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EFFECT OF NITROGEN ON THE BIOCHEMICAL CONSTITUENTS AND ANTIOXIDANT PRODUCTION BY TWO GREEN UNICELLULAR ALGAE

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Abstract

In this study, two green microalgae, *Dunaliella bardawil* and *Chlorella ellipsoidea*, were cultured in different concentrations of nitrogen as sodium nitrate. These concentrations were 0.0 (nitrogen-free), 2.5, 5.0, 10.0, 25.0 and 50.0 mM N in case of *D. bardawil* and 0.0, 0.25, 0.50, 1.00, 2.5, 5.0 and 10.0 mM N in case of *C. ellipsoidea*. The results revealed that dry weight, protein, carbohydrates, chlorophyll a and chlorophyll b of *D. bardawil* were highly reduced in N-deficient cultures, whereas carotenoids were unaffected. Glycerol production by *Dunaliella* decreased to about 25 % of control in the absence of nitrogen. Similarly, dry weight, protein and carbohydrate contents of *C. ellipsoidea* were greatly reduced in N-deficient culture, whereas chlorophyll a and chlorophyll b of the same alga seemed to be slightly affected by the absence of nitrogen. Supplementation of nitrogen caused an obvious increase in dry biomass gain and biochemical components of the two algae. *D. bardawil* grown in N-deficient culture produced massive amounts of β-carotene reached to about 227.2 % of control. On the other hand, the accumulation of β-carotene in *Chlorella* cells was unaffected by the absence of nitrate. The content of β-carotene significantly decreased in both microalgae at the higher doses of nitrate. The contents of vitamin E and vitamin C were greatly reduced in both microalgae grown in N-deficient cultures. Supplementation of nitrate caused an obvious increase in the contents of both vitamins in *Dunaliella*. Contrarily, In case of *Chlorella*, while vitamin E was not detected, the content of vitamin C was greatly reduced in all doses of nitrate.

Key words: Antioxidants, β-carotene, *Chlorella ellipsoidea*, *Dunaliella bardawil*, Green microalgae, Nitrogen, vitamin C, vitamin E.

Introduction

Algae are pivotal organisms in aquatic ecosystems as they contribute substantially to primary photosynthetic production. Several unicellular algae become increasing attractive for use by commercial food companies, as they

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produce a great variety of metabolites that are essential for food health (**Kay, 1991**). However, the major interest in recent years has been in microalgae as a source of high value products such as carotenoids, phycobilins and antioxidant vitamins (**Ip and Chen, 2005**). Furthermore, the research performed on algae confirms their therapeutic effects for humans. When algae are exposed to a variety of environmental stresses including excess of irradiance, extreme temperature, salinity, desiccation, starvation and exposure to pollutants, they may respond by a burst of reactive oxygen species (ROS) (**Küpper *et al*., 2001**). In the plant system, ROS are always formed by the inevitable leakage of electron on molecular oxygen from electron transport activities of chloroplasts, mitochondria and the plasma membrane (**Foyer, 1997**). ROS generally include singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), superoxide radical anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (**Mallick, 2004**). The toxicity of ROS arises from their ability to initiate reactive cascade reactions that lead to protein damage, lipid peroxidation, DNA damage and finally cell death (**Davey *et al*., 2002**). Upon oxidative conditions, plants, macro- and microalgae, as many other aerobic organisms respond by developing antioxidant defense mechanisms (both enzymatic and non-enzymatic means) to combat the danger posed by the presence of ROS.

Dunaliella is a unicellular marine green alga that has been cultivated intensively in the last years as a commercial source of natural β-carotene and glycerol, when grown under appropriate conditions (**Borowitzka and Borowitzka, 1988; Gómez-Coronado *et al*., 2004**). The green alga Chlorella has attracted considerable interest for commercial production of functional foods such as polyunsaturated fatty acids by *Chlorella sorokiniana* (**Chen and John, 1991**) and protein, total lipids, carbohydrate and antioxidants of vitamin C and vitamin E by *Chlorella pyrenoidosa* (**Hu *et al*., 2007**).

β-carotene is one of the most widespread and well studied carotenes with antioxidant abilities (**Phadwal and Singh, 2003**). β-carotene protects the cells against oxidative damage by inhibiting or quenching free radicals and ROS (**Hunter and Willer, 1994**). Epidemiological evidences have shown β-carotene to prevent cancer of various organs by means of antioxidant ability (**Hirvonen *et al*., 2000**).

Tocopherols (Vitamin E) are lipid-soluble antioxidants with a widespread occurrence in nature (**Kruk and Strzalka, 1995**). Many microorganisms were screened for the existence of tocopherols, among them, *Dunaliella* (**Brown *et al*., 1999**), *Chlorella*, *Chlamydomonas*, *Ochromonas* (**Taketomi *et al*., 1983; Tani and Tsumura, 1989**) and Euglena (**Kusmic *et al*., 1999**) attracted the attention of many scientists due to their potential role for antioxidant production. Vitamin E represents the most important lipid-soluble antioxidants in plants and animals by scavenging ROS and breaking radical chain reaction (**Burton and Traber, 1990**). Recently, it is believed that vitamin E is involved in the regulation of cellular signaling processes and gene expression (**Frank, 2005**).

L-ascorbic acid (L-AA), commonly known as vitamin C, is one of the most important water-soluble vitamins in the human diet, because it helps the body in forming connective tissues, bone, teeth, blood vessel walls, and assists the body in assimilating iron and amino acids (**Parviainen, 1995**). Generally, L-AA occupies a central role in the protection of plant cells against abiotic and biotic stresses (**Davey *et al*., 2002**).

Nitrogen deficiency which is known to increase the oxidative damage in microalgae, significantly increased total carotenoids in *D. bardawil* (**Shaish *et al*., 1993; Salguero *et al*., 2003; Ghoudhari *et al*., 2008**). Nitrogen starvation in *D. tertiolecta* was characterized by a loss of cell protein and chlorophyll and decline in photosynthetic function (**Young and Beardall, 2003**). Both *D. salina* Teod. and *D. bardawil* were recorded to accumulate large amounts of β-carotene reaching 10 % of the algal dry weight under nitrate or sulphate deficiency (**Ben-Amotz and Avron, 1983; Borowitzka and Borowitzka, 1988**).

The aim of this study is to elucidate the effect of nitrogen deficiency or nitrogen supply on growth, some biochemical responses as well as the production of three important antioxidants namely, β-carotene, vitamin E and vitamin C by two green microalgae, *Dunaliella bardawil* and *Chlorella ellipsoidea* grown under conditions of different nitrogen supply.

Materials and Methods

Two unicellular green microalgae were used in this investigation, *Dunaliella bardawil* and *Chlorella ellipsoidea*. *Dunaliella bardawil* was kindly supplied by Prof. Abdel-Fattah Khaleafa, professor of Phycology, Botany Department, Alexandria University, Alexandria, Egypt, while *Chlorella ellipsoidea* was obtained from the algal collection centre of Mansoura University, Mansoura, Egypt. The axenic cultures of *D. bardawil* and *C. ellipsoidea* were maintained in MH (**Loeblich, 1982**) and MBL (**Nichols, 1973**) nutrient media, respectively.

In order to examine the effect of nitrogen on growth, biochemical components as well as antioxidant production, the two tested microalgae were cultured in different concentrations of nitrogen as sodium nitrate. These concentrations were 0.0 (nitrogen-free), 2.5, 5.0, 10.0 (control medium), 25.0 and 50.0 mM N in case of *D. bardawil* and 0.0, 0.25, 0.50, 1.00(control medium), 2.5, 5.0 and 10.0 mM N in case of *C. ellipsoidea*. The axenic cultures of both algae were aseptically inoculated into 50ml fresh nutrient media in 250 ml Erlenmeyer flasks. The algal inoculum density was adjusted to arbitrary standard cell number of 0.5×10^5 and 1.5×10^6 cells ml^{-1} of *D. bardawil* and *C. ellipsoidea*, respectively. All the culture flasks were incubated at 28±2ºC under continuous illumination (using fluorescent lamps) at 78 $\mu Em^{-2}s^{-1}$. The duration of the experiments was 10

days as determined from the growth curves of both microalgae. All the experiments were carried out in triplicates.

At the expiry of the experimental period, the culture biomass was separated from its medium by centrifugation for 20 min. at 100x g and directly put in a hot dry oven at 70-80˚C for 24 hrs or till constant weight to determine the changes in the dry weight. The pigments (chlorophyll a, chlorophyll b and carotenoids) were estimated following the method described by **Metzner *et al*.** (**1965**). The method of **Lowry *et al***. (**1951**) was applied to estimate the protein content, using bovine serum albumin as standard. Total carbohydrate content was estimated in algal biomass as glucose according to the method described by **Dubois *et al.*** (**1956**), while glycerol was estimated in *D. bardawil* cultures according to the method recommended by **Chitlaru and Pick** (**1989**).

The contents of β-carotene, vitamin E and vitamin C in the algal biomass were measured using high performance liquid chromatography (HPLC) analysis. Analysis of the three antioxidants was performed on a model "HP1050" HPLC equipped with UV detector. Separation and determination were performed on a C18 (ODS) column (4.6mm × 250mm). The gradient mobile phase consisted of acetonitrile/ chloroform (29:8, v/v), in case of β-carotene and vitamin E, while in case of vitamin C, the column was eluted with a mixture of methanol and water (1:1, v/v). Total run time for separations was approximately 15 min. at a flow rate of one ml min^{-1}. β-carotene, vitamin E and vitamin C were detected at 254, 470 and 254 nm, respectively (**Gertz, 1990**).

Statistical analysis: Data are expressed as mean ± SE from three independent experiments. One-way analysis of variance (ANOVA) was performed using SPSS version 12, followed by Duncan's test (**Christensen, 1996**).

Results

Data represented in Table (1) show that the dry weight of *D. bardawil* grown in control medium (10.0 mM N) recorded the highest value of dry weight gain. Increasing or decreasing the nitrate concentration than control value resulted in a significant decrease in the dry weight of *D. bardawil*, except at 25.0 mM N, which is not significantly different from control value. It is worthy to notice that the drop in dry weight is significantly the same at 5.0 and 50.0 mM N. Among all the doses of nitrate, 2.5 mM induced the lowest dry weight gain (46 % with respect to control). A severe drop in dry weight of *D. bardawil* is occurred when the alga was grown in nitrate-deficient medium, where it attained only 20.7 % of that of control. On the other hand, the dry weight of *C. ellipsoidea* attained its maximum value (43.33 mg in 50 ml) at 5mM nitrate. This value is about 1.5 times of that of control culture (1mM N). The dry weight increased as nitrate dose increased to 10 mM nitrate, but this increment is non-significantly different from that at 5 mM N. The dry weight of this alga grown either in nitrate-deficient

medium or in low concentrations of nitrate (0.25 and 0.5 mM) are non-significantly different from control.

Table (1): Effect of different concentrations of nitrogen on some growth parameters of *D. bardawil* and *C. ellipsoidea*. Data are averages of three separate experiments of triplicates each. Each value represents the mean ± SE (p< 0.05). Means followed by the same letters are not significantly different.

Organism	Nitrogen Conc. (mM)	Dry Weight (mg/50ml)	Protein Content (mgg^{-1}dry weight)	Carbohydrate Content (mgg^{-1}dry weight)	Glycerol (%control)
D. bardawil	0.00	8.47^{d}±0.20	74.02^{f}±1.59	73.35^{b}±1.70	25.00^{f}±0.35
	2.50	18.87^{c}±0.09	251.45^{e}±3.85	117.78^{b}±1.12	56.35^{e}±0.59
	5.00	25.10^{b}±0.35	301.19^{d}±6.74	111.72^{b}±36.99	58.44^{d}±0.45
	10.00 (control)	40.97^{a}±1.27	390.53^{c}±4.75	229.15^{a}±45.61	100.00^{b}±0.00
	25.00	39.47^{a}±0.68	507.67^{a}±8.06	252.23^{a}±7.86	102.02^{a}±0.62
	50.00	23.47^{b}±1.11	490.21^{b}±5.74	104.50^{b}±44.97	87.12^{c}±0.34
C. ellipsoidea	0.00	21.67^{c}±0.67	115.65^{b}±5.11	137.02^{c}±10.18	-
	0.25	22.67^{c}±1.20	140.44^{b}±5.89	139.20^{c}±6.03	-
	0.50	23.67^{c}±1.67	192.24^{a}±15.95	159.67^{c}±5.81	-
	1.00 (control)	26.00^{c}±1.0	216.05^{a}±9.42	222.91^{a}±14.63	-
	2.5	31.00^{b}±0.58	210.31^{a}±2.42	192.49^{b}±4.51	-
	5.0	43.33^{a}±2.19	209.10^{a}±12.72	195.17^{b}±11.72	-
	10.0	40.33^{a}±2.67	206.04^{a}±14.47	163.11^{c}±1.96	-

The same table demonstrates that protein content of *D. bardawil* attained its maximum value at the higher nitrate concentrations, 25mM and 50mM N recording 130.0 and 125.5% compared to control, respectively. At lower concentrations of nitrate, 2.5 and 5.0mM N, protein content significantly decreased reaching 64.4 and 77.1 % of control, respectively. Under N-deficient condition, the protein content of *D. bardawil* recorded only 19 % of control. On the other hand, the protein content of *C. ellipsoidea* attained its maximum value at control culture (1.0 mM N). The total protein content recorded 65.0, 89.0, 97.3, 96.8 and 95.4 % as compared with control, at 0.25, 0.5, 2.5, 5.0 and 10 mM N, respectively. N-deficient culture showed the lowest accumulation of protein in *C. ellipsoidea* reaching 53.5 % of control.

The content of total carbohydrates was significantly enhanced in *D. bardawil* cells grown in 10.0 mM N (control) and 25 mM N. Increase or decrease of nitrate concentration from these doses caused significant decrease in the accumulation of carbohydrates. The contents of carbohydrates amounted 51.4, 48.8 and 45.6 % as compared with control at 2.5, 5.0 and 50 mM N, respectively. The algal cells grown in N-free culture had the lowest value of total carbohydrate (32.0 % of control).

Furthermore, the production of glycerol by *D. bardawil* attained the highest value at 25 mM N (102.02 % of control), but significantly decreased to 87.12 % as the nitrate concentration arose up to 50 mM N. Glycerol production significantly decreased to 56.35 and 58.44 % of control, respectively, at lower nitrate concentrations (2.5 and 5.00 mM). The lowest value of glycerol (25 %) was obtained from the N-deficient culture (Table 1). On the other hand, the carbohydrate content significantly accumulated by the *C. ellipsoidea* cells at 1.0 mM N (control culture). The carbohydrates content significantly decreased to 62.4% of control at 0.25 mM N. Carbohydrate also significantly decreased to 61.5% in the N-free culture, but this value is non-significantly different from that at 0.25 mM N. With increasing the dose of nitrate to 2.5, 5 and 10 mM N, a slight significant reduction in carbohydrate content was observed.

Figure (1a) shows that *D. bardawil* grown in N-deficient culture exhibited the lowest contents of chl a, chl b and carotenoids. Supplementation of the nitrate dose (from 2.5 to 50 mM) significantly and progressively increased the contents of the three pigments reaching the highest values at the highest nitrate dose (50mM). These values are 13.47, 5.55 and 5.21 mgg^{-1} dry weight corresponding to 135.4, 122.0 and 115.5 % of control for chl a, chl b and carotenoids, respectively.

Figure (1b) reflects that chlorophyll a content of *C. ellipsoidea* is significantly enhanced at 1.0 (control), 2.5 and 5.0 mM N recorded 8.80, 8.78 and 7.91 mg g^{-1} dry weight. These values are non-significantly different. Reduction of the nitrate dose to 0.25 and 0.5 mM caused a significant reduction in chl a content of *C. ellipsoidea* reached to 32.4 and 19.1 % of control, respectively. Similarly, the increase of nitrate concentration to 10 mM caused a significant drop of chl a content reached to approximately 50 % of control. Chlorophyll b content of *C. ellipsoidea* exhibited a similar trend with chlorophyll a with the highest value at 2.5mM N. The lowest value of chlorophyll b was also attained at 10 mM N (58.4 % of control). Fig. (1b) further shows that the absence of nitrate caused significant decrease in the contents of chl a and chl b to about 68.1 and 75.2 % of control, respectively. These values are insignificantly different from that recorded at 0.25 mM N. N-deficient or N-supplemented cultures (from 0.25 mM up to 2.5 mM) have no significant effect on the carotenoids accumulation in Chlorella. At the highest doses of nitrate, carotenoids content significantly decreased to about 75 and 55.0 % of control at 5.0 and 10.0 mM N, respectively.

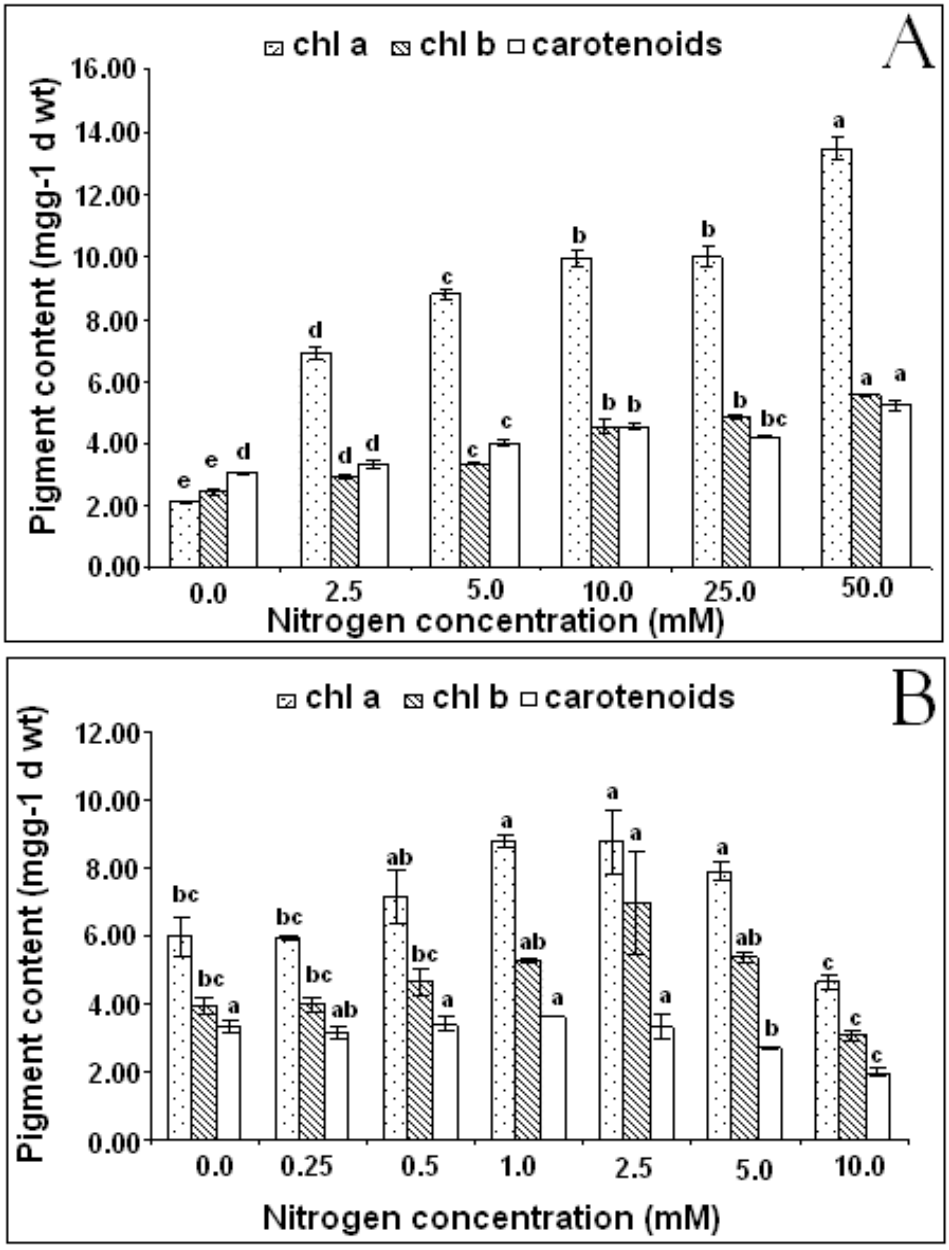

Figure (1): Effect of different concentrations of nitrogen on photosynthetic pigment content of *D. bardawil* (a) and *C. ellipsoidea* (b) after 10 days-growth period. Data are the average of three separate experiments, each experiment being performed in triplicate. Error bars denote SE of the mean. Different letters at the top of the bars indicate significant differences at $p< 0.05$

Table(2) and Fig.(2a) demonstrate that *D. bardawil* grown in nitrogen-free culture significantly accumulated massive amounts of β-carotene reached to about 277.2 % of that of control culture. Among the nitrate concentrations used, the accumulation of β-carotene was still significantly higher than control at 5 mM N (122.3 % of control). As the nitrate concentration increased to 25.0 and 50.0 mM, the amount of β-carotene significantly dropped to 33.3 and 26.5 % as compared with control, respectively. The production of vitamin E by *D. bardawil* grown in N-deficient culture, decreased to about half that of control. In N-supplemented culture, vitamin E significantly accumulated to 234.2 % of control at 25 mM N then significantly decreased to 139.8 and 114.5 % control at 5 and 50 mM N, respectively. Vitamin C content in *D. bardawil* was significantly enhanced to 138.2, 154,6 and 149.7 % of control at 5, 25, and 50 mM N, respectively. The lowest value of this vitamin (78.8 % of control) was attained when the alga grew in N-free culture.

Table (2): Accumulation of antioxidants (β-carotene, vitamin E & vitamin C) in *D. bardawil* and *C. ellipsoidea* grown in the presence of different concentrations of nitrogen. Data are averages of 3 different experiments of triplicate each. Each value represents the mean ± SE ($p<0.05$). Means followed by the same letters are not significantly different.

Organism	Nitrogen Concentration (mM)	Antioxidants concentration (µg /100 mg fresh weight)		
		β-carotene	vitamin E	vitamin C
D. bardawil	0.0	1738.0^{a}±21.94	36.6^{e}±2.60	1.3^{b}±0.10
	5.0	767.0^{b}±38.68	108.1^{b} ±2.89	2.3^{a}±0.11
	10.0 (control)	627.0^{c}±15.59	77.3^{d}±1.96	1.7^{b}±0.17
	25.0	209.0^{d}±6.20	181.0^{a}±6.95	2.6^{a}±1.17
	50.0	166.0^{d}±8.51	88.5^{c}±2.04	2.5^{a}±1.05
C. ellipsoidea	0.0	98.0^{a}±4.04	nd.±0.00	0.72^{c}±0.023
	0.5	91.0^{a}±1.73	nd.±0.00	0.94^{c}±0.012
	1.0 (control)	94.0^{a}±1.73	4.30^{a}±0.36	1.57^{a}±0.035
	5.0	77.0^{b}±3.46	nd.±0.00	1.32^{b}±0.092

(nd. = not detected).

On the other hand, the accumulation of β-carotene in *C. ellipsoidea* was significantly unaffected by the absence or presence of nitrate, except at the highest dose of nitrate (5.0 mM), where β-carotene accumulation significantly decreased to about 81.9 % as compared with control.

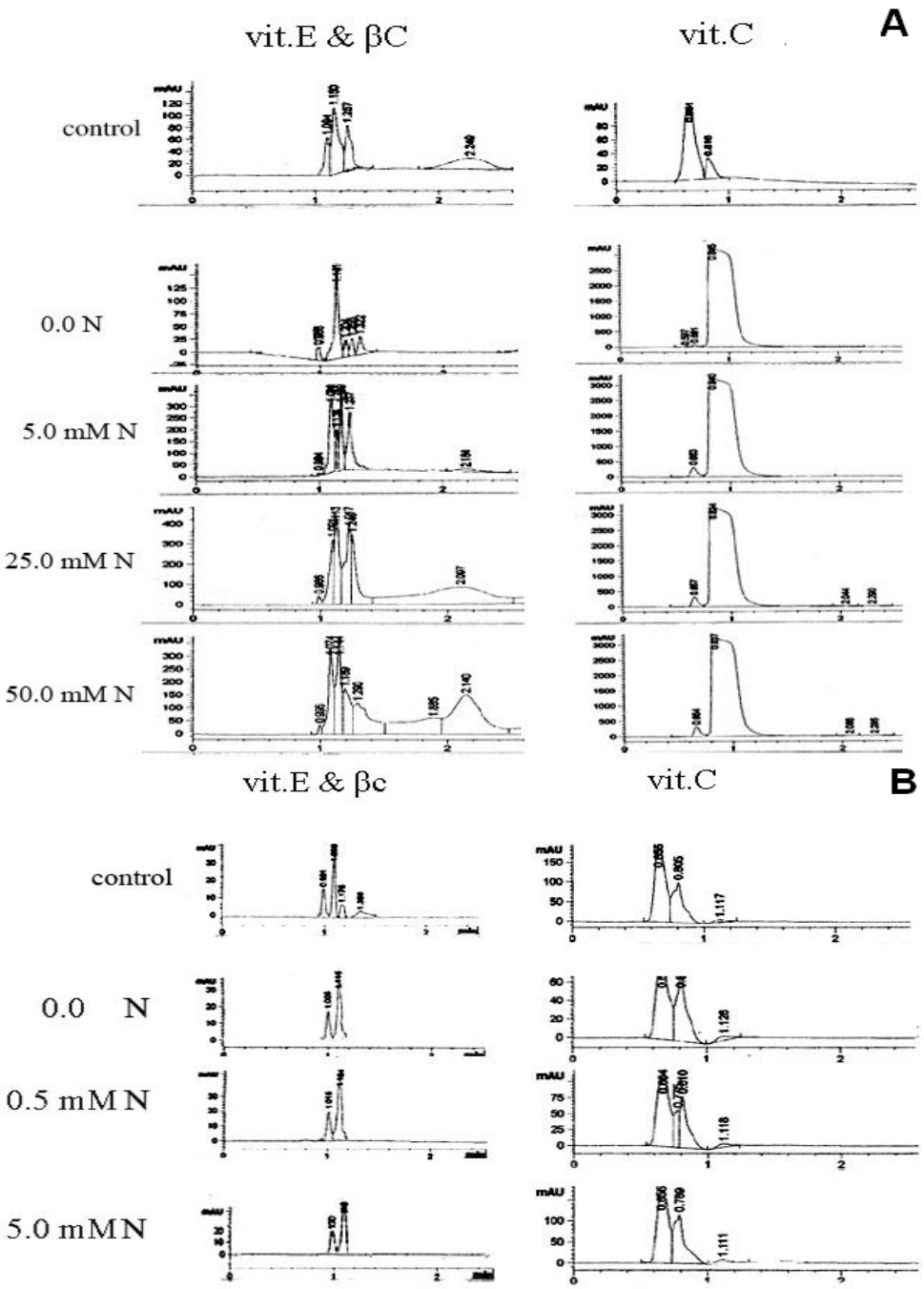

Figure (2): HPLC chromatograms of β-carotene (βC), vitamin E (vit. E) and vitamin C (vit. C) of *D. bardawil*. (a), and *C. ellipsoidea* (b) grown under different concentrations of nitrogen

Furthermore, vitamin E was not detected in the extracts of *C. ellipsoidea* grown either in N-deficient culture or in 0.5 and 5.0 mM N. Vitamin E, however, accumulated only when the alga was grown in 1 mM N (control culture). The accumulation of vitamin C by *C. ellipsoidea* attained its maximum value at 1 mM N (control culture). The amount of vitamin C decreased to 59.9 and 84.1 % of control at 0.5 and 5.0 mM N, respectively. In N-deficient culture, vitamin C recorded the lowest accumulation value (45.9 % of control) (Table 2 and Fig. 2b).

Discussion

Nitrogen is an important component of culture medium; it can provoke important changes in the growth of the microalgal species. The results of this investigation clearly show that nitrogen deficiency had a dramatic effect on all tested growth parameters dry weight of the two chlorophycean microalgae, *D. bardawil* and *C. ellipsoidea*. This effect is more prominent in case of *D. bardawil*, i.e., *C. ellipsoidea* seemed less sensitive to absence of nitrate in the nutrient medium. In N-free cultures, the dry weight gain of *D. bardawil* and *C. ellipsoidea* reached 20.7 and 83.4 % as compared with control cultures, respectively. These results appear to be consistent with the findings of **Ben-Amotz and Avron** (**1983**) who reported that the alga ceases to divide when available nitrate is depleted. Similarly, **Abe *et al***. (**2007**) reported that the N-free medium inhibited cell division of *Coelastrella striolata* var. *multistriata*. According to **Arad *et al***. (**1993**) and **El-Baz *et al***. (**2002**) it seems that the division of *Dunaliella* and *Chlorella* cells grown under nitrogen deficiency is blocked.

Data obtained from the present study also revealed that 10.0 mM nitrate enhanced the dry weight yield of *D. bardawil*. With increasing or decreasing this dose the dry biomass gain significantly decreased. In case of *C. ellipsoidea*, the dry weight increased as the concentration of nitrate increased, reaching the highest value at 5 mM N and 10 mM N, (166.7 and 155.1 % of that of control culture), respectively. These findings are in agreement with the findings of **Fabregas *et al***. (**1989**) who reported that the growth of *D. tertiolecta* tended to increase with increasing of nitrogen concentration. At higher concentration of nitrate there was no significant difference in growth was observed. Similarly, **Uriarte *et al***. (**1993**) reported that the growth rate and cell size of *D. primolecta* were correlated positively with the nitrogen concentration of the medium. A higher biomass of *Coelastrum striolata* var. *multistriata* was also obtained at higher nitrate concentration (**Abe *et al***., **2007**). Contrary, **Taha** (**2002**) found that higher doses of nitrate may reduce the growth of *Dunaliella* species. On the other hand, **Jimenez and Niell** (**1991**) mentioned that nitrogen content affects the total cell biomass but not cell division. The marked differences in the growth of the two tested organisms in the present study can be attributed to the amount of available nitrogen. Generally, microalgal cultures at higher nitrogen concentrations are usually made to obtain maximum production of total biomass or of a particular product (**Mc Lachlan**, **1964**).

The results of the present study showed that N-deficient culture exhibited the lowest values of protein and carbohydrates of both *D. bardawil* and *C. ellipsoidea*. The reduced value of protein was more significantly lower in case of *D. bardawil*. Similarly, *Dunaliella* grown in N-deficient medium exhibited a significant decrease in the content of chlorophyll a and chlorophyll b, whereas carotenoids are hardly being affected by the absence of nitrogen. On the other hand, the three pigments of *C. ellipsoidea* were hardly affected when this alga grew in N-free culture. In this connection, **Kaplan *et al*.** (**1986**) generalized that nitrogen deficiency is coupled to protein and chlorophyll decrease. **Jimenez and Niell** (**1991**) and **Uriarte** (**1993**) also reported a significant decrease in the synthesis of photosynthetic pigments under N-deficient condition. Moreover, **Ben-Amotz** (**1987**) showed that when *D. bardawil* was grown under nitrogen depletion, the chlorophyll content decreased to a minimal value. **Ip and Chen** (**2005**) reported that absence of nitrogen can alter the normal metabolism such as protein synthesis and hence indirectly affects the pigment formation in algae. According to several studies (**Rice *et al*., 1994**; **El-Baz *et al*., 2002**) it seems that photosynthesis of *Dunaliella* and *Chlorella* grown under N-deficiency continues leading to specific compounds such as carotenoids, carbohydrates and triglycerides. Generally, nitrogen deficiency was characterized by a loss of cell protein and a reallocation of protein resources to ameliorate the effect of N-deprivation (**Geider *et al*., 1998**).

The data obtained herein also revealed that both protein and carbohydrate contents of *D. bardawil* increased as the concentration of nitrate increased then began to decrease at the highest nitrate level. In case of *C. ellipsoidea*, an obvious enhancement of protein content was almost occurred under all the used doses of nitrate, while the carbohydrates increased as the nitrogen increased to 1.0 mM then began to decrease significantly at higher concentrations.

Variation in the composition of the culture medium can cause changes in the biochemical content of microalgae, especial protein and carbohydrates. Among the different components of the culture medium, the source and concentration of nitrogen induce important changes in the biochemical composition of microalgal species (**Kaplan *et al*., 1986**). **Fabregas *et al*.** (**1989**) emphasized that the carbohydrate content of D. tertiolecta increased proportionally to nitrogen concentration and these carbohydrates were more independent of the protein content. On the contrary, **Uriarte *et al*.** (**1993**) mentioned that the amount of protein/cell decreased significantly with reduced nitrate availability while the associated carbohydrate content increased significantly. In marine microalgal cultures, in any condition where protein content decreased, lipid and/or carbohydrates might be expected to increase depending on the species of the alga (**Utting**, **1985**).

Furthermore, the results of the present study revealed that the photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) of *D. bardawil* increased as the nitrate concentration increased recorded the highest values of the three pigments were recorded at 50 mM N. In case of *C. ellipsoidea*, the three pigments increased as the nitrate concentration increased to 5.0 mM N then their amounts decreased at the higher dose.

These data are in accordance with those of **Fabregas *et al***. (**1989**), who showed that chlorophyll a content of *D. tertiolecta* per ml culture increased with the increase in the nitrogen concentration but decreased at high nitrogen values. **Pei-Ram and Wei-Hua** (**1999**) concluded that nitrogen concentration had a large effect on cell biomass and pigment production. Similarly, **Abe *et al***. (**2007**) reported that increasing of nitrate concentrations in culture medium, promoted the production and the accumulation of large amounts of carotenoids.

Nitrogen as a component of chlorophyll molecule can stimulate the production of chlorophyll without affecting the growth (**Shutter**, **1979**). **Fabregas *et al***. (**1989**) claimed that chlorophyll only accounts for a small part of the cytoplasm in microalgae and, therefore, changes in chlorophyll are not necessarily indicative of changes in biomass or vise versa. This is the case in our results as the chlorophyll content of both *D. bardawil* and *C. ellipsoidea* showed no relation to the dry biomass gain of both microalgae. It is worthy to mention that chlorophyll was more sensitive than carotenoids to N-availability especially in case of *D. bardawil*. A similar distinction was observed in *D. tertiolecta* (**Young and Beardall**, **2003**).

Furthermore, the findings of the present study revealed that glycerol production by *D. bardawil* was markedly decreased in N-deficient culture. Addition of nitrate to the medium promoted the production of glycerol only at the higher doses. This result is in agreement with those of **Taha** (**2002**) who reported that nitrogen is among the main elements responsible for the synthesis of glycerol. Contrary to the present result, **Phadwal and Singh** (**2003**) showed that nutrient depletion did not seem to have a profound impact on glycerol accumulation in the cells of *Dunaliella* species.

The obtained results in the present study revealed that *D. bardawil* accumulated massive amounts of β-carotene when grown in N-free medium. The production of β-carotene sharply dropped reaching only 33.3 and 26.5 % as compared with control, when the alga grown at 25 and 50 mM N, respectively. On the contrary, β-carotene content of *C. ellipsoidea* seemed to be unaffected by absence or presence of various doses of nitrogen. This might be related to the fact that the biosynthesis of carotenoid molecules does not require a nitrogen source.

The above for mentioned results appear to be consistent with the findings of some other studies. **Lerch** (**1937**) found that N-deficiency caused the cells of *D. salina* to redden, due to an increase in β-carotene content. Two strains of

Dunaliella, *D. salina* Teod. and *D. bardawil*, produce and accumulate large amounts of β-carotene, reaching more than 10 % of the algal dry weight under appropriate stress conditions, which include nitrate deficiency (**Loeblich**, **1982**; **Ben-Amotz and Avron**, **1983**; **Salguero *et al*.**, **2003**). In this connection, **Abd El-Baky *et al*.** (**2003**) showed that the highest level of carotenoids was obtained by 2 species of *Spirulina* when grown in N- free medium. Generally, it has been reported that stress conditions such as nitrogen deficiency may induce the accumulation of carotenoids in green algae (**Leach *et al*.**, **1998**).

β-carotene is an effective endogenous scavenger, it quenches both triplet-state of chlorophyll and singlet oxygen, preventing initiation of lipid peroxidation (**Parker and Joyce**, **1967**) and dissipating the excess energy as heat (**Osmond *et al*.**, **1997**). **Shaish *et al*.** (**1993**) suggested that photosynthetically produced oxygen radicals are involved in triggering massive β-carotene accumulation in *D. bardawil*. An oxygen-tolerant mutant of *Azospirillium brasilense* could produce more carotenoids in response to a high dissolved-oxygen concentration in culture (**Hartmann and Hurek**, **1988**).

The persistence of carotenoids under nitrogen stress may relate to reorganization of the photosynthetic apparatus to maximize chlorophyll-specific light-harvesting efficiency under N-stress (**Sosik and Mitchell**, **1991**). The accumulation of carotenoids in algal cells under N-stress was attributed to the fact that carotenoids do not require nitrogen for their synthesis (**Abd El-Baky *et al*.**, **2003**).

The data obtained from the present study also revealed that the accumulation of vitamin E in the cells of *D. bardawil* grown in N-free culture, significantly decreased to about 50 % of the control culture. The highest value of vitamin E accumulation was obtained at 25 mM N, whereas at 5 and 50 mM N, the vitamin content decreased to values which are higher than that of control. On the other hand, vitamin E was not detected in the extracts of *C. ellipsoidea* grown in N-free or N-supplemented culture. The cells of *C. ellipsoidea* grown at 1mM N (control) accumulated 4.3 µg α-tocopherol per 100 g fresh weight. **Abd El-Baky *et al*.** (**2003**) found that, under N-deficincy, *Spirulina platensis* and *S. maxima* accumulated α-tocopherol to values of 960 and 1325 µg Kg^{-1} dry weight, respectively, whereas minimum values were found in the cyanobacteria grown in adequate nitrogen medium, with values of 144.8 and 266.5 µg kg^{-1} dry weight .

Early studies on vitamin E production by microorganisms showed that some algae such as *Euglena gracilis* and *Dunaliella* are considered as a potential source for tocopherols under conditions of N-stress (**El-Baz *et al*.**, **2002**). On the other hand, **Abd El-Baky *et al*.** (**2003**) showed that the production of α-tocopherol in two isolates of *Spirulina* increased with decreasing nitrogen level in nutrient medium. α-tocopherol (or vitamin E) is one of the acknowledged

antioxidants in biological systems (**Polle and Rennenberg**, **1994**). This lipid-soluble vitamin functions as ROS scavenger and plays an important role in protecting and maintaining the integrity of cell membranes, especially in the chloroplasts, where it quenches $^1O_2$ (**Mano**, **2002**). Vitamin E also protects membrane proteins from oxidation. Being a lipid-soluble molecule, it is very important as a chain terminator of free-radical reactions that cause lipid peroxidation (**Burton *et al***., **1982**).

As shown from the results in the present study, vitamin C content of both *D. bardawil* and *C. ellipsoidea* grown in N-deficient cultures significantly decreased as compared with that of control culture. Addition of different doses of nitrate significantly enhanced the accumulation of vitamin C in *D. bardawil* cells. On the contrary, the content of this vitamin in *C. ellipsoidea* grown in 0.5 mM N was greatly reduced then it increased at higher nitrate doses (1mM and 5 mM). It is worthy to notice that the content of vitamin C is approximately the same in both algal cells grown under controlled conditions (1.65 and 1.56 µg 100 mg^{-1} fresh weight for *D. bardawil* and *C. ellipsoidea*, respectively.).

L-Ascorbic acid (L-AA) performs a range of important biochemical and physiological functions in plants and animals. The biological function of L-AA is based upon the well-known antioxidant character of this molecule, which occupies a central role in the protection of plant cells against abiotic and biotic stresses (**Davey *et al***., **2002**). It was also shown that vitamin C is an important antioxidant that reacts not only with H_2O_2, but also with $O_2^{\bullet -}$, $HO^{\bullet}$ and lipid hydroperoxides (**Yu**, **1994**). In aqueous solution, $^1O_2$ is also quenched by L-AA (**Mano**, **2002**). Furthermore, because L-AA is midly electronegative, it can accept electrons from a wide range of substrates (**Davey *et al***., **2002**).

Bayanova and Trubachev (**1981**) analyzed 75 species for their vitamin C content and found that the levels ranged from 0.054 % (*Platymonas virdis*) to 0.24 % (*Chlorella vulgaris*). *Dunaliella tertiolecta* contained 347 fg $cell^{-1}$ (**Hapette and Poulet**, **1990**). On the other hand, studies carried with the freshwater microalga *Scenedesmus acutus* showed ascorbic acid values between 750 and 1653 mg kg^{-1} (**Becker**, **1980**). It is worthy to mention that the vitamin C content of the two tested microalgae in the present study grown in different doses of nitrate ranged from 2.28 to 2.55 and 0.72 to 1.32 µg 100 mg^{-1}fresh weight for *Dunaliella* and *Chlorella*, respectively.

Conclusion

Stress treatments consisting in unbalanced inorganic nitrogen supply that affect the algal cell's biochemical composition, will have a major impact on its nutritional value.

This investigation has attempted to illustrate a rational approach to the evaluation of microalgal processes for the production of fine chemicals and other products for possible commercialization.

Maximum amount of β-carotene was accumulated in *D. bardawil* under nitrogen deficiency. However, this nutrient stress condition causes a sharp decrease in the accumulation of glycerol by this microalga. *D. bardawil* was found to have a higher β-carotene accumulation capacity better than *C. ellipsoidea*, and can be commercially exploited by employing a medium established on the basis of the nutrient deficiency studies.

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تأثير النتروجين على المكونات البيو كيميائية وانتاج مضادات الأكسدة بواسطة اثنين من الطحالب الخضراء الدقيقة

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تم فى هذه الدراسة زراعة اثنين من الطحالب الخضراء الدقيقة هما دونـاليلا برداويل وكلوريلا إليبسويدا فـى تركيـزات مختلفـة مـن النتـروجين والـذى تمـت إضـافته فـى صـورة نتـرات الصـوديوم. هـذه التركيزات هى: 0.0, 2.5, 5.0, 10.0, 25.0 and 50.0 فى حالة دونـاليلا برداويل و 0.0, 0.25, 0.5, 1.0, 2.5, 5.0 and10.0 ملى مول فى حالة كلوريلا إليبسويدا. وقد أثبتت النتائج حدوث نقص كبير فى قيم الوزن الجاف والبروتين والكربوهيدرات وكلوروفيل ا وكلوروفيل ب فى حالة طحلب دونـاليلا برداويل فـى المزارع الخالية من النتروجين بينما لم تتأثر قيم الكاروتينات. كمـا وجـد أيضـا أن إنتـاج الجليسـرول بواسطة نفس الطحلب قل بنسبة 25% فـى المـزارع خاليـة النتـروجين مقارنـة بـالكنترول. بالمثل فـى حالـة كلوريلا إليبسويدا حـدث نقـص شـديد فـى قيم الـوزن الجـاف والبـروتين والكربوهيـدرات فـى المـزارع الخاليـة مـن النتروجين بينما قيم كلوروفيل ا وكلوروفيل ب لنفس الطحلب لم تتأثر بشكل ملحوظ بغياب النتروجين. ولقد وجد أن إمداد هذه المزارع بـالنتروجين قد سبب زيـادة ملحوظـة فـى قيم الـوزن الجـاف والمكونـات الحيويـة للكائنين. دلت النتائج أيضا على أن زراعة طحلب دونـاليلا فى أوساط غذائيـة خاليـة مـن النتـروجين أدى إلـى إنتاج كمية كبيرة من البيتا-كاروتين بواسطة هذا الطحلب وصلت إلـى حوالى 227% مـن الكنتـرول. ولكـن علـى العكـس تمامـا أثبتـت الدراسـة أن تـراكم البيتـا-كـاروتين فـى طحلـب كلـوريلا إليبسـويدا لـم يتـأثر بغيـاب النتروجين. أظهرت النتائج أن محتوى البيتا-كاروتين فى الكائنين قل بشكل ملحوظ فى المزارع التـى تحتـوى على كميات كبيرة من النترات. أما محتوى الكائنين من فيتامين E و فيتـامين C فقد وجـد أنـه قل بشـدة فـى حالة نقص النتروجين بينمـا وجد أن إمداد مزرعـة الـدونـاليلا بكميـات زائـدة مـن النتـروجين قد سـبب زيـادة ملحوظة فى محتوى كلا من الفيتامنين. وعلى العكس تمامـا فبـالرغم مـن أن فيتـامين E لـم يسـتدل عليـه فـى طحلب كلوريلا فان فيتامين C قل بشدة فى كل تركيزات النترات.