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## The Effect of Low Light Intensities on Oxidative Stress Induced by Short-term Chilling in *Dunaliella salina* Teod

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**Abstract:** Under circumstances where electron transport is restricted, such under low temperature condition, oxidative stress may occur even at optimal or low-light intensities. Short-term-effects of light intensities (20 or 100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ), on the levels of 6 enzymatic, two nonenzymatic antioxidants, chl a, chl b, total carotenoid and  $\beta$ -carotene, on the antioxidant response of *Dunaliella salina* under cold temperature (13°C) were quantified after 24 h stress treatments. The activity of superoxide dismutase (SOD) increased, under 13°C/100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ , whereas ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), superoxide dismutase and pyrogallol peroxidase activities were induced under 13°C/20  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ . The cells exhibited an increase in reduced ascorbate and reduced glutathione (GSH) coincident with a marked increase in oxidized glutathione (GSSG), at 13°C/100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ . There were no marked changes in ascorbate or glutathione pools at 13°C/20  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ , which are similar to those at 28°C/100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ . Chlorophylls and carotenoids reduction were also observed under chilling treatments, which were more reduced by the higher light intensity (13°C/100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ). The results of present study indicated various antioxidants responds to different combinations of chilling and low light intensities, in *D. salina*. These responses are very sensitive to small increase in the light intensity.

**Key words:** Antioxidant response, *Dunaliella salina*, low light, Photon Flux Densities (PFDs), photooxidative stress, short-term chilling

### INTRODUCTION

There are many reports that excess irradiance alone (Hideg, 1996; Baroli and Melis, 1996; Herrmann *et al.*, 1997) or in combination with chilling (Wise, 1995) can cause photooxidative stress. But oxidative stress may happen under moderate to low photon flux densities (PFDs) under chilling. Low temperature caused a decrease in the quantum efficiency of PSII, the activity of PSI, the activation of some of carbon fixation enzymes and also a decline in ATP synthase (Sonoike, 1998; Allen and Ort, 2001). Therefore, light intensities that would not cause injury under physiological conditions may appear as excess irradiations at low temperature (Hideg, 1996).

There is evidence, that  $\text{O}_2$  dependent PSI photoinactivation is most evident under chilling at low to moderate PFDs, because during chilling at high PFD, PSII inactivation should protect PSI (Sonoike, 1996).

The halotolerant green alga, *Dunaliella*, has received much attention as an ideal organism and a model system (Cowan *et al.*, 1992) in physiological researches. This algae has high tolerance under changing environmental factors (Jiménez and Niell, 1990) and an ability for  $\beta$ -carotene accumulation under stress

conditions (Grobbelar, 1995; Jahnke, 1999; Mendoza *et al.*, 1999). There is some data about *Dunaliella* response to high irradiance (Lers *et al.*, 1990) and to low temperature, in relation to carotenoids (Jiménez and Niell, 1991; Cowan *et al.*, 1992; Ben-Amotz, 1996; Mendoza *et al.*, 1996). But there appears to be no investigation about ROS and the induction of other antioxidant components (enzymatic and nonenzymatic) in response to chilling particularly under low PFDs in this alga. Therefore the aim of this research was, to study the short-term effect of cold stress on the response of antioxidant components of *Dunaliella salina* at low light intensities and to investigate the possible differences between antioxidant responses to various low PFDs.

### MATERIALS AND METHODS

#### Cultures, experimental set up for growth and stress conditions:

The algal material used for this experiment was a strain of *Dunaliella salina* (Teoredesco) Gh-U, according to Preisig (1992), obtained from Biotechnology Research Center of Gheshm Island. The experiment was accomplished during years 2004 to 2005 in the school of biosciences, University of Exeter, UK and in the Biology Department of the University of Isfahan, Iran. The

cultures were grown in a Johnson medium (Johnson *et al.*, 1968), modified according to Shariati and Lilley (1994). The alga were aseptically inoculated as pure suspended cultures into 250 mL Erlenmeyer flasks, containing 100 mL of fresh medium, to give  $\sim 24 \times 10^4$  cells per mL (using hemocytometer). All cultures were placed in an incubator at a photon flux density (PFD) of  $90\text{-}100 \mu\text{mol m}^{-2} \text{sec}^{-1}$  provided by cool-white fluorescent lamps, under a 16 h light ( $28 \pm 0.5^\circ\text{C}$ )/8 h dark ( $28 \pm 0.5^\circ\text{C}$ ) cycle with shaking at 96 rpm. Cultures were harvested in the exponential growth phase (referred as unstressed control, initial hour of stress) and transferred to the following continuous light and temperature conditions:  $13^\circ\text{C}/100 \mu\text{mol m}^{-2} \text{sec}^{-1}$  (13/100),  $28^\circ\text{C}/100 \mu\text{mol m}^{-2} \text{sec}^{-1}$  (28/100, referred as stressed control) and  $13^\circ\text{C}/20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  (13/20) for 24 h. The last condition was designed to have a combination of chilling and lower PFD, to compare with the combination of low temperature and the higher PFD (13/100).

For all enzyme and substrate assays, 40 mL aliquots of cultures were sampled at initial hour and 24 h after transfer to the temperature and light treatments and used to measure ascorbate and dehydroascorbate (DHA), GSH and GSSG, the enzymatic activities of APX, MDHAR, DHAR, GR, SOD, total peroxidase and pigment contents including chlorophyll a (Chl a), Chl b, total carotenoid and  $\beta$ -carotene. Three replicate samples were taken for each assay.

**Enzyme and substrates extractions:** Forty milliliter of cultures were pelleted at 1500 g (SANYO MSE MISTRAL 3000i) for 10 min at  $13^\circ\text{C}$ . The pellet was resuspended in a small volume of its supernatant and transferred to preweighed 1 mL microcentrifuge tube and re-centrifuged at 12000 g for 3 min. After calculating the pellet weight, it was resuspended in either extraction solution A or B. Solution A and B were prepared according to the methods of Smirnov and Colombé (1988) and Baker *et al.* (1990), respectively.

**Enzyme determinations:** The supernatant prepared as described earlier, was used to measure APX, MDHAR and GR activities (Smirnov and Colombé, 1988), DHAR activity (Nakano and Asada, 1981) and total peroxidase activity was measured by  $\text{H}_2\text{O}_2$  dependent pyrogallol (Groppa *et al.*, 1999). SOD activity was measured according to Ukeda *et al.* (1997). All enzymes were assayed at  $24 \pm 1^\circ\text{C}$ .

**Ascorbate/DHA and GSH/GSSG determinations:** Cell pellets suspended in extraction medium B and stored at  $-80^\circ\text{C}$ , were thawed for 2 or 3 min and centrifuged at 12000 g for 5 min. Total ascorbate and reduced ascorbate, were determined based on the method of Kampfenkel *et al.* (1995). Changes in absorbance were

monitored at 520 nm for total ascorbate and reduced ascorbate separately with a microplate reader (VERSA, S/NB 02963). DHA content is estimated from the difference between assays with and without NEM. Part of the same supernatant was used for glutathione assay according to the method of Baker *et al.* (1990). Changes in absorbance followed for 2 min, with the mentioned recording microplate reader at 415 nm. Reduced glutathione is estimated from the difference between total glutathione and oxidized form.

**Pigment assay:** Pigments (Chl a, Chl b and also  $\beta$ -carotene) were measured spectrophotometrically (Shimadzu, UV-160) based on the method of Eijkelhoff and Dekker (1997). Total carotenoid was quantified according to Lichtenthaler and Buschmann (2001) equation Camejo *et al.* (2005).

**Statistical analysis:** Statistical comparisons were made using ANOVA with Tukey multiple comparisons and paired-samples T test ( $p < 0.001, 0.01$  and  $0.05$ ).

## RESULTS

**Antioxidant enzyme responses:** Enzyme activities were influenced by the low temperature treatments in *Dunaliella* cells during 24 h. In Fig. 1A, the initial activities of total peroxidase, APX, MDHAR, DHAR, GR and in Fig. 1B, the activity of SOD, at initial hour of stress, initial values (before the stress treatments) shown in Fig. 1A and B. Compared to unstressed control, 13/100 condition caused an increase about 220% in SOD activity (Table 1), indicating that SOD is particularly responsive to low temperature under this light intensity. A very small increase observed in DHAR activity under the above condition, compared to unstressed control. However there appears to be no induction in the activity of APX,

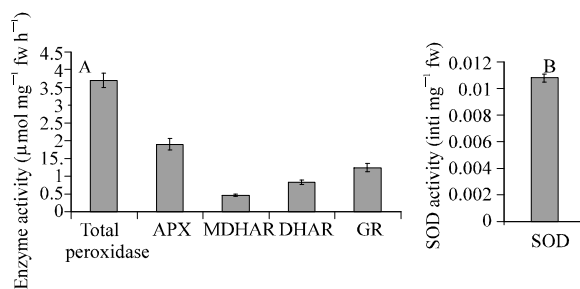


Fig. 1: Initial enzyme levels in *D. salina* (Gh-U), before stress (0 h) at  $28 \pm 0.5^\circ\text{C}$ , 16 h light/8 h dark cycle. (A) Total peroxidase, APX (ascorbate peroxidase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase) and GR (glutathione reductase); (B) SOD (superoxid dismutase). Data for all are mean  $\pm$  SEM from fifteen independent experiments

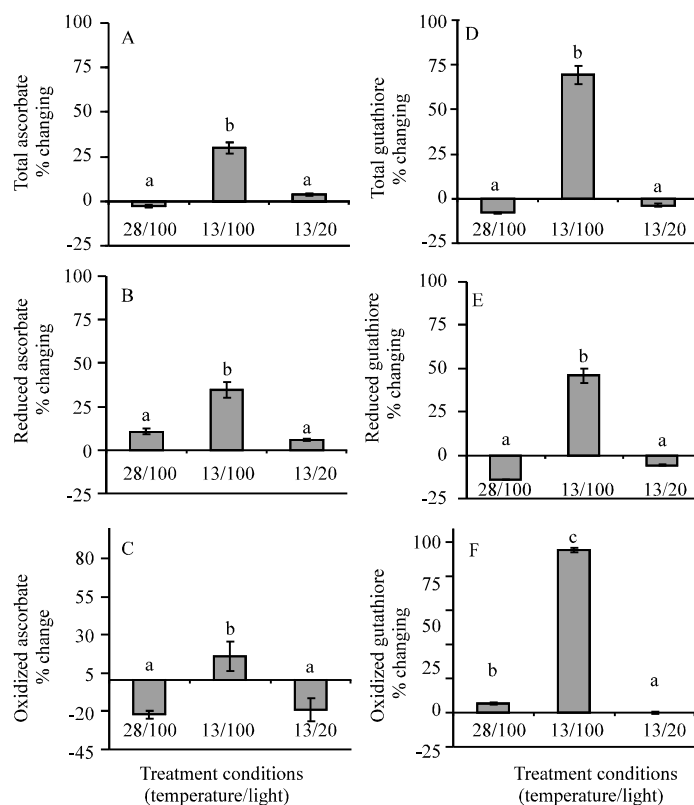


Fig. 2: Changes of ascorbate and glutathione in *D. salina* (Gh-U), after 24 h under temperature/light treatment conditions: 28°C/100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ , 13°C/100 and 13°C/20  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ . The data are expressed as % change from the initial values (before stress at 28±0.5°C, 16 h light/8 h dark cycle); (A) Total ascorbate, 100% = 5.18±0.26 nmol mg<sup>-1</sup> f.wt; (B) Reduced ascorbate, 100% = 3.82±0.36 nmol mg<sup>-1</sup> f.wt; (C) Oxidized ascorbate, 100% = 1.28±0.55 nmol mg<sup>-1</sup> f.wt; (D) Total glutathione, 100% = 1.09±0.29 nmol mg<sup>-1</sup> f.wt; (E) Reduced glutathione, 100% = 0.71±0.21 nmol mg<sup>-1</sup> f.wt and (F) Oxidized glutathione, 0.36±0.09 nmol mg<sup>-1</sup> f.wt. Results are mean±SEM from three independent experiments. Different letters indicate significant differences among three stress conditions at p<0.05 (according to Tukey test)

Table 1: Changes of antioxidant enzyme activities; APX (ascorbate peroxidase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase) and GR (glutathione reductase), SOD (superoxid dismutase) and total peroxidase, as % changing on control in *D. salina*, after 24 h under 28°C/100  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , 13°C/100 and 13°C/20  $\mu\text{mol m}^{-2}\text{s}^{-1}$  treatment conditions

Temperature/light (°C $\mu\text{mol m}^{-2}\text{sec}^{-1}$ )	Treatment conditions		
	(28/100)	(13/100)	(13/20)
APX	-33.21±3.76 <sup>a</sup>	-12.20±0.98 <sup>b</sup>	27.74±4.65 <sup>c</sup>
MDHAR	-16.93±3.76 <sup>b</sup>	-49.44±10.96 <sup>a</sup>	-28.88±0.97 <sup>ab</sup>
DHAR	-41.57±4.79 <sup>a</sup>	10.02±5.19 <sup>b</sup>	11.19±1.14 <sup>b</sup>
GR	-15.71±1.17 <sup>a</sup>	1.85±1.42 <sup>b</sup>	-1.77±0.73 <sup>b</sup>
SOD	79.76±4.72 <sup>a</sup>	221.32±6.41 <sup>b</sup>	240.79±7.72 <sup>b</sup>
Total peroxidase	-33.29±1.59 <sup>a</sup>	5.12±3.52 <sup>b</sup>	46.48±4.88 <sup>c</sup>

Values are mean±SEM of three independent experiments. Different letters indicate significant differences at p<0.05 (according to Tukey test) among three stress conditions for each enzyme

MDHAR, GR and total peroxidase, under the above condition (13/100), nevertheless these enzymes showed

a reduced decline in the activity than those in 28/100. Some decreases in enzyme activities under 28/100, might be caused by transfer from light/dark cycles to continuous light for 24 h. Low temperature in combination with a lower PFD (13/20), induced the activity of APX (nearly 27%), DHAR (>10%), SOD (about 240%) and total peroxidase (>40%) in comparing with initial hour of stress.

Overall, these results suggest that, the enzyme activities were affected under low temperature treatment, but it seems that these activities were more induced and less declined under 13/20 compared with 13/100.

**Ascorbate/DHA and GSH/GSSG couples under stress treatment conditions:**

Low temperature also affected ascorbate and glutathione pool sizes at 100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  (Fig. 2A and D). Reduced ascorbate and glutathione

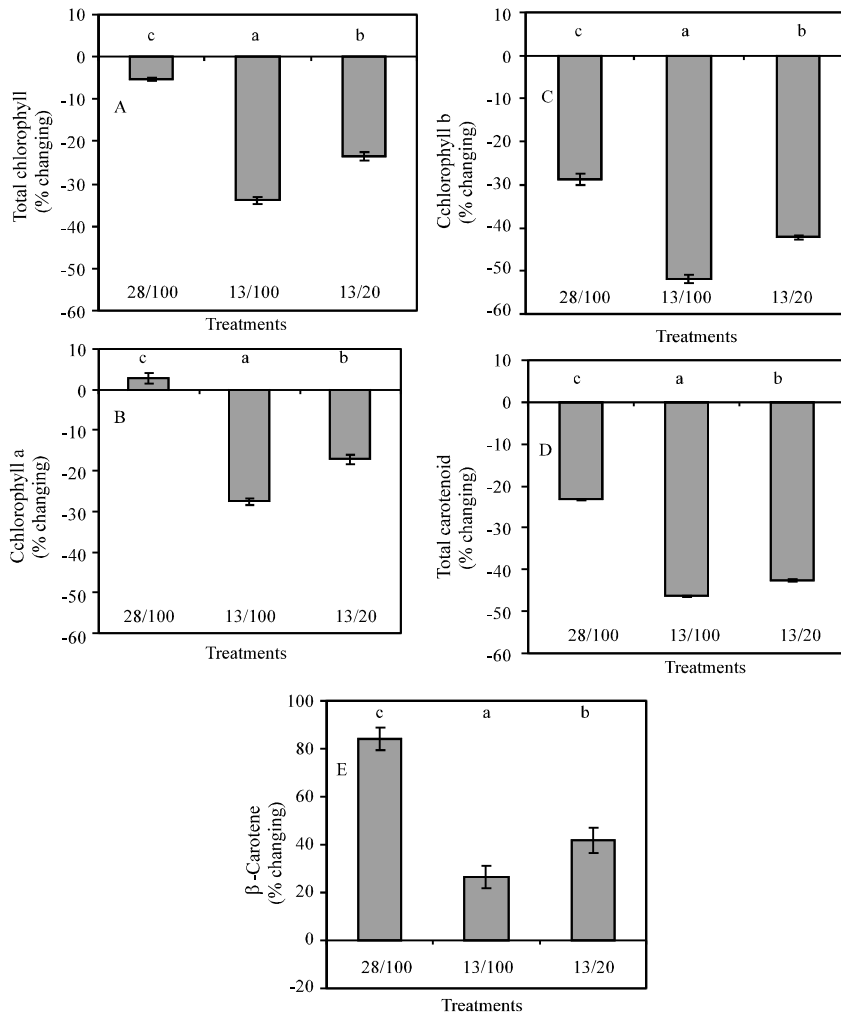


Fig. 3: Changes of pigments in *D. salina*, after 24 h under designed temperature/light treatments: 28°C/100  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ , 13°C/100  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  and 13°C/20  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . The data are expressed as % change from the initial values (before stress at 28±0.5°C, 16 h light/8 h dark cycle); (A) Total chlorophyll, 100% = 3.71  $\mu\text{g mg}^{-1}$  f wt; (B) Chlorophyll a, 100% = 2.75  $\mu\text{g mg}^{-1}$  f. wt; (C) Chlorophyll b, 100% = 0.97  $\mu\text{g mg}^{-1}$  f wt; (D) Total carotenoid, 100% = 1.54  $\mu\text{g mg}^{-1}$  f.wt and (E)  $\beta$ -carotene 100% = 0.68  $\mu\text{g mg}^{-1}$  f.wt. Results are mean±SEM. from three independent experiments. Different letters indicate significant differences among three stress conditions at  $p < 0.05$  (according to Tukey test).

exhibited an increase under 13/100 condition (Fig. 2B and E). However there was no significant increase in DHA under 13/100 (Fig. 2C), but GSSG was strongly (~120%) increased by this treatment condition (Fig. 2F). There were no marked changes in ascorbate and glutathione contents between 28/100 and 13/30 conditions (Fig. 2A-F).

The results show that, higher PFD (100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at low temperature caused a stronger elevation in ascorbate and glutathione pools size. The lower PFD (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at low temperature, showed a similar effect to 28/100 condition.

**The changes in chlorophyll and carotenoid contents under stress conditions:** The low temperature treatments

resulted in chlorophyll reduction, but chilling at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, caused a greater reduction in total Chl, Chl a, and Chl b compared to 13/20 condition (Fig. 3A-C). The changes in total carotenoids, showed the same pattern as Chlorophylls (Fig. 3D).  $\beta$ -carotene had also a smaller concentration under both low temperature treatment conditions (13/100 and 13/20, Fig. 3E), compared to 28/100. Such reduction in photosynthetic pigments were much stronger in 13/100 condition. Generally, the above results suggest that, photosynthetic pigments at low temperature is very sensitive to increasing in the light intensity in *Dunaliella*.

## DISCUSSION

PFD response curve (0 - 2000  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ) (of net photosynthesis ( $\text{O}_2$  evolution) in *D. salina* strain Gh-U (data not shown), indicated that light intensities required to saturate photosynthesis (saturation irradiance) is about 400-500  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ .

Therefore the experiment was designed to determine whether low light intensities, much lower than those needed for photosynthesis saturation (20 and 100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ) produce oxidative stress in chilled *Dunaliella* cells and if this, even at low light irradiances, depends on the intensity of light. Another aim was to determine which selected antioxidant components are particularly responsive to stress under designed conditions.

In our experiments, significant induction of APX and other peroxidases concomitant with increased SOD and DHAR activities were observed in short-term chilled cells at 20  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  light intensity (13/20).

Ascorbate/DHA and GSH/GSSG are involved in ROS scavenging. According to the results of Streb *et al.* (1999), ascorbic acid increased at low temperature in chilling-tolerant cereal leaves but it decreased in chilling-sensitive maize (Leipner *et al.*, 2000). Increases in glutathione content also improved chilling tolerant in mung bean (Yu *et al.*, 2002). Present results showed that both ascorbate and glutathione pools in *Dunaliella* cells were increasingly affected by the 13/100 condition, during 24 h, which was not observed at 13/20 and 28/100 treatment conditions. It should be noted that cell physiological condition in lower PFD/chilling (13/20) may be different from the condition at a higher PFD/chilling (13/100), which in combination, may result in a greater imbalance between light energy absorption and utilization via  $\text{CO}_2$  assimilation. Under 13/20 condition, most quantified antioxidant enzymes were induced in the activity and no marked increases in DHA or GSSG were observed under this condition during 24 h, whereas increased GSSG and the reduction of enzyme activities appeared under 13/100, along with the higher chlorophylls and carotenoids reduction. These findings are nearly similar to Wise and Naylor (1987) results in which, under low temperature ( $5^\circ\text{C}$ ) and light intensity (1000  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ) in cucumber, a dramatic increase in GSSG along with chlorophylls, bulk xanthophylls and  $\beta$ -carotene degradation, were observed during initial h of stress. However a decrease in total glutathione was also observed in chilled and light treated cucumber, that there was not in *Dunaliella* cells at 13/100 condition, in our experiment. Chl degradation should have physiological importance in protecting cells from the oxidative damage due to the reactive species of oxygen. Kudoh and Sonoike (2002)

showed that the bleaching of *Cucumis sativus* leaves was happen during the recovery period of both chilling/growth light or weak light. This was caused by the degradation of PSI complexes (which bind approximately a half of chlorophylls) along with chlorophyll molecules. They implicated that, chlorophyll content does not decrease during chilling treatment where the activity of proteases or other enzymes is low. In contrast, in our experiment chlorophyll degradation was occurred during chilling and the extent of it was affected by the photon flux densities during treatment which was higher at 13/100, suggesting that this fast response during 24 h, might limit excited energy on photosystems to prevent damage cells, efficiently. This response is also reported about the LHCI mRNA in *Chlamydomonas reinhardtii* transferred of 26 to 18°C (Teramoto *et al.*, 2002).

Carotenoids (xanthophylls and carotenes), as non-photochemical quenchers, at low temperature could also be involved in anti-oxidative defense. However the importance of increased non-photochemical energy dissipation in the recovery of *D. salina* (Król *et al.*, 1997) and also *Zea mays* plants grown under chilling stress has been reported (Haldimann *et al.*, 1995), but it seems that a short-term chilling has a different effect on carotenoid content. Carotenoids reduction under low temperature treatments (13/20 and 13/100), indicated the impaired energy-dissipating system by the short-term chilling.

In conclusion, we found that, the light intensity, even at low PFDs, could be resulted in oxidative stress in chilled *Dunaliella* cells. Interestingly, small increases in light intensity from 20 to 100  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ , seems cause significant differences in antioxidative response in *D. salina*, during short term chilling. SOD level concomitant with the other enzymes was induced under lower PFD in chilled cells. This enzyme also along with ascorbate and glutathione contents, was responsive to oxidative stress under chilling at higher PFD,

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