as vitamin E, vitamin C and β-carotene in higher concentrations than in conventional foods (Brown and Framer, 1994). *Dunaliella* has unique ability to accumulate large amounts of β-carotene either in nature or under laboratory conditions (El Baz et al., 2002, Del Campo et al*.,* 2007). β-Carotene plays an important role in medicine, nutrition and as colouring agent and is in high demand in global markets (Ye et al., 2008). *Dunaliella* species accumulate β-carotene at least 8 to 13% of their dry weight under stress conditions such as high light intensity, high salt concentration, nitrate deficiency or extreme temperatures (Gomez-Pinchetti et al., 1992).

Salinity stress leads to a series of changes in photosynthesis, photorespiration, osmotic adjustment and compartmentation, amino acid and carbohydrate synthesis (Kawasaki et al*.,* 2001; Ozturk et al., 2002). Studies on the effects of salinity on the production of reactive oxygen species (ROS) and the regulation of antioxidant defence systems are scarce in algae. It has been suggested that, the immediate responses of marine plants to adverse environmental conditions are mediated through a common mechanism, namely the excessive production of ROS (Dring, 2006). These include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) (Lesser, 2006). The production of ROS can be enhanced by stress and if the accumulation of ROS exceeds the capacity of plants to remove them, it will lead to oxidative stress (Kreslavski et al., 2007). As a result, photosystems could be damaged because of DNA mutation, protein denaturation, lipid peroxidation and chlorophyll bleaching as well as the loss of membrane integrity (Leshem et al*.,* 2007). Plants possess a number of low-molecular-weight antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols) and various antioxidative enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX) or catalase (CAT) are involved in the detoxification of ROS and the avoidance of damage under salt stress (Cavalcanti et al., 2007; Sekmen et al*.,* 2007). Moreover, compatible solutes may also play regulatory roles in mitigating damaging effects caused by oxidative stress (Cuin and Shabala, 2007a).

Under normal conditions, ROS production and scavenging is well regulated. In this way, this enzyme system can eliminate the damaging effects of toxic oxygen species (Meloni et al, 2003). High salinity is known to cause hyperionic and hyperosmotic effects in plants, leading to membrane disorganization, increases in reactive oxygen species and metabolic toxicity. The correlation between antioxidant capacity and salt tolerance is well known. Plants with high levels of constitutive or induced anti-oxidants have been reported to have greater resistance to oxidative damage, as reviewed by Parida and Das (2005).

In this paper, the response to salinity stress is studied with emphasis on physiological and biochemical mechanisms through comparing the adaptability of the two species *Dunaliella tertiolecta* and *Dunaliella salina* to varied range of hypo and hyper saline environment and their effect on growth, β-carotene and glycerol content. Attempts to elucidate whether antioxidant resistance mechanism is a strategy for these chlorophytes algae to counteract salinity changes are made by the determination of the levels of lipid peroxidation as estimated by thiobarbituric acid reacting substance (TBARS) contents, the contents of H_2O_2 and the water-soluble antioxidants ascorbic acid (AsA) and glutathione (GSH) were also determined. The activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase and glutathione *S*-transferase were also determined.

MATERIALS AND METHODS

Algae and growth conditions

D. salina and *D. tertiolecta* were kindly supplied by Dr. Khalefa Prof. of phycology, Alexandria University, Egypt. The organisms were grown in MH medium (Loeblich, 1982) containing variable NaCl concentration (0.05, 0.1, 0.5, 1.0, 2.5, 3.0, 3.5 and 4.0 M NaCl) beside the control which was 1.25 M NaCl. The experiments were carried out in 250 ml Erlenmeyer flasks. Illumination was in 12 h light/12 h dark cycle, 150 µ mol m^{-2} s^{-1}, temperature was 25 ± 2°C and pH 8.0 ± 0.2.

Measurement of growth and pigment content

Growth of algae was measured as change of culture turbidity at 687 nm using a Perkin Elmer spectrophotometer in proportional to the cell count (measured by haemocytometer) at each growth salinity level (Jahnke and White, 2003). The amounts of the chlorophyll a, chlorophyll b and β-carotene were measured spectrophotometrically as described by Jeffery and Humphrey (1975) and Jaspers (1965).

Analytical methods

Carbohydrates were analyzed using the phenol sulphuric acid method (Dubois et al*.,* 1956). Proteins were extracted according to Rausch (1981) and estimated according to Hartree (1972). Glycerol was extracted and determined according to Chitlaru and Pick (1989) and the developed yellow colour was measured at 410 nm. Total free amino acids were extracted and determined according to Speckman et al. (1958). Results were expressed as (g/100 ml culture) and are means ± S.D. of three replicates.

Determination of lipid peroxidation and hydrogen peroxide production

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by thiobarbituric acid reactive substance (TBARS) as described by Health and Parker (1968), using 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid

(TCA) and the absorbance was measured at 532 and 600 nm. H_2O_2 levels were prepared and estimated as described by Mukherjee and Choudhuri (1983). The intensity of yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient of 0.28 $\mu mol^{-1}cm^{-1}$.

Estimation of ascorbate and total glutathione

Ascorbic acid (AsA) concentration was measured by using the 2, 6-dichlorophenol-indophenol (DCPIP) photometric method (Guri, 1983), the absorbance was measured at 600 nm and the concentration of AsA was determined from a standard curve with ascorbic acid.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined according to the method of Griffiths (1980) and the absorbance at 412 nm was monitored. A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione, reduced GSH (total GSH-oxidized GSSG) and GSSG.

Enzyme extraction

Enzyme extract was prepared following the method of Gossett et al. (1994). Protein concentrations were determined by the method of Bradford (1976) using defatted bovine serum albumin as a standard.

Estimation of antioxidant enzymes activity

Superoxide dismutase (SOD) was assayed following the method of Beauchamp and Fridovich (1971), by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) and the enzyme activity was calculated by determining the percentage inhibition per min. Fifty percent of inhibition was taken as equivalent to 1 unit of SOD (mg^{-1} protein h^{-1}).

Catalase (CAT) activity was measured as described by Cakmak and Marschner (1992) and calculated using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ and expressed as enzyme unit (mg^{-1} protein h^{-1}).

Guaiacol peroxidase (GPX) activity was measured using the method of Egley et al. (1983). The enzyme activity was calculated using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ and expressed as enzyme units mg^{-1} protein h^{-1}.

Ascorbate peroxidase (APX) was assayed according to Nakano and Asada (1981). APX was determined using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ by measuring the decrease in absorbance at 290 nm for 1 min. It was expressed as enzyme unit (mg^{-1} protein h^{-1}).

Glutathione reductase (GR) activity was determined spectrophotometrically by monitoring GSSG (glutathione oxidized)-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm as described by Foyer and Halliwell (1976). Enzyme activity was measured in terms of the NADPH left unoxidized using an extinction coefficient, 6.224 $mM^{-1}cm^{-1}$ and expressed as enzyme unit mg^{-1} protein h^{-1}.

Glutathione-S-transferase (GST) activity was measured as described by Mannervik and Guthenberg (1981) by following the changes in the absorbance at 340 nm in a mixture containing 0.17 mM sodium phosphate buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol and enzyme extract. EU= the amount of enzyme that catalyses the formation of 1 µmol of S- 2,4-dinitrophenylglutathione mg^{-1}protein h^{-1}.

Statistical analysis

All data were subjected to standard one-way analysis of variance (ANOVA) using COSTAT 2.0 statistical analysis software manufactured by CoHort Software Company (1986). Comparison of the main effects was performed using the least significant difference (LSD) and a significant level of $P < 0.05$ by Duncan's new multiple range test.

RESULTS AND DISCUSSION

Effect of different salinity levels on the growth of *D. salina* and *D. tertiolecta*

The growth of *D. salina* and *D. tertiolecta* expressed as optical density was measured at normal condition (1.25 M NaCl). It was considered as the control (Figure 1A). *D. salina* and *D. tertiolecta* differed in their ability to grow at varying NaCl concentrations. The two species showed a remarkable tolerance to hypo and hyper saline conditions and survived in nearly all tested concentrations (0.05 to 4.0 M NaCl). It was noticed that the growth was higher under the high salt conditions (2.5 to 4.0 M NaCl) than in the low ones (1.0 to 0.05 M NaCl). Growth was significantly reduced to 39 and 11% for *D. salina* and *D. tertiolecta* at 4.0 M salinity stress, respectively, when compared with control. Concerning the behaviour in hypo saline condition (0.05 M NaCl), the optical density was reduced to 5 and 16% for *D. salina* and *D. tertiolecta*, respectively.

Hypo and hyper salinity caused decrease in pigment content. Chlorophylls a and b were found to be high in the control. Taking into consideration, the levels of chlorophyll a and b were originally higher in *D. tertiolecta* (8.47 and 1.95 mgl^{-1}, respectively) than in *D. salina* (4.36 and 1.75 mgl^{-1}). Higher and lower concentrations of NaCl adversely affected the amount of chlorophyll a and b. The chlorophyll a content of *D. salina* was significantly reduced by about 38 and 12% at 4.0 and 0.05 M NaCl, respectively (Figure 1B). The corresponding value in *D. tertiolecta* was about 23 and 18% compared with control. At 4.0 and 0.05 M NaCl chlorophyll b content of *D. salina* was significantly reduced by about 43 and 13%, respectively. The chlorophyll b values for *D. tertioletca* were 44 and 25%, respectively (Figure 1C). Reduction in photosynthetic pigments during haloadaptation of the studied species is probably due to non adaptability of the organism to salinity stress and a decrease in photosynthetic rate. Ben-Amotz et al. (1985) found low contents of protein, carbohydrates and pigments except lipid in *Botryococcus braunii* cells when grown in 0.5 M NaCl.

Effect of different salinity levels on metabolite production of *D. salina* and *D. tertiolecta*

Because in most cases, microorganisms use a rapid and reversible increase in the concentration of some physiological solutes to counteract osmotic fluctuations in the medium, the level of glycerol was measured in the

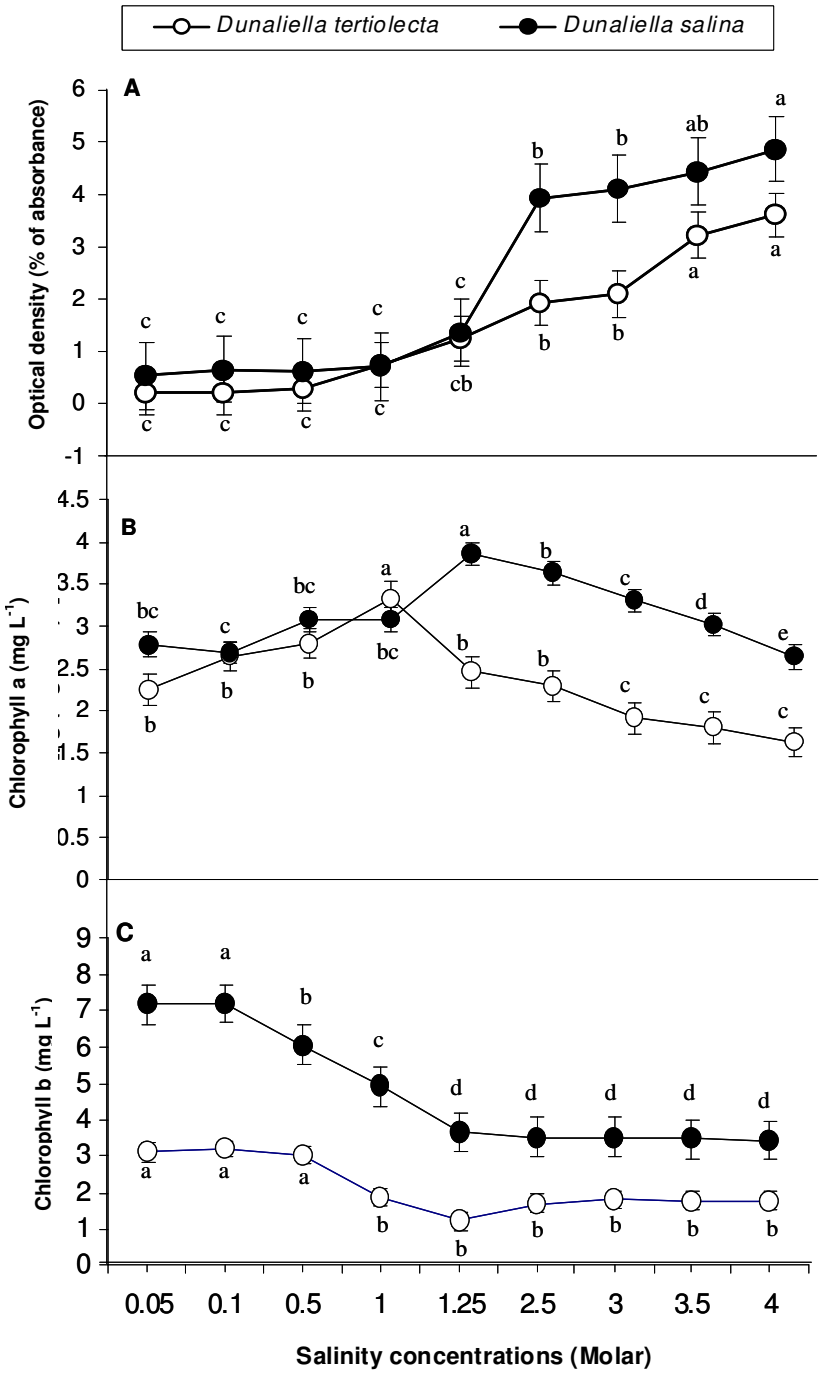

Figure 1. Effect of different salinity concentrations on (A) optical density as percentage of absorbance, (B) chlorophyll a and (C) chlorophyll b content of *D. tertiolecta* and *D. salina* growing in media containing an extremely wide range of salt concentrations (0.05 to 4.0 M NaCl). The vertical bars represent the standard deviation; different letters above the bars indicate significant level at $p < 0.05$.

examined *Dunaliella* species. After the exposure to salt stress conditions a massive change in the glycerol concentrations was evident. The maximum production of glycerol value was achieved at 4.0 M salinity (Figure 2A). It seems that, the major function of glycerol in *Dunaliella* is to serve as an osmotic regulator and it is a compatible solute at high concentrations which protects enzyme activity. Avron and Ben-Amotz (1992) recorded that gly-

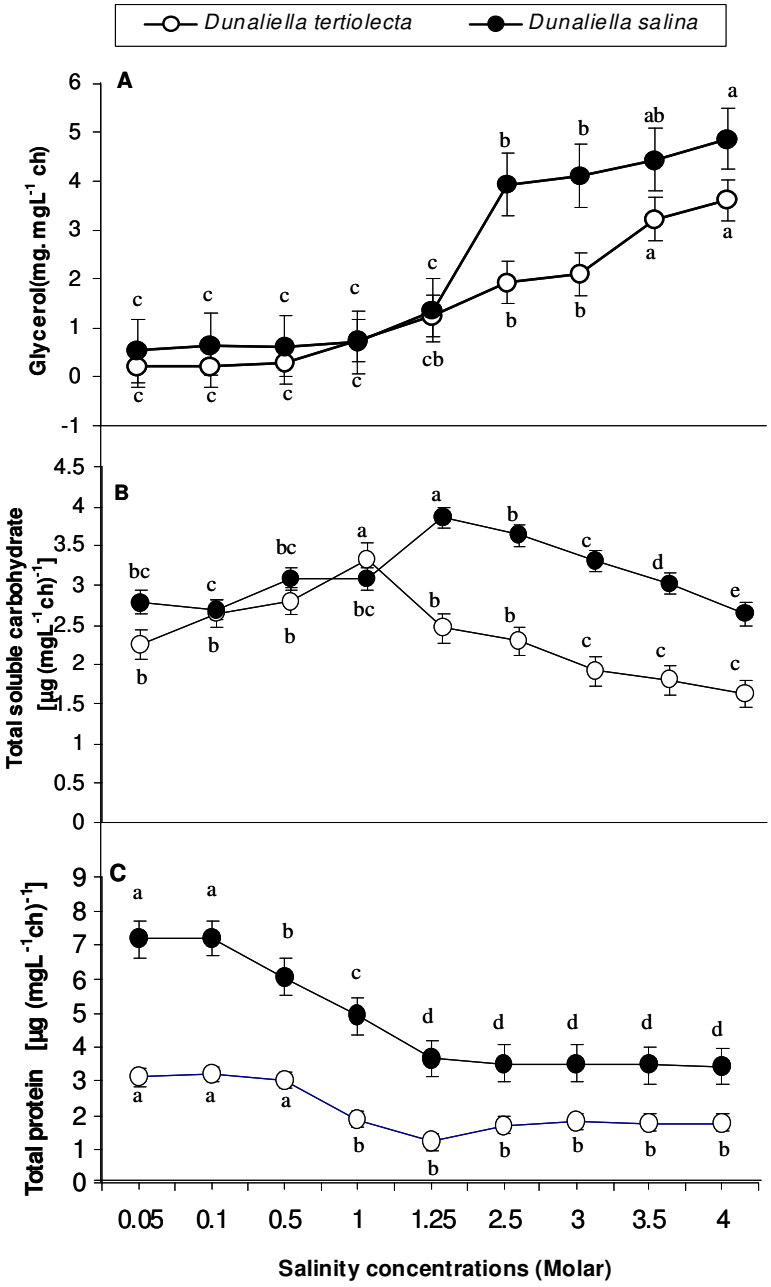

Figure 2. Effect of different salinity concentrations on (A) glycerol synthesis, (B) soluble carbohydrate and (C) total protein of *D. tertiolecta* and *D. salina*. Cultures were grown in medium containing wide range of salinity (0.05 to 4.0 M Nacl). The vertical bars represent the standard deviation; different letters above the bars indicate significant level at $p < 0.05$.

cerol is a compound important for osmotic regulation in *Dunaliella*. On the contrary, for the two tested species of *Dunaliella* a notable decrease in glycerol content was observed at 0.5, 0.1 and 0.05 M salinity cultures under that of the control (Figure 2A). Low concentration of NaCl in growth medium especially for *D. salina* (1.0 to 0.05 M) significantly decreased glycerol contents. Moreover, at low external salinities, the existing glycerol is converted to starch to abolish its osmotic potential as reported by Ben-Amotz (1975) and Chitlaru and Pick (1991). Under these conditions, the internal glycerol levels approached minimum values but a constitutive level of solutes is maintained within the cell (Pick et al., 1986)

The soluble carbohydrates content of *Dunaliella* grown

at different concentration of NaCl (0.05 to 4.0 M) and in controlled conditions (1.25 M NaCl) was shown in Figure 2B. It is noticed that, the increase and decrease in salt stress over and below the 1.25 M NaCl was accompanied by a significant decline in soluble carbohydrate content. It was notable that, the carbohydrate contents were originally higher in *D. salina* than in *D. tertiolecta.* The decrease in carbohydrate content under salt stress could be attributed to the decrease in chlorophylls content (Figure 1B and C) and increase in glycerol content of the two algal species (Figure 2A). The flux of carbon between starch production in the chloroplast, synthesis of glycerol in the cytoplasm and accumulation of carotenoids are some of the important physiological responses produced under stress conditions (Raja et al., 2007). Goyal (2007) observed that, the contribution of products of starch breakdown to glycerol synthesis increased progressively with increasing salt stress. On the contrary, Rao et al. (2007) reported that a marginal increase in carbohydrate content was observed when the alga was grown at different salinity stress.

Results in Figure 2C show that, protein in control was 3.66 and 1.20 µg mgchl^{-1} for *D. salina* and *D. tertiolecta,* respectively. When cells were exposed to 0.05 M NaCl, protein increased by about 2.0 and 2.6 –fold for *D. salina* and *D. tertiolecta,* respectively. Lower growth associated with high protein content may be due to the fact that, organism divert some of the protein from state of growth to state of osmoregulation. On the other hand, protein content of hyper salinity treated cells did not vary significantly.The specific nitrogen-containing compounds that accumulates in saline environments varies with plant species. Osmotic adjustment, protection of cellular macromolecules, storage of nitrogen, maintenance of cellular pH, detoxification of the cells and scavenging of free radicals are proposed functions of these compounds under stress conditions (Parida and Das, 2005).

Effect of salinity stress on total free amino acids profiles of *D. salina and D. tertiolecta*

Total free amino acids were measured in *D. salina* and *D. tertiolecta* after seven days exposure to 0.1, 2.5 and 1.25 M NaCl as control. Free amino acids vary in cells stressed with either hypo or hyper saline concentrations and in cells grow in controlled condition (Table 1). Under normal growth conditions, the total amino acids contents were markedly higher in *D. tertiolecta* (1.587 g/100 ml culture) than in *D. salina* (0.876 g/100 ml culture). In untreated *D. tertiolecta* and *D. salina*, aspartate, glutamate, glycine, alanine and leucine altogether accounted for 58 and 68% of total amino acids, respectively. In hypo and hyper saline conditions, some amino acids increased, some others decreased whereas the rest exhibited almost no change. Data strongly suggested that, the level of free amino acids In *D. salina* is not influenced by external hyper saline conditions. On the contrary, when the alga grows in hypo saline conditions, a marked increase in amino acid contents was notable which reach 150% of the control value. When *D. tertiolecta* was grown under hypo and hyper saline conditions, amino acids content increased by 44% and decreased by about 43%, respectively. Ammonia exhibited a marked decrease in hyper saline conditions. It showed an increase which ranged from 2- to 4- fold in *D. tertiolecta* and *D. salina* in hypo saline media. It seemed that, accumulation of nitrogen containing compounds is usually correlated with plant salt tolerance (Mansour, 2000). Agastian et al. (2000) recorded that, free amino acids increased at low salinity while decreased at high salinity in mulberry. Cuin and Shabala (2007b) showed that, free amino acids modulate salt stress-induced K^+ efflux possibly by affecting the activity of key membrane transporters.

Influence of salinity on lipid peroxidation (TBARS) and hydrogen peroxide production (H_2O_2) of *D. salina* and *D. tertiolecta*

Lipid peroxidation (TBARS contents) as oxidative stress parameters were significantly affected by salinity (Figure 3A). When compared with control, the contents of TBARS were significantly increased in both hypo and hypersaline media for both *Dunaliella* species. However, the contents of TBARS were originally higher in *D. salina* (0.1 µmol L^{-1}) than in *D. tertolicta* (0.06 µmol L^{-1}). Concerning the hypo saline conditions, data revealed that the malondialdehyde (MDA) concentration for the studied species reached its maximum value at 0.5 M NaCl where it increased significantly by about 3-fold compared with control. For hyper saline environment, on the other hand, the results showed that the degree of accumulation of MDA was the maximum at 4 M NaCl where it was 0.14 and 0.31 µmolL^{-1} for *D. tertiolecta* and *D. salina,* respectively. In this study, the highest degree of lipid peroxidation occurred in both species. In *D. tertiolecta,* it was more than double while in *D. salina* it was three fold higher than in the control. These experimental results support that *D. salina* is damaged more rapidly by ROS than *D. tertiolecta* and it can be classified as a more stress-tolerant species, while *D. salina* as a more stress susceptible species. $^{\cdot}OH$ is one of the most reactive oxygen radicals and has sufficient reactivity to initiate membrane peroxidation (Halliwell and Gutteridge, 1989). Low lipid peroxidation in stressed algae can also result from a protective effect of non enzymatic antioxidant compounds which protect the photosynthetic apparatus from photo-damage by quenching triplet chlorophyll and singlet oxygen and dissipating the excess energy as heat in addition to the different strategies in the enzymatic system (Young and Frank, 1996). NaCl treatments led to a significant increase in H_2O_2 content in both hypo and hyper saline medium (Figure 3B).

Table 1. Total free amino acids profile in *D. salina* and *D. tertiolecta* cells in controlled condition and after exposure to salt stress condition. Values are mean ± SD of three replicates.

A.A	Control (1.25 M)	%	0.1 M	%	2.5 M	%
Dunanliella salina						
Aspartic	0.131±0.098	13.13	0.150±0.123	12.75	0.119±0.111	11.90
Threonine	0.031±0.013	3.13	0.134±0.079	4.48	0.039±0.018	3.93
Serine	0.032±0.009	3.25	0.150±0.099	5.02	0.052±0.012	5.29
Glutamic	0.114±0.005	11.46	0.337±0.038	11.25	0.100±0.002	10.04
Glycine	0.127±0.110	12.76	0.375±0.235	12.52	0.139±0.119	13.93
Alanine	0.134±0.015	13.44	0.329±0.102	10.97	0.125±0.012	12.53
Valine	0.047±0.026	4.74	0.122±0.124	4.09	0.045±0.023	4.56
Isoleucine	0.025±0.009	2.56	0.079±0.025	2.64	0.026±0.008	2.60
Leucine	0.092±0.058	9.24	0.200±0.156	6.68	0.085±0.053	8.52
Tyrosine	0.024±0.019	2.42	0.052±0.091	1.74	0.020±0.017	2.08
Phenylalanine	0.035±0.005	3.58	0.082±0.019	2.76	0.030±0.003	3.03
Histidine	0.0089±0.006	0.89	0.014±0.015	0.49	0.010±0.004	1.08
Lysine	0.047±0.019	4.70	0.094±0.056	3.16	0.061±0.025	6.15
Arginine	0.028±0.019	2.80	0.106±0.0101	3.56	0.036±0.019	3.60
Total	0.876±0.096		2.22±0.198		0.888±0.098	
Ammonia	0.117±0.125	11.77	0.533±0.201	17.79	0.105±0.115	10.57
Dunanliella tetriolecta						
Aspartic	0.18±0.005	9.0	0.471±0.062	15.73	0.127±0.031	12.47
Threonine	0.087±0.097	4.35	0.099±0.100	3.33	0.046±0.56	4.62
Serine	0.098±0.058	4.92	0.132±0.078	4.43	0.061±0.025	6.15
Glutamic	0.17±0.001	8.52	0.275±0.098	9.19	0.101±0.048	10.10
Glycine	0.228±0.192	11.43	0.297±0.198	9.91	0.156±0.121	15.63
Alanine	0.188±0.090	9.40	0.259±0.128	8.64	0.119±0.058	11.96
Valine	0.093±0.085	4.66	0.128±0.101	4.28	0.043±0.085	4.33
Isoleucine	0.054±0.015	2.74	0.061±0.018	2.05	0.024±0.009	2.48
Leucine	0.158±0.110	7.92	0.174±0.125	5.83	0.080±0.98	8.08
Tyrosine	0.042±0.032	2.14	0.081±0.069	2.71	0.017±0.022	1.76
Phenylalanine	0.066±0.010	3.30	0.084±0.025	2.80	0.028±0.009	2.88
Histidine	0.028±0.004	1.42	0.028±0.004	0.96	0.010±0.001	1.00
Lysine	0.109±0.126	5.47	0.105±0.121	3.53	0.055±0.059	5.59
Arginine	0.07±0.012	3.52	0.091±0.009	3.06	0.038±0.005	3.84
Total	1.587±0.182		2.28±0.568		0.906±0.112	
Ammonia	0.407±0.258	20.38	0.706±0.298	23.55	0.0864±0.552	8.64

D. tertiolecta showed lower H_2O_2 content than *D. salina* when salinity decreased or increased. Figure 3B revealed that, hyper saline medium has steady state levels of H_2O_2 irrespective to NaCl level in *D. tertiolecta*. In contrast, *D. salina* showed significant enhancement in the H_2O_2 content. High levels of H_2O_2 can accelerate processes like Haber-Weiss reaction, resulting in the formation of hydroxyl radicals that can cause lipid peroxidation (Loggini et al., 1999).This is clear from the greater degree of lipid peroxidation in *D. salina* up on exposure to NaCl stress. In contrast, *D. tertiolecta* exihibited lower degree of lipid peroxidation and a steady level of H_2O_2 content under hypo and hypersaline conditions.

Status of antioxidant pool under salinity stress

β-Carotene content of *D. tertiolecta* and *D. salina* grown under normal culture condition (1.25 M NaCl) was 2.88 and 2.82 mgl^{-1}, respectively (Figure 4A). Increased concentration of NaCl in growth medium to 4.0 M, increased β-carotene contents to 3.19 and 3.98 mgl^{-1} for *D. Tertiolecta* and *D. salina* which represents 11 and 41% increase, respectively compared with control. The cell

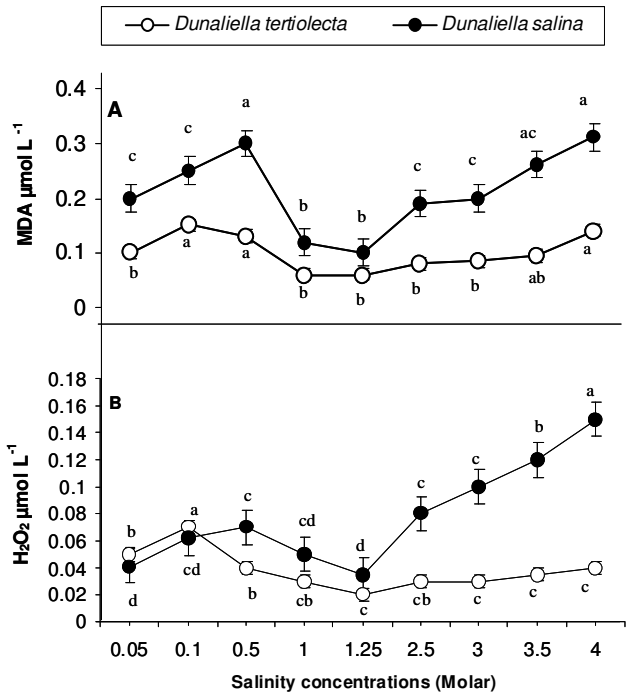

Figure 3. Changes in the content of (A) TBARS as MDA and (B) H_2O_2 in *D. tertiolecta* and *D. salina* in response to different salinities. Different letters above the vertical bars indicate significant level at $p < 0.05$.

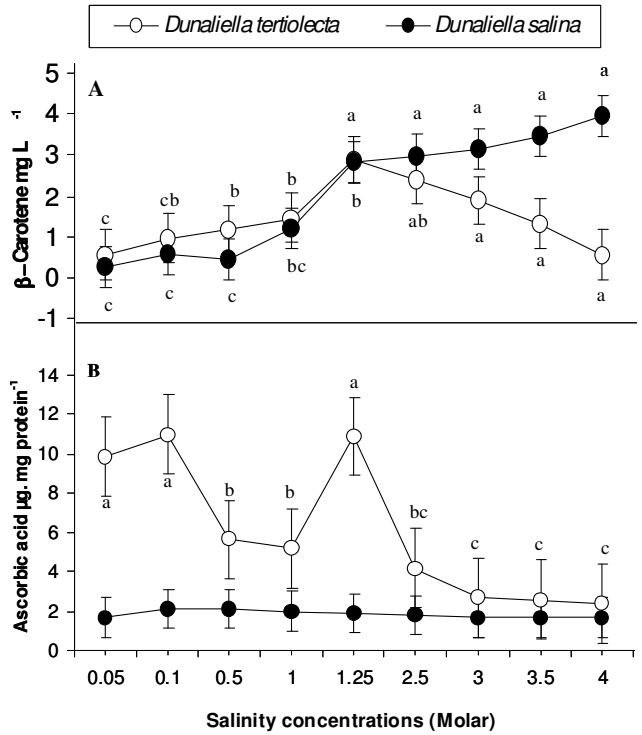

Figure 4. Changes in (A) the content of β-carotene and (B) total ascorbic acid in *D. tertiolecta* and *D. salina* in response to salinities. Different letters above the vertical bars indicate significant level at $p < 0.05$.

color of *D. salina* changed from green to orange-red following accumulation of carotenoid, but the color of *D. tertiolecta* was not changed on a large scale under the same conditions. The ability to accumulate large amounts of β-carotene content under adverse conditions, especially for *D. salina* may be due to that β-carotene quenching singlet oxygen and free radicals. This is in accordance with the results of Telfer (2002) who reported that β-carotene protect the alga from damage by preventing the formation of reactive oxygen species. In contrast, β-carotene was reduced at low salinity stress indicating that the algae were stressed. This reduction may be attributed to those β-carotene functions in this case as accessory light-harvesting pigments rather than scavenging the free radicals sufficiently. Thereby, it seems that under hyposaline condition, *Dunaliella* has a mechanism of salt tolerance that does not correlate with the accumulation of β-carotene. Johnson and Schroeder (1995) revealed that the percentage of β- carotene in *D. salina* even comes up to 13.8% under natural conditions; however, the percentage falls to only about 0.3% in unsuitable conditions.

The contents of ascorbate and glutathione were determined to test whether water soluble antioxidants are involved as the defence system against salinity-induced oxidative stress in *Dunaliella* species. Concerning *D. tertiolecta* (Figure 4B) the contents of ascorbic acid were significantly decreased at 1.0 and 0.5 NaCl by about 50% then increased at 0.1 M NaCl by 6%, whereas at 4 M NaCl it decreased by 77% relative to the control value. However, *D. salina* the content of ascorbic acid exhibited a steady state level on exposure to hypo and hyper salinity. The decreased level of ascorbate in *D. tertiolecta* is due to its participation in reducing H_2O_2 to H_2O catalyzed by increasing activity of ascorbate peroxidase. It reacts directly with hydroxyl radicals, superoxide, singlet oxygen and H_2O_2 and it is also used for regeneration of the lipophilic antioxidant α-tocopherol (Smirnoff, 1996; Noctor and Foyer, 1998). Ascorbic acid is also involved in photoprotection, in the regulation of photosynthesis and in preserving the activities of enzymes that contain prosthetic transition metal ions (Smirnoff, 1996). Jahnke and White (2003) working with *Dunaleilla* found that, increasing salinity produced more than 35% increase in both reduced and total ascorbate, while under hyposaline conditions showed reduction in total ascorbate compared with moderate and high salinities.

Reduced glutathione significantly increased at hyposaline and decreased at hyper saline condition in *D. tertiolecta* (Figure 5A). In contrast, *D. salina,* the reduced glutathione decreased significantly in hypo saline, while it increased in hyper saline conditions. The oxidized glutathione was found to decrease significantly in both species and the decrease was more obvious in hyper saline conditions (Figure 5B). Ratio of reduced to oxidized glutathione GSH/GSSG increased in *D. salina* cultured in hypersaline condition, while increased in *D. tertiolecta* grown under hypo saline media (Figure 5C). Glutathione is involved in the defense system by increasing the glutathione pool for scavenging H_2O_2. An increase in glutathione reductase activity reflects that in the ability to regenerate glutathione, the GSH/GSSG ratios increased hyposaline in *D. tertiolecta*. This is because it has been documented that, GSH functions as an antioxidant to react none enzymatically with singlet oxygen, superoxide ion and hydroxyl radical and also protect proteins against denaturation caused by the oxidation of protein thiol groups (Noctor et al., 2002). Furthermore, GSH is also utilized by glutathione peroxidase in the removal of lipid and alkyl peroxide (Eshdat et al., 1997) or by glutathione-S-transferase to reduce peroxide (Cummins, 1999).

This study suggest that the decreased level of GSSG in both *Dunaliella* species might be due to its reduction to GSH by increased activity of glutathione reductase (GR), thus maintaining constant GSH level. The increase in ratio of GSH/GSSG in salt-treated *Dunaleilla* was only due to a decreased level of GSSG. Lu et al. (2006) recorded that, GSH/GSSG ratios did not increase; possibly, GSH is consumed at a faster rate than the regeneration rate, thus, leading to notable reduction of GSH/GSSG ratios in salinity-stressed *Ulva fasciata*. In response to hyposalinity, a halotolerant microalga *D. tertiolecta* increases GSH and thylakoid α tocopherol contents instead of antioxidant enzymes as an antioxidant defense system (Jahnke and White, 2003).

Salinity induced modulation in the activities of ROS scavenging enzymes

Often, increase in antioxidant activity is identified as the key in the prevention of salt damage, while sensitive species typically exhibit either no change or a decrease in activity (Hernandez et al., 2000; Shalata et al., 2002).

Salinity significantly affected the activity of ROS scavenging enzymes (Figure 6A to F). Catalase activity is insignificantly changed by increasing salinity with the maximum activity at 4 M NaCl in *D. salina*. However, a steady state was noticed in hyposaline condition ranging from 9.06 to 11.03. In *D. tertiolecta*, the response of catalase activity to hypersalinity decreased by about 36% at 4 M NaCl. Under hyposaline condition, catalase activity decreased at 1 and 0.5 M NaCl and increased at 0.1 and 0.05 M NaCl (Figure 6A).

Peroxidase activity in control and stressed *D. tertiolecta* was higher than those of *D. salina*. The specific activity of peroxidase (GPX) under hyposaline media (0.05 M NaCl) was half that of hypersaline (4 M NaCl) for *D. salina,* since it recorded 7.18 and 14.89 unit mg protein^{-1} h^{-1}, respectively. For *D. tertiolecta,* it recorded 64.90 at 0.1 M NaCl and 61.97 unit mg protein^{-1} h^{-1} at 4 M NaCl (Figure 6B).

The specific activity of superoxide dismutase in *D. tertiolecta* was six times more than that of *D. salina*. With respect to control, the specific activity of SOD was nearly

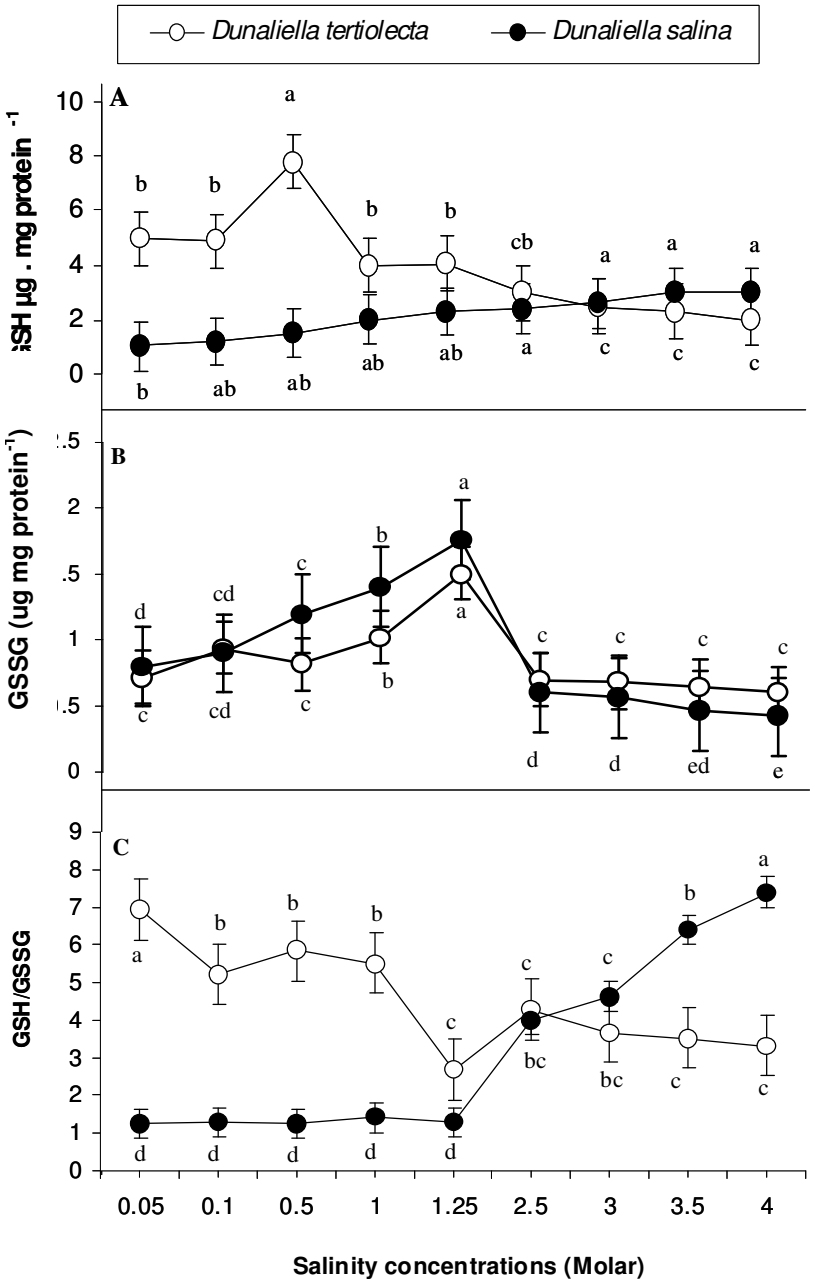

Figure 5. Changes in (A) the content of GSH, (B) GSSG and (c) GSH/GSSG ratio in *D. tertiolecta* and *D. salina* in response to different salinities. Different letters above the vertical bars indicate significant level at $p < 0.05$.

doubled at 4 M NaCl in *D. salina* and this value was one third of that of *D. tertiolecta* at the same concentration. For hypo saline medium, the results showed that there is approximately no change in SOD activity for *D. salina*. On the other hand, *D. tertiolecta* showed significant increase in SOD activity by about 32% at 0.1 M NaCl. Many studies have demonstrated that, upper limit salinity tolerance in higher plants is correlated with the ability to scavenge reactive oxygen species formed under stress conditions (Gossett et al., 1994; Hernandez et al., 2000).

In this investigation, *D. salina* revealed insignificant changes in superoxide dismutase, catalase and peroxidase activities throughout the entire range of growth salinities. This implies that *D. salina* does not majorly depend on SOD activity for detoxification of $O_2^{\cdot}$; it has alternative, non enzymatic routes for conversion of $O_2^{\cdot}$ to H_2O_2 using antioxidants like GSH. In support of this view, upon exposure to hypersaline condition, significant enhancement in the total level of glutathione and β carotene was observed. Salinity induced increased in SOD, are often

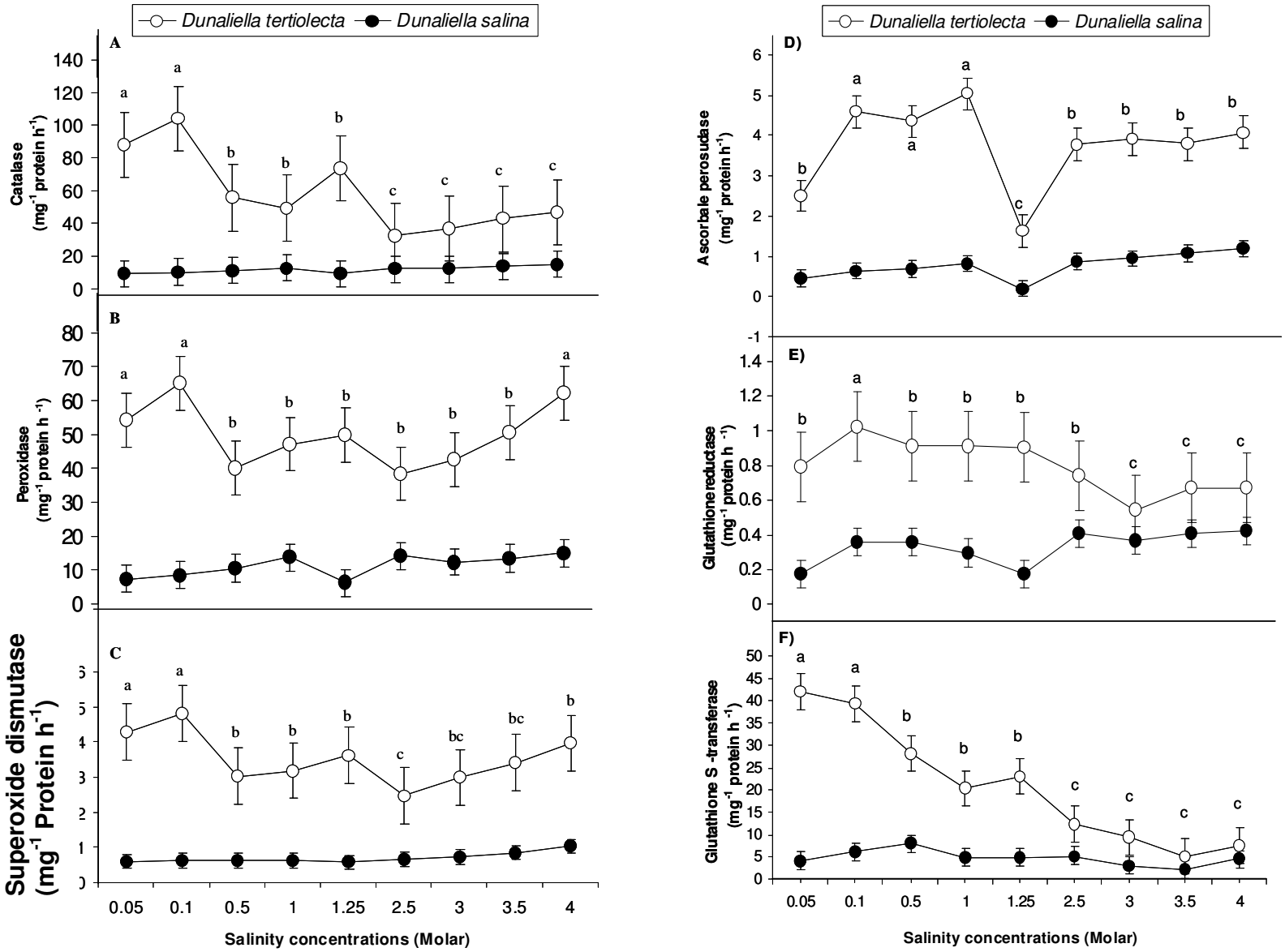

Figure 6. The activities of (A), CAT (Catalase), (B), GPX (Guaiacol peroxidase), (C), SOD (Superoxide dismutase), (D), APX (Ascorbate peroxidase), (E), GR (Glutathione reductase) and (F), G.S.T (Glutathione-S-transferase) in *D. tertiotecta* and *D. salina* in response to different salinities. Letters above vertical bars indicate significant level at $P < 0.05$.

seen in many plants (Hernandez et al., 1999; Shalata et al., 2002), but others did not find changes in SOD levels (Gossett et al., 1994). Likewise, catalase responses to salinity stress are variable from large increases in activity (Gossett et al., 1994; Shalata et al., 2002) to no changes (Olmos et al., 1994; Fadzilla et al., 1997).

In contrast, *D. tertiolecta* had significant increase in SOD, CAT and GPX on exposure to extreme range of salinity indicating that these enzymes are utilized for enzymatically scavenging of ROS. Decreased activities of SOD, CAT and GPX under salinity stress conditions is possibly due to enhancement of H_2O_2 levels since catalase activity was linked to H_2O_2 accumulation (Lee et al., 2001). Evidently, the antioxidant defense system is differently regulated between algal species. It is likely that, SOD activity in *D. tertiolecta* increased for dismutation of superoxide ion and the generated H_2O_2 is subsequently degraded by CAT and GPX together with ascorbate-glutathione cycle in extreme range of salinity. Portune et al. (2010) suggested that, SOD and CAT play important protective roles against ROS during growth of several rapidophytes.

High activities of ascorbate peroxidase (APX) were maintained at high salinities during the growth of the studied alga. Figure 6D, demonstrated a significant increase in APX which reached to about 3 and 2.5 folds compared with the control value at 1 and 4 M NaCl, respectively, for *D. tertolicta*. In *D. salina* there was insignificant change in APX activity in both hypo and hyper saline media but the maximum increase was obvious in hypersaline one (6- fold the control value). High activities of APX were stimulated under salinity stress for both studied species. This is probably due to the fact that, APX may be involved in the degradation of H_2O_2 generated under salinity condition. A similar result has been observed in a green micro alga *D. tertiolecta* (Jahnke and White, 2003) in response to high salt stress that APX and MDHAR activities increased as defensive responses to remove ROS and keep cellular ascorbic acid level constant.

Glutathione reductase (GR) activity increased above the control value in *D. salina* and decreased in *D. tertiolecta* through the exposure to hypersaline conditions (Figure 6E). However, in the hypo saline environments it increased by about 2- and 1.2-folds at 0.1 M NaCl for *D. salina* and *D. tertiolecta,* respectively. Glutathione reductase is activated under hyposaline environment; this probably could increase the ratio of $NADP^+$/NADPH, thereby ensuring the availability of $NADP^+$ to accept electrons resulting into less flow of electrons to O_2 for generation of ROS (Reddy et al., 2004). Under such a situation, the flow of electrons to O_2 and therefore, the formation of ROS can be minimized. Moreover, the suppression of activities of GR demonstrates that AsA and GSH are consumed without regeneration. Lu et al. (2006) working with *U Ulva fasciata* illustrated that, increasing AsA and GSH contents and enhancing regeneration of these antioxidants were indicated by increased GSH/GSSG ratios and increased GR, DHAR and MDHAR activities. It has been reported in several higher plants that total AsA and GSH contents decreased under high salt stress (Gossett et al., 1994; Hernandez et al., 2002), but in some cases, they are increased by high salt conditions (Lechno et al., 1997).

The response of glutathione -S-transferase (GST) differed in different salinity- stressed cells. By decreasing salinity condition, the activity of GST increased for D. *tertiolecta,* it recorded 42.07, whereas its value was 5.19 unit mg $protein^{-1}$ h^{-1} at 0.05 M NaCl. Concerning *D. salina,* GST activity showed approximately steady state by decreasing NaCl concentration. While it's catalytic activity decreased in *D. tertiolecta* and *D. salina* under hypersaline condition by about 67 and 9.0% compared with their corresponding control, respectively. The enhancement of GST expression has been considered as a marker for plant response to environmental stress. In addition to its catalytic property, this enzyme also functions as binding proteins and is capable of accommodating a broad range of endogenous and xenobiotics ligands. Glutathione S-transferase was significantly elevated under NaCl stress condition in mulberry cultivars, nevertheless, the activity was higher in tolerant than in susceptible cultivars (Sudhakar et al., 2001). On the contrary, hypersaline conditions interfere with the synthesis of GSH which may diminish plant defense against hydroxyl radicals and oxidative stress.

Conclusions and recommendations

The green alga *Dunaliella* can adapt to saline environments and it is considered a model organism for salinity tolerance. This work demonstrates that, the mechanisms of salt tolerance in *D. tertiolecta* are unique from those of *D. salina*. Under salt conditions, two features for glycerol are evident in this study: (1) both synthesis and degradation of glycerol exists(osmotic pressure); (2) a significant stimulation in the synthesis of glycerol is observed under hyper-osmotic conditions.

As a general mechanism, *Dunaliella* grown under salinity stress correlated with the accumulation of more efficient non enzymatic and enzymatic antioxidant. The alga increased the production of antioxidant or elevated activities of protective enzymes to detoxify and eliminate the highly ROS. Under stress conditions, reactive oxygen species molecules are not controlled by protective systems and destroy membrane lipids, pigments and proteins.

This study strongly suggests that, induction of antioxidants defense is one component of the tolerance mechanism of *Dunaliella* species to salinity as evidence by its growth behaviour. This study provide base-line information and engineering of the alga for oxidative stress tolerance need to target gene coding for enzymatic

and non enzymatic reactive oxygen species cavenging component to achieve greater degree of success.

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