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Stress responses of the green microalga, *Dunaliella salina* to PEG-induced drought

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Abstract

Drought stress was evaluated with polyethylene glycol 6000 (PEG 6000) treatment in *Dunaliella salina*, a microalga known for its great ability to withstand salinities of more than 30%. The aim was to explore the acclimation mechanisms used by the microalga to regulate its growth and physiology during coping with drought stress. The microalga was subjected to culture mediums containing 2 and 5% PEG for 25 days and was compared with a control culture medium. Significant decrease in growth parameters such as specific growth rate, biomass and number of divisions per day was demonstrated in PEG-treated algae. During PEG treatment, chlorophylls slightly increased, while β-carotene and total protein were not affected. Osmolytes, as well as carbohydrates, were found to be significantly higher in PEG-treated algae than in control. Increased catalase and ascorbate peroxidase activities were proportionally related to PEG concentrations in the cultures. The PEG-treated cells accumulated a considerable amount of hydrogen peroxide and malondialdehyde, especially at higher PEG concentrations. Electrolyte leakage increased, regardless of the PEG concentrations applied, while DNA fragmentation was not observed after 25 days of treating with PEG. It was concluded that *Dunaliella* cells could respond to the drought stress, probably by using a higher accumulation of a range of osmolytes and also more stimulation of the antioxidant enzymatic system.

Introduction

Marine lineages living intertidally, or in ephemeral pools, are exposed to varying periods of dehydration. Many green algae also live under a high salinity condition in the marine environment which induces an oxidative stress similar to dehydration. Salinity and desiccation are considered as different types of water deficiency. In this regard, salinity stress is recognized as 'physiological drought' which may reduce the water potential of algal cells that are still in full contact with water, while desiccation stress could increase an intensive dehydration in the cells (Kirst, 1990). Although both types of stress similarly cause loss of turgor as an important agent for cell elongation and growth; they might induce different changes in the ratio of ions selectively taken up by the cells (Holzinger & Karsten, 2013).

Desiccation leads to oxidative stress and, eventually, generation of reactive oxygen species (ROS) in algae. ROS are a potent cause of destruction to biological macromolecules such as nucleic acids, proteins and lipids (Scheibe & Beck, 2011). Numerous studies concerning desiccation tolerance in cyanobacteria, lichens, mosses and plants have been addressed in the literature (Kranner *et al*., 2008; Gechev *et al*., 2013). The ability of organisms to survive in dry conditions is called desiccation tolerance. Desiccation tolerance in algae is a promising field for research, as suggested by Bewley ~40 years ago (Bewley, 1979). Davis (1972) reported desiccation tolerance in vegetative and reproductive cells of 119 species of green algae. Several recent studies have reviewed physiological, biochemical and molecular impacts of desiccation (Tardieu *et al*., 2018; Bremer & Krämer, 2019; Oliver *et al*., 2020). However, the mechanisms of desiccation tolerance in green algae, especially microalgae are not fully understood. For instance, Holzinger & Karsten (2013) have reviewed the mechanisms of desiccation tolerance in green algae at the ultra-structural, physiological and molecular levels. More recently, Lin & Wu (2014) compared the PEG-induced drought responses of some drought-tolerant and non-tolerant algal species and suggested that the tolerance to drought stress might be indicated by physiological parameters such as the proline level and the enzymatic and non-enzymatic antioxidant system.

Dunaliella as one of the most tolerant eukaryotic and photosynthetic organisms is a suitable microalgal model to clarify the tolerance mechanisms of plant and algal cells to different stresses (Cowan *et al*., 1992). The genus *Dunaliella* belongs to the Volvocales order and Polyblepharidaceae family. The members of the *Dunaliella* are unicellular, biflagellate, ovoid or spherical algae without a rigid cell wall. Several researchers have investigated the effects of different abiotic stresses such as heavy metals, salinity, low temperature, acidic environment, UV and oxidative stress on *D. salina* (Yilancioglu *et al*., 2014; Belghith *et al*., 2016). For instance, a proportional relationship was found between the accumulation of stress metabolites such as proline, glycine betaine, glycerol and total carbohydrates and the salt concentration of

the medium ranging from 0.5 to 5.5 M (Mishra *et al.*, 2008). The synergistic effects of salinity and PEG on the growth of *D. salina* have been previously reported in a preliminary study (Kumar & Dharmaraj, 2003); however, the sole effect of PEG on the growth and physiology of *D. salina* has not been studied yet. This study aimed to investigate the effect of different concentrations of PEG on the growth, morphology and physiology of *D. salina* as an attempt to shed some light on the mechanisms underlying the effects of drought stress on this microalga.

Materials and methods

Cell culture and PEG treatment

The alga, *D. salina* (UTX LB 2538) was obtained from the Culture Collection of Algae in UTEX, USA and maintained and cultivated in a modified Johnson's medium (Johnson *et al.*, 1968). The cells were grown at 25°C in 500 ml Erlenmeyer flasks containing 250 ml of the medium on a rotary shaker stirred at 110 rpm under continuous white light with a photon flux density of 100 µmol m^{-2} s^{-1}. In a preliminary experiment, the cells were treated with different concentrations of PEG 6000 (0, 2.5, 5, 7.5, 10, 15, 20, 25 and 30%) to select the appropriate medium for a subsequent experiment. Finally, two concentrations of PEG (2.5 and 5%) and a control medium were used to simulate drought stress treatment in *D. salina*. For all PEG treatments as well as control, 5 ml of freshly grown cells were inoculated into the medium with a fixed amount of NaCl (1.5 M) and the cultures monitored for 25 days. The PEG-induced osmotic potential was calculated by using the equation previously formulated by Michel & Kaufmann (1973):

$$\mathrm{OP(Mpa)} = (-1.18 \times 10^{-2}) \times C - (1.18 \times 10^{-4}) \times C + (2.67 \times 10^{-4}) \times C.T + (8.39 \times 10^{-7}) \times C^2 \times T$$

where C is PEG concentration, and T is temperature.

The NaCl-induced osmotic potential was calculated using the Van't Hoff equation and total osmotic potential was calculated by summing PEG- and NaCl-induced osmotic potentials.

Growth analysis

The cell number was counted using a hemocytometer under an Olympus light microscope at 400×. The dry biomass was recorded after filtration of 10 ml of culture through a pre-weighed 45 mm glass filter, rinsed with 10 mL ammonium formate (0.5 M) and dried in an oven at 70°C until constant.

Specific growth rate was determined using the equation previously used by Levasseur *et al.* (1993) as follows: $K' = \ln(N2/N1)/t2 - t1$ (where, $N1$ and $N2$ were biomass (mg ml^{-1}) at the first time ($t1$) (day 5) and the second time ($t2$) (day 15), respectively). The number of divisions per day (Div./day) and the doubling time of the cells (D.time) were also calculated as indicated below:

$$\mathrm{Div./day} = \frac{K'}{\ln 2}$$

$$\mathrm{D.time} = \frac{1}{\mathrm{Div./day}}$$

ImageJ software was used to measure the relative changes of cell surface area under different PEG concentrations compared with the control.

The Speed index of cells, as an indicator of the cell motility, scored from 0 (no motility) to 5 (maximum motility) under the light microscope.

Glycerol determination

Glycerol content was measured according to Chen *et al.* (2011) method. The algal pellets were collected from 10 ml of the cultures centrifuged at 6000 rpm for 5 min and then were resuspended in 1.0 ml of distilled water and 0.2 ml of chloroform. After homogenizing the suspension for 8 min, the supernatant was harvested by centrifugation at 11,000 rpm for 10 min. Next, 100 µl of the supernatant was mixed with 200 µl distilled water and 1 ml of sodium periodate reagent. After 5-min, 2.5 ml of acetylacetone reagent was added. The suspension was placed in a water bath at 60°C for 30 min. After cooling at room temperature, absorbance was read at 410 nm. Glycerol content was determined using a standard curve for pure glycerol as reference and results were expressed as micrograms of glycerol per 10^6 cells.

Proline determination

Proline was extracted and measured using the method of Bates *et al.* (1973) based on the reaction of proline with ninhydrin. About 10 ml of cell culture were centrifuged at 6000 rpm for 5 min. The pellet was homogenized in 3 ml sulphosalicylic acid 3% and then centrifuged at 13,200 rpm for 20 min. A 1:1:1 (v:v:v) solution of the supernatant, ninhydrin acid and glacial acetic acid was boiled for 1 h and transferred on ice for 10 min. The reaction mixture was extracted with 2 ml of toluene by vortexing. The absorbance of the upper phase (coloured solution) was determined at 520 nm using toluene as the blank and L-proline as the standard. Results were expressed as nanomolar of proline per gram fresh weight.

Glycine betaine determination

The amount of glycine betaine (GB) was determined following the method of Sairam & Srivastava (2002). The cells were harvested by centrifugation of 10 ml of the cultures at 6000 rpm for 5 min and then resuspended in 10 ml of distilled water. The extract was recentrifuged and equal volumes of 2 N H_2SO_4, and 0.8 ml cold KI–I_2 reagent (1.75 g I_2 and 2 g KI in 10 ml deionized water) was added to the supernatant and was thoroughly mixed. The homogenized sample was held in ice for 16 h, centrifuged and then 1 ml of supernatant was carefully mixed with 1 ml of 1, 2-dichloroethane. After 3 h of incubation at room temperature, the absorbance was read at 365 nm. The concentration of glycine betaine (GB) was estimated using the known amount of GB as standard and results were expressed as micrograms of GB per 10^6 cells.

Protein quantification

For protein extraction, 10 ml of cell suspensions were centrifuged at 6000 rpm for 5 min. Cell pellets were homogenized in 80% cold hydrous ethanol and the extract was held at −20°C for 30 min after centrifuging. The precipitated protein pellets were dissolved in 0.1 N NaOH. Total soluble protein content was determined by the Bradford (1976) method using crystalline bovine serum albumin (BSA) as a standard reference. Results were expressed as micrograms of protein per 10^6 cells.

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Determination of soluble carbohydrates

Total soluble sugars were estimated according to the phenol sulphuric acid method, as described by Kochert (1978). For extraction of carbohydrates, 10 ml of 70% ethanol was added to ~0.1 g dry weight of the algal pellet. 1 ml of the extract was brought to a volume of 2 ml with deionized water and then mixed well with 1 ml of phenol solution (5%). After adding 5 ml concentrated sulphuric acid (80%), the optical density of the sample was measured at 480 (rhamnose), 485 (glucose), and 490 (mannose). The concentration of the sugars was calculated by comparison with their standard curves, and results were expressed as micrograms of carbohydrates per 10^6 cells.

H_2O_2 determination

H_2O_2 measurement was performed according to the method of Sergiev *et al.* (1997). Cell suspension (10 ml) was centrifuged at 6000 rpm for 5 min. The supernatant was removed carefully, and the pellets were resuspended in 2 ml 0.1% (w/v) cold trichloroacetic acid (TCA) in an ice bath. The mixture was centrifuged at 12,000 rpm for 15 min. Next, 1 ml of the supernatant was transferred to a test tube containing 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. After mixing thoroughly in a vortex mixer (model SA 8, Stuart, Britain), the absorbance of the supernatant was recorded at 390 nm. The concentration of H_2O_2 was calculated by comparison with a standard curve. A mixture without the supernatant served as a blank, and results were expressed as micromole of H_2O_2 per 10^6 cells.

Lipid peroxidation

The level of lipid peroxidation was estimated through the amount of malondialdehyde (MDA). The MDA content was measured according to the method of Heath & Packer (1968). First, 10 ml suspension culture was centrifuged at 6000 rpm for 5 min, and the resulting pellet was resuspended by pipetting in 3 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was then centrifuged, and 500 µl supernatant was mixed with 500 µl of 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 95°C for 30 min and immediately cooled in an ice bath to stop the reaction. The absorbance was read at both 532 and 600 nm. The MDA concentration was calculated using the molar extinction coefficient of 155 $mM^{-1}cm^{-1}$. Results were reported as nanomoles of MDA per 10^6 cells.

$$\text{MDA equivalent} = [(A532 - A600)/155000] \times 10^6$$

The 532 and 600 nm levels were the maximum absorbances of the TBA-MDA complex and the correction for non-specific turbidity, respectively.

Membrane permeability

Electrolyte leakage (EL) was determined for assaying algal membrane permeability using a conductivity meter (istek, model C65, Korea) according to the method of Dionisio-Sese & Tobita (1998). Samples (10 ml) were centrifuged at 6000 rpm for 5 min and the pellets were washed twice with double distilled water. After dissolving the pellets in 10 ml deionized water and holding on a shaker for 6 h, the initial conductivity of the solution was measured ($C1$). The samples were then boiled for 40 min and cooled to room temperature to measure the second conductivity ($C2$). The percentage of electrolyte leakage was measured as follows:

$$\%EC = \frac{C1}{C2} \times 100$$

Enzymes extraction and assay

For assaying antioxidant enzymes, the cells were harvested from 10 ml of culture by centrifugation at 6000 rpm for 5 min. The pellet was homogenized in 1 ml of cold potassium phosphate buffer (50 mM, pH 7.4), containing 1% (w/v) polyvinylpyrrolidone (PVP) in an ice bath with mortar and pestle. The homogenate was centrifuged at 4°C for 15 min at 4000 rpm and the supernatant was used as enzyme extract in order to assay the activities of both catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11), spectrophotometrically.

The CAT activity was determined as previously described by Aebi (1983). The reaction mixture contained 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.4). The reaction was started with the addition of 100 µl enzyme extract to 900 µl reaction mixture. The decline in absorbance at 240 nm was recorded for 1 min, and catalase activity was calculated by following the dissociation of hydrogen peroxide and using an absorbance coefficient of 0.039 mM^{-1} cm^{-1} for H_2O_2. One unit of catalase was defined as the amount of enzyme that is required for the disintegration of 1 µmol of H_2O_2 in 1 min at 25°C.

Analysis of APX activity was performed based on the Nakano & Asada (1981) method. The reaction mixture contained 625 µl phosphate buffer with 0.2 mM EDTA (pH 7.4), 175 µl ascorbic acid (0.5 mM), 50 µl H_2O_2 (25 mM), 50 µl BSA and 100 µl enzyme extract. The reaction was initiated by the addition of H_2O_2 (25 mM) and a reduction in absorbance at 290 nm was monitored for 1 min. Enzyme activity was measured by an absorbance coefficient of 2.6 mM^{-1} cm^{-1} for ascorbic acid. One unit of ascorbate peroxidase was expressed as the oxidation of 1 µmol ascorbic acid in 1 min at 25°C.

DNA fragmentation test

The effect of PEG on DNA fragmentation was studied as an indicator of damage to the nucleic acid under stress conditions by running and investigating the extracted DNA on an agarose gel. DNA was extracted from the cell suspension (10 ml) using a silica-based technique previously described by Li *et al.* (2010). DNA integrity was checked by electrophoresis on 1.5% agarose gel. Lambda DNA/HindIII (Thermo Scientific) was used as a size marker.

Determination of pigments

To determine pigments including chlorophylls (*a* and *b*) and *β*-carotene, 10 ml of the cell suspension was centrifuged for 5 min and the pellet was homogenized in 80% acetone solvent. After centrifugation, the absorbance of the supernatant was measured at 431, 460, 480 and 412 nm. The pigments were estimated by the equations previously derived (Eijckelhoff & Dekker, 1997). The tolerance index (TI) was measured as described by Kohl & Losch (2004) using the following formula:

$$\text{TI}(\%) = \frac{\text{chlorophylls content of PEG treated cells}}{\text{chlorophylls content of control}} \times 100$$

Statistical analyses

The experimental design was entirely randomized, with three repeats per treatment. The obtained data and difference between the mean values were analysed statistically by conducting ANOVA (analysis of variance) followed by Fisher's LSD test at the $P < 0.01$ level. Statistical analyses and graphs were produced in Excel and Graph Pad Prism 6 software.

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Table 1. The PEG-induced and total osmotic potentials (OPs) of the media with different concentrations of PEG 6000 and their effects on the growth kinetic parameters of microalgae, *D. salina*

	PEG-induced OP (Mpa)	Total OP (Mpa)	Specific growth rate (mg day.ml^{-1})	Div./day	D.time
Control	0.0	−72.32	3.63 ± 0.46^{a}	5.11 ± 0.24^{a}	0.19 ± 0.04^{a}
PEG 2%	−0.14	−72.46	4.45 ± 0.57^{a}	6.43 ± 0.51^{b}	0.15 ± 0.07^{b}
PEG 5%	−0.50	−72.82	1.34 ± 0.15^{b}	1.72 ± 0.23^{c}	0.51 ± 0.15^{c}
PEG 7.5%	−0.93	−73.25	0.51 ± 0.13^{c}	0.91 ± 0.12^{d}	0.68 ± 0.13^{d}
PEG 10%	−1.48	−73.80	0.12 ± 0.04^{c}	0.20 ± 0.11^{d}	5.01 ± 0.53^{e}

The values shown are means followed by ± SD, N = 3. Different superscript letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level. Div./day and D.time indicate the number of divisions per day and the doubling time of the cells, respectively.

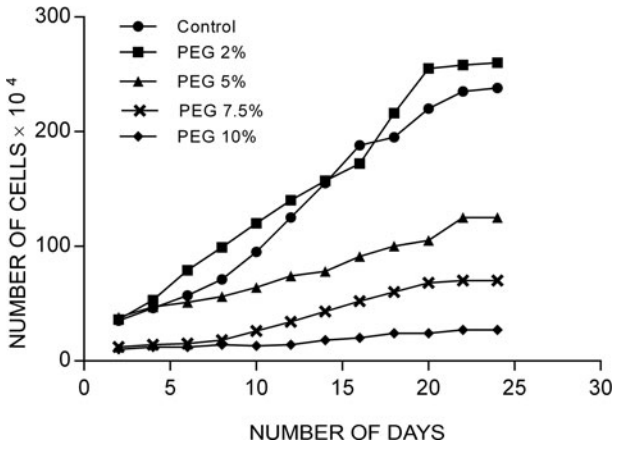

Fig. 1. The effect of PEG 6000 concentrations (0, 2, 5, 7.5 and 10%) on growth of *D. salina*. Growth kinetics determined from the number of cells counted every 2 days during 25 days of culture. The cells treated with 2% PEG showed a slightly higher biomass than the PEG-free control. The growth of microalgae was significantly inhibited when the dose of PEG increased more than 2%.

Results

The effect of PEG on algal growth and morphology

The threshold of drought tolerance in *D. salina* was initially assayed by cultivating the alga in a wide range of PEG doses. Generally, we did n't observe any cell growth or culture greening in PEG doses higher than 10%. With the increasing PEG concentration up to 10%, both the PEG-induced potential and the total osmotic potential of media as indicators of stress intensity decreased from 0.0 to −1.48 Mpa and from −72.32 to −73.80 Mpa, respectively (Table 1). The kinetic growth of *D. salina* (Figure 1) indicated that all the treatments reached a stationary phase over 24 days of cultures. According to the results, a slight increase in cell growth was observed for 2% PEG-treated cells compared with the PEG-free control. However, increase in PEG up to 5% or more, had significant negative effects on the rate of cell growth. The 2% PEG treated cells and PEG-free control reached their stationary phases on days 20 and 22 of culture with the maximum values of 238×10^4 and 260×10^4 cells ml^{-1}, respectively. The stationary phase of *D. salina* growth in 5% PEG took place on the day 24 with a maximum number of 125×10^4 cells ml^{-1}. The growth of *Dunaliella* in 7.5 or 10% PEG was not significant and only plateau-like curves were observed in both cases. As depicted in Table 1, other growth kinetic parameters were also influenced by PEG treatment. Specific growth rates and a number of divisions per day were found to be 4.45 mg day ml^{-1} and 6.4 for 2% PEG treatment, respectively and were slightly higher than those for control. The doubling time of the cells decreased from 0.19 in control to 0.15 in 2% PEG. PEG treatment higher than 2% reduced the specific growth rate and the number of divisions per day, while it significantly increased the doubling time of the cells.

Microscopy studies were performed to investigate the morphology of *Dunaliella* cells under different doses of PEG stress. Although no clumping cells in PEG-free control or PEG 2% treatment were observed, large cell clumps were noticed in the PEG treatments higher than 2% (Figure 2). On the other hand, the cell sizes increased in the various doses of PEG, such that compared with the control cells, the relative area surface of the cells treated with 2, 5, 7.5 and 10% PEG, raised by 1.21-, 1.51-, 1.69- and 1.72-fold, respectively. The shape of cells changed mainly from normal-ellipsoid in control to more irregular shapes in high concentrations of PEG (Figure 2 and Table 2).

The speed index, as an indicator of the cell motility, was affected by the PEG treatment. The motility of the cells in the control medium was the maximum with a mean speed index of 5, whereas the application of any dose of PEG to the medium proportionally reduced the speed index. The cells treated with 10% PEG had no motility and had the minimum speed index.

Glycerol content increased dramatically during PEG treatment

Intracellular glycerol content of *D. salina* raised from 6.73 ± 0.51 in control up to 16.74 ± 2.63 µg/10^6 cells in 5% PEG treatment. Based on the results, a logarithmic relationship existed between the cellular accumulation of glycerol and the elevation of PEG concentration (Figure 3A). The maximum value of glycerol was observed in cells treated with 5% PEG, which was about 1.8- and 2.5-fold more than those in PEG-free control and 2% PEG, respectively. By increasing PEG from 0 to 2%, a slight increase in glycerol was observed in the cells; however, more PEG treatment up to 5% led to a logarithmic enhancement of this compound in *Dunaliella*.

Significant accumulations of proline and glycine betaine were observed under PEG treatment in D. salina

The findings showed that drought stress rising due to PEG treatment intensified higher production of compatible osmolytes such as proline and glycine betaine in *Dunaliella* compared with control (Figure 3B, C). A considerable increase was detected in the proline content of *D. salina* cells after increasing PEG. The highest accumulation of proline was in 5% PEG treated cells with a mean of 4 nmol g^{-1} FW, being ~4- and 2-fold more than those in control or PEG 2% treated cells, respectively.

The results also indicated that another vital osmolyte, glycine betaine, substantially increased with a mean from 100.6 µg/10^6 cells in control up to 145.2 and 225.8 µg/10^6 cells in PEG 2 and 5%, respectively. The highest value of glycine betaine was again found for the 5% PEG-treated cells, which were ~2.2-fold more than the value of the control.

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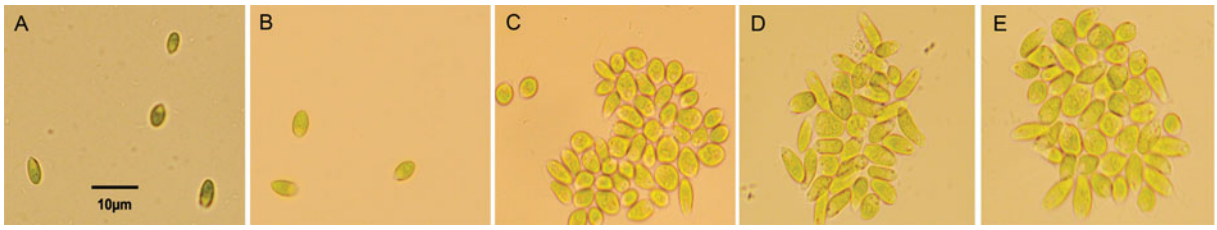

Fig. 2. The morphology of microalga, *D. salina* cultured under different PEG 6000 concentrations. A: *D. salina* cultured in PEG-free control medium; B, C, D and E: *D. salina* cultured in media with 2, 5, 7.5 and 10% PEG, respectively; Optical zoom ×400.

Table 2. The effects of different concentrations of PEG 6000 on morphology parameters and motility of microalgae, *D. salina*

	Relative surface area to control	Cell shape	Speed index	Number of colonies per microscopic field ×10	Number of cells per colony
Control	1	Ellipsoid	5	0	0
PEG 2%	1.21	Ellipsoid	3	0	0
PEG 5%	1.51	Ellipsoid Irregular	1	12	30.82 ± 11.34^{a}
PEG 7.5%	1.69	Ellipsoid Irregular	1	11	31.71 ± 7.78^{a}
PEG 10%	1.72	Ellipsoid Irregular	0	11	56.50 ± 18.50^{b}

The values shown are means followed by ± SD, N = 3. Different superscript letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level. The Speed index of cells scored from 0 (no motility) to 5 (maximum motility).

A relatively constant amount of protein was produced in D. salina *resulting from PEG treatment*

Treatment with 2% or 5% PEG compared with the control, did not change protein content in *D. salina* cells meaningfully (Figure 3D). The protein content of the control (518 ± 103 mg g^{-1} FW) only was 1.18- and 1.20-fold more than those in 2% (439 ± 34 mg g^{-1} FW) and 5% PEG (406 ± 40 mg g^{-1} FW), respectively.

Soluble carbohydrates increased in D. salina *over PEG treatment period*

According to our data, treating the cells with different concentrations of PEG led to increasing trends in soluble carbohydrates, including glucose, mannose and rhamnose (Figure 4). In this context, higher concentrations of PEG induced more sugar accumulations in *Dunaliella*, such that the highest mean content of glucose, mannose and rhamnose was obtained in 5% PEG-treated cells, which were 18.80, 10.05 and 10.82 $\mu g/10^6$ cells, respectively.

Very large H_2O_2 production resulting from PEG-induced drought stress in D. salina

The amount of hydrogen peroxide significantly increased in PEG-treated cells, especially in 5% PEG treatment. As indicated in Figure 5A, a slight increase in H_2O_2 was observed upon exposure of *Dunaliella* cells to 2% PEG, however by changing the PEG concentration from 2% to 5%, a vast amount of H_2O_2 accumulation was observed in the cells. The mean value of H_2O_2 content in PEG 5%-treated cells was 0.066 µmol/10^6 cells which showed a 33-fold increase relative to untreated cells (0.002 µmol/10^6 cells).

PEG treatment-induced lipid peroxidation in D. salina

The MDA content as an indicator of lipid peroxidation level is shown in Figure 5B. A negligible amount of MDA was seen in the PEG-free control. However, a PEG-dependent increase in MDA contents was also observed, such that the higher the PEG level applied, the more MDA content increased. These results demonstrated that compared with control, application of 2 and 5% PEG induced 25- and 74-fold increase of lipid peroxidation, respectively.

PEG induced leakage in the cell membrane

As another part of the article shows, it was found that the PEG treatment of *Dunaliella* induced significant leakage in the cell membrane; however, there were no meaningful differences in electrolyte leakage of cells treated with 2% or 5% PEG (Figure 5C). Generally, PEG-induced drought stress increased leakage in *D. salina* cells up to 30% compared with control.

Antioxidant enzyme activities

As seen in Figure 6A and B, following PEG treatment, enzymatic antioxidant systems of *D. salina*, including catalase (CAT) and ascorbate peroxidase (APX) were strongly affected. The data indicated that in comparison with control, by increasing PEG from 2% to 5%, the mean activity of CAT rose by ~2.0- to 3.9-fold, respectively (Figure 6A). A similar trend was also revealed for APX. The mean activity of this enzyme increased 1.7-fold in 2% PEG and up to 2.9-fold in 5% PEG compared with control (Figure 6B).

Chlorophylls increase did not coincide with significant changes in β-carotene during PEG-induced drought stress

An increase in chlorophyll *a* and *b* pigments compared with the control were triggered by drought stress, regardless of the PEG concentrations applied. As depicted in Table 3, the content of chlorophyll *a* and *b* was higher in *D. salina* at 2% or 5% PEG

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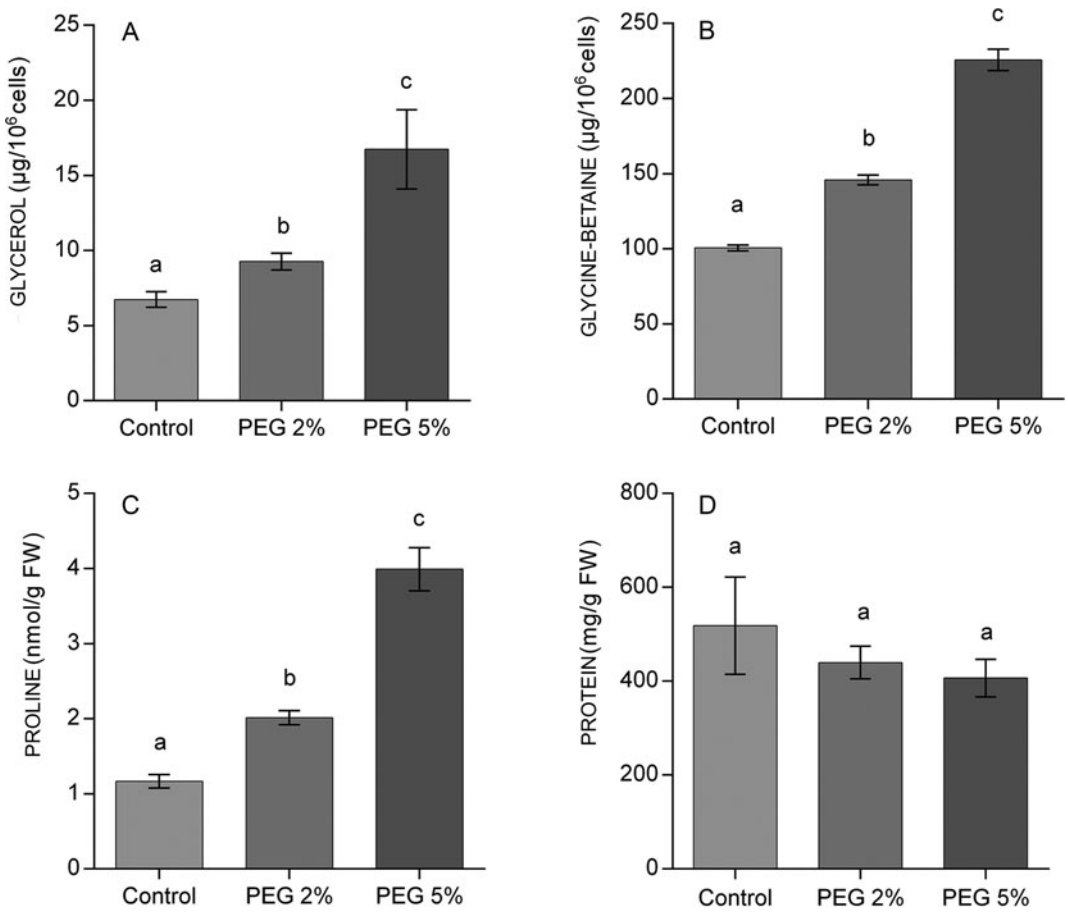

Fig. 3. The effect of drought stress on compatible solutes and protein production in *D. salina* cultured under different PEG 6000 concentrations (0, 2 and 5%). The amounts of glycerol (A), proline (B) and glycine betaine (C) significantly increased by exposing the algae to higher content of PEG. The content of protein (D) was not meaningfully different among the treatments. Different lower case letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level, Means ± SEs, N = 3.

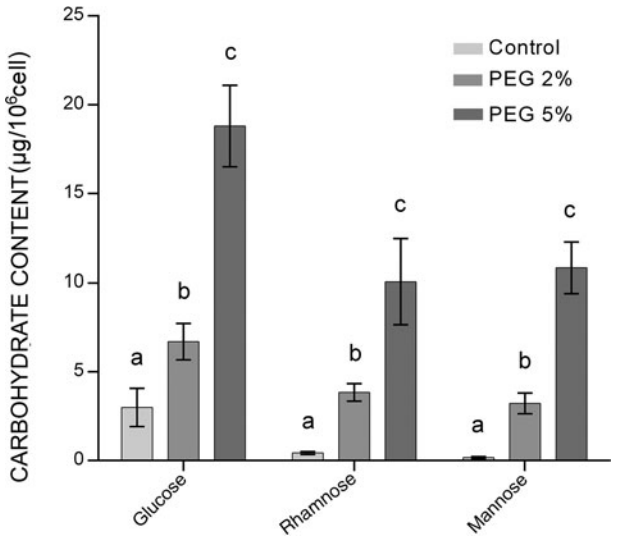

Fig. 4. The effect of drought stress on carbohydrates accumulation in *D. salina* cultured under different PEG 6000 concentrations (0, 2 and 5%). The amounts of glucose, rhamnose and mannose significantly were increased by exposing the algae to the higher content of PEG. Different lower case letters indicate significant difference between the means according to Fisher's LSD test at the $P < 0.01$ level, Means ± SEs, N = 3.

compared with the control. The algae exposed to 2% or 5% of PEG had approximately identical TI.

No meaningful difference was found in β-carotene content between the control and PEG treatments. Other results also indicated that the ratio of total chlorophyll/β-carotene increased by PEG treatments. However, there was no difference in the ratios between 2% or 5% PEG treatments.

DNA fragmentation

The use of DNA laddering evaluated the effect of PEG on nucleic acid fragmentation, as an indicator of programmed cell death (PCD). A single band from an intact genomic DNA of alga was observed at a size of ~20,000 base pairs (bp) in all DNA samples extracted from the control (Figure 7, lane 1) and PEG-treated cells (Figure 7, lanes 2 and 3). The absence of DNA laddering showed no damage to nucleic acids even at the high concentration of PEG.

Discussion

Although there are numerous reports focused on the defence mechanisms in response to drought stress in higher plants and some species of algae (Gechev *et al.*, 2013; Lin & Wu, 2014), to the best of our knowledge, there has been no study about physiological responses of the microalga *Dunaliella* to drought stress to date. Recently, Lee & Yeh (2014) evaluated the effects of PEG concentrations on cell growth, size variation and lipid production of microalgae, *Nannochloropsis oculata*. The simultaneous effects of salinity and PEG on *D. salina* growth have been previously studied by Kumar & Dharmaraj (2003); however, the effect solely of different concentrations of PEG on the growth and physiology of this alga remained under-investigated. The effect of different concentrations of PEG on *D. salina* growth was first studied by

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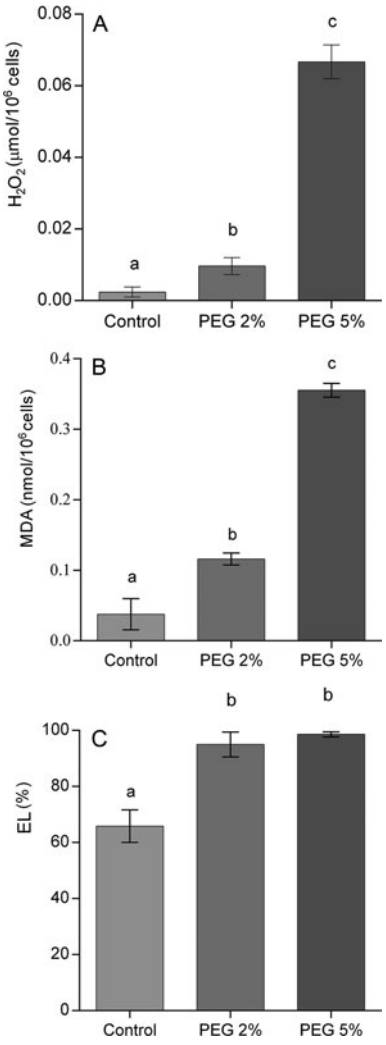

Fig. 5. The effect of drought stress on the amounts of H_2O_2 (A), malondialdehyde (MDA) (B) and electrolyte leakage (C) in *D. salina* cultured under different PEG 6000 concentrations (0, 2 and 5%). The amount of H_2O_2 and MDA dramatically increased after exposing the algae to the higher content of PEG, especially 5%. However, there was no significant difference in the electrolyte leakage among the treatments. Different lower case letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level, Means ± SEs, N = 3.

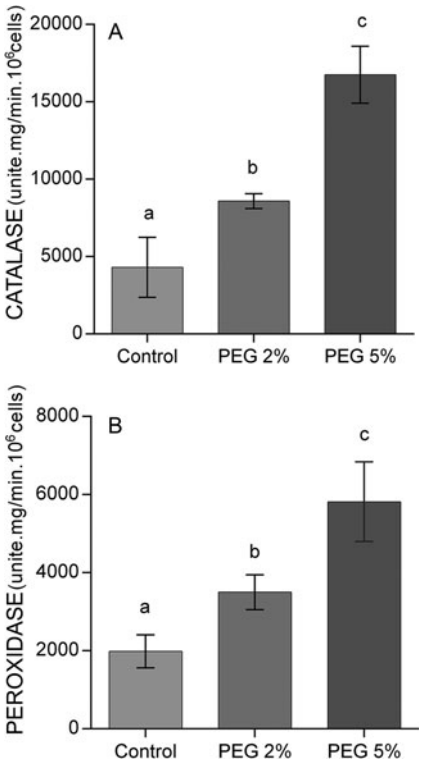

Fig. 6. The effect of drought stress on the enzymatic antioxidant systems in *D. salina* cultured under different PEG 6000 concentrations (0, 2 and 5%). The activities of catalase (CAT) (A) and ascorbate peroxidase (APX) (B). MDA were strongly increased after exposing the algae to the higher content of PEG, especially 5%. Different lower case letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level, Means ± SEs, N = 3.

measuring specific growth rate, doubling time, cell divisions per day and finally, the growth kinetic. Our overall results showed that although *D. salina* grew slightly better in 2% PEG than the control, the elevation of the PEG concentrations had adverse effects on cell growth. This was in accordance with the results obtained by Lee & Yeh (2014), which demonstrated that growth of the microalgae, *N. oculata*, could be facilitated only in PEG-6000 concentrations ≤0.5% (w/v). In the present study, PEG concentrations of more than 5% induced significant mortality rates and finally quickly reaching a plateau phase. Lin & Wu (2014) stated that the total inhibition in the growth of *Chlorella vulgaris* did not take place even at PEG concentrations of more than 40%. Other microalgae in the same study, including *Chlamydomonas reinhardtii*, *Klebsormidium flaccidum* and *Leptolyngbya boryana*, also continued to grow in PEG-6000 concentrations by more than 20%, 30% and 40%, respectively. The higher inhibition of algal growth under lower PEG concentrations in our study might be related to the effective formation of NaCl-PEG complexes in the culture medium. The surface adsorption of these complexes on the microalgal cell wall would lead to further complex formation caused by cross-linkages, which eventually might result in the production of cell clumping and the reduction of the speed indices of the cells (Chan *et al.*, 1981). *Dunaliella salina* cells grown in PEG doses higher than 2% had bigger cell sizes and the majority of them tend to be highly attached and formed larger cell clumps. The PEG tolerance of *D. salina* in this study was shown to be significantly lower compared with the microalgae tested in Lin & Wu's (2014) study, which might be related to the fact that a higher concentration of NaCl (1.5 M) used in our culture medium, strongly accelerated the effect of PEG by the formation of NaCl-PEG complexes, whereas the maximum concentration of NaCl in Lin and Wu's study did not exceed more than 200 mM. On the other hand, the high concentration of NaCl in combination with 5% PEG induced a massive total osmotic potential of −73.8 Mpa, while this maximum total value in Lin and Wu's study was only −2.7 Mpa.

In response to various stresses such as salt stress, drought stress, cold stress, heavy metal toxicity and UV or gamma radiation, cells tend to accumulate a considerable amount of compatible solutes like low-molecular-weight compounds such as proline, glycine betaine and glycerol as well as other kinds of sugars. Solutes accumulation is well known to be a species-dependent character, such that the resistant plants generally produce larger amounts of compatible compounds in response to stresses (Delauney *et al.*, 1993; Molinari *et al.*, 2007; Lin & Wu, 2014; Toghyani *et al.*, 2020). The compatible solutes could help osmotic and volume control and act as an osmoprotectant (Molinari *et al.*, 2007), a redox buffer (Hare & Cress, 1997), protecting enzymes and proteins against destruction (Matysik *et al.*, 2002), a regulator of cytosolic acidity (Venekemp, 1989), a remover of free radicals (Matysik *et al.*, 2002) and a membrane stabilizer (Lin & Wu, 2014). Additionally, solutes such as proline are considered to play an essential role in the defence mechanism

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Table 3. The effect of drought on the accumulation of pigments including chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and *β*-carotene (*β*-car) and the ratio of total chlorophylls (TChl) to *β*-carotene (TChl:*β*-car) in microalgae, *D. salina* cultured under different concentrations of PEG 6000. Tolerance Index (TI) also measured on the basis of chlorophylls content

	Chl *a*	Chl *b*	*β*-car	TChl:*β*-car	TI (%)
Control	0.846 ± 0/019[a]	0.191 ± 0/002[a]	0.348 ± 0/024[a]	2/986 ± 0.159[a]	—
PEG 2%	1.019 ± 0/028[b]	0.261 ± 0/030[b]	0.330 ± 0/022[a]	3/877 ± 0.443[b]	122.20
PEG 5%	1.027 ± 0/038[b]	0.250 ± 0/016[b]	0.316 ± 0/013[a]	4/128 ± 0.357[b]	122.40

The values shown are means followed by ± SD, N = 3. Different superscript letters indicate significant differences between the means according to Fisher's LSD test at the $P < 0.01$ level.

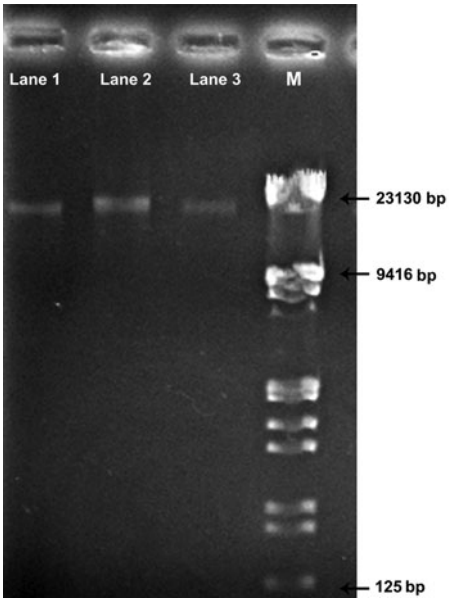

Fig. 7. The effect of drought stress on the DNA fragmentation in *D. salina* cultured under different PEG 6000 concentrations (0, 2 and 5%). M: Lambda DNA/HindIII (Thermo Scientific), lane 1: control, lane 2: algae treated with PEG 2%, lane 3: algae treated with PEG 5%.

of stressed cells by providing carbon, nitrogen and an energy source (Kuznetsov & Shevykova, 1999). The amounts of proline have been previously reported to increase in *D. salina* cells under salinities up to 5.5 M (Mishra *et al.*, 2008). According to our results, *D. salina* indicated a high ability to accumulate more intracellular proline contents in response to drought stress, which was highly correlated with the increased doses of PEG. This was compatible with the results of Delauney *et al.* (1993) and Molinari *et al.* (2007) for higher plants and Lin & Wu (2014) for soil algae and cyanobacteria. In the current study, it was also found that the proline accumulation co-occurred with a significant PEG-induced H_2O_2 generation, especially in 5% PEG-treated cells. This compound was produced 33-fold more than in untreated cells. The compatible solutes such as proline could protect proteins, especially enzymes, from denaturing under stress conditions (Anaraki *et al.*, 2018). In such a condition, the activity of antioxidant enzymes, CAT and APX, simultaneously rose with a positive correlation with H_2O_2 generation and proline accumulation. These enzymes seemingly act as scavenger and detoxifier of stress-generated ROS, such as hydrogen peroxide (Arora *et al.*, 2002) and have been demonstrated to protect the plant cells from programmed cell death (PCD), especially under stress conditions (Tattini *et al.*, 2009). In this study, the absence of DNA fragmentation in *D. salina* cells showed no occurrence of PCD under PEG treatment, which could be related to the significant activation of antioxidant enzymes of the alga.

Several species of marine algae have been reported to accumulate glycine betaine as a stabilizing osmolyte (Mishra *et al.*, 2008). In the present study, the amount of glycine betaine increased in *Dunaliella* cells in proportion to the amount of the PEG level. Mishra *et al.* (2008) reported a glycine betaine accumulation of about 5.7 µg per 10^5 cells during the culture of *D. salina* in salt concentrations up to 5.5 M. According to our results as the first report regarding the effect of drought stress on glycine betaine accumulation in *Dunaliella*, cells are grown under 1.5 M NaCl and 5% PEG contained about 22.6 µg per 10^5 cells glycine betaine, which suggests that glycine betaine contributes to PEG-induced stress. Glycine betaine reportedly functioning as an osmoprotectant caused a greater acclimation capacity of plants under drought stress. This osmolyte is mainly accumulated in chloroplasts and especially has essential roles in protecting the thylakoid membrane of chloroplasts; thus it might affect photosynthesis (Rogers *et al.*, 2009).

Glycerol production is considered as one of the most essential features of *Dunaliella* upon different stress conditions. A positive correlation was reported between glycerol production and salt concentration by Arun and Singh in *D. salina* and *D. tertiolecta*. They demonstrated that glycerol was induced in the cells with maximum values of 30.5 pg cell^{-1} for *D. salina* and 19.36 ± 0.13 pg cell^{-1} for *D. tertiolecta* achieved at 30% and 25% NaCl concentration, respectively. So, they concluded that under low concentration of salt (around 10%), negligible amounts of glycerol would be accumulated in *D. salina* with a mean value of about 5.46 pg cell^{-1} (Arun & Singh, 2013). In the present study, a similar level of salinity (8%) induced similar amounts of glycerol in *D. salina* in a PEG-free control with a a mean value of 6.74 pg cell^{-1}. However, when the cells exposed to 8% NaCl (1.5 M) were treated with 2% or 5% PEG, the mean amount of glycerol significantly increased to 9.27 or 16.74 pg cell^{-1}, respectively. This indicates that *D. salina* has a strong ability to adjust its intracellular osmotic potential when coping with high levels of PEG. Although this is the first report of importance of glycerol accumulation in *D. salina* upon drought stress, other studies have widely reported the higher accumulation of intracellular glycerol in *Dunaliella* as a significant mechanism for adjusting osmotic potential against abiotic stress including temperature, metal toxicity, nutrient depletion, as well as different ranges of salinity (Chitlaru & Pick, 1991; Phadwal & Singh, 2003; Mishra *et al.*, 2008). It was also indicated that glycerol acts as a 'compatible solute' to protect enzymes against inactivation or inhibition under stress conditions (Chitlaru & Pick, 1991). In addition to low-molecular solutes, for the first time, we reported that the levels of soluble sugars (glucose, mannose and rhamnose) were also elevated under PEG-induced drought conditions in *D. salina*. Mishra *et al.* (2008) previously reported that the total sugars significantly increased up to 3-fold when *D. salina* was exposed to high salinity stress (5.5 M NaCl). It has been concluded that the accumulation of soluble sugars in plant cells plays an essential role in response to various abiotic stresses during vegetative and reproductive development.

The alga, *D. salina* has a well-known ability to accumulate a large amount of *β*-carotene in the chloroplast (up to 14% of

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dry weight) to protect the photosynthetic pigments and chlorophylls against photo-damage, especially if the alga is exposed to extreme stress conditions (Aasen *et al*., 1969). It was previously indicated that *β*-carotene synthesis was significantly induced up to several fold by the various ranges of stresses (Phadwal & Singh, 2003; Hosseini Tafreshi & Shariati, 2008). Lin & Wu (2014) reported that during PEG treatment, some microalgae such as *C. vulgaris* and *L. boryana* accumulated a high amount of carotenoids; meanwhile, some other species had limited ability to synthesize additional carotenoids in such conditions. Carotenoids are known to effectively quench the singlet excited chlorophylls (1Chl*) and chlorophyll triplets. The excess excitation energy resulting from stress could finally be dissipated via the xanthophyll cycle (Das & Roychoudhury, 2014). The algal carotenoids such as astaxanthin have previously been recognized as the potent light-attenuating molecules that were able to weaken desiccation intensity (Oliver *et al*., 2020). The carotenoids are also known to have protective contributions to stabilizing the plasma membrane against membrane lipid peroxidation (Havaux, 1998). Our results showed that the PEG-induced drought did n't trigger *β*-carotene accumulation in *D. salina*. Therefore, the accumulation of carotenoids such as *β*-carotene probably plays no critical role in drought stress in this microalga, which could explain the high degree of lipid peroxidation and a high percentage of EL in *D. salina* under PEG treatment. Both the content of chlorophylls and the ratio of total chlorophyll/*β*-carotene increased in the cells treated with PEG. It has been previously shown that chlorophyll content is significantly induced by salinities of more than 3.0 M in *D. tertiolecta* and *D. salina* (Mishra *et al*., 2008). A similar tolerance index (TI) between the cells treated with 2% or 5% PEG showed a remarkable ability of *D. salina* to protect its pigments at a steady-state under a high degree of drought. Additionally, the protein content in PEG-treated cells remained relatively constant in response to stress and this might be correlated again with a high degree of *Dunaliella* tolerance to drought stress.

Conclusion

The present study demonstrated that the green microalga, *D. salina*, intrinsically employed various strategies during coping with drought stress, including increase of a range of osmolytes and stimulation of the activity of some antioxidant enzymes. A significant amount of osmolytes such as proline, glycerol, glycine betaine and soluble sugars accumulated in *Dunaliella* during drought stress which might show that these solutes acted as osmoprotectants to protect the enzymes and other proteins from stress-related injuries. This might be supported by a higher activity of antioxidant enzymes such as catalase and ascorbate peroxidase under PEG-induced drought compared with PEG-free conditions. The microalgae may also consume PEG to break it down and use it as an alternative source of carbon during stress which should be further studied in detail. On the other hand, it was concluded from our study that in contrast to salinity stress, PEG-induced drought did not stimulate a higher carotenoid accumulation in *D. salina*. Therefore, the responsive mechanisms against these two stressors at least in part may be different in this microalga. Overall, the present study provided an insight into how eukaryotic algal cells can tolerate high intensities of drought stress. Future investigations could be directed towards a full consideration of protective mechanisms of microalgae against drought stress at the genomics, proteomics and metabolomics levels. This may help us to discover gene or protein candidates with potential of reducing the negative effect of drought on eukaryotic cells.

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Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Aasen AJ, Eimhjellen KE and Liaaen-Jensen S (1969) An extreme source of *β*-carotene. *Acta Chemica Scandinavica* **23**, 2544–2545.

Aebi HE (1983) Catalase in vitro. In Keating C (ed.), *Methods in Enzymology*. Amsterdam: Elsevier, pp. 121–126.

Anaraki ZE, Hosseini Tafreshi SA and Shariati M (2018) Transient silencing of heat shock proteins showed remarkable roles for HSP70 during adaptation to stress in plants. *Environmental and Experimental Botany* **155**, 142–157.

Arora A, Sairam RK and Srivastava GC (2002) Oxidative stress and antioxidative system in plants. *Current Science* **82**, 1227–1238.

Arun N and Singh DP (2013) Differential response of *Dunaliella salina* and *Dunaliella tertiolecta* isolated from brines of Sambhar Salt Lake of Rajasthan (India) to salinities: a study on growth, pigment and glycerol synthesis. *Journal of the Marine Biological Association of India* **55**, 65–70.

Bates L, Waldren RP and Teare ID (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205–207.

Belghith T, Feki AE, Athmouni K, Ayadi H and Bellassoued K (2016) Physiological and biochemical response of *Dunaliella salina* to cadmium pollution. *Journal of Applied Phycology* **28**, 991–999.

Bewley JD (1979) Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiology* **30**, 195–238.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Bremer E and Krämer R (2019) Responses of microorganisms to osmotic stress. *Annual Review of Microbiology* **73**, 313–34.

Chan K, Wong KH and Ng SL (1981) Effects of polyethylene glycol on growth and cadmium accumulation of *Chlorella salina* CU-I. *Chemosphere* **10**, 985–991.

Chen H, Lao YM and Jiang JG (2011) Effects of salinities on the gene expression of a (NAD^+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. *Science of the Total Environment* **409**, 1291–1297.

Chitlaru E and Pick U (1991) Regulation of glycerol synthesis in response to osmotic changes in *Dunaliella*. *Plant Physiology* **96**, 50–60.

Cowan AK, Rose PD and Horne LG (1992) *Dunaliella salina*: a model system for studying the response of plant cells to stress. *Journal of Experimental Botany* **43**, 1535–1547.

Das K and Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers of Environmental Science & Engineering* **2**, 1–13.

Davis JS (1972) Survival records in the algae, and the survival role of certain algal pigments, fats and mucilaginous substances. *Biologist (Columbus, Ohio)* **54**, 52–93.

Delauney AJ, Hu CAA, Kavi Kishor PB and Verma DPS (1993) Cloning of ornithine d-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. *Journal of Biological Chemistry* **268**, 18673–18678.

Dionisio-Sese ML and Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Science Journal* **135**, 1–9.

Eijckelhoff C and Dekker JP (1997) A routine method to determine the chlorophyll a, pheophytin a and carotene contents of isolated Photosystem II reaction center complexes. *Photosynthesis Research* **52**, 69–73.

Gechev TS, Dinakar C, Benina M, Toneva V and Bartels D (2013) Molecular mechanisms of desiccation tolerance in resurrection plants. *Cellular and Molecular Life Sciences* **70**, 689–709.

Hare PD and Cress WA (1997) Metabolic implications of stress induced proline accumulation in plants. *Plant Growth Regulation* **21**, 79–102.

Havaux M (1998) Carotenoids as membrane stabilizers in chloroplasts. *Trends in Plant Science* **3**, 147–151.

Heath RL and Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* **125**, 189–198.

https://doi.org/10.1017/S0025315420000971 Published online by Cambridge University Press

Holzinger A and Karsten U (2013) Desiccation stress and tolerance in green algae: consequences for ultrastructure, physiological, and molecular mechanisms. *Frontiers of Plant Science* **4**, 1–18.

Hosseini Tafreshi SA and Shariati M (2008) *Dunaliella* biotechnology: methods and applications. *Journal of Applied Microbiology* **107**, 14–35.

Johnson MK, Johnson EJ, McElrory RD, Speer HL and Bruff BS (1968) Effects of salt on the halophilic alga *Dunaliella viridis*. *Journal of Bacteriology Research* **95**, 1461–1468.

Kirst GO (1990) Salinity tolerance of eukaryotic marine algae. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 21–53.

Kochert G (1978) Carbohydrate determination by the phenol-sulfuric acid method. In Helebust JA and Craigie JS (eds), *Handbook of Physiological Methods – Physiological and Biochemical Methods*. Cambridge: Cambridge University Press, pp. 96–97.

Kohl KI and Losch R (2004) Experimental characterisation of metal tolerance. In Prasad MNV (ed.), *Heavy Metal Stress in Plants: From Biomolecules to Ecosystem*, 2nd Edn. Berlin: Springer-Verlag, pp. 434–454.

Kranner I, Beckett R, Hochman A and Nash TH (2008) Desiccation-tolerance in lichens: a review. *Bryologist* **111**, 576–593.

Kumar PS and Dharmaraj S (2003) Studies on the growth of the marine microalga *Dunaliella salina* (Teodoresco). *Indian Journal of Fisheries* **50**, 259–262.

Kuznetsov VI and Shevykova NI (1999) Proline under stress: biological role, metabolism, and regulation. *Russian Journal of Plant Physiology* **46**, 274–287.

Lee YH and Yeh YL (2014) Using polyethylene glycol as nonionic osmoticum to promote growth and lipid production of marine microalgae *Nannochloropsis oculata*. *Bioprocess and Biosystems Engineering* **37**, 1669–1677.

Levasseur M, Thompson PA and Harrison PJ (1993) Physiological acclimation of marine-phytoplankton to different nitrogen-sources. *Journal of Phycology* **29**, 587–595.

Li JF, Li L and Sheen J (2010) Protocol: a rapid and economical procedure for purification of plasmid or plant DNA with diverse applications in plant biology. *Plant Methods* **6**, 1–8.

Lin CS and Wu JT (2014) Tolerance of soil algae and cyanobacteria to drought stress. *Journal of Phycology* **50**, 131–139.

Matysik J, Alia A, Bhalu B and Mohanty P (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Current Science* **82**, 525–532.

Michel B and Kaufmann M (1973) The osmotic potential of polyethylene glycol 6000. *Plant Physiology* **51**, 914–916.

Mishra A, Mandoli A and Jha B (2008) Physiological characterization and stress-induced metabolic responses of *Dunaliella salina* isolated from salt pan. *Journal of Industrial Microbiology & Biotechnology* **35**, 1093–1101.

Molinari HBC, Marur CJ, Daros E, De Campos MKF, Carvalho JFRP, Filho JCB, Pereira LFP and Vieira LGE (2007) Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. *Physiologia Plantarum* **130**, 218–229.

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

Oliver M, Farrant JM, Hilhorst HWM, Mundree S, Williams B and Bewley JD (2020) Desiccation tolerance: avoiding cellular damage during drying and rehydration. *Annual Review of Plant Biology* **71**, 435–460.

Phadwal K and Singh PK (2003) Effect of nutrient depletion on β carotene and glycerol accumulation in two strains of *Dunaliella* sp. *Bioresource Technology* **90**, 55–58.

Rogers ME, Colmer TD, Frost K, Henry D, Cornwall D, Hulm E, Hughers SR, Nichols PGH and Craig AD (2009) The influence of NaCl salinity and hypoxia on aspects of growth in *Trifolium* species. *Crop and Pasture Science* **60**, 71–82.

Sairam RK and Srivastava GC (2002) Changes in antioxidant activity in subcellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. *Plant Science* **162**, 897–904.

Scheibe R and Beck E (2011) Drought, desiccation, and oxidative stress. In Lüttge U (ed.), *Plant Desiccation Tolerance*. Heidelberg: Springer, pp. 209–232.

Sergiev I, Alxieva V and Karanov E (1997) Effect of spermone, atrazine and combination between them on some endogenous protective systems and stress markers in plants. *Comptes Rendus de l'Academie Bulgare des Sciences* **51**, 121–124.

Tardieu T, Simonneau T and Muller B (2018) The physiological basis of drought tolerance in crop plants: a scenario-dependent probabilistic approach. *Annual Review of Plant Biology* **69**, 733–759.

Tattini M, Traversi ML, Castelli S, Biricolti S, Guidi L and Massai R (2009) Contrasting response mechanisms to root-zone salinity in three co-occurring Mediterranean woody evergreens: a physiological and biochemical study. *Functional Plant Biology* **36**, 551–563.

Toghyani MA, Karimi F, Hosseini Tafreshi SA and Talei D (2020) Two distinct time dependent strategic mechanisms used by *Chlorella vulgaris* in response to gamma radiation. *Journal of Applied Phycology* **32**, 1677–1695.

Venekemp JH (1989) Regulation of cytosol acidity in plants under conditions of drought. *Physiologia Plantarum* **76**, 112–7.

Yilancioglu K, Cokol M, Pastirmaci I, Erman B and Cetiner S (2014) Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. *PLoS ONE* **9**, e91957.

https://doi.org/10.1017/S0025315420000971 Published online by Cambridge University Press