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ORIGINAL ARTICLE

Evaluation of radical scavenging system in two microalgae in response to interactive stresses of UV-B radiation and nitrogen starvation

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Abstract The effects of UV-B radiation and/or deprivation of nitrogen stresses on growth rate, some antioxidant compounds, and activities of some antioxidant enzymes, superoxide dismutase (SOD; EC1.15.1.1), ascorbate peroxidase (APx; EC1.11.1.11), guaiacol peroxidase (GUPx; EC1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2), as well as the levels of total glutathione pool, UV-B absorbing pigments, malondialdehyde (MDA) and H_2O_2 concentrations were studied in *Spirulina platensis* and *Dunaliella salina*. Less damage was observed in response to the combined UV-B and nitrogen deprivation as shown by growth rate and photosynthetic pigments especially in *Dunaliella salina*. A significant increase in flavonoids and phenolics under dual stress was observed. Conversely, a great reduction in malondialdehyde (MDA) and H_2O_2 concentrations were recorded under the combined stress compared to the effect of each stress. Furthermore, a significant increase in GSH/GSSG ratio toward the control was recorded in response to combined stresses, whereas a significant reduction in this ratio was observed in both microalgae in response to each stress. Increased activities of antioxidant enzymes were recorded under UV-B and nitrogen deprivation stresses.

1. Introduction

Several studies proved that the availability of non-mineral micro and macronutrients greatly affect the biochemical composition of microalgae (Visviki and Santikul, 2000; Fábregas et al., 2004). Nitrogen is considered to be the most important key nutrient and its availability significantly affect algal growth and its metabolism. For instances, nitrogen is the main component of functional and structural proteins and accounts 7–20% of cell dry weight (Hu, 2004). Moreover, phosphorus is the main constituents of the most important energy molecule ATP which is involved in the most biochemical reactions in the living cells. Limitation of nitrogen in growth culture of cyanobacteria as well as red algae resulted in the degradation

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of phycobilisomes which is considered as the antenna of light harvesting in photosystem II (Collier and Grossman, 1992). Gordillo et al. (1998) exposed *Spiriluna platensis* cells to nitrogen limitation and they found a great reduction in photosynthesis and disruption in the enzyme balance of cells as a result chlorophyll synthesis was drastically affected and lipid content was increased significantly. Nitrogen limitation also enhance the accumulation of carotenoids and astaxanthin in addition to their acyl-esters in *Dunaliella* sp. and *Haematococcus pluvialis* as clarified by Borowitzka et al. (1991). Zhekisheva et al. (2002) stated that when *Haematococcus pluvialis* was exposed to nitrogen depletion, the ratio of produced fatty acids/astaxanthin increased to five. Consequently, the oil globules formed from astaxanthin esters could have resulted from the production of the oleic acid and the esterification of the astaxanthin. Under normal conditions, active oxygen species (AOS) are competently excluded by non-enzymatic and enzymatic antioxidants, whereas during cumulative stress conditions the production of AOS exceeds the capacity of the antioxidative systems to remove them, oxidative stress (Martindale and Holbrook, 2002; Dickinson and Chang, 2011). The antioxidant constituents includes ascorbic acid and glutathione, the two major constituents of the antioxidant ascorbate–glutathione cycle, which detoxify H_2O_2 in the chloroplasts (Asada, 1999).

The orchestrated action of antioxidant enzymes; superoxide dismutase (SOD; EC1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (GPX; EC1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2), that operate in the chloroplast and cytosol, are able to control the cellular concentrations of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), thereby preventing the formation of reactive radicals (Noctor and Foyer, 1998; Zhekisheva et al., 2002. Higher glutathione content was observed under UV Stress in several works (Dash and Mohanty, 2002; Martindale and Holbrook, 2002), allowing the maintenance of a high GSH/GSSG ratio.

Nitrogen assimilation by various algae was affected by the environmental conditions, as well as, the nitrogen source varied among species (Soletto et al., 2005; Converti et al., 2006). The aim of the present study was to investigate the interactive effects of exposure to UV-B radiation and/or nitrogen limitation on photosynthetic and UV absorbing pigments in two microalgae. Oxidative stress indices (lipid peroxidation and hydrogen peroxide), antioxidant defense system including non-enzymatic metabolites (Carotenoids, and glutathione), as well as, activities of antioxidant enzymes were examined.

2. Materials and methods

2.1. Experimental design

Cyanobacteria microalgae cultures of *Spirulina platensis*. and micro green algae *Dunaliella salina* were obtained from the Algae stock Culture Collection of the Botany and Microbiology Department, Science College, King Saud University, Riyadh, Saudi Arabia. Microalgal Cultures were grown in 3 l glass flasks containing 2 l BG-11 culture medium at 25–30 °C. A continuous air stream was supplied via 0.2 μm cellulose acetate filter at rate of 1 l min^{-1}. A complete set of light squares in the form of chessboard alignment of cool-white fluorescent light (manufactured by Future Lighting Solutions, Montreal, QC, Canada) were used for illumination. Cell density was determined at 550 nm using UV–Vis spectrophotometer (JENWAY, Staffordshire, UK) for monitoring the growth curve. For all microalgal cultures the log cell number achieved from the linear portion of a curve as a function of time was used in calculating the maximum specific growth rate. Subcultures of the two microalgae were incubated in a clean conical flasks containing 400 ml BG-11 medium at 32 °C under white light of 85 μmol (photons) m^{-2} s^{-1} supplemented with 20 μmol (photons) m^{-2} s^{-1} of light, 1.5% CO_2 enriched air was supplied using an air pump and used for aeration to increase the contact between culture and medium. Autoclaved air supplementation and filter were applied to prevent culture contamination The cultivation of microalgal cells was continued until the middle of the exponential phase previously identified at OD_{680} between 0.35 and 0.7 (see Image 1).

The mass culture of microalgae was carried out in three different sets of BG-11 medium, the first set containing complete nutrient components of the BG-11 medium, in the second set, nitrogen was withdrawn from the BG-11 medium and in the third, phosphorus was removed from the BG-11 medium. All sets were exposed to UV-B lamp (290–320 nm) at irradiance of 0.50 W m^{-2} for three days (2 h day^{-1}). All microalgae grown under experimental conditions were performed in triplicate.

At each sampling time after exposure to UV-B irradiation, each flask was vigorously mixed and 10 ml for determining the cell density and pigment analysis. The rest of culture was centrifuged at 2500 rpm for 20 min and aliquot amounts, from each microalgae culture, was collected for subsequent analysis. For each microalgae, control experiments were supplemented in triplicates under complete BG-11 culture in addition to standard growth conditions minus UV-B irradiation. The initiative cell density without inoculation for control microalgae was 1×10^6 cell/ml. Growth rate, (cell/ml/day) was calculated by the equation:

$$\text{Growth rate} = \frac{C_x - C_0}{t_x}$$

where C_x (cell ml^{-1}) is the cell density obtained at the time t_x (days) and C_0 (cell ml^{-1}) is the inoculums density.

2.2. Pigment analysis

Samples of 4.5 ml were withdrawn for pigment analysis in the following order: just before UV-B stress (control, time 0), after 2, 4 and 6 h of UV-B stress. Absorption spectra for wavelengths from 350 to 750 nm were measured with UV–Vis spectrophotometer (JENWAY, Staffordshire, UK). Chlorophyll *a* and carotenoid contents were measured as described previously. The amount of pigments present were calculated according to the formula of Lichtentaler and Wellburn (1985).

$$\text{Chlorophyll } a = 10.05\ A662 - 0.766\ A644$$

$$\text{Chlorophyll } b = 16.37\ A644 - 3.140\ A662$$

$$\text{Carotene} = 1000\ A470 - 1.280\ Ca - 56.7\ Cb/230$$

2.3. Oxidative stress indices

2.3.1. Lipid peroxidation

Malondialdehyde (MDA) an indicator of lipid peroxidation products was quantified in algal cell extracts according to the

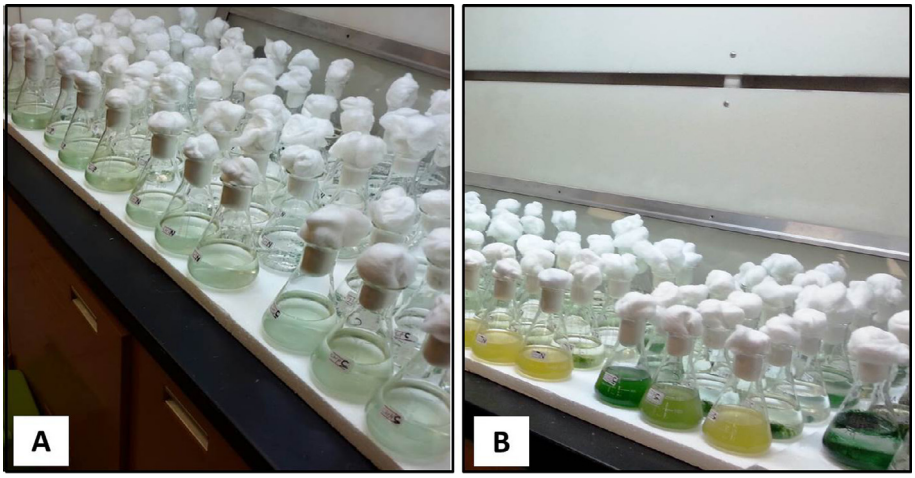

Image 1 Exposure of *Spirulina platensis*. and *Dunaliella salina* UV-B and/or N_2 starvation treatment (A) before treatment and (B).

method described by Haraguchi et al. (1997). A fresh algal sample (0.5 g) was homogenized in 5 ml of 10% (W/V) trichloroacetic acid (TCA), and centrifuged to obtain a homogenate at 10,000 rpm for 10 min. One ml of the supernatant was mixed with 2 ml of 0.5% Thiobarbituric acid (TBA) solution (in 10% TCA). The mixture was kept at 95 °C for 45 min, and cooled at room temperature, then centrifuged at 5000 rpm for 10 min to remove the interfering substances. Absorbance was read at 532 nm using UV–Vis spectrophotometer (JENWAY, Staffordshire, UK) and corrected for the nonspecific absorbance by subtracting the value obtained at 600 nm (Cakmak and Horst, 1991). MDA concentration was calculated using the extinction coefficient, 155 mM^{-1} (Dai et al., 1997).

2.3.2. Hydrogen peroxide content

The H_2O_2 level was measured colorimetrically as described by Sergiev et al. (1997). $H_2O_2$was extracted by homogenizing 0.5 g of fresh algae in 5 ml of 10% (W/V) iced trichloroacetic acid (TCA). After that, centrifugation was made at 10,000 rpm for 10 min. Add 1.5 ml potassium phosphate buffer (50 mM, pH 7.0) to 0.5 ml of the supernatant and 1 ml of 1 M potassium iodide (KI). The intensity of the yellow color of the supernatant was measured at 390 nm using UV–Vis spectrophotometer (JENWAY, Staffordshire, UK). H_2O_2 level was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}.

2.4. Antioxidant defense system

2.4.1. Non enzymatic constituents

2.4.1.1. Determination of algal total carotenoids. Spectrophotometric determination of total carotenoids was determined according to the method described by Semenenko and Abdullaev (1980) at 450 nm using UV–Vis spectrophotometer (JENWAY, Staffordshire, UK).

2.4.1.2. Determination of total phenolics and flavonoids. Free phenolic compounds were estimated according to Pritchard et al. (1997) using the Folin-Ciocaltens reagent. Flavonoids were extracted and determined as described by Mirecki and Teramura (1984). Leaves were ground to a powder in liquid nitrogen before extraction in 10 ml of acidified methanol (HCl: methanol, 1:99, v/v), Absorbance was measured at 300 nm.

2.4.1.3. Determination of glutathione. Glutathione (GSH) was determined as described by Anderson (1985). "Collected cells were homogenized with cold mortar and pestle with ice-cold 5% sulfosalicylic acid (1:10 w/v) and centrifuged at 15,000*g* for 10 min". The supernatant were used for (GSH + GSSG) determination. Total (GSH + GSSG) was determined by adding 1.0 ml potassium phosphate buffer (0.5 M pH 7.5), 0.5 ml sodium phosphate buffer (0.1 M with 5 m M EDTA, pH 7.5), 0.1 ml NADPH,0.6 mM DTNB (2 mM in sodium phosphate buffer) and 0.1 ml glutathione reductase (2.5 units ml^{-1}) to 0.5 ml supernatant. The reaction was monitored as an increase in absorbance at 412 nm, and total glutathione was calculated from a standard curve. Oxidized glutathione (GSSG) was determined by adding 1.5 potassium buffer (0.5 M, pH 7.5) and 0.2 ml 2-vinylpyridine to 1 ml supernatant, allowing the mixture to react for 1 h. to remove GSH. GSSG was measured using the same procedure as for total glutathione determination with a GSSG standard curve. GSH was determined by subtracting GSSG from the total glutathione content. Quantification of glutathione levels was based on reduced form of glutathione (GSH) calibration curve.

2.4.2. Antioxidant enzymes

2.4.2.1. Preparation of enzyme extracts. Frozen algal samples (0.5 g) were homogenized using mortar and pestle in 5 ml of ice cold extraction buffer (250 mM sucrose and 25 mM Tris, pH 7.2). containing 0.1% (V/V) Triton X-100 and 1% (W/V) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and supernatants were used for analyses of enzyme activities.

2.4.2.2. Superoxide dismutase (SOD, EC1.15.1.1). SOD (EC1.15.1.1) activity was evaluated according to the method described by Stewart and Bewley (1980) applying the photochemical nitroblue tetrazolium (NBT). 0.5 g fresh algae was homogenized in 5 ml extraction buffer consisting (50 mM phosphate buffer pH 7.8, 0.1% (w/v) bovine serum albumin,

0.1% (w/v) ascorbate, and 0.05% (w/v) β-mercaptoethanol). The assay mixture in 3 ml contained 50 mM phosphate buffer (pH 7.8), 9.9 mM methionine, 57 mM NBT, 0.025% (w/v) Triton X-100, and 0.005% (w/v) riboflavin. The formation of purple formazan (photo-reduction of NBT) was measured at 560 nm. One unit of SOD is defined that causes 50% inhibition of the photo-reduction of NBT.

2.4.2.3. Guaiacol peroxidase (GPX, EC1.11.1.7). GPX activity was determined by the method of Titiana et al. (1999). Guaiacol peroxidase was measured spectrophotometrically at 25 ± 2 °C and the reaction mixture (2 ml) is composed of 50 mM phosphate buffer pH 6.1, 0.4% H_2O_2, and 1% guaiacol. The reaction was started by the addition of an enzyme extract equivalent to 5 μg protein. The increase in absorbance due to oxidation of guaiacol ($E = 25.5\ mM^{-1}\ cm^{-1}$) that resulted in the formation of tetraguaiacol was measured at 470 nm.

2.5. Statistical analysis

All data were statistically analyzed as a split plot (factorial experiment) in a randomized complete block design for each individual microalgae. UV-B doses were allocated to the main plots while nitrogen deprivation was assigned to the subplots. Means separation within treatments using paired comparison *t*-test ($P \leqslant 0.05$) were carried by using software of SPSS program (V. 20 IBM, USA).

3. Results

The cell density of *S. platensis* and *D. salina* decreased significantly under the exposure to UV-B and/or N_2 starvation for 3 days. The cell density for *S. platensis* after UV-B and N_2 starvation was decreased by 50% and 45% relative to the control after 3 days of stress treatment (Table 1). The corresponding values for *D. salina* under the same experimental conditions were 58 and 64, respectively. The growth of *Dunaliella salina* was much better than *S. platensis*. Therefore, *S. platensis* was markedly sensitive to exposure to nitrogen deprivation and/or UV-B radiation stress than *D. salina*. The cumulative stresses reduction of the cell density in both *S. platensis* and *D. salina* showed a less remarkable reduction in cell density in comparison with reduction under single stress.

Photosynthetic pigments such as chl *a*, chl *b* and carotenoids were affected in a similar pattern like growth rate. For example the exposure of *S. platensis* to UV-B or N_2 starvation alone for three days resulted in a significant reduction in chl *a* reached 36% and 49% relative to the control. The corresponding values for *D. salina* were 32% and 42%. Similar pattern was observed for chlorophyll *b* in both treated algae. On the other hand the carotenoid content was significantly increased under the same experimental condition in the two tested algae and reached to approximately 2.8 fold increase under exposure for 3 days to UV-B and N_2 starvation, respectively (Table 1).

However, the cumulative stresses (UV-B and nitrogen deprivation) appears to have less suppressive effect than either of the separate treatments. The decrease in chlorophyll *a* in *Spirulina platensis* and *Dunaliella salina* in response to cumulative stresses of both UV-B and nitrogen deprivation for 3 days was 10% and 4%, respectively in comparison with control. Similar pattern was observed for chlorophyll *b*. On the other hand the combined stresses resulted in a significant increase in carotenoids content that reached 1.8 in *Spirulina platensis* and *Dunaliella salina*.

The increment of carotenoids/total chlorophyll ratio under the exposure to single stress indicated that both treated algae increased the non enzymatic antioxidant defense metabolites to ameliorate the drastic effect of each stress.

The effects of UV-B and/or nitrogen deprivation stresses on pigments were almost similar to that on growth rate. Chlorophyll *a* content in N_2-deprivation stressed microalgae was 58% of the control in *D. salina*; the corresponding value *S. platensis* was only 51%.

Under the prevailing conditions, the effect of N_2-deprivation stress was markedly severe than that of UV-B (Table 1).

Irradiating the N_2-deprivation stressed microalgae with UV-B increased their pigment content as compared to their response to each individual treatment, but the values were still lower than non-treated controls.

Total phenolics in *S. platensis* and *D. salina* was increased significantly under the exposure to UV-B or N_2-deprivation stress separately. Additionally, the exposure of both tested algae to cumulative stresses exhibited a highly significant increment in total phenolic compounds (Table 2). The change in flavonoid content in both treated microalgae was slightly significant under the exposure to separate UV-B and N_2 starvation treatment. On the other hand, the cumulative stress induced a highly significant increment in flavonoids content which amounted to 2.8 and 2.5 fold compared with the control value *of S. platensis* and *D. salina* respectively (Table 2).

The occurrence of an oxidative stress induced by UV-B and/or N_2-deprivation stresses was monitored by analyzing membrane damage through measurement of MDA levels. MDA production was increased significantly in both microalgae. Under the exposure of *S. platensis* to UV-B and N_2 starvation stress, separately, the MDA content reached 97 and 132.3 μMg^{-1} (d.m) which represent 2.3 and 3.2 fold more than control value. The corresponding values for *D. salina* were 78.6 and 107.3 representing 2.3 and 3.1 fold control values. The cumulative stresses, however, exhibited a significant reduction in the control value. A similar trend was recorded concerning H_2O_2 content (Table 2).

In control microalgae the GSSG content was nearly a tenth of the reduced form. Both reduced and oxidized forms of glutathione were increased significantly in *Spirulina platensis* and *Dunaliella salina* under exposure to UV-B and N_2-deprivation. Concerning GSH/GSSG ratio under the UV-B or N_2-deprivation stress, a significant decrease in both microalgae was induced and the cumulative stress treatment resulted in a significant increment in both ratio and enzyme activity toward the control microalgae (Table 3).

Superoxide dismutase activity was increased under the effect of UV-B or N_2-deprivation treatment when applied individually, a significant increase was shown in response to the combined UV-B and N_2 deprivation stresses (Table 4). Concerning Gupx activity, significantly in both microalgae under all treatments (Table 4).

4. Discussion

Under the interactive effect of N_2-deprivation and UV-B irradiation, the data of growth criteria revealed that *Spirulina*

Table 1 Effect of UV-B and nitrogen limitation on cell density and photosynthetic pigments *Spirulina platensis* and *Dunaliella salina*. Values are means ± SE ($n = 5$).

Treatment 72 h	Cell density (%)		Chlorophyll *a*		Chlorophyll *b*		Total carotenoids		Total carotenoids/ chlorophyll *a* + *b*	
	S. platensis	*D. salina*	*S. platensis*	*D. salina*	*S. platensis*	*D. salina*	*S. platensis*	*D. salina*	*S. platensis*	*D. salina*
Optimum nutrient + zero UV-B	100	100	4.31 ± 0.58[a] (100%)	4.12 ± 0.36[a] (100%)	2.30 ± 0.57[a] (100%)	2.65 ± 0.49[a] (100%)	1.21 + 0.30[a] (100%)	1.28 + 0.37[a] (100%)	0.18 (100%)	0.19 (100%)
Optimum nutrient + UV.B dose (kJ m^{-2} d^{-1})	50	58	2.77 ± 0.57[b] (64%)	2.81 ± 0.60[b] (68%)	1.45 ± 0.20[b] (63%)	1.61 ± 0.23[b] (61%)	3.34 ± 0.57[b] (276%)	3.72 ± 0.58[b] (290%)	0.79 (439%)	0.84 (422%)
Nitrogen supplied + Zero UV-B	45	64	2.21 ± 0.04[b] (51%)	2.41 ± 0.100[c] (58%)	1.12 ± 0.12[b] (49%)	0.95 ± 0.42[c] (37%)	2.69 ± 0.39[c] (222%)	2.74 ± 0.55[b] (214%)	0.81 (450%)	0.82 (432%)
Nitrogen starvation + UV.B dose (kJ m^{-2} d^{-1})	66	76	3.88 ± 0.48[b] (90%)	3.95 ± 0.467[b] (96%)	1.32 ± 0.20[b] (57%)	1.48 ± 0.41[b] (56%)	2.12 ± 0.48[b] (175%)	2.26 ± 0.48[b] (177%)	0.41 (228%)	0.42 (221%)

Table 2 Effect of UV-B and nitrogen starvation for 3 days on phenolics, flavonoids, H_2O_2 and malondialdehyde MDA of *Spirulina platensis* and *Dunaliella salina*. Values are means ± SE ($n = 5$).

Treatment	Phenolics (mg g^{-1} (d.m))		Flavonoids (mg g^{-1} (d.m))		H_2O_2 (μM g^{-1} (d.m))		MDA (μM g^{-1} (d.m))	
	Spirulina platensis	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*
Optimum nutrient + zero UV-B	2.93 ± 0.55[a]	3.22 ± 0.35[a]	0.70 ± 0.577[a]	0.93 ± 0.490[a]	0.46 ± 0.058[a]	0.43 ± 0.06[a]	41.5 ± 0.92[a]	34.8 ± 0.70[a]
Optimum nutrient + UV-B dose (kJ m^{-2} d^{-1})	4.31 ± 0.57[b]	4.71 ± 0.46[b]	1.48 ± 0.200[b]	1.72 ± 0.210[b]	0.76 ± 0.058[b]	0.64 ± 0.058[b]	97.0 ± 0.57[b]	78.6 ± 0.58[b]
Nitrogen starvation + Zero UV-B	3.88 ± 0.04[b]	3.95 ± 0.10[c]	1.23 ± 0.120[b]	1.53 ± 0.120[c]	0.83 ± 0.06[a]	0.68 ± 0.07[a]	132.3 ± 0.59[c]	107.3 ± 0.61[b]
Nitrogen starvation + UV-B dose (kJ m^{-2} d^{-1})	5.33 ± 0.57[d]	5.87 ± 0.57[d]	1.95 ± 0.220[d]	2.29 ± 0.23[d]	0.65 ± 0.06[b]	0.54 ± 0.06[b]	78.6 ± 2.73[d]	55.4 ± 0.08[d]

Table 3 Effect of UV-B and nitrogen starvation on reduced glutathione, GSH; oxidized glutathione, GSSG, GSH/GSSG ratio and glutathione reductase (GR) activity *Spirulina platensis* and *Dunaliella salina*. Values are means ± SE ($n = 5$).

Treatment 72 h	GSH (nmol/g (dm))		GSSG (nmol/g (dm))		GSH/GSSG		GR (U/mg protein)	
	Spirulina platensis	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*
Optimum nutrient + zero UV-B	166 ± 7.05[a]	154 ± 6.21[a]	21 ± 0.91[a]	21.4 ± 0.65[a]	7.90	7.19	0.19 ± 0.041[a]	0.21 ± 0.033[a]
Optimum nutrient + UV-B dose (kJ m^{-2} d^{-1})	241 ± 5.52[b]	189.4 ± 5.44[b]	63.7 ± 4.67[b]	43.6 ± 2.21[b]	3.78	4.34	0.37 ± 0.051[a]	0.42 ± 0.040[a]
Nitrogen starvation + Zero UV-B	195.7 ± 1.51[c]	182.5 ± 1.45[c]	52.4 ± 4.21[c]	46.3 ± 3.23[c]	3.73	3.93	0.31 ± 0.056[c]	0.28 ± 0.061[c]
Nitrogen starvation + UV-B dose (kJ m^{-2} d^{-1})	217.8 ± 1.36[d]	188.4 ± 1.45[d]	39.3 ± 3.47[d]	28.2 ± 2.17[d]	5.54	6.68	0.29 ± 0.039[d]	0.25 ± 0.037[d]

platensis responded more readily than *Dunaliella salina*. This suggests that UV-B exposure might have offset some of the effects of N_2-deprivation associated with the ability to increase its osmotic pressure.

In this investigation reduction in cell density due to cumulative stresses was only for *Spirulina platensis* compared with 50% and 55% decrease under UV-B exposure or N_2-deprivation stress. *Dunaliella salina* showed less effect than *Spirulina platensis* under cumulative stress which may have delayed and reduced the severity of N_2-deprivation stress through a reduction in water – loss rates and through an increase growth rate. In this connection, ultraviolet light

Table 4 Effect of UV-B and nitrogen starvation on superoxide dismutase, SOD; ascorbate peroxidase, APX and guaiacol peroxidase, GUPX activities *Spirulina platensis* and *Dunaliella salina*. Values are means ± SE (n = 5).

Treatment 72 h	SOD (U/mg protein)		APX (U/mg protein)		GUPX (U/mg protein)	
	Spirulina platensis	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*	*Spirulina platensis*	*Dunaliella salina*
Optimum nutrient + zero UV-B	9.10 ± 0.56[a]	11.2 ± 0.39[a]	2.43 ± 0.57[a]	2.89 ± 0.55[a]	384 ± 5.77[a]	412 ± 5.74[a]
Optimum nutrient + UV-B dose (kJ m^{-2} d^{-1})	19.44 ± 0.58[b]	24.8 ± 0.49[b]	5.76 ± 0.67[b]	6.14 ± 0.46[b]	523 ± 5.76[b]	601 ± 5.73[b]
Nitrogen starvation + Zero UV-B	17.54 ± 0.45[c]	22.63 ± 0.43[c]	4.93 ± 0.45[c]	5.22 ± 0.47[b]	507 ± 5.74[c]	573 ± 5.72[c]
Nitrogen starvation + UV-B dose (kJ m^{-2} d^{-1})	13.45 ± 0.48[d]	14.25 ± 0.45[d]	3.64 ± 0.39[d]	4.00 ± 0.32[d]	464 ± 5.78[d]	511 ± 5.65[d]

adversely affects the alga primarily due to the damage to the photosynthetic machinery in the cells (Pessoa, 2012). Similarly increased exposure to UV-B alleviated N_2-deprivation in green algae as described by Moazami-Goudarzi and Colman (2012). These reports indicated that genotypic differences, assimilation and utilization and stage of growth are involved in the interaction of UV-B and N_2-deprivation stresses.

Low level of cell damage in this investigation can be explained by the accumulation of phenolics and flavonoids in response to UV-B and N_2-deprivation stresses together which, occur more rapidly than would response to N_2-deprivation or UV-B when imposed alone. This probably indicates that phenolics may act synergistically with flavonoids to stabilize macromolecules, thereby stabilizing the protoplasm. Under cumulative stresses of UV-B and N_2-deprivation, the flavonoids content was increased by 246% in *Dunaliella salina* compared with only 165% under N_2-deprivation stress and by 185% under the effect of UV-B radiation. This result is supported by the observations of Srinivas and Ochs, 2012. These results also agree with a study on *Nannochloropsis* illustrating, that flavonoids act as sunscreen against potentially damaging UV-B radiation (Forján Lozano et al., 2007). Hydrogen peroxide enhancement in N_2-deprivation or UV-B treatment especially *Spirulina platensis* revealed the occurrence of an oxidative stress which catalyzes lipid peroxidation and consequently MDA, and resulted in membrane damage and electrolyte leakage indicating that *Spirulina platensis* showed lower antioxidant enzyme activities and lower phenolics. These different effects between the two microalgae under the experimental treatments are judged by the criteria and behaviors of enzymatic and non-enzymatic antioxidants. Cumulative treatments resulted in a significant increase in GSH/GSSG ratio toward the control treatment, as a result of higher GR activity which is required for regulation of glutathione threshold level and activation of several enzymes. These results are correlated with the changes in the GSH levels. The decrease in GSH/GSSG ratio in response to individual stress is probably due to both the decrease in glutathione synthesis and its consumption to protect cells from oxidative stress damage. This assumption is supported by the observations of Saragosti et al. (2010) and Sharma et al. (2012).

Based on these short term studies the combination of both stresses improved the growth pattern much more in the *Dunaliella salina*. This improvement was mirrored by the observed elevation in the activity of antioxidant enzymes such as GuPx, SOD and GR and accompanied with lowering H_2O_2 and MDA contents; whereas the MDA content in the N_2-deprivation-stressed microalgae was increased significantly compared with the control.

High scavenging potential of H_2O_2 is a prerequisite for tolerance of microalgae and an increase in GuPx activity is a common response to various oxidative stress factors. The increase in GuPx activity with UV-B under N_2-deprivation stress in *Dunaliella salina*, with a concomitant decline in H_2O_2 level suggest the scavenging role through GuPx. This result agrees with the findings of Khotimchenko and Yakovleva (2005) who found greater GuPx activity after UV-B exposure in *Tichocarpus crinitus*. The activity of ascorbate peroxidase was increased significantly by UV-B irradiation and decreased toward the control under cumulative stresses. APX and GR induction was consistent with data found by Takagi and Yoshida (2006). It may be added that the high positive responses of *Dunaliella salina* to the combined treatment is the unexpected increase in SOD activity which scavenges superoxide radicals and protect the biomolecules from such radicals. Thereafter, a significant reduction in H_2O_2 and MDA contents resulted and consequently the membrane permeability and growth rate were ameliorated. Under the cumulative stresses, the increase in superoxide dismutase and GUPx confers to the two used microalgae an increased capacity for oxygen radical scavenging and maintenance of the integrity of cellular membranes and all sub-cellular structures. The present results in SOD activity are supported by Saragosti et al. (2010). In *Stylophora pistillata*, UV-B exposure increased guaiacol peroxidase, ascorbate peroxidase and superoxide dismutase activities. Such response are considered important for protection against UV-B stress. The present results on *Spirulina platensis* and *Dunaliella salina* are confirmed by Srinivas and Ochs (2012).

According to the present results a non- specific signaling pathway could have taken place in microalgae exposed to UV-B irradiation and induced an enhancement of hydrogen peroxide and lipid peroxidation. This result in stimulating non enzymatic and enzymatic antioxidant defense system probably due to temporal gene expression. This assumption is supported by the observations of Liang et al. (2006) who observed the effect of UV-B on mediating signal transduction. The increased defense mechanisms observed after the combination of both UV-B and N_2-deprivation stresses in the present study ensure that the two treated microalgae undoubtedly activate several defense mechanisms, contributing to the maintenance of the structural integrity of the cell components and presumably alleviate (to some extent) the photooxidation damage.

Our results are in agreement with the results obtained by Converti et al. (2009), who examined the different effects of abiotic stresses including temperature and nitrogen source concentration on cell growth and metabolic constituents of *Chlor-*

ella and *Nannochloropsis*. On reduction of NO_3 concentrations in the growth culture by 75% (1.5–0.375 g l^{-1} for *Chlorella vulgaris* and 0.3–0.075 l^{-1} for *Nannochloropsis*), lipid concentration became 3 and 2 fold, respectively, with only a small reduction in growth rate at optimal growth temperature. This outcome shows that it may be promising to obtain a higher lipid content for biofuel fabrication by limiting the nitrogen source with controlling temperature.

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