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RESEARCH ARTICLE

Trehalose treatment alters carbon partitioning and reduces the accumulation of individual metabolites but does not affect salt tolerance in the green microalga *Dunaliella bardawil*

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Abstract The effects of trehalose (Tre), a non-reducing disaccharide, on metabolic changes, antioxidant status, and salt tolerance in *Dunaliella bardawil* cells were investigated. Algal suspensions containing 1, 2, and 3 M NaCl were treated with 5 mM Tre. While the content of pigments, reducing sugars, proteins, glycerol, and ascorbate pool accumulated with increasing salinity, the content of non-reducing sugars, starch, amino acids, proline, hydrogen peroxide, and lipid peroxidation level decreased significantly. Tre-treated cells showed a decrease in pigments content, reducing sugars, starch, proteins, amino acids, proline, glycerol, and the activity of non-specific peroxidase and polyphenol oxidase, but an increase in non-reducing sugars, oxidized ascorbate, and ascorbate peroxidase activity occurred unchanged in the ascorbate pool. However, the density and fresh weight of the cells remained statistically unchanged in all Tre-treated and untreated cultures. These results suggest that *D. bardawil* cells potentially tolerate different salt levels by accumulating metabolites, whereas Tre treatment changes carbon partitioning and significantly reduces beneficial metabolites without altering salt tolerance. Therefore, the regulation of carbon partitioning rather than the amount of assimilated carbon may play an important role in inducing salinity tolerance of *D. bardawil*. However, Tre is not able to enhance the salt tolerance of halotolerants and is even economically damaging due to the reduction of unique metabolites such as glycerol and β-carotene.

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Introduction

Among the environmental stresses that plants face, salinity is highly complex and difficult due to its multiple effects (Henry et al. 2015). The occurrence of osmotic stress followed by the accumulation of ions to the point of toxicity is known as the primary effect of salinity stress (Carillo et al. 2011; Nounjana et al. 2012). Osmotic stress restricts plant water uptake, while subsequent ion toxicity increases the toxic level of Na^+ and Cl^- and decreases the uptake of potassium ions, which are essential for the function of several enzymes (Munns et al. 2006; Nounjana et al. 2012). As a secondary effect, oxidative stress due to the production of reactive oxygen species (ROS) under salt stress can also be developed (Parida and Das 2005; Nounjana et al. 2012; Abdallah et al. 2016; Ramadan et al. 2019). Therefore, salt stress tolerance in plants is a complex process that is achieved by different strategies through various mechanisms of molecular, biochemical, and physiological (Zhang and Shi 2013; Abdallah et al. 2016). Metabolic adjustment as an effective mechanism in tolerating the salt stress of photosynthetic eukaryote organisms is involved in the accumulation of compatible solutes and the development of antioxidant systems. These systems include non-enzymatic antioxidants such as ascorbate and carotenoids, as well as antioxidants enzymes such as catalase (CAT), ascorbate peroxidase (APX), and pyrogallol peroxidase (PPX) (Sairam and Tyagi 2004; Chen and Jiang, 2010; Mishra and Jha 2011; Einali and Valizadeh 2015; Einali 2018).

Trehalose (Tre) is a non-reducing disaccharide of glucose that is widely found in many organisms, from bacteria

to higher plants and animals (Elbein et al. 2003). It has a key role in controlling metabolism during plant growth and development, including signal transduction, detoxification and ROS scavenging, membrane protection, and stabilization of proteins and enzymes (Bae et al. 2005; Paul 2007; Luo et al. 2010; Abdallah et al. 2016). Tre acts as an osmoprotectant in stress tolerance of some organisms, including plants (Almeida et al. 2007; Iordachescu and Imai 2008). However, the role of this molecule in algae under environmental stress remained unclear.

Dunaliella, a unicellular green alga, is a halotolerant organism that is able to survive in a wide range of salt concentrations while maintaining its intracellular sodium level (Ben-Amotz and Avron 1983; Garcia et al. 2007; Chen et al. 2009). However, the optimum growth typically occurs for most *Dunaliella* species at the concentration of 1–1.5 M salt (Avron and Ben-Amotz 1992; Takagi and Karseno 2006; Mishra et al. 2008; Mishra and Jha 2011). *Dunaliella* cells respond to salinity stress by accumulating intracellular glycerol as an osmoregulatory agent through photosynthesis in light or starch mobilization in the dark (Shariati and Lilley 1994; Chen et al. 2009). It also has the ability to produce β-carotene as an antioxidant photoprotector in large amounts in response to high salinity (Ben-Amotz et al. 1982; Jahnke and White 2003; Salguero et al. 2003; Abd El-Baky et al. 2004). In addition to these responses, the up-regulation of antioxidant enzymes activity in *Dunaliella* cells grown under salt stress has been demonstrated (Stepien and Klobus 2005; Haghjou et al. 2009; Einali 2018). Our previous studies showed that the strategy of stress tolerance in *Dunaliella* cells could be different depending on the species, type of stress, and exogenously applied molecule for possible amelioration of the adverse effects of stress (Einali and Valizadeh 2015; Einali 2018; Mirshekari et al. 2019; Bahador et al. 2019). Even though Tre as an osmoprotectant plays an essential role in plant stress tolerance, the response of *Dunaliella* cells to exogenous Tre under environmental stress has not been reported yet. Accordingly, in the present study, the role of Tre in growth, antioxidant status, and metabolic modifications was assessed in *D. bardawil* cells under long-term salt stress to find out its possible effect on salt toleration.

Materials and methods

Algal cultures and experimental treatments

Dunaliella bardawil Ben-Amotz *et* Avron (UTEX 2538) was obtained from The Culture Collection of Algae at the University of Texas at Austin. The cells were inoculated in a fresh modified Johnson medium (pH = 7.5) containing concentrations of 1, 2, and 3 M NaCl (Einali and Valizadeh 2015). The inoculated cultures were incubated in a growth chamber at 25 °C with a light regime of 16 h light (70 µmol photons m^{-2} s^{-1})/8 h dark under continuous shaking (100 rpm) to reach the exponential growth phase. Due to optimum growth at the concentration of 1 M NaCl, the algal cells grown at this concentration served as the control treatment for the salt stress experiments (Einali and Valizadeh 2015). Each suspension was poured into two flasks, one treated with 5 mM Tre, and the other received only distilled water. All suspensions were transferred to the growth conditions mentioned earlier. Tre-untreated suspensions were used as control. All experiments were run in triplicate from three separate flasks, and algal cells sampling was performed just before (time zero) and 48 h after Tre treatment.

Determination of cell density and fresh weight

The density of algal cells was determined by recording their absorbance at 680 nm against alga-free media. The fresh weight of cells was determined by harvesting the algal pellet (AP) through centrifugation (2000 × g for 10 min) of 30 ml of cell suspensions. The cell mass was resuspended in an isotonic medium, transferred to a pre-weighed micro-tube, and centrifuged at 10,000 × g for 5 min to be harvested again. The cell mass was desalinated by washing with 0.2 M NaCl culture medium and reweighed (Haghjou et al. 2014).

Pigments determination

Chlorophyll (Chl) and β-carotene as photosynthetic pigments were extracted from the AP (harvested from 1 ml of suspension) with 80% (v/v) acetone (Einali 2018). Arnon (1949) method was used to determine the content of Chl. The absorbance of the extract at 480 nm was used for the calculation of β-carotene concentration assuming $E_{1\%}^{1\ cm}$ of 2273 (Ben-Amotz and Avron 1983).

Determination of soluble sugars, starch, and glycerol

For soluble sugars extraction, the acetone-decolorized AP (harvested from 30 ml of algal suspension) was extracted with 80% (v/v) ethanol (Mirshekari et al. 2019). Reducing (RS) and non-reducing sugars (NRS) were measured spectrophotometrically by the methods of Miller (1959) and Handel (1968), respectively. Total soluble sugars (TSS) were obtained by the sum of RS and NRS content.

Starch was extracted from the sugar-free cell mass according to the modified method of acid hydrolysis (Mirshekari et al. 2019). Starch content was quantified by determining the liberated glucose through the anthrone

method and calculated by multiplying the glucose equivalent of 0.9 (McCready et al. 1950).

Glycerol was extracted from the AP (harvested from 30 ml of algal suspension) with 1.5 ml of distilled water and 0.2 ml of chloroform (Chen et al. 2009). Sonication of the cells was carried out twice for the 30 s at 2-min intervals. The suspension was centrifuged (12,000 × g for 10 min), and the resulting supernatant was used for glycerol determination. For glycerol determination, 0.2 ml of supernatant was mixed with 1 ml of sodium periodate reagent (3 mM sodium periodate and 100 mM ammonium acetate in 100 ml of 6% acetic acid). After 5 min, 2.5 ml of acetylacetone reagent (acetylacetone and isopropanol, 1:99, v/v) was added to the mixture. The samples were incubated at 60 °C for 30 min, and the absorbance was recorded at 410 nm. Glycerol concentration was calculated from a calibration curve of glycerol (Chen et al. 2009).

Determination of total protein, free amino acids, and proline

After extraction of pigments, AP was resuspended with 0.5 ml of sample buffer (60 mM Tris–HCl buffer (pH 6.8), 10% (v/v) glycerol, and 2% (w/v) SDS) and incubated at 90 °C for 60 min (Stone and Gifford 1997). The suspension was then centrifuged at 10,000 × g for 15 min, and the resulting supernatant was used to determine the total protein content according to the method of Markwell et al. (1981).

To extract total amino acids and proline, fresh AP (harvested from 30 ml of cell suspension) were resuspended with 80% (v/v) ethanol (Mirshekari et al. 2019). The resulting ethanolic extract was concentrated through evaporation and decolored by chloroform (1:5; v/v). The content of proline and the free amino acid was determined by the ninhydrin methods described by Bates et al. (1973) and Yemm and Cocking (1955), respectively.

Ascorbate determination

The modified method of Shigeoka et al. (1979) was used to determine total ascorbate and dehydroascorbate (DHA) by measuring the hydrazone complex produced in two samples with or without 2,6-dichlorophenol indophenol at 530 nm (Einali and Valizadeh 2015). The difference between total ascorbate and DHA was considered as reduced ascorbate (RAS).

Enzyme extraction and assay

Total soluble protein (TSP) containing crude enzymes was extracted from fresh AP (harvested from 30 ml of cell suspension) with 1 ml of extraction buffer (Bahador et al. 2019). To extract ascorbate peroxidase (APX), 5 mM ascorbic acid was also added to the extraction buffer. When using TSP to assay protease(s) activity, EDTA and PMSF were not present in the extraction buffer (Einali and Valizadeh 2017). The extract was incubated for 1 h at 4 °C followed by twice 30 s sonication with the 2-min interval. The decolorization of the suspension was carried out by adding 10 mg of charcoal and then centrifuging at 12,000 × g for 10 min at 4 °C. The 12,000 × g supernatant was used for TSP determination and enzyme analyses. TSP was measured using the Bradford (1976) colorimetric method.

CAT activity was measured according to Luck (1965) method. The assay mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, and crude enzyme extract. The activity of CAT was determined using the extinction coefficient of 0.0394 cm^2 μmol^{-1} at 240 nm for H_2O_2.

APX activity was measured using the method of Chen and Asada (1992). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 1 mM H_2O_2, and enzyme extract. APX activity was calculated assuming the extinction coefficient of 2.8 cm^2 μmol^{-1} at 290 nm for dehydroascorbate production.

The activity of PPX and polyphenol oxidase (PPO) was quantified as described by Nakano and Asada (1981). The assay mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2, 40 mM pyrogallol, and enzyme extract for the PPX assay, while H_2O_2 was excluded from the reaction mixture for the PPO assay. The activities of PPX and PPO were calculated using the extinction coefficient of 2.47 cm^2 μmol^{-1} at 430 nm for purpurogallin production.

The activity of protease(s) was quantified by measuring the liberated soluble amino nitrogen (Peoples and Dalling 1978). The assay mixture contained 0.5 ml of substrate (1% bovine serum albumin in 50 mM phosphate buffer (pH 4.5, 7.5, 9)), 0.1% (v/v) 2-mercaptoethanol, and 0.1 ml of enzyme extract. The reaction was developed by incubating samples at 37 °C for 2 h and terminated by adding 0.7 ml of 15% (w/v) trichloroacetic acid (TCA). The mixture was then centrifuged at 5,000 × g for 10 min, and the supernatant was analyzed for measuring the amount of soluble nitrogen using Yemm and Cocking (1955) method.

Lipid peroxidation and hydrogen peroxide determination

The extraction of hydrogen peroxide and Malonyldialdehyde (MDA) was carried out by adding 5 ml of 0.1% (w/v) cold TCA to AP (harvested from 30 ml of algal suspension) in an ice bath. The mixture was placed in an

ultrasonic bath for two cycles of 30 s with an interval of 2 min and then centrifuged at 12,000 × g for 15 min. The 12,000 × g supernatant was analyzed for H_2O_2 and MDA estimation.

The potassium iodide method of Alexieva et al. (2001) was used to determine hydrogen peroxide content. MDA concentration was determined as an indicator of lipid peroxidation using the Heath and Packer (1968) method.

Statistical analysis

Results were expressed as mean and standard deviation (SD) from three independent analyses. All data were tested for normality and equal variance. Data were analyzed by two-way factorial analysis of variance (ANOVA) at $P < 0.05$, and statistically significant differences between the treatments were determined by the Tukey test.

Results

Changes in density, fresh weight, and pigment contents of *D. bardawil* cells in response to Tre and salt treatment

The density and fresh weight of algal cells did not change significantly in 2 and 3 M NaCl suspensions compared to the control. This was also true in Tre-treated cells whose density or fresh weight did not show a significant change compared to untreated controls (data not shown).

Chl contents of Tre-untreated cells in 2 and 3 M NaCl suspensions were higher than the control (Fig. 1). The concentrations of Chl *a* and total Chl were negatively affected by Tre treatment in all cell suspensions compared to untreated cultures (Fig. 1A, C). Chl *b* content of the Tre-treated cells grown at 3 M NaCl was the same with the untreated control, whereas in 2 and 3 M NaCl suspensions containing Tre were 29 and 58% lower, respectively (Fig. 1B). The ratio of Chl *a/b* increased by 20 and 25% in the Tre-untreated suspensions in relation to the control (Fig. 1D). This ratio was positively changed by Tre treatment in the cells collected from 1 M NaCl suspension relative to untreated control. However, Chl *a/b* ratio did not change with Tre treatment in 2 M NaCl but decreased at 3 M NaCl suspension (Fig. 1D).

The β-carotene content of algal cells in 2 and 3 M NaCl suspensions was significantly increased by 47 and 50%, respectively, against control (Fig. 1E). The concentration of β-carotene in the Tre-treated cells significantly dropped by 23, 67, and 30% in 1, 2, and 3 M NaCl suspensions, respectively (Fig. 1E). The ratio of total Chl to β-carotene increased in 2 M NaCl suspensions compared to the control but did not change at 3 M NaCl. Tre treatment did not affect this ratio in all cell suspensions (Fig. 1F).

Effects of Tre and salt treatment on soluble sugars, starch, and glycerol contents

TSS and RS contents of algal cells increased in response to salt concentration (Fig. 2A, B). The level of these sugars was not affected by Tre treatment in 1 M NaCl suspension, while it decreased in 2 M NaCl grown cells (Fig. 2A, B). Tre treatment did not significantly change TSS in 3 M NaCl (Fig. 2A) but decreased RS content by 19% (Fig. 2B). Salt treatment alone significantly decreased NRS and starch contents of *D. bardawil* cells compared to the control (Fig. 2C, D). NRS concentration decreased by 23% in the Tre-treated cells in 1 M NaCl compared to untreated control while enhanced in other suspensions (Fig. 2C). Tre treatment significantly decreased the starch content of cells in 1 and 2 M NaCl against untreated controls but was ineffective in 3 M NaCl suspension (Fig. 2D).

Glycerol production in *D. bardawil* cells showed a salt-dependent increase (Fig. 2E). Glycerol concentration was decreased by Tre treatment in suspensions of 1 and 3 M NaCl compared to untreated controls while in 2 M NaCl suspension remained unchanged (Fig. 2E).

Changes in protein and amino acid contents of *D. bardawil* cells in response to Tre and salt treatment

Total protein content in cells grown in 2 M NaCl increased by 24% compared to the control but did not change in cells grown in 3 M NaCl. Tre treatment significantly decreased the protein level of all cell suspensions (Fig. 3A). The concentration of TSP gradually increases in response to the salt level. While TSP levels increased by 36% in cells grown in 1 M NaCl treated with Tre, in cells grown in 2 and 3 M NaCl they decreased 22 and 27%, respectively (Fig. 3B). Total amino acid content decreased with increasing salt levels. Free amino acid levels were significantly reduced in all Tre-treated cells (Fig. 3C). The proline levels of algal cells in 2 and 3 M NaCl cultures were 43 and 53% lower, respectively, than the control. Proline content in Tre-treated cells collected from 1 and 3 M NaCl cultures decreased by 22 and 48%, respectively, compared to untreated suspensions, but remained unchanged at 2 M NaCl (Fig. 3D).

Changes in hydrogen peroxide and MDA contents of *D. bardawil* cells treated with Tre and salinity

Hydrogen peroxide content in cells grown in 3 M NaCl was lower than other salinities (Fig. 3E). Tre treatment did not affect the level of hydrogen peroxide in 1 and 2 M

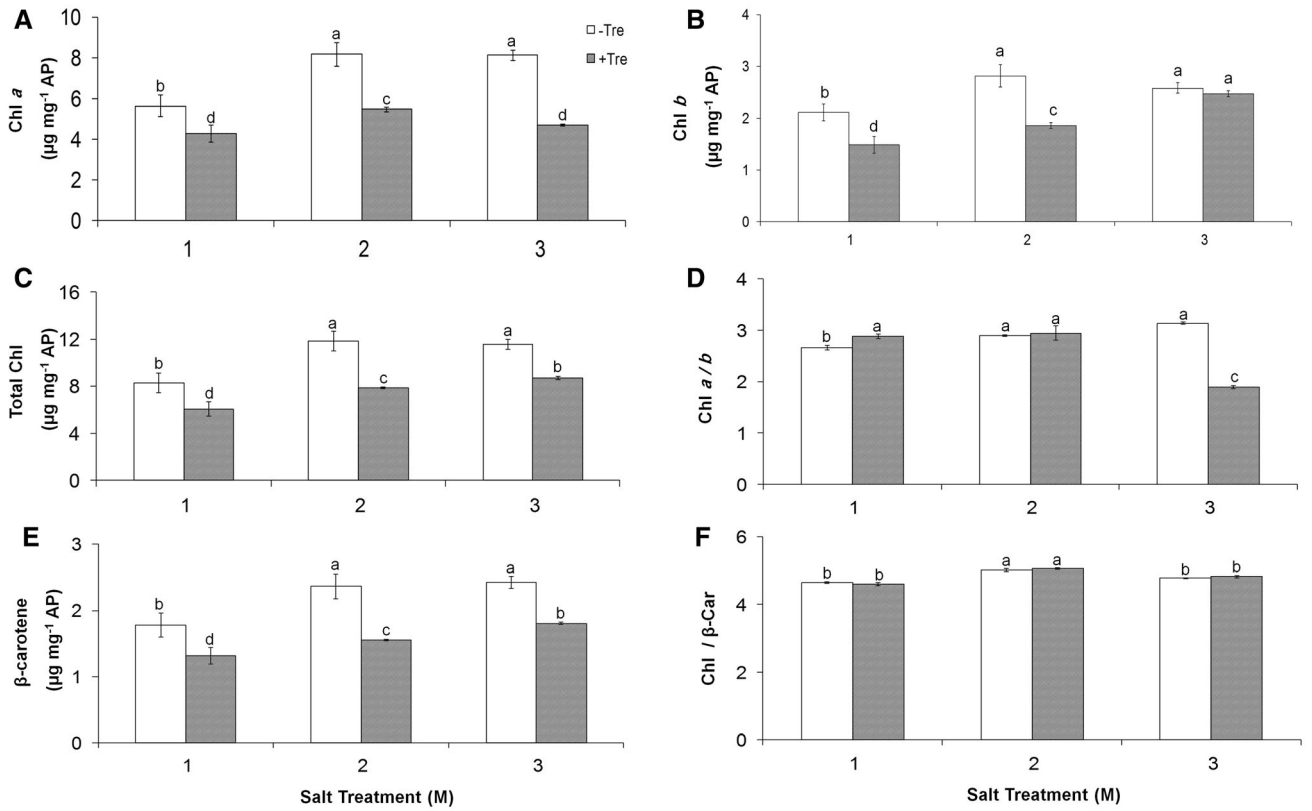

Fig. 1 Pigments content of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h. (**A**) Chl *a*, (**B**) Chl *b*, (**C**) total Chl, (**D**) Chl *a/b*, (**E**) β-carotene, and (**F**) total Chl: β-carotene. Results were expressed as the mean ± SD of three independent measurements. Statistically significant changes at $P < 0.05$ with Tukey test was shown by different letters

NaCl suspensions but significantly reduced it in suspensions containing 3 M NaCl (Fig. 3E). Lipid peroxidation level decreased with increasing salt concentration (Fig. 3F). Tre treatment significantly increased by 76% the MDA level of cells in 1 M NaCl relative to untreated control. However, lipid peroxidation in Tre-treated cells grown in 3 M NaCl did not change against untreated cultures (Fig. 3F).

Changes in the ascorbate pool content of *D. bardawil* cells in response to Tre and salt treatment

Total ascorbate and RAS of algal cells significantly increased in response to salt levels (Fig. 4A, B). Tre treatment increased the total ascorbate of 1 M NaCl-grown cells by 19% but did not change it at other salinities (Fig. 4A). However, the content of RAS remained statistically unchanged in Tre-treated cells against untreated controls (Fig. 4B). DHA content in cells grown in 3 M NaCl was 4.8 times higher compared to other untreated cultures. Salinity in combination with Tre treatment increased DHA levels in 1 and 2 M NaCl suspensions but significantly decreased it in 3 M NaCl culture (Fig. 4C). The RAS: DHA ratio increased by 33% in 2 M NaCl suspensions but did not change in 3 M NaCl. This ratio increased fivefold in Tre-treated cells at 3 M NaCl while was negatively affected by Tre in other suspensions (Fig. 4D).

Changes in enzymes activity of *D. bardawil* cells in response to Tre and salt treatment

Salt treatment differently affected the activity of all enzymes studied in algal cells compared to the control (Fig. 5A–D). CAT and PPX activity increased in 2 M NaCl suspension but decreased abruptly in 3 M NaCl (Fig. 5A, C). CAT activity was significantly increased by 66 and 34% in Tre-treated cells grown at 1 and 3 M NaCl, respectively, compared to the control but was 23% lower in cells grown at 2 M NaCl (Fig. 5A). APX activity increased in response to salt levels. Tre treatment significantly enhanced APX activity in 1 and 2 M NaCl suspensions compared to untreated controls but decreased it in cultures containing 3 M NaCl (Fig. 5B). Tre treatment reduced the PPX activity of cells in 2 M NaCl by 42% compared to untreated cultures while did not affect 1 and 3 M NaCl suspensions (Fig. 5C). PPO activity increased with salt level. Its activity increased by 58% in Tre-treated cells in

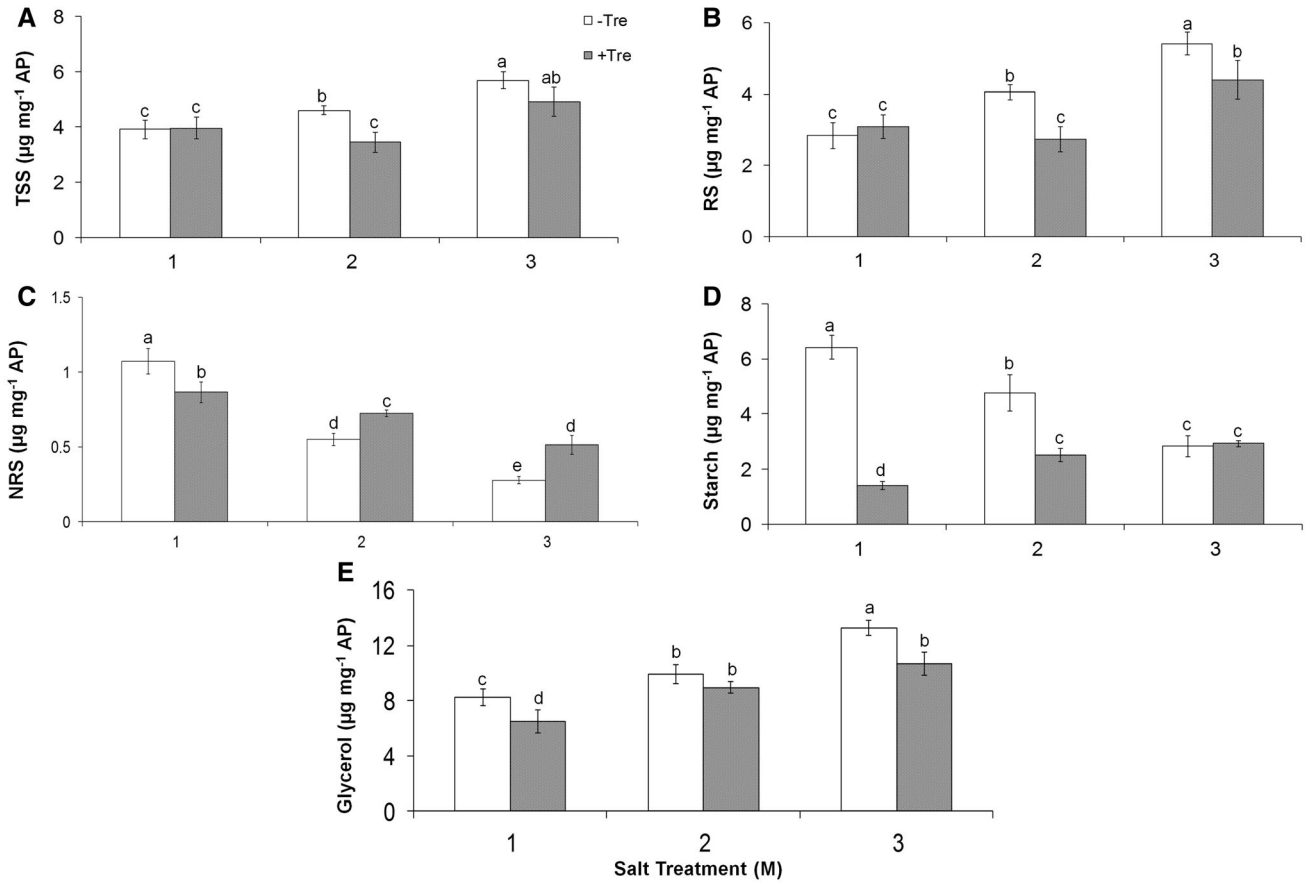

Fig. 2 Changes in total soluble sugars, TSS (**A**), reducing sugar, RS (**B**), non-reducing sugar, NRS (**C**), starch (**D**), and glycerol (**E**) contents of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h. Results show the mean ± SD of three separate analyses. Different letters show statistically significant changes at $P < 0.05$ with Tukey test

1 M NaCl relative to untreated control, but decreased by 73 and 85% in 2 and 3 M NaCl suspensions, respectively (Fig. 5D).

The proteolytic activity of algal cells increased at acid pH in response to the salt level, while the activity at neutral and alkaline pHs was lower in 3 M NaCl suspensions. (Fig. 5E). Tre treatment did not change the acidic or alkaline proteolytic activity of cells in 1 M NaCl but decreased its activity at neutral pH. The proteolytic activity of Tre-treated cells in 2 M NaCl dropped in acid pH compared to untreated controls while remained unchanged at other pHs. However, increased activity of proteases at neutral and alkaline pHs occurred in 3 M NaCl suspensions treated with Tre (Fig. 5E).

Discussion

Cell density as an indicator of cell division in *D. bardawil* suspension was not affected by salt level, which indicates that this species of *Dunaliella* is more tolerant of salinity levels than *D. salina*, a strain that shows the negative effect of high salinity on cell division (Takagi and Karseno 2006; Mishra et al. 2008; Einali and Valizadeh 2015). The density of cells was not affected by Tre treatment, which means that this sugar has no beneficial effect on the cell number of *Dunaliella* as a halotolerant organism. This is consistent with a study showing that Tre treatment did not reduce the adverse effects of salinity on salt-tolerant rice seedlings (Theerakolpisut and Gunnula 2012). Similarly, the fresh weight of *D. bardawil* cells did not change at salinity alone or in combination with Tre treatment. The positive role of Tre in enhancing growth parameters, including fresh weight in stressed or unstressed plants, has been previously reported (Zeid 2009; Chang et al. 2014; Abdallah et al. 2016). Given that the level of metabolites in cell suspensions fluctuate by salinity alone or combined with Tre treatment, the lack of change in fresh weight of cell can be attributed to the coordinated changes in metabolites during treatments and the ineffectiveness of Tre on *D. bardawil* cell growth.

Photosynthetic pigment contents were higher in 2 and 3 M NaCl suspensions. In contrast, pigments content of Tre-treated cells not only did not increase but also

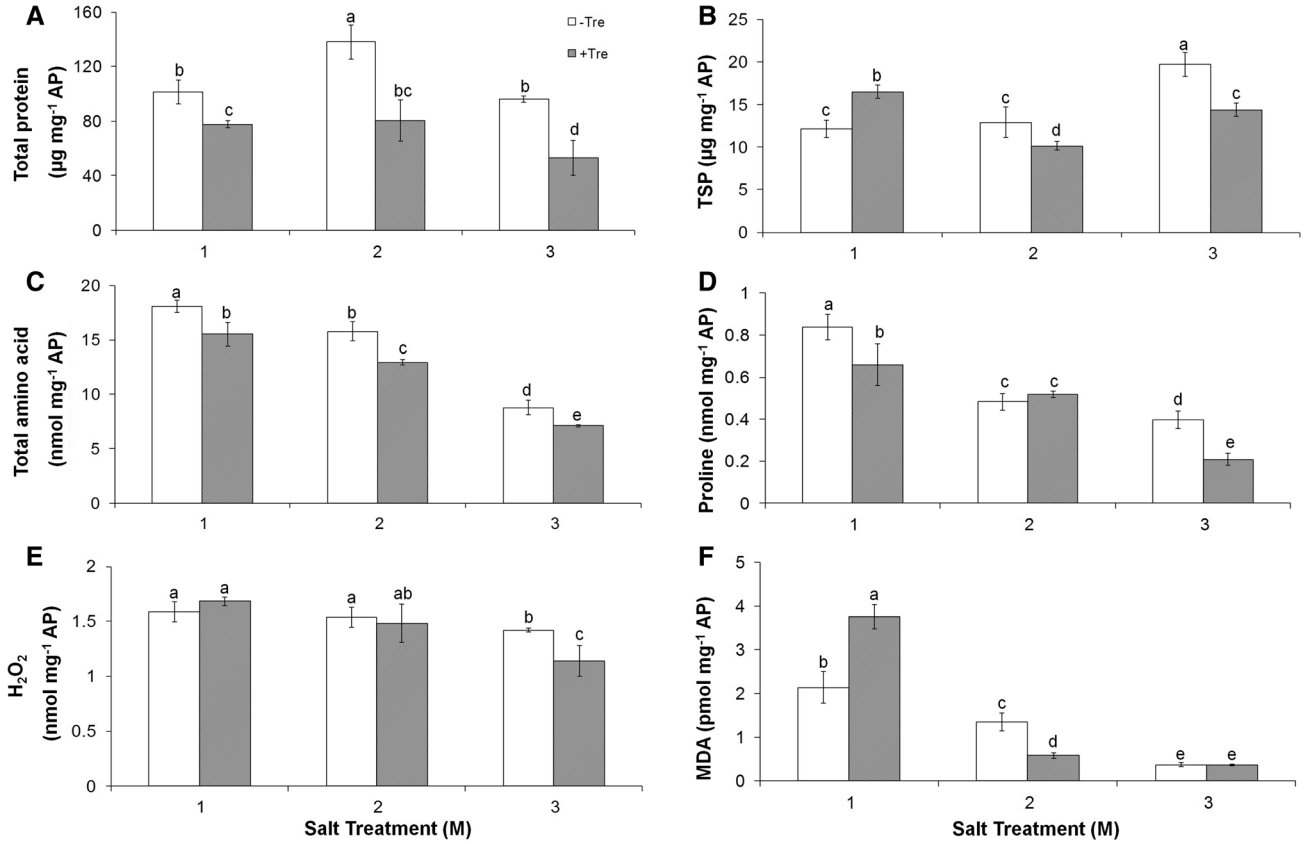

Fig. 3 Total protein (**A**), total soluble protein, TSP (**B**), free amino acid (**C**), proline (**D**), Hydrogen peroxide (**E**), and lipid peroxidation (**F**) levels of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h. Data were calculated from three independent experiments and expressed as the mean ± SD. Statistically significant changes at $P < 0.05$ with Tukey test was shown by different letters

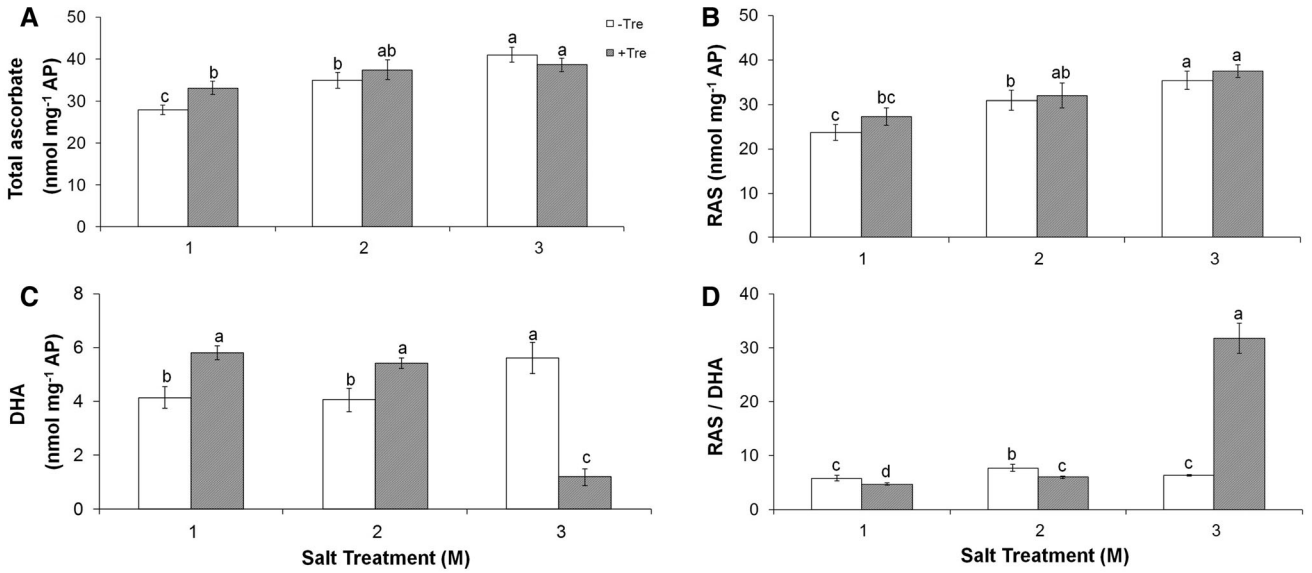

Fig. 4 changes in ascorbate pool content of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h. (**A**) Total ascorbate, (**B**) reduced ascorbate, RAS, (**C**) dehydroascorbate, DHA, and (**D**) RAS: DHA. The bars are mean ± SD of three separate measurements. Different letters indicate statistically significant changes at $P < 0.05$ with Tukey test

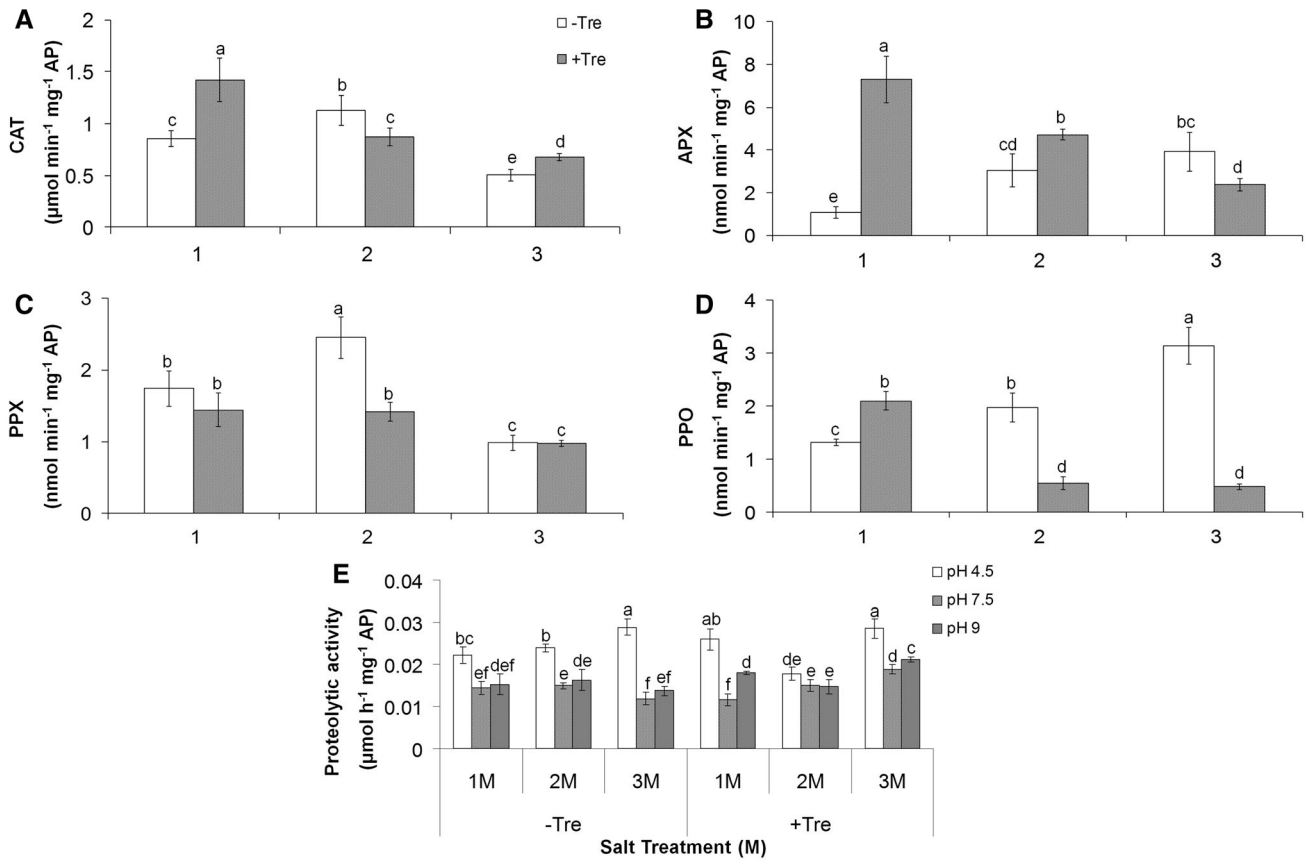

Fig. 5 Changes in the activity of CAT (**A**), APX (**B**), PPX (**C**), PPO (**D**), and proteolytic (**E**) of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h. Data were shown as the mean ± SD of three independent experiments. Statistically significant changes at $P < 0.05$ with Tukey test was shown by different letters

decreased significantly. Unlike numerous studies that have shown the enhancing role of Tre in pigments content under both stable and unstable conditions (Zeid 2009; Theerakulpisut and Phongngarm 2013; Abdallah et al. 2016), our work indicates that Tre has a decreasing effect on pigment contents of all suspensions. Decreased Chl *a/b* ratio in 3 M NaCl suspension treated with Tre shows a higher Chl *b* content against Chl *a* in this treatment. This indicates that at high salinity, Chl *b* is less sensitive to Tre treatment than Chl *a*. However, the ratio of total Chl to β-carotene indicates a coordinated change in the pigments of Tre-treated cells relative to untreated cultures.

Changes in the pattern of soluble sugars in *D. bardawil* cells indicate a more or less similar strategy in salt tolerance of this alga with other plants in which the accumulation of soluble sugars under salinity is a common occurrence (Dubey and Singh 1999; Prado et al. 2000; Flowers 2004; Pattanagul and Thitisaksakul 2008). Decreased levels of NRS and starch versus increased RS pool in cell suspensions may be attributed to increased carbon demand for the biosynthesis of other metabolites, as evidenced by increased glycerol and some antioxidant molecules such as ascorbate and β-carotene. As reported in *Arabidopsis* (Borsani et al. 2001) and two rice cultivars (Walia et al. 2007), this pattern may occur due to increased activity of sucrose and starch-hydrolyzing enzymes in 2 and 3 M NaCl suspensions. Because glycerol synthesis is the most common way for salt tolerance in *Dunaliella* cells (Ben-Amotz and Avron 1981; Ben-Amotz et al. 1982; Chen et al. 2009; Oren 2017), a decrease in NRS and starch by increasing salt level can be evidently attributed to the production of this metabolite. The association of glycerol accumulation with salt level confirms the direction of carbon flow towards synthesizing characteristic metabolites. Tre treatment decreased RS while increased NRS content in 2 and 3 M NaCl suspensions, suggesting that Tre may have a role as a signal molecule in the carbon partitioning of *D. bardawil* cells. The finding that Tre acts as a signal molecule in stress tolerance rather than as an osmoprotectant (Iturriaga et al. 2009) further supports this suggestion. The increase in NRS in Tre-treated cells could not be due to the uptake of Tre by algal cells, as it did not occur in the 1 M NaCl suspension. Decreased glycerol accumulation in Tre-treated cells due to increased

decomposition during the glycerol cycle (Oren 2017), which is associated with sucrose accumulation and reduced or no change in starch synthesis, confirms the role of Tre in changing carbon dividing.

Total and soluble proteins of algal cells increased under salinity while free amino acid and proline contents decreased significantly. This suggests that, unlike other plants (Velitcukova and Fedina 1998; Manan et al. 2016), protein synthesis in *D. bardawil* cells not only does not reduce by salinity but also enhances by increasing salt levels. Increased proteolytic activity at acidic pH of 3 M NaCl-grown cells, which is associated with increased soluble proteins, implies that acidic proteases may be involved in increasing protein solubility. Because the catalytic activity of enzymes depends on their solubility (Han et al. 2020), increasing the solubility of protein with salt level in *D. bardawil* cells can be referred to as increasing enzyme activity. Increased activity of some antioxidant enzymes such as CAT and APX may further support this suggestion. However, the decrease in solubility of proteins in Tre-treated cells grown at high salinity is associated with a reduction in total protein and an increase in proteolytic activity at neutral and alkaline pHs, indicating the role of these proteases in protein degradation. The different pattern of free amino acid and proline accumulation in *D. bardawil* cells with most plants under salinity (Gadallah 1999; Claussen 2005; Khadri et al. 2006; Mohamedin et al. 2006; Yoon et al. 2009) confirms the distinct salt tolerance strategy in this alga. The decrease in these metabolites in algal cells with increasing salinity may be attributed to the synthesis of other osmolytes such as glycerol and antioxidant molecules, including β-carotene and ascorbate. Tre treatment decreased or did not affect the proline content of algal suspensions. This is consistent with the reported results on the negative effect of Tre on proline content in rice seedlings under salinity (Nounjana et al. 2012) and two maize cultivars under water stress (Ali and Ashraf 2011). However, unlike these plants, Tre does not affect cell density, confirming that proline is not a determining factor in the salt tolerance of *D. bardawil* cells. Because the decrease in amino acids and proline content of Tre-treated cells was associated with less accumulation of glycerol and β-carotene, a partial diversion of carbon flow to NRS synthesis emphasizes the shift in carbon dividing by Tre.

Ascorbate, as an antioxidant molecule, plays an essential role in mitigating the adverse effects of ROS under stress conditions (Gallie 2013). Increased ascorbate pool with salt level, which in our study was associated with decreased lipid peroxidation and hydrogen peroxide, indicates increased salinity tolerance of cells. The role of ascorbate in the salt tolerance of *D. tertiolecta* (Jahnke and White 2003) and *D. salina* (Einali and Valizadeh 2015) has been previously reported. Tre treatment did not affect total ascorbate and RAS contents. However, DHA was positively affected by Tre treatment in 1 and 2 M NaCl while drastically decreased in 3 M NaCl. This change in DHA level is well matched with APX activity in all Tre-treated and untreated cultures. Despite the high RAS: DHA ratio due to a low DHA in 3 M NaCl suspension treated with Tre, it is not associated with any change in RSA as an important index of stress tolerance (Foyer and Noctor 2011). This indicates that *D. bardawil* cells have a high capacity to recycle ascorbate that is not affected by Tre, so they tolerate salinity and the resulting oxidative stress. However, despite the change in the activity pattern of antioxidant enzymes, the Tre treatment did not affect the salt tolerance of the algal suspension, indicating that a change in enzyme activity is compensated by a corresponding change in other enzymes. The changes in APX activity as a specific peroxidase in 2 and 3 M suspensions, which was associated with reciprocal changes in CAT and PPX activity, further support this suggestion and may indicate a possible association between antioxidant enzymes activity and the status of ascorbate pool. Similarly, different changes in the activity of antioxidant enzymes have been previously reported in *D. salina* cells during salinity (Mishra and Jha 2011; Einali and Valizadeh 2015). These mutual changes observed in the activity of antioxidant enzymes could explain the different effects of Tre on the enzymatic activity of 2 and 3 M NaCl suspensions.

As summarized in Fig. 6, the extraordinary salinity tolerance of *D. bardawil* cells is achieved by accumulating glycerol, total soluble sugars, and antioxidant molecules such as β-carotene and ascorbate. However, the activity of antioxidant enzymes showing their role in achieving salt tolerance, and some osmoprotectants such as amino acids and proline have no involvement in this mechanism. Tre treatment reduced the content of pigments and the accumulation of some metabolites, including proteins, proline, amino acids, and glycerol. These alterations were associated with changes in antioxidant enzyme activity, carbon partitioning, and DHA levels but no significant change in ascorbate pool content. However, Tre-induced changes did not affect the density and fresh weight of the cells, indicating unchanged salt tolerance of *D. bardawil* cells. Although the Tre treatment reduces the beneficial metabolites produced due to salinity in *D. bardawil*, such as glycerol and β-carotene, the algal tolerance to salt remains unchanged, which could be due to changes induced by Tre in carbon partitioning. This means that the proper regulation of carbon partitioning instead of the amount of photosynthates may be involved in salt toleration. Thus, Tre as a signal molecule is not only ineffective in increasing the salinity tolerance of halotolerant

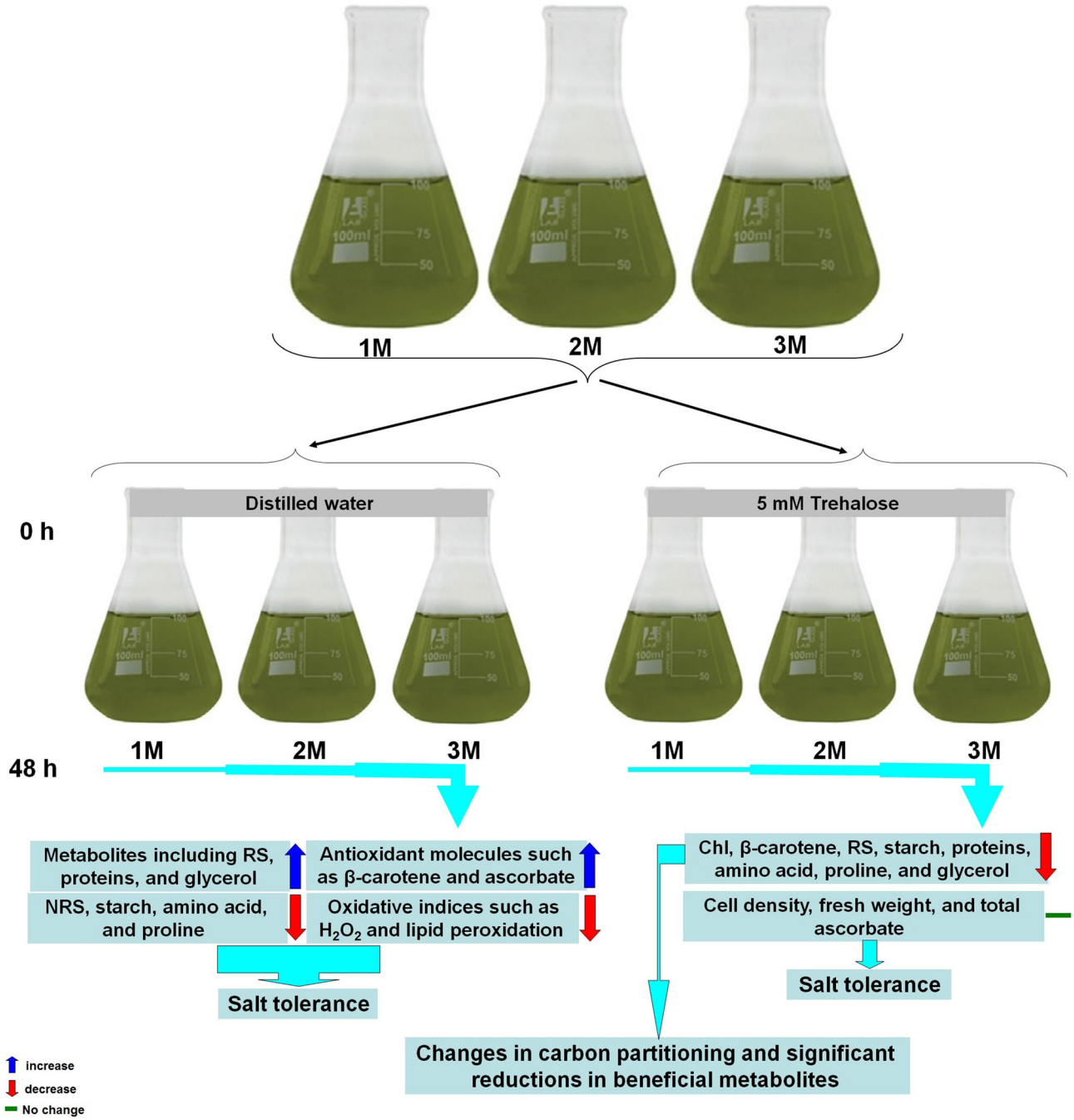

Fig. 6 Schematic illustration for experimental design and metabolic changes of *Dunaliella bardawil* cells grown at 1, 2, and 3 M NaCl in the presence or absence of 5 mM Tre after 48 h

organisms such as *Dunaliella* but is also biotechnologically harmful due to reduced biosynthesis of metabolites.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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References

Abd El-Baky HH, El Baz FK, El-Baroty GS (2004) Production of antioxidant by the green alga *Dunaliella salina*. Int J Agric Biol 6:49–57

Abdallah MS, Abdelgawad ZA, El-Bassiouny HMS (2016) Alleviation of the adverse effects of salinity stress using trehalose in two rice varieties. S Afr J Bot 103:275–282

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Ali Q, Ashraf M (2011) Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defense mechanism. J Agron Crop Sci 197:258–271

Almeida AM, Cardoso LA, Santos DM, Torné JM, Fevereiro PS (2007) Trehalose and its applications in plant biotechnology. In Vitro Cell Dev Biol 43:167–177

Arnon D (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Avron M, Ben-Amotz A (1992) *Dunaliella*: physiology, biochemistry and biotechnology. CRC Press, Boca Raton

Bae H, Herman E, Bailey B, Bae HJ, Sicher R (2005) Exogenous trehalose alters *Arabidopsis* transcripts involved in cell wall modification, abiotic stress, nitrogen metabolism, and plant defense. Physiol Plant 125:114–126

Bahador E, Einali A, Azizian-Shermeh O, Sangtarash MH (2019) Metabolic responses of the green microalga *Dunaliella salina* to silver nanoparticles-induced oxidative stress in the presence of salicylic acid treatment. Aquat Toxicol 217:105356

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free Proline for water stress studies. Plant Soil 39:205–208

Ben-Amotz A, Avron M (1981) Glycerol and β-carotene metabolism in the halotolerant alga *Dunaliella*: a model system for biosolar energy conversion. Trends Biochem Sci 6:297–299

Ben-Amotz A, Avron M (1983) On the factors which determine massive β-carotene accumulation in the halo-tolerant alga *Dunaliella bardawil*. Plant Physiol 72:593–597

Ben-Amotz A, Katz A, Avron M (1982) Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotenerich globules from *Dunaliella bardawil* (Chlorophyceae). J Phycol 18:529–537

Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. Plant Physiol 126:1024–1030

Bradford M (1976) A rapid and sensitive method for the quantitation of microgramquantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carillo P, Grazia Annunziata M, Pontecorvo G, Fuggi A, Woodrow P (2011) Salinity stress and salt tolerance. In: Shanker AK, Venkateswarlu B (eds) Abiotic stress in plants—mechanisms and adaptations. InTech, Rijeka, pp 21–38

Chang B, Yang L, Cong W, Zu Y, Tang Z (2014) The improved resistance to high salinity induced by trehalose is associated with ionic regulation and osmotic adjustment in *Catharanthus roseus*. Plant Physiol Biochem 14:140–148

Chen G, Asada K (1992) Inactivation of ascorbate peroxidase by thoils requires hydrogen peroxide. Plant Cell Physiol 33:117–123

Chen H, Jiang JG (2010) Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. Environ Rev 18:309–319

Chen H, Jiang JG, Wu GH (2009) Effect of salinity changes on the growth of *Dunaliella salina* and its isozyme activites of glycerol-3-phosphat dehydrogenas. J Agric Food Chem 57:6178–6182

Claussen W (2005) Proline as a measure of stress in tomato plants. Plant Sci 168:241–248

Dubey RS, Singh AK (1999) Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolizing enzymes in rice plant. Biol Plant 42:233–239

Einali A (2018) The induction of salt stress tolerance by propyl gallate treatment in green microalga *Dunaliella bardawil*, through enhancing ascorbate pool and antioxidant enzymes activity. Protoplasma 255:601–611

Einali A, Valizadeh J (2015) Propyl gallate promotes salt stress tolerance in green microalga *Dunaliella salina* by reducing free radical oxidants and enhancing b-carotene production. Acta Physiol Plant 37:83

Einali A, Valizadeh J (2017) Storage reserve mobilization, gluconeogenesis, and oxidative pattern in dormant pistachio (*Pistacia vera* L.) seeds during cold stratification. Trees 31:659–671

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R-27R

Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Gadallah MAA (1999) Effects of proline and glycine betaine on *Vicia faba* response to salt stress. Biol Plant 42:249–257

Gallie DR (2013) The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. J Exp Bot 64:433–443

Garcia F, Freile-Pelegrin Y, Robledo D (2007) Physiological characterization of *Dunaliella* sp. (Chlorophyta, Volvocales) from Yucatan Mexico. Bioresour Technol 98:1359–1365

Haghjou MM, Shariati M, Smirnoff N (2009) The effect of acute high light and low temperature stresses on the ascorbate-glutathione cycle and superoxide dismutase activity in two *Dunaliella salina* strains. Physiol Plant 135:272–280

Haghjou MM, Colville L, Smirnoff N (2014) The induction of menadione stress tolerance in the marine microalga, *Dunaliella viridis*, through cold pretreatment and modulation of the ascorbate and glutathione pools. Plant Physiol Biochem 84:96–104

Han X, Ning W, Ma X, Wang X, Zhou K (2020) Improving protein solubility and activity by introducing small peptide tags designed with machine learning models. Metab Eng Commun 11:e00138

Handel EV (1968) Direct microdetermination of sucrose. Anal Biochem 22:280–283

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Henry C, Bledsoe SW, Griffiths CA, Kollman A, Paul MJ, Sakr S, Lagrimini LM (2015) Differential role for trehalose metabolism in salt-stressed maize. Plant Physiol 169:1072–1089

Iordachescu M, Imai R (2008) Trehalose biosynthesis in response to abiotic stresses. J Integr Plant Biol 50:1223–1229

Iturriaga G, Suarez R, Nova-Franco B (2009) Trehalose metabolism: from osmoprotection to signalling. Int J Mol Sci 10:3793–3810

Jahnke LS, White AL (2003) Long-term hyposaline and hypersaline stresses produce distinct antioxidant responses in the marine alga *Dunaliella tertiolecta*. J Plant Physiol 160:1193–1202

Khadri M, Tejera NA, Liuch C (2006) Alleviation of salt stress in common bean by exogenous abscisic acid supply. J Plant Growth Regul 25:110–119

Luck H (1965) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlage Chemie, Weinheim, pp 885–894

Luo Y, Li F, Wang GP, Yang XH, Wang W (2010) Exogenous supplied trehalose protects thylakoid membranes of winter wheat from heat-induced damage. Biol Plant 54:495–501

Manan A, Ayyub CM, Pervez MA, Ahmad R (2016) Methyl jasmonate brings about resistance against salinity stressed tomato plants by altering biochemical and physiological processes. Pak J Agric Sci 53:35–41

Markwell MAK, Hass SM, Tolbert NE, Bieber LL (1981) Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol 72:296–303

McCready RM, Guggolz J, Silviera V, Owens HS (1950) Determination of starch and amylose in vegetables. Anal Chem 22:1156–1158

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428

Mirshekari M, Einali A, Valizadeh J (2019) Metabolic changes and activity pattern of antioxidant enzymes induced by salicylic acid treatment in green microalga *Dunaliella salina* under nitrogen deficiency. J Appl Phycol 31:1709–1719

Mishra A, Jha B (2011) Antioxidant response of the microalga *Dunaliella salina* under salt stress. Bot Mar 54:195–199

Mishra A, Mandoli A, Jha B (2008) Physiological characterization and stress-induced metabolic responses of Dunaliella salina isolated from salt pan. J Ind Microbiol Biotechnol 35:1093–1101

Mohamedin A, El-Kader AA, Badran NM (2006) Response of sunflower (Helianthus annuus L.) to plants salt stress under different water table depths. J Appl Sci Res 2:1175

Munns R, James RA, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57:1025–1043

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Nounjana N, Nghiab PT, Theerakulpisuta P (2012) Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. J Plant Physiol 169:596–604

Oren A (2017) Glycerol metabolism in hypersaline environments. Environ Microbiol 19:851–863

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Safe 60:324–349

Pattanagul W, Thitisaksakul M (2008) Effect of salinity stress on growth and carbohydrate metabolism in three rice (*Oryza sativa* L.) cultivars differing in salinity tolerance. Indian J Exp Biol 46:736–742

Paul M (2007) Trehalose 6-phosphate. Curr Opin Plant Biol 10:303–309

Peoples MB, Dalling MJ (1978) Degradation of ribulose 1,5-bisphosphate carboxylase by proteolytic enzymes from crude extracts of wheat leaves. Planta 138:153–160

Prado FE, Boero C, Gallardo M, Gonzalez JA (2000) Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinoa* Willd. Bot Bull Acad Sin 41:27–34

Ramadan AA, Abd Elhamid EM, Sadak MS (2019) Comparative study for the effect of arginine and sodium nitroprusside on sunflower plants grown under salinity stress conditions. Bull Natl Res Centre 43:118

Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421

Salguero A, de la Morena B, Vigara J, Vega JM, Vilchez C, Leon R (2003) Carotenoids as protective response against oxidative damage in *Dunaliella bardawil*. Biomol Eng 20:249–253

Shariati M, Lilley MC (1994) Loss of intracellular glycerol from *Dunaliella* by electroporation at constant osmotic pressure: subsequent restoration of glycerol content and associated volume changes. Plant Cell Environ 17:1295–1304

Shigeoka S, Yokota A, Nakano Y, Kitaoka S (1979) The effect of illumination on the L-ascorbic acid content in *Euglena gracillis* Z. Agric Biol Chem 43:2053–2058

Stepien P, Klobus G (2005) Antioxidant defense in the leaves of C3 and C4 plants under salinity stress. Physiol Plant 125:31–40

Stone SL, Gifford DJ (1997) Structural and biochemical changes in loblolly pine (*Pinus taeda* L.) seeds during germination and early seedling growth: I. Storage protein reserves. Int J Plant Sci 158:727–737

Takagi M, Karseno YT (2006) Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J Biosci Bioeng 101:223–226

Theerakulpisut P, Gunnula W (2012) Exogenous sorbitol and trehalose mitigated salt stress damage in salt-sensitive but not salt tolerance rice seedlings. Asian J Crop Sci 4:165–170

Theerakulpisut P, Phongngarm S (2013) Alleviation of adverse effects of salt stress on rice seedlings by exogenous trehalose. Asian J Crop Sci 5:405–415

Velitcukova M, Fedina I (1998) Response of photosynthesis of *Pisum sativum* to salt stress as affected by methyl jasmonate. Photosynthetica 35:89–97

Walia H, Wilson C, Zeng L, Ismail AM, Condamine P, Close TJ (2007) Genome-wide transcriptional analysis of salinity stressed japonica and indica rice genotypes during panicle initiation stage. Plant Mol Biol 63:609

Yemm EW, Cocking EC (1955) The determination of amino acids with Ninhydrin. Analyst 80:209–213

Yoon JY, Hamayun M, Lee SK, Lee IJ (2009) Methyl jasmonate alleviated salinity stress in soybean. J Crop Sci Biotechnol 12:63–68

Zeid IM (2009) Trehalose as osmoprotectant for maize under salinity-induced stress. Res J Agric Biol Sci 5:613–622

Zhang JL, Shi H (2013) Physiological and molecular mechanisms of plant salt tolerance. Photosynth Res 115:1–22