INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2004/06–1–49–57
http://www.ijab.org

Production of Antioxidant by the Green Alga *Dunaliella salina*

HANAA H. ABD EL-BAKY[1], FAROUK K. EL BAZ AND GAMAL S. EL-BAROTY†
Agricultural Division, National Research Center, Dokki, Cairo, Egypt
†*Department of Biochemistry, Faculty of Agriculture, Cairo University, Egypt*
[1]Corresponding author's e-mail: Abdelbakyh@hotmail.com

ABSTRACT

The variation of the lipophilic (carotenoids and α-tocopherol) and hydrophilic (glutathione and ascorbic acid) antioxidant contents, and the activities of antioxidant enzyme such superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), as well as cellular malonaldehyde and stable radicals of *D. salina* in response to ultraviolet B (UV-B radiation 290-320 nm) and secondary carotenoid induction conditions (nitrogen starvation and high NaCl concentration) were examined. The results indicate that nitrogen deficiency combined with NaCl stress and UV-B irradiated is potential increase of both lipophilic and hydrophilic antioxidant contents. Also, HPLC analysis of carotenoids extracts showed that *D. salina* accumulated significant quantities of β–carotene and secondary carotenoids, mainly astaxanthin and zeaxanthin. Furthermore, the activity of antioxidant enzymes CAT, SOD and POD showed a positive significant correlation with the antioxidant content and with the exposured UV-B irradiance. Cellular malondialhyde content and quantities of alkyl radical-PER signal indicators of lipid peroxidation were much higher in irradiant cells compared to unirradiant cells. These result revealed that *D. salina* had high resistance to environmental conditions. These qualities therefore make *D. salina* good candidates for successful culture in open ponds to production of useful materials, such as β–carotene, astaxanthin, zeaxanthin, ascorbic acid and α-tocopherol. Also, it could be used to provide a rich source of such antioxidant for health foods.

Key Words: *Dulinaliella salina*; Nitrogen; Salt stress; Carotenoids; Tocopherols; UV-B radiation; Antioxidant enzyme

INTRODUCTION

Use of *microalgae* for human consumption as a source of high value health food, functional foods and for production of biochemical products, such as vitamins, *carotenoids*, *phycocyanin* and polyunsaturated fatty acids including the omega-3 fatty acids have been developed (Richmond, 1986; Borowitzka, 1992; Pugh *et al.,* 2001). Among the various *microalgae* that have been explored for their suitability for commercial potential: *Dunaliella* species, *Chlorella* species and *Spirulina* species are three major type that have been used successfully to produced high concentrations of valuable compounds such as lipids, protein and pigments (Abe *et al.,* 1999; El-Baz *et al.,* 2002; Abd El-Baky *et al.,* 2002). *Dunaliella* species are able to accumulate large amount of carotenoids (Ben-Amotz *et al.,* 1982; El-Baz *et al.,* 2002). *D. salina* contains up to 10% β-carotene on dry weight basis when grown under stress conditions including: high salt concentration, high light intensity and nitrogen limitation (Ben-Amotz & Avron, 1988; El-Baz *et al.,* 2002). β-carotene and other carotenoids (astaxanthin & lutein) are integral part of the photosynthetic apparatus in algae and functions as accessory pigments in the harvesting complex and as protective agents against the active oxygen products (AOS) that are formed from photo-oxidation. These oxygen radicals can react with macromolecules and lead to cellular damages (Malanga *et al.,* 1997). The mechanism of biological effect of illumination (including near-UV-B) appear to involve endogenous photosensitization and formation of AOS, such as from singlet oxygen ($^1O_2$), superoxide radical (O^-_2), hydroxyl radical (^-OH) and hydrogen peroxide (H_2O_2) (Martin & Burch, 1990; Malange & Puntarulo, 1995). The algae have developed defiance system against photo-oxidative damage by antioxidative mechanisms to detoxify and eliminate these highly reactive oxygen species. These antioxidant defiance system includes hydrophobic (carotenoids & α-tocopherol) and hydrophilic antioxidant (ascorbic acid & glutathione) and antioxidant enzymes likes superoxide dismutase (SOD), catalsae (CAT), glutathione reductase (GR) ascorbic peroxidase (APX) and peroxidase (POD) (Rao *et al.,* 1996; Malanga *et al.,* 1997; Rijstenbil, 2002). The author previously reported that *D. salina* accumulated large amount of carotenoids, α-tocopherol and ascorbic acid, and enhanced the activities of the antioxidant enzymes when grown under high light intensity, in media containing high salt concentration and/or limiting nitrogen (El-Baz *et al.,* 2002). These results suggested that the adaptive response of these algae to high illumination could depend on the activity of antioxidant enzyme and the ability to accumulate simultaneously large quantities of carotenoids, vitamin E and vitamin C. However, these antioxidant products are mainly aimed at the health food market for direct human consumption which recognized as safe (Abe *et al.,* 1999; El-Baz *et al.,* 2002). Since many authors postulated that a high intake of antioxidant compounds might decrease the risk of cancer, aging, inflammation, stroke disease and neurodegenerative disease

(Parkinson's & Alzheimer's) in human and experimental animals (Schwartz, 1996, Abd El-Baky *et al.,* 2002; Abouel Enein *et al.,* 2003).

The aim of this work was to study the used of *D. salina* cells to production of antioxidant substances by expose to low-dose of UV-B radiation when grown in media containing deferent level of NaCl and nitrogen concentrations. The profile of the activity of antioxidant enzymes (SOD, CAT & POD), lipid peroxidation, lipid – soluble antioxidant (vitamin E, β-carotene & other carotenoids) and water-soluble antioxidant (ascorbic acid & glutathione) were examined.

MATERIALS AND METHODS

Algal source. Marine microalgae *Dunaliella salina* was obtained from the Culture Collection of Botany Department, Texas University, Austin, Texas, U.S.A.

Growth conditions. *Dunaliella salina* was cultured in a 4 L flask with 2.5 L of culture medium containing 8% NaCl and 5 mM nitrogen, pH 8.5 during spring season in National Research Center (NRC). Media and nutrients were sterilized. All glass and plastic ware were washed with 10% HNO_3 and rinsed with distilled water. The cultures were gassed with 1.5% volume CO_2 in air and were grown at 20-25ºC. The mass culture of *D. salina* were carried out with KNO_3 as nitrogen source with two different concentrations 5 and 2.5 mM N, in media containing 8, 12 and 16% NaCl, respectively. The cultures illuminated with continuous cool white fluorescent lamps (Philips 40 W) at light intensity levels were approximately 200 W m^{-2} (El-Baz *et al.,* 2002). An ultraviolet- B lamp (radiation 290-320 nm, UVM-57 Chromatovue) at irradiance of 0.50 W m^{-2} was used for irradiated the culture for 4 h^{-1}. All algal grown under experimental conditions were performed in triplicate for 15 days.

Growth measurements. The growth of *Dunaliella salina* was measured by dry weight methods and optical density (O.D) as described by Payer (1971).

Harvesting. Under all experimental conditions the cells culture at stationary-phase were harvested at 4°C by centrifugation at 6000 x g for 15 min and frozen at -20°C.

Extraction of carotenoids and tocopherols. Carotenoids and tocopherols were extracted from algal cells with 1: 10 (w/v) tetrahydrofuran (THF) in present 30 mg^{-1} of BHT (2,6 ditertra-butyl-p-cresol) and magnesium carbonate (1 g10 g^{-1} sample). After 24 h, the aliquot of the clear extracted pigments was filtered and evaporated to sample value 5 mL under a stream of nitrogen. The extracted pigments were saponified with 25 mL of 10% methanolic potassium hydroxide for 2 h at room temperature, then carotenoids and tocopherols were extracted with dichloromethane. The solvent layer was then separated by separator funnel, several time washed with distilled water, then dried on Na_2SO_4, and dryness under nitrogen (Farag *et al.,* 1998).

Determination of algal total carotenoids. The total carotenoids was determined by spectrophotometric method at 450 nm, β-carotene served as a standard compound was used for preparing the calibration curve (Semenenko & Abdullaev, 1980).

Identification of carotenoids. Carotenoids were separated by thermoseparation Liquid Chromatography system consisted of a spectra system UV 2000 detector (hold at 438 nm) and Spectra System P2000 pump, on a 250x4.6 mm (i.d) column packed with Chromosil C_{18} material, 5 μm particle size and eluted with 80:10 (v/v) acetonitrile: methanol, at flow rate of 1 mL min^{-1}. Some available standard carotenoids: β-carotene, zeaxanthin, lutein, astaxanthin and cryptoxanthin (Sigma) were also run by the same HPLC method (Honya *et al.,* 1994; Abd El-Baky, 2003).

Determination of algal tocopherols. Tocopherols were determined by HPLC equipped with Spectra System UV2000 detector at 290 nm and sparated on a 250 x 4.6 nm (i.d) column packed with Vydac and eluted with 90:10 acetonitrile: methanol (v/v) at a flow rate of 1 mL min^{-1}. Standard of α tocopherol (Sigma) was ran under the same conditions (Abd El-Baky, 2003).

Extraction and determination of ascorbic acid. Ascorbic acid (vitamin C) was extracted from the cells with 2% meta-phosphoric acid, and determined by spectrophotometric methods using 2, 6 di-chlorophenol indophenol dye (Augustin *et al.,* 1985).

Preparation of cytosolic fraction. Algae cells were excised and homogenized using mortar and pestle in 5 mL of ice-cold extraction buffer (250 mM sucrose & 25 mM Tris, pH 7.2). The homogenate was centrifuged at 16000 x g for 20 min at 4°C and supernatant (extracts) was used for analyses of enzyme activities, glutathione (GSH), lipid peroxidation and protein content.

Determination of glutathione (GSH). The GSH content of algal cell extracts was measured by reaction with 5,5 dithiobis-2-nitrobenzoic (DTNB) reagent to give a compound that absorbed at 412 nm (Silber *et al.,* 1992). GSH was expressed as μM.

Enzymes assays. The activity of cytosolic SOD (EC 1.15.1.1) was determined by photochemical method (Ginnopolitis & Ries, 1977). Spectrophotometric method of Chance and Maehly (1955) was used to assay the POD activity (EC 1.11.1.7)**.** The CAT enzyme (EC 1.11.1.6) activity was assayed Spectrophotometrically by the decomposition of H_2O_2 at 240 nm in a reaction mixture containing of a 10 mM H_2O_2 and 25 mM phosphate buffer, pH 7.0 (Hans- Luck, 1970).

Determination of lipid peroxidation products. The lipid peroxidation products in algal cells extracts was estimated by the formation of thiobarbaturic acid reactive substances (TBARS) and quantified in term of malonaldhyde (MDA) as described by Haraguchi *et al.* (1997). The lipid peroxidation was expressed as micromoles of MDA. The extinction coefficient of TBARS was taken as $1.56x10^{5}$ at wave length 532 nm.

Detection of free radical by ESR-Spain. The algal cells (0.2 g) from both control and treatment were lyophilized in a speed Vac Sc 100 (savant). The powdered cells (0.05 g) were prepared in 1,1 diphenyl-2 picrylhydrazyl (DPPH). No increased in back ground ESR signal occurred when DPPH alone was exposed to UV-light. ESR spectra were obtained at room temperature using a Bruker (Karlsruhe, Germany), Spectrometer ELEXSYS E-500, operating at 9.808 GHz with 100 kH_z modulation frequency. EPR instruments setting for the Spin trapping experiments were: microwave power, 20.2 mW; modulation amplitude 1.0 G; time constant, 51 ms; receiver gain 60.0. EPR for all samples were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double-integrated to obtain the area intensity.

Determination of protein. Protein content in algal buffer extracts was assayed spectrophotometrically at 595 nm, using comassein blue G 250 as a protein binding dye (Bradford, 1976). Bovine serum albumin (BSA) was used as a protein standard. Protein concentrations in the samples were calculated from the calibration curve, in mg protein in mL extract. Activities of SOD, CAT and POD were normalized to protein concentration.

Statistical analysis. Data represent the means ± SD. Results were analyzed by one- way ANOVA and Scheffe' *F*-test to identify significant differences between groups. *P*-values < 0.01 were considered significant. All analyses performed using Co Stat software version 4 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS AND DISCUSSION

Influence of growth conditions on antioxidant contents of *D. salina.* The lipophilic (α-tocopherol and total careotenoids), and hydrophilic (glutathione & ascorbic acid) antioxidant contents were determined in algae cells grown under all experimental conditions are presented in Table I and II.

Lipophilic antioxidant. The cellular carotenoid contents in irradiated cells was increased gradually by decrease nitrogen level combined with increase NaCl concentration in nutrient medium (Tables I, II). According to the level of total carotenoid contents in algae cells grown under experimental conditions, the highest level of total carotenoids content mg g^{-1} cells and percentage of d.w (in parentheses) was obtained in UV-Birradiated cells grown in media containing 2.5 mM nitrogen and 16% NaCl (N deficiency & NaCl stress), with value 115.2 mg g^{-1} cells (11.5%). Whereas, these values were 14.5 (1.45%) and 9.2 mg g^{-1} cells (0.92%) in UVB-irradiated cells and unirradiated cells grown under optimum conditions (5 mM N and 8% NaCl), respectively. Therefore, UV-B-exposure and the cells growth conditions affected the accumulation of carotenoids in *D. salina*.

The carotenoid profiles in unirradiated and irradiated *D. salina* cells grown under all experimental conditions were verified by HPLC separation on the RP-18 silica gel (Table I, & Fig. 1). The unirradiated *D. salina* culture grown under optimum conditions was characterized by the lower content of β-carotene as compared with other culture exposed to UVB-radiation grown under the same conditions. However, the results indicated a direct relationship between β-carotene percent in irradiated cells and increasing salinity and decrease N levels in the nutrient medium. In general, in all treated cells, β-carotene, astaxanthin and zeanxanthin were identified as major carotenoids. The concentration of these carotenoids ranged from 3.2 to 62.31, 2.13 to 21.31 and 1.41 to 14.51 mg g^{-1} of cells d.w., respectively. These mean that the ratio of these carotenoids to total caroteoids under stress condition was

Table I. Effect of UV- irradiation, nitrogen limitation and salt stress on carotenoids profile of *Dunaliella salina*

	Total carotenoids			β-carotene			Astaxanthin			Zeaxanthin			Lutein			Cryptoxanthin		
Treatment	mg g^{-1}	%	Ratio	mg g^{-1}	%	% of Total car.	mg g^{-1}	%	% of Total car.	mg g^{-1}	%	% of Total car.	mg g^{-1}	%	% of Total car.	mg g^{-1}	%	% of Total car.
Optimum nutrients not exposed to UV	9.2	0.92	1.0	3.2	0.32	34.78	2.13	0.213	23.1	1.41	0.14	15.3	0.92	0.092	1	0.21	0.02	2.28
Optimum nutrients exposed to UV 5mM Nitrogen + 8 % NaCl	14.5	1.45	1.58	5.11	0.51	35.24	3.21	0.321	22.1	2.01	0.20	13.9	1.11	0.11	7.6	0.42	0.04	2.89
2.5 mM Nitrogen +8 % NaCl	31.1	3.11	3.38	12.91	1.29	41.15	4.51	0.45	14.5	3.10	0.31	9.96	1.51	0.15	4.8	0.81	0.08	2.6
Zero Nitrogen + 8 % NaCl	45.5	4.55	4.94	20.33	2.03	44.68	6.21	0.62	13.6	4.21	0.42	9.2	2.13	0.21	4.7	1.01	0.10	2.2
12 % NaCl + 5 mM Nitrogen	54.1	5.41	5.88	25.45	2.54	47.04	8.95	0.89	16.5	5.31	0.53	9.8	2.31	0.23	4.27	1.15	0.11	2.12
12 % NaCl + 2.5 mM Nitrogen	105.1	10.5	11.42	57.11	5.71	55.29	18.34	1.83	17.4	12.31	1.23	11.71	6.57	0.65	6.2	2.48	0.24	2.35
16% NaCl + 5 mM Nitrogen	72.9	7.29	8.05	35.42	3.54	48.58	12.31	1.23	16.8	7.35	0.73	10.1	4.42	0.44	5.7	1.6	0.16	2.26
16% NaCl + 2.5 mM Nitrogen	115.2	11.5	12.6	62.31	6.23	54.08	21.31	2.13	18.49	14.51	1.45	12.59	7.14	0.71	6.19	2.73	0.27	2.37

All values are significant at (P< 0.5); Car. = Carotenoid

Table II. Effect of UV- irradiation, nitrogen limitation and salt stress on antioxidant substances of *Dunaliella salina*

Treatment	α-Tocopherol			Ascorbic acid			GSH	
	mg g^{-1}	%	Ratio	mg g^{-1}	%	Ratio	µM	Ratio
Optimum nutrients not exposed to UV	0.368	0.036	1.0	2.7	0.27	1.0	97.12	1.0
Optimum nutrients exposed to UV	0.427	0.042	1.24	0.7.2	0.72	2.67	174.11	1.80
2.5 mM Nitrogen +8 % NaCl	0.584	0.058	1.61	11.9	1.19	4.42	185.24	1.91
Zero Nitrogen + 8 % NaCl	0.896	0.089	2.47	15.4	1.54	5.70	209.51	2.16
12 % NaCl + 5 mM Nitrogen	1.91	0.191	5.19	9.5	0.95	3.52	190.97	1.97
12 % NaCl + 2.5 mM Nitrogen	2.67	0.267	7.25	21.3	2.13	4.96	240.0	2.27
16% NaCl + 5 mM Nitrogen	2.38	0.238	6.47	13.4	1.34	7.89	220.84	2.47
16% NaCl + 2.5 mM Nitrogen	3.83	0.383	10.4	25.41	2.54	9.41	253.4	2.61

Table III. Effect of UV- irradiation, nitrogen limitation and salt stress on antioxdant enzyme system catalase, peroxidase and superoxid dimutase of *Dunaliella salina*

Treatment	Catalase			Peroxidase			Superoxide dismutase		
	U mg^{-1} protein	Ratio[a]	Ratio[b]	U mg^{-1} protein	Ratio[a]	Ratio[b]	U mg^{-1} protein	Ratio[a]	Ratio[b]
Optimum nutrients not exposed to UV	3.04	1.0		1.38	1.0		18.0	1.0	
Optimum nutrients exposed to UV	20.13	6.62	1.0	9.32	6.75	1.0	20.36	1.13	1.0
2.5 mM Nitrogen +8 % NaCl	23.24	7.64	1.15	11.45	8.29	1.22	22.32	1.24	1.11
Zero Nitrogen + 8 % NaCl	26.35	8.67	1.31	13.22	9.56	1.42	26.40	1.47	1.29
12 % NaCl + 5 mM Nitrogen	27.11	8.92	1.35	15.41	11.17	1.65	29.21	1.62	1.43
12 % NaCl + 2.5 mM Nitrogen	35.62	11.7	1.77	24.11	17.47	2.59	36.41	2.02	1.79
16% NaCl + 5 mM Nitrogen	31.45	10.3	1.56	18.21	13.19	1.95	33.61	1.87	1.65
16% NaCl + 2.5 mM Nitrogen	39.55	13.0	1.96	26.41	19.14	2.83	40.11	2.23	1.97

Ratio[a]:Optimum nutrients not exposed to UV / treatment; Ratio[b]:Optimum nutrients exposed to UV / treatment; All values are significant at ($P< 0.5$)

about 20, 10 and 10, times that in cells grown under optimum condition. However, depending on growth conditions lutein and cryptoxanthin were detected in *D. salina.* The amount of these carotenoids quantity increased in irradiated *D. salina* cells gradually under a variety conditions as a function of the N limitation and high NaCl concentration in growth medium. For instance, the concentration of lutein and cryptoxanthin in irradiated cells grown in media containing 16% NaCl in present 2.5 and 5 mM N (in parentheses) were 7.14 (4.42) and 27.35 mg g^{-1} (16.50 mg g^{-1}), respectively in comparison to 0.92 and 21 mg g^{-1} respectively, in unirradiated cells grown under optimum conditions (5 mm N & 8% NaCl). Thus, it seems that UV-B exposed, nitrogen limitation and high salt concentration play an important role in controlling carotenogenesis in *D. salina.* Accordingly, the production of β-carotene, astaxanthin, zeanxanthin, lutein and cryptoxanthin could be enhanced with cultured *D. salina* under environmental stress conditions.

Algae of the genus *Dunaliella* can be accumulate large amount of carotenoids per cell reaching up to 10% of dry weight when grown under stress conditions such as high intensity irradiation, high salt concentration and nutrient limitation (Ben-Amotz & Avron 1988; Bar *et al.,* 1995; El Baz *et al.,* 2002). However, carotenoids play a highly effective role in protecting photosynthesis pigment, enzyme and membrane against photoxidative damage (Götz *et al.,* 1999). Under high irradiation, the photosynthetic apparatus dose not sufficient utilizes light energy, and the excess energy lead to the formation of free radicals rather than active oxygen molecules (singlet oxygen). These radicals are responsible for peroxidation reactions that destroy various compounds of photosynthesis apparatus. Thus, the algae such as *Dunaliella* and *Chlorella* accumulate large amount of β-carotene, astaxanthin, and zeanxanthin for scavenge or eliminating as well as for reducing the radicals, reaching the cell component (Rise *et al.,* 1994; El-Baz *et al.,* 2002). In this study exposed of *D. salina* to UV-B irradiation caused significant increase in the amount of carotenoids, as compared with unexposed cells. Thus, a positive relationship between carotenoids content and relief from UV-B inactivation was observed. Also, the results showed that in UV-B exposed cells, the carotenoids biosynthesis is shifted toward formation of astaxanthin and cryptoxanthin as shown in Table II. It was shown recently that astaxanthin and zeananthin are the most effective protection against UV-B radiation in some microorganism, and that it prevents radical peroxidation processes in liposomes much better than β-carotene (Götz *et al.,* 1999). Furthermore, these results are evidence for a mechanism for protecting the cells against irradiation damage. However, Götz *et al.* (1999) reported that β-carotene and astaxanthin in cyanobacterium exposed to UV-B radiation exert their protective function as antioxidants to inactive UV-B-induced radicals in photosynthetic membrane. On other hand, the carotenoids in the cell membrane of microalgae

Table IV. Effect of UV-irradiation, nitrogen limitation and salt stress on lipid peroxidation of *D. salina*

Treatment	MAD mM mg^{-1} protein	Ratio
Optimum nutrients not exposed to UV	1.04	1.00
Optimum nutrients exposed to UV	4.97	4.77
2.5 mM Nitrogen +8 % NaCl	6.70	6.42
Zero Nitrogen + 8 % NaCl	7.31	7.02
12 % NaCl + 5 mM Nitrogen	8.93	8.58
12 % NaCl + 2.5 mM Nitrogen	17.12	16.44
16% NaCl + 5 mM Nitrogen	10.50	10.08
16% NaCl + 2.5 mM Nitrogen	19.60	18.83

Table V. Effect of UV-irradiation, nitrogen limitation and salt stress on free radical levels of *D. salina*

Treatment	Relative free radical		
	%	Ratio[a]	Ratio[b]
Optimum nutrients not exposed to UV	100%	1.00	
Optimum nutrients exposed to UV	295.8	2.95	1.00
2.5 mM Nitrogen +8 % NaCl	213.7	2.13	0.72
Zero Nitrogen + 8 % NaCl	274.7	2.74	0.928
12 % NaCl + 5 mM Nitrogen	340.6	3.40	1.15
12 % NaCl + 2.5 mM Nitrogen	591.0	5.91	1.99
16% NaCl + 5 mM Nitrogen	82.0	0.82	0.277
16% NaCl + 2.5 mM Nitrogen	97.6	0.97	0.33

Ratio[a] :Optimum nutrients not exposed to UV / treatment; Ratio[b]: Optimum nutrients exposed to UV / treatment

could act as a filter for UV-B- radiation (Jürgens & Weckesser, 1985).

Tocopherol. The cellular content of tocopherols in unirradaited and irradiated *D. salina* grown under all experimental condition is show in Table II and Fig 2. The results indicate that *D. salina* responds to stress condition by accumulated significant amount of α-tocopherol. Tocopherol values obtained in irradiated cells grown at 16% NaCl in present of 2.5 and 5 mM N were 3.83 and 2.38 mg g^{-1}, respectively, whereas these values were 2.67 and 1.91 mg g^{-1} at 12% NaCl, respectively. Therefore changes are due to differences in the α-tocopherol production in function of NaCl and nitrogen concentration.

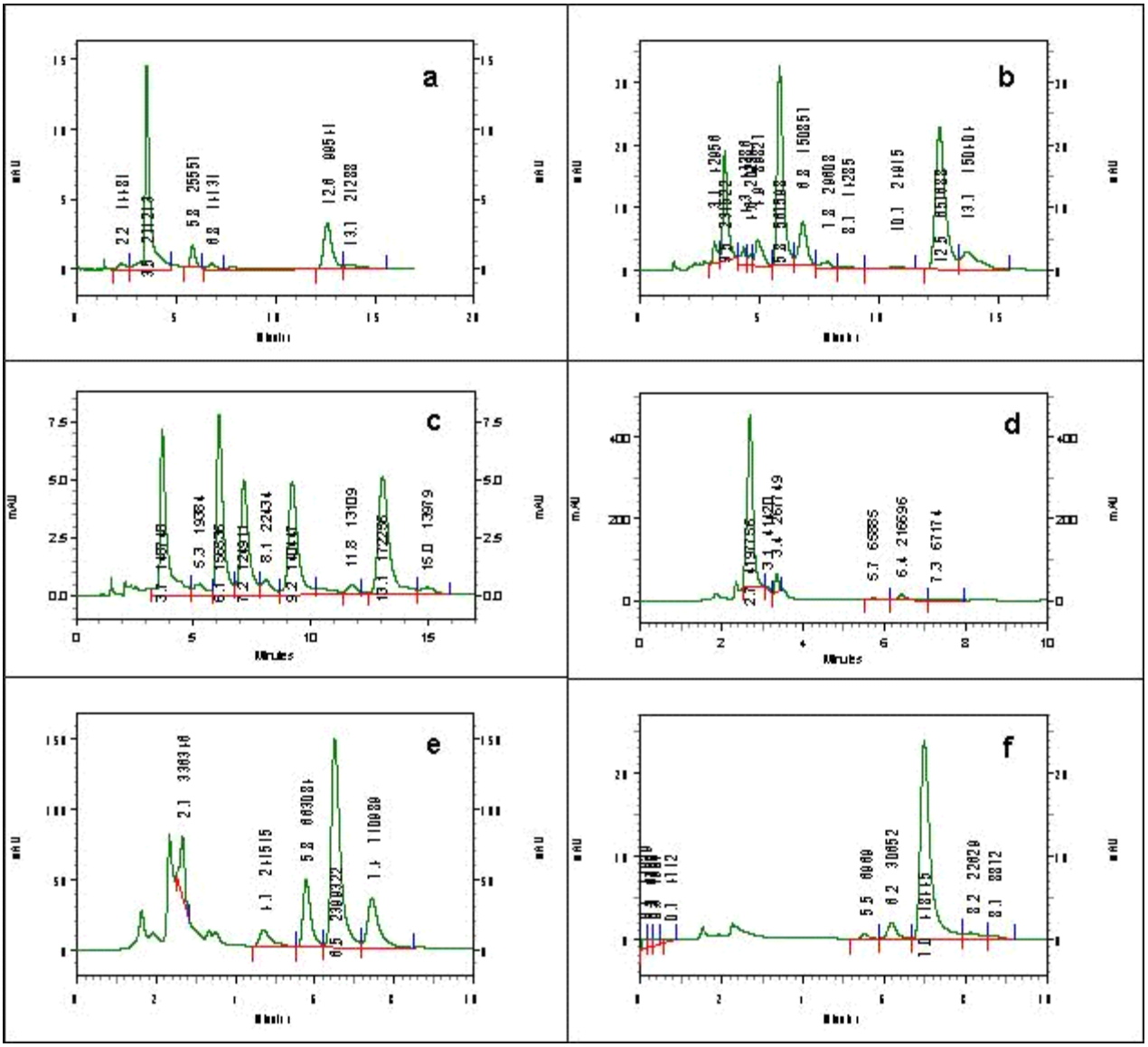
Hydrophilic antioxidants. With respect to the content of hydrophilic antioxidants, both ascorbic acid and glutathione were detected in *D. salina.* The concentration of these antioxidants significant increased as results to expose UV-B, and grown under nitrogen limitation and high NaCl concentration in growth media (Table II). Maximum values were obtained in irradiated cells grown in medium containing 2.5 mM nitrogen and 16% NaCl with 25.41 mg g^{-1} cells d.w and 253.4 μM, respectively. Whereas, minimum values were found in unirradiated cells grown under optimum condition, with 2.7 mg g^{-1} and 97.12 μM, respectively, compared with value 7.2 mg g^{-1} and 174.11 μM in irradiated cells grown under the same condition.

In general, the variation in hydrophilic and lipophilic antioxidant content in irradiated *Dunaliella* cells depending upon nitrogen and NaCl concentration. Similar finding obtained by El-Baz *et al.* (2002), they reported that the cellular antioxidant levels increased in *D. salina* when grown under nitrogen limitation and high NaCl concentration and exposure to high light intensity. Abe *et al*. (1999) reported that the *Trentepohlic aurea* cells was accumulated 2.1 mg β-carotene, 0.3 mg L-ascorbic acid and 2.4 mg tocopherols, respectively, g^{-1} d.w when the culture exposed to high light intensity (430 μmol photon m^{-2} S^{-1}). While in *T. Pseudonana*, UV-B caused a significant increase in GSH content (Rijstenbil, 2000). However, the variations in β-carotene, vitamin C and E content of *D. tertiolecta* have been shown to results concentration in culture from nitrogen sources and media (Abalde *et al.,* 1991).

As a general mechanism the present results revealed that the *D. salina* exposed to UV-B radiation grown under combined stress conditions (high NaCl concentration, & nitrogen deficiency) is usually correlated with accumulation of more efficient antioxidative compounds such as ascorbic acid, glutathion, tocopherol and carotenods (UV-B screening compounds). These compounds can inactivate oxygen radicals, or the effective replacement of damaged constituents can resist UV-B stress (Götz *et al.,* 1999). The negative effect of the various environmental stresses is at least partially due to the generation of active oxygen species (AOS) (Shalata & Tal, 1998). The AOS are produced during normal aerobic metabolism by the interaction between O_2 and electrons leaks from electron transport chains in the chloroplasts and mitochondria (Halliwell, 1997). The AOS molecule $^{\bullet}OH$ (hydroxyl), H_2O_2 (hydrogen peroxide), O_2 (superoxide) and $^1O_2$ (singlet oxygen) are not controlled by protective systems and may destroy proteins, lipid and pigments such as chlorophyll under stress conditions. Thus, algae increased the production of antioxidant or elevated activities of protective enzymes to detoxify and eliminate the highly reactive oxygen species. The antioxidant defense system includes hydrophobic molecules such as carotenoids and tocopherols to remove the singlet oxygen. While the hydrophilic antioxidant ascorbic acid and glutathione are effective chemical scavengers of oxygen radicals (Shalate & Tal, 1998).

Activities of antioxidant enzyme system. The levels of antioxidant enzyme activities CAT, SOD and POD in *D. salina* grown under all experimental conditions are shown in Table III. These enzyme activities in radiated *D. salina* cells were enhanced under all experimental conditions. UV-B exposure, increase the SOD, POD and CAT activities in *D. salina* as compared with unexposed cells, but the induced in SOD level was not significant ($P>0.05$). The variation in these enzyme activities in all experiment was caused by variation in cell growth conditions. However, these enzyme activities were

Fig. 1. HPLC profile of carotenoids and tocopherol of *Dunaliella salina* expoused with UV-B. a: Control not treated with UV-B (Carotenoids); b:Control treated with UV-B (carotenoids);c: Standard of Carotenoids; d: Control not treated with UV-B (Tocopherol); e: Control treated with UV-B (Tocopherol); f: Standard Alpha tocopherol **Compound (RT):** Astaxanthin (3.6-4.2); Lutein (5.5-6.2); Cryptoxanthine (8.9-9.5); b-carotene (12.0- 13.5); Zeaxanthin (7.2 - 8.2)



significantly increased as results to nitrogen limitation and increase the NaCl concentration in growth media. For instances, irradiated cells grown under high salt concentration (16%) combined with nitrogen limitation (2.5 mM N), had an induce in CAT, POD and SOD activities approximately 1.96, 2.83 and 1.97 times as high as that in irradiated cells grown under optimum conditions. Whilst these values were 6.62, 6.75 and 1.13 in irradiated cells grown in 5 mM N and 8% NaCl. At 2.5 mM N. with 12% and 16% NaCl (in parenthesis), the cellular CAT, POD and SOD levels were being 1.77 (1.96), 2.59 (2.83) and 1.79 (1.97) times over than that in irradiated cells grown under optimum condition. These results collectively suggested that an UV-B enhanced these enzyme activities correlated with decrease N level and an increased in NaCl concentration in the growth media. However, several investigator reported that the plant have evolved a better antioxidant mechanisms including antioxidant enzyme like SOD, CAT and POD to increased the resistance to environmental stresses including salt, nitrogen and carbon limitation, drought and UV-irradiation (Rao *et al.,* 1996; Zhang & Kirhham, 1996; Shalata & Tal, 1998; Abd El-Baky, 2003). In which, plants or algae subject to environmental stress, the

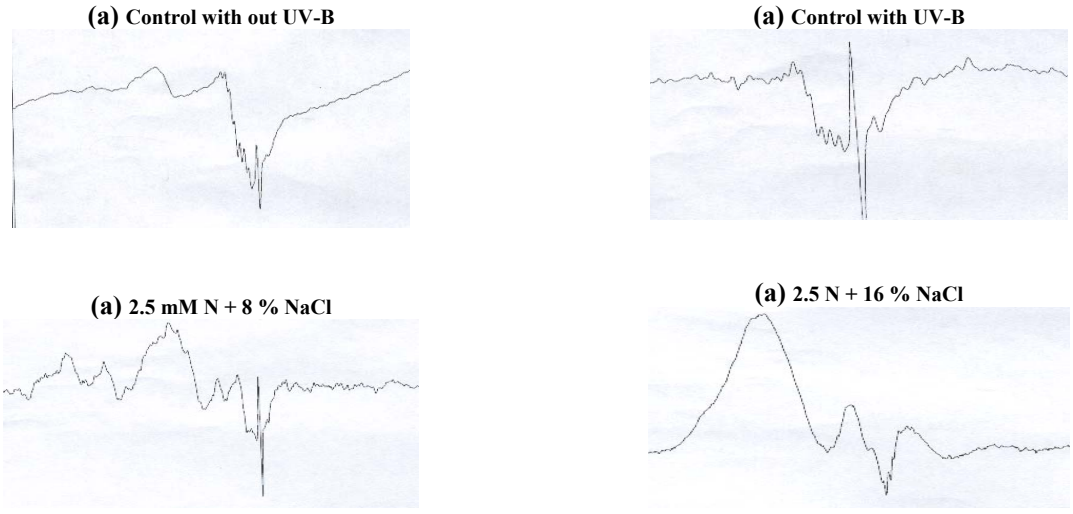
balance between the production of AOS and the quenching activity of antioxidants may be upset and oxidative damage may result (Cakmak & Marschner, 1992). Rijstenbil (2002) found that exposure diatom *Thalassiosira pseudonana* to UV-b and UV-A caused, a significant increase in SOD activity. Gossett *et al*. (1996) suggested that protection from oxidative damage induced under salt stress by more active ascorbare- glutathine cycle and a higher level of antioxidant enzymes like CAT, SOD and POD. However, Strid (1993) and Hernandez *et al*. (1995) reported that Cu, Zn-SOD and ascorbate, peroxidase have a role in preventing NaCl or UV-B irradiation induced oxidation stress in some plants.

Generally, enzymes and secondary compounds of higher plants have been showed using *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. For example, vitamin C, Vitamin E and β-carotene all have antioxidant activity, and thus provide a cellular defense against reactive oxygen species which damage DNA (Peto *et al.,* 1981; Barclay *et al.,* 1983; Mckersie *et al.,* 1990; Hunter *et al.,* 1994).

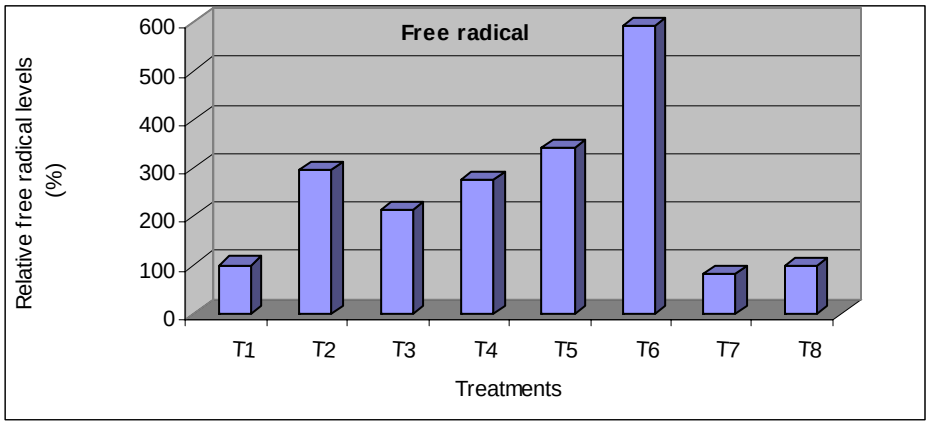
The cellular content of lipid and water-soluble antioxidants and the antioxidant enzyme level were affected in whole cells exposed to UV, as compared to control value. The increase in carotenoids, α-tocopherol, ascorbic acid and glutathion content and enzyme level of SOD, CAT and POD in the irradiated algae reported here, suggests that the adaptation to photooxidative stress in algae cells by regulation the synthesis and repair of proteins and enhanced antioxidative protection system (Malanga *et al.,* 1997).

Lipid peroxidation. The content of MAD (a by-product of the lipid peroxidation) (Table IV) is currently used as an index of lipid oxidation caused by UV-B in unicellular algae, bacterial, higher plants and animals tissues. It is also, widely used as a biomarker for oxidation damage (Malanga

Fig. 2. Electron paramgnatic spectra (a) and free radicals level (b) of control and UV-B treated *D. salina* cells as effected by nitrogen limitation and salt stress



(b)



et al., 1997; Rijstenbil, 2002). The content of MAD in non-irradiate *Dunaliella* cells were 1.04 mM mg^{-1} protein and significant ($P<0.05$) increased in irradiate cells grown under all experimental conditions ranged from 4.97 to 19.60 mM mg^{-1} protein (Table IV). The higher MAD level with values 17.12 and 19.60 mM mg^{-1} protein was obtained when cells exposed to UV-B and grown in medium containing low nitrogen level (2.5 mM) coupled with increased in NaCl concentration 12 and 16%, respectively. The large increases in MDA level in *D. salina* grown under all experimental conditions indicate that UV-B enhanced active oxygen production ˙OH (hydroxyl radicals), which led to lipid peroxidation process (MAD). The enhancement lipid peroxidation in *Dunaliella* culture exposed to UV-B, when grown under nitrogen limitation and salt stress conditions has been reported by Shelly *et al.* (2002). In general, the enhanced of lipid peroxidation in many microorganisms may results from a complexity of environmental factors including light, nutrient limitations (nitrogen or carbon) and high solar radiance (Butow *et al.,* 1998; Rijstenbil, 2002).

Alkyl radical. The direct detection of stable free radical in lyophilized of all algal treated cells was measured by more sensitive technique, PER spectrometer at room temperature. The EPR stable free radical recorded with g-value at a maximum absorption of 2.006 is shown in Fig. 2 and relative % of free radical is under the pecks is shown in Table 5. The data revealed that UV-B exposure significantly increased radical content in the algal cells grown under optimum conditions to 295.5% of the unexposed cells (100%). The value quantitation of stable free radicals in algal cells grown in nitrogen limitation 2.5 mM and zero combined with 8% NaCl media was 213.7 and 274.7% respectively, of the control. In contrast, at 12% NaCl, the amount of free radicals being 3.4 and 5.9 times over than that in the control and these values were decreased to 0.97 and 0.82 of control in algal cells grown at 16% NaCl. There is well-supported evidence that the antioxidant compounds found in algae cell possess free radical scavenging properties. However, these results indicated that the significant increase in stable free radical in algal cells after UV-B exposure and these are in agreement with previous reports from Malanga *et al.* (1997) in cells, microalgae *Chlorella vulgaris.* In which, quantitation of alkyl radical ESR signals in chloroplasts of *C. vulgaris* indicated that UV-B exposure significantly increased radical content in the membranes.

REFERENCES

Abd El-Baky, H.H., A. Moawd, A.N. El-Behairy and G.S. El-Baroty, 2002. Chemoprevention of benzo[a]pyrene-induced carcinogen and lipid peroxidation in mice by lipophilic algae extracts (phycotene). *J. Med. Sci.,* 2: 185–93

Abd El-Baky, H.H., 2003. Over production of Phycocayanin pigment in blue green alga Spirulina Sp .and its inhibitory effect on growth of Ehrlich ascites carcinoma cells. *J. Med. Sci*., 3: 314–24

Abalde, J.J. Fabregas and C. Herrero, 1991. β-carotene, vitamin C and vitamin E content of the marine microalgae *Dunaliella tertiolecta* cultured with different nitrogen sources. *Bioresource Tech*., 38: 121–5

Abe, K., N. Nishmura and M. Hirano, 1999. Simultaneous production of βcarotene, vitamin E and vitamin C by the aerial microalga Trentepohia aurea. *J. Appl. Phycol*., 11: 33–6

Aboul-Enein, M.A., F.K. El-Baz, G.S. El-Baroty, A.M. Yousse and H.H. Abd El-Baky, 2003. Antioxidant activity of algal extracts on lipid peroxidation. *J. Med. Sci*., 3: 87–98

Augustin J., P.B. Klein, D. Becker and B.P. Venugopal, 1985. Vitamin C. *In: Methods of Vitamin Assay.* p. 323. Academic Press, Now York, USA

Bar, E., M. Rise, M. Vishkautsan and A. Shoshana, 1995. Pigment and structural changes in Chlorella zofingiensis upon light and nitrogen stress. *J. Plantphysiol*., 146: 527–34

Barclay, L.R.C., S.J. Locke and J.M. Macneil, 1983. The autoxidation of unsaturated in micelles synergism of inhibitors vitamins C and E. *Canadian J. Chem*., 61: 1288–90

Ben-Amotz, A., Z.A Kay and M. Avron, 1982. Accumulation of β-carotene in halotorant algae: purification and characterization of β-carotene rich globules from *Dunaliella baradwil*. *J. Phycol*., 18: 529–37

Ben-Amotz, A. and M. Avron, 1988. The wavelength dependence of massive of carotene synthesis in Dunaliella bardawil. *J. Phycol*., 25: 178–83

Borowitzka, M.A., 1992. Algal biotechnology products and processes matching science and economics. *J. Appl, Phycol*., 4: 267–79

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram of protein utilizing of protein– day binding. *Anal. Biochem*., 72: 248–54

Butow, B., D. Wynne, A. Sukenik, O. Hadas and E. Tel-Or, 1998. The synergistic effect of carbon concentration and high temperature on lipid peroxidation in peridinium gatunense. *J. Plankton Res*., 20: 355–69

Cakmak, I. and H. Marschner, 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. *Plant Physiol*., 98: 1222–7

Chance, B. and A.C. Maehly, 1955. Assay of catalase and peroxidase. *In:* Colowic, S.P. and N.O. Kaplan (eds), *Methods of Enzymology,* Vol. 2. p. 764. Academic Press, New York, USA

El- Baz, F.K., M.A Aboul-Enein, G.S. El-Baroty, A.M. Youssef and H.H. Abd El-Baky, 2002. Accumulation of antioxidant vitamins in *Dunaliella salina*. *Online J. Biol. Sci*., 2: 220–3

Farag, R.S., K.H. Elkhwas and M. Mohamed, 1998. Distribution of carotenoids in some fresh and boiled foods. *Ads. Food Sci*., 20: 1–6

Ginnopolitis, N.C. and S.K. Ries, 1977. Superoxide dismutase occurrence in higher plants. *Plant Physiol*., 59: 309–14

Gossett, D.R., S.W. Banks, E.P. Millhollon and C. Luceas, 1996. Antioxidant response to NaCl stress in a control and NaCl-tolerant cotton cell line grown in the presence of paraquat buthionine sulfoximine, and exogenous glutathione. *Plant Physiol*., 112: 803–9

Götz, T., U. Windhövel, P. Böger and G. Sandman, 1999. Protection of photosynthesis against ultraviolet-B radiation by carotenoids in transformants of the cyanobacterium Synechococcus PCC7942[1]. *Plant Physiol*., 120: 599–604

Halliwell, B., 1997. Antioxidants and human disease: a general introduction. *Nut. Rev*., 55: S44–S51

Hans-Luck, 1970. Catalase. *In:* Verlag Chemie Gmb H. (ed.), *Method of Enzymatic Analysis.* pp: 885–8. *Weinheim Bergstr,* Academic Press, New York and London

Haraguchi, H., H. Ishikawa and I. Kubo, 1997. Antioxidative action of di-terpenoids from Podocarpus nagi. *Planta Medica,* 63: 213–5

Hernandez, J.A., E. Olmos, F.J. Corpas, F. Sevilla and L.A. Del-Rio, 1995. Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Sci.,* 105: 151–67

Honya, M.K., T. Kinoshita, M. Ishikawa, H. Mori and K. Nisizw, 1994. Seasonal variation in lipid content of cultured Laminaria japonica fatty acids, srerols, βcarotene and tocopherol. *J. Appl. Phyco*. 6: 25–9

Hunter, D.J. and W.C. Willett, 1994. Diet, body build, and breast cancer. *Ann. Rev. Nutr.*, 14: 393–418

Jürgens U.J. and J. Weckesser, 1985. Carotenoids containing outer membrane of *Synechocystis* sp. strain PCC6714. *J. Bacteriol.*, 164: 384–9

Malanga G., G. Calmanovici and S. Puntarulo, 1997. Oxidative damage to chloroplasts from *Chlorella vulgaris* exposed to Ultraviolet β-radiation. *Physiol. Plant.*, 101: 455–62

Malanga, G. and S. Puntarulo, 1995. Oxidative stress and antioxidant content in Chlorella vulgaris after exposure to ultraviolet β-radiation. *Physiol. Plant.,* 94: 672–9

Martin, J.P. and P. Burch, 1990. Production of oxygen radicals by photosensitzation. *Methods Enzymol.*, 186: 635–45

Mckersie B.D., F.A. Hoekstra and L.C. Kreg, 1990. Differences in the susceptibity of plant membrane lipids to peroxidation. *Biochim. Biophys. Acta,* 1030: 119–26

Payer, H.D., 1971. First report upon the organization and experimental work of the Thailand German project on the production and utilization of single cell green algae as a protein source for human nutrition. Inst. of Food Res. And Product Development Kasetsar Univ., Bangkok, Thailand.

Peto R., R. Doll and J.D. Buckley, 1981. Can dietary beta-carotene materially reduce human cancer rate. *Nature,* 290: 201–8

Pugh N., S.A. Ross, H.N. ElSohly, M.A. ElSohly and D.S. Pasco, 2001. Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity from *Spirulina platensis*, *Aphanizomenon flos*-aquae and *Chlorella pyrenoidosa. Planta Med.*, 67: 737–42

Richmond A., 1986. *Handbook of Microalge Mass Culture.* CRC Press Inc. Bocaraton, Florida.

Rao, M.V., G. Paliyath and D.P. Ormrod, 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol.,* 110: 125–36

Rise M.E, M. Cohen, M. Vishkautsan, H.E. Cojocau, E. Gotrlieb and S. Arad, 1994. Accumulation of secondary carotenoids in *Chlorella zofingiensis. J. Plant Physiol.,* 144: 287–92

Rijstenbil, J.W., 2002. Assessment of oxidative stress in the planktonic diatom *Thalassiosira pseudonana* in response to UV-A and UV-B radiation. *J. Plankton Res.,* 12: 1277–88

Schwartz, J.L., 1996. The dual roles of nutrients as antioxidant and prooxidation: Their effect on tumor cell growth. *J. Nutr.*, 126: 12215–75

Shelly, K., P. Heraud and J. Beardall, 2002. Nitrogene limitation in *Dunaliella Tertiolecta*) Chlorophyceae) leads to increased susceptibility to damage by Ultraviolet-B radiation but also increased repair capacity. *J. Phycol.,* 38: 713–20

Semenenko, E.V. and A.A. Abdullaev, 1980. Parametric control of β-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation. *Fizioloiya, Rastenii*, 27: 31–41

Shalata, A. and M. Tal, 1998. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerative *Lycopersicon pennelli*. *Physiol. Plant.*, 104: 169–74

Silber, R., M. Farber, E. Papopoulos, D. Nervla, L. Liebes, M. Bruch and R. Bron, 1992. Glutathione depletion in chronic lymphocytic leukemia B-lymphocytes. *Blood,* 80: 2038–40

Strid, A., 1993. Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet β-radiation. *Plant cell Physiol.*, 34: 949–53

Zhang, J. and M.B. Kirkham, 1996. Lipid peroxidation in sorghum and sunflower seedling as affected by ascorbic acid, benzoic acid and propyl gallate. *J. Plant Physiol.,* 149: 489–93

(Received 01 November 2003; Accepted 10 December 2003)