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Nitrogen Deficiency Maximizes the Production and Accumulation of β-Carotene via Induction of Different Macromolecule Derivatives in *Dunaliella salina* (Dunal) Teodoresco

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THIS STUDY aims at investigating the effects of nitrogen deficiency and NaCl stress on β-carotene production and growth of *Dunaliella salina* (Dunal) Teodoresco. *D. salina* was exposed to different levels of N: 2.5, 4.9, 7.4 and 9.9mM KNO_3 or Na^+: 1.3, 1.7, 2.6, 3.4 and 4.3M NaCl. β-carotene production and accumulation were significantly better in nitrogen deficiency-treated *D. salina* than that in NaCl-treated cells, with a maximum increase of 5.5mg g DW^{-1} at 2.5mM KNO_3. Algal growth, in terms of cell number and the relative growth rate (RGR), was increased with the increase in the availability of nitrogen but decreased by NaCl stress. In contrast, the production of β-carotene by *D. salina* was enhanced under nitrogen deficiency and excessive salinity, suggesting the opposite effect of nitrogen availability and stressful NaCl on the production and accumulation of β-carotene concomitant with the inhibition of algal growth. GC analysis confirmed that nitrogen deficiency-treated *D. salina* accumulates β-carotene higher than untreated cells (control), suggesting the great potential of nitrogen deficiency-treated *D. salina* to produce different β-carotene macromolecule derivatives compared to those in the control. Such different hydrocarbons could be converted into safe β-carotene. Therefore, *D. salina* could be used as a reliable biological system for β-carotene production. The study recommended cultivating *D. salina* under low salinity to obtain *D. salina* cells, but high salinity to obtain their β-carotene.

Keywords: β-carotene, *Dunaliella salina*, Growth, NaCl stress, Nitrogen deficiency.

Introduction

Marine algae, including microalgae and seaweeds, are used as a unique, sustainable, and alternative source of carotenoids (such as fucoxanthin, lutein, β-carotene, and siphonaxanthin) (Safafar et al., 2015; Khalid et al., 2018). The industrial interest towards the production of natural carotenoids, particularly β-carotene using algae, has substantially augmented, as they offer cost, scale, time, and yield advantages over terrestrial plants. Carotenoids in microalgae can be categorized into two groups, primary and secondary carotenoids, based on their metabolism and function. Primary carotenoids are structural and functional components in the photosynthetic apparatus, which take a direct part in photosynthesis. Secondary carotenoids refer to extra-plastic pigments produced in large quantities, through carotenogenesis, after exposure to specific environmental stimuli (Grung et al., 1992; Minhas et al., 2016). β-carotene, as the most prominent of carotenoids, has many benefits for plants and humans. For the plants, including algae, β-carotene accumulates as lipid globules in the inter-thylakoid spaces of the chloroplasts in algae (Vorst et al., 1994; Polle et al., 2020), forming a component of the photosynthetic

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reaction center and functions as an auxiliary light-harvesting pigment, thereby preventing photo-damage for photosynthetic apparatus (Ben-Amotz et al., 1987). β-carotene can be obtained during excessive irradiances across the formation of reactive oxygen species (ROS), by quenching the triplet-state chlorophyll or by reacting with singlet oxygen ($^1O_2$), where it acts as potent ROS scavengers and inhibitors of lipid per-oxidation, also acts as a light filter (Telfer, 2002). The amount of accumulated β-carotene depends on the light absorbed by the *Dunaliella* cells during one division cycle (Metting, 1996). The formation of lipid globules is considered as the driving force for β-carotene overproduction or β-carotene sequester; moreover, de novo fatty acid synthesis is indispensable for β-carotene accumulation (Rabbani et al., 1998). With regard to human life, β-carotene is used in a wide array of pharmaceutical applications such as antioxidant, immune-modulatory, and anticancer agents (Raja et al., 2004, 2007).

Natural β-carotene from algae is similar to that found in most fruits and vegetables where there are more than 600 types of carotenoids and 50 pro-vitamin-A includes natural β-carotene (Faure et al., 1999). The β-carotene content can rise to more than 10% of *Dunaliella* dry weight when it is subjected to stress conditions such as high salinity, high light intensity, nutrient deprivation, and extreme temperature (Krol et al., 1997; Kleinegris et al., 2009; Badr et al., 2014; Senousy et al., 2020). *Dunaliella* species respond to high salinity by enhancement of photosynthetic CO_2 assimilation, causing the orange color of salt lakes and salterns (Oren, 2002).

Among the important physiological responses produced under stress conditions in *Dunaliella*, β-carotene can be accumulated when carbon availability (Oren-Shamir et al., 1989). In *D. salina*, the optimum salinity for growth lies between 18 and 22% NaCl whereas; the optimum salinity for carotenoid production is > 27% NaCl (Borowitzka et al., 1984). Similarly, nitrate starvations cause the accumulation of β-carotene in the wild type of *Dunaliella* (Becker, 2004). The ratio α-carotene and β-carotene to chlorophyll a increased under nitrogen (Geider et al., 1998) and phosphorus limited conditions (Krom & Brenner, 1991). The carotene/chlorophyll a ratio increased by 33 times under nitrogen deficiency conditions in aqueous two-phase systems (León et al., 2003).

The genus *Dunaliella* (Chlorophyta) is a unicellular green alga, ovoid or ellipsoid in shape; lacks a rigid cell wall with variable size. The cells are enclosed by an elastic plasma membrane covered by a mucus surface coat and shrink or swell rapidly when exposed to hypertonic and hypotonic conditions, respectively (Ben-Amotz, 1993). *Dunaliella* possesses some characteristics such as the absence of a rigid cell wall, high concentrations of some intracellular solutes, highly acidic cell proteins, and regions of the cell acting as buffer zones between the medium and certain cell compartments. One or more of these characteristics enables *Dunaliella* to survive in concentrated salt solutions. The cell is motile with two equal flagella and contains a single cup-shaped chloroplast which contains chlorophyll a and b and valuable carotenoid pigments such as α- and β-carotene, violaxanthin, neoxanthin, zeaxanthin, and lutein. These pigments are responsible for the color of *Dunaliella* blooms (Ben-Amotz, 1980, 1993).

Dunaliella, as a natural source of β-carotene, has the ability to extensively grow in natural conditions, forming colored blooms in many ponds and lakes in Egyptian habitats, particularly El-Agamy, Alexandria. The natural β-carotene has advantages more than synthetic β-carotene that does not reduce the occurrence of diseases, including cancer in humans as much as natural β-carotene does (FDA, Federal Register, USA; Ben-Amotz & Fishler, 1998). In addition, the world consumption of natural β-carotene products increases more than natural sources. Many studies reported that *D. salina* can extensively grow in a polluted environment, giving unsafe β-carotene source that adversely affects man health, consequently, *D. salina* growing under controlled conditions produces safe β-carotene, also β-carotene production can be maximized via exposure to salt stress or nitrogen starvation (Becker, 2004). To the best of our knowledge, which is more productive to β-carotene, NaCl-treated *D. salina*, or N deficiency-treated *D. salina* under controlled conditions? Also, what is the difference in chemical composition between nitrogen deficiency-treated *D. salina* and untreated cells? These questions are not well investigated. So, we investigated the effect of nitrogen depletion and NaCl stress on the growth and β-carotene productivity of *D. salina*, also we compared the chemical composition, GC-MS analysis, of nitrogen deficiency-treated *D. salina*

with that in untreated cells.

Materials and Methods

Sampling

Water and algal samples were collected from a hyper-saline lagoon located in El-Agamy, Alexandria, Egypt in 2018. It is located between 31°7` 89"N and 29° 46` 7.40"E. The samples were transferred to the laboratory under iced conditions to carry out the experiments.

Water analysis

Electrical conductivity (EC) was measured using EC meter Jenway (Model 4320), soluble Na^+ and K^+ ions were estimated using a Jenway PFP7 flame photometer. Calcium and magnesium (Ca^{2+} and Mg^{2+}), carbonate, bicarbonate (CO_3^{2-} and HCO_3^-), and chlorides were determined according to APHA (2005).

Preparation of algal sample

Dunaliella was isolated by diluting the water sample to make serial dilutions from 10^{-1} to 10^{-10}. One mL, of the 10^{-2} to10^{-6} dilutions, was poured on the solid Johnson medium (Johnson et al., 1968). The plates were incubated at 30°C under continuous light (50µmol photons PAR m^{-2} s^{-1}) for 3 weeks. *Dunaliella* colonies were purified as described by Pringsheim (1946) and Hoshaw & Rosowski (1973) and identified according to Rippika et al. (1979). The culture was renewed at regular intervals to maintain the alga in the exponential phase of growth.

Experimental design

One mL of the starting *Dunaliella* maintenance culture was inoculated in a 500-mL Erlenmeyer flask containing 250mL of Johnson medium. The stock Johnson solution (JS) contained (g/L): NaCl, 73; $MgCl_2.6H_2O$, 1.5; KCl, 0.2; $CaCl_2.2H_2O$, 0.2; $NaHCO_3$, 0.043; KH_2PO_4, 0.035; 10mL of Fe-solution (mg/L) (Na_2EDTA, 189; $FeCl_3.6H_2O$, 244), and then 10mL of microelements solution (mg/L): H_3BO_4, 61; $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 38; $CuSO_4.5H_2O$, 6; $CoCl_2.6H_2O$, 5.1; $ZnCl_2$, 4.1; $MnCl_2.4H_2O$, 4.1. The medium pH was adjusted at 7.5 ± 0.1. Salinity stress was imposed by adding NaCl to the medium at concentrations of 1.3, 1.7, 2.6, 3.4, and 4.3M NaCl for 18 days. Nitrogen deficiency was imposed by adding KNO_3 to the medium at concentrations of 2.5, 4.9, 7.4, and 9.9mM KNO_3 and incubated at 28±2°C under continuous illumination (50µmol photons PAR m^{-2} s^{-1}) for 18 days. Three replicates were used for each treatment.

Algal cell counting and relative growth rate (RGR)

After the intervals of treatments, the *D. salina* cells were counted using a haemocytometer according to the method described by Grigoryev (2014). The relative growth rate (RGR) of the alga, also known as the efficiency index, was estimated from the following equation:

$$\text{RGR} = \frac{ln\, M_2 - ln\, M_1}{t_2 - t_1}\,\text{day}^{-1}$$

where, M_2 and M_1 are the algal biomass estimated as cell number at times t_2 and t_1, respectively.

Estimation of β-carotene

Two mL of algal culture was taken after intervals (3, 6, 9, 12, 15, and 18 d) from each treatment unit. The cells were centrifuged at 4000rpm for 10min. at room temperature. The supernatants were discarded, and the pellets were re-suspended in 5mL of 80% acetone. β-carotene extraction from cellular debris was done by centrifuging at 4000rpm for 10min. A cuvette containing acetone was used as a blank to calibrate the spectrophotometer to the zero point. The concentration of β-carotene in the supernatant was determined spectrophotometrically at 455nm according to the method described by Javeria et al. (2013).

Sample preparation for GC Analysis

A known weight (1g) of dried algal mass was extracted using a serial extraction method by dissolving in 10mL of methanol (99.5%) for 3-4hrs., then centrifuged at 10000rpm for 6-7min., and the supernatant was separated by filter with 0.5 µm pore size (Lefort-Tran et al., 1988). After concentrating, the crude extract was kept in fresh glass vials and stored in the dark at 4°C till GC-MS analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical composition of the alga was performed using Trace GC-TSQ Quantum Mass Spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m × 0.25mm × 0.25µm film thickness). The column oven temperature was initially held at 50°C and then increased at a rate of 5°C/min to 200°C, the latter temperature was held for 2min,

and then increased to the final temperature of 290°C at a rate of 30°C/min and held for 2min.

The injector and MS transfer line temperatures were kept at 270 and 260°C, respectively; Helium was used as a carrier gas at a constant flow rate of 1mL/min. The solvent delay was 3min and diluted samples of 1µL were injected automatically using AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70eV ionization voltages over the range of m/z 50–500 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and the mass spectra with those of WILEY 09 and NIST 11 mass spectral databases.

Statistical analysis

Each experiment was carried out at least in triplicate. The data were subjected to two-way ANOVA with Fisher's least significant difference (LSD) using SPSS v 22 at a significant level of $P \leq 0.05$ to assess the effect of the main factors (Age of culture and level of NaCl or KNO_3) and their interaction on the algal growth and β-carotene production.

Results

Chemical properties of water

The water sample was characterized by high EC (180mmohs/cm). The concentrations of soluble Na^+ and K^+ ions recorded 1.52 and 0.05M, respectively. Moreover, the concentrations of Ca^{2+} and Mg^{2+} amounted to 0.16 and 0.29M, respectively. Whereas CO_3^{2-} was absent, HCO_3^- recorded 0.005M, in addition to chlorides and sulfate recorded 1.55 and 0.38M. , respectively

Morphological and microscopic identification of the studied alga

The experimental species was primarily identified as *Dunaliella*, according to its morphological characteristics. It is a free-floating alga, forming bloom in a hyper-saline lagoon with red color (Fig. 1A). This preliminary identification was further confirmed by microscopic analysis, *D. salina*. It is a unicellular green alga characterized by ovoid shape; lacks rigid cell wall with size varying from 5 to 25µm in length and from 3 to 13µm in width. The cell is motile with two equal long flagella and contains a single cup-shaped chloroplast (Fig. 1B).

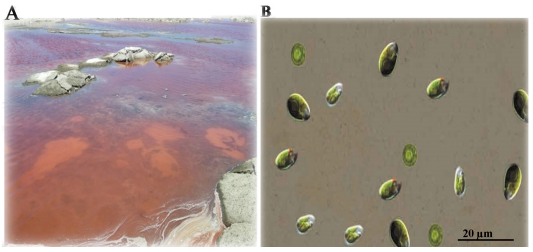


Fig. 1. A) Bloom-forming *D. salina* at the sampling site. B) A microscopic photo of *D. salina* (1000 X).

Two-way ANOVA showed very highly significant effects of the main factors (NaCl or KNO_3 level and age of algal culture) and their interaction on β-carotene production and cell number (algal growth). Whereas the main factor KNO_3 level exerted a strong effect on β-carotene production (with greater F ratio) relative to NaCl level, the main factor NaCl level exerted a greater effect on cell number (with greater F ratio) relative to KNO_3 level (Table 1).

Change in D. salina cell number in response to NaCl stress

The time course of *D. salina* growth exhibited a sigmoidal pattern, with a negligible lag period, followed by a rapid exponential phase, a stationary phase, and eventually a death phase at NaCl treatments (Fig. 2A). The exponential phase was markedly increased with decreasing the level of salinity. The response of algal growth to NaCl treatment varied according to the level of NaCl and age of culture. Where the *D. salina* cell number at the lower levels, 1.3M (equals media JS) and 1.7M NaCl, was significantly increased by 6.2 and 5.2 folds, respectively, by the 3rd day compared to zero time, this increase was followed by an extended stationary phase from the 3rd d and 6th d, respectively to the 15th d which followed by significant reduction where death phase onset. Whereas the cell number at 2.6M NaCl was significantly increased by 3.4 folds at the 3rd d compared to zero time; this was followed by a transient stationary phase from the 3rd d to 6th d, followed by gradual reduction. Similarly, the cell number at 3.4 and 4.3M NaCl was increased by 2.1 and 1.4 folds at the 6th d compared to zero time; this was followed by a negligible stationary phase with a progressive reduction after the 6th d of the treatment ($P<0.001$) (Fig. 2A).

TABLE 1. Two-way ANOVA shows the effects of the main factors (NaCl or KNO_3 levels and age of culture) and their interaction on β-carotene production and growth of *D.salina*

Source of variation	df	F	P	F	P
		β- Carotene		Cell Number	
NaCl	4	105.9	0.000	65.09	0.000
Age	6	144.9	0.000	27.11	0.000
NaCl × Age	24	14.70	0.000	2.583	0.001
		β- Carotene		Cell Number	
KNO_3	3	5392	0.000	35.25	0.000
Age	6	1812	0.000	27.50	0.000
KNO_3 × Age	18	589.7	0.000	2.720	0.002

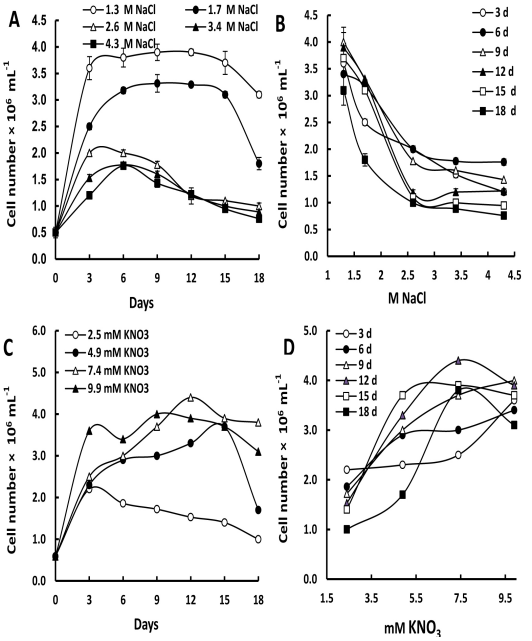


Fig. 2 . Effect of NaCl and N deficiency on cell number of *D. salina:* Time course of cell number at (A) NaCl stress and (C) N deficiency. The response of cell number at (B) NaCl stress and (D) N deficiency at different growth stages [Each value is the mean of 3 replicates ±SE]

Salinity led to a marked reduction in cell number, and the magnitude of reduction varied according to the salinity level. Increasing salinity from 1.3 to 4.3M NaCl led to a progressive and consistent decline in *D. salina* cell number at the early stages of growth (the 3rd day) by 33% (P<0.001). Whereas increasing in salinity from 1.3 to 2.6M NaCl exhibited a progressive reduction of 59%, 45%, 31%, 30%, and 32% at the stages of growth from the 6th to 18th day, respectively, this reduction was followed by steady values with further increase in the salinity level up to 4.3M NaCl (P<0.001) (Fig. 2B).

Changes in D. salina cell number in response to N deficiency

Time course of nitrogen deficiency-treated *D. salina* exhibited a different sigmoidal pattern compared to NaCl-treated cells, with a negligible lag period, followed by a rapid exponential phase, a stationary phase, and an eventually death phase at all KNO_3 levels. The timing of onset of these phases was markedly changed with increasing the level of KNO_3. The response of algal growth to KNO_3 treatment varied according to the level of KNO_3 and age of culture (Fig. 2C). Where, 2.5mM KNO_3 significantly increased the cell number by 3.2 folds at the 3rd day compared to zero time, this increase was followed by a sluggish reduction from the 3rd d to 18th d. Also, 4.9mM KNO_3 significantly increased the cell number by 5.3 folds at the 15th day compared to zero time; this increase was followed by a sluggish reduction by 45% from the 15th to 18th day. Likewise, 7.4mM KNO_3 significantly increased the cell number by 6.5 folds at the 12th day compared to zero time, this increase was followed by a sluggish reduction by 16% from the 12th to 18th day (P<0.001). Similarly, 9.9mM KNO_3 significantly increased *D. salina* cell number by 5.1 folds during the 3rd day compared to zero time, this increase was maintained from the 3rd to 15th day (P<0.001) and was followed by a 19% reduction.

The cell number of *D. salina* was progressively increased with increasing KNO_3 level reaching the maximum increase at 7.4mM KNO_3, particularly by the 12th day. During the early stages of growth

(the 3rd day), increasing KNO_3 level from 2.5 to 7.4mM didn't show any change in cell number, but from 7.4 to 9.9mM KNO_3 increased cell number by 44% on the same day. By the 6th day, cell number was increased by 83% upon increasing in KNO_3 level from 2.5 to 9.9mM KNO_3. Meanwhile, at the moderate stage of growth (i.e., the 9th day of the growth), cell number was increased by 1.33 folds, upon increasing KNO_3 from 2.5 to 9.9mM. However, by the 12th day, cell number was increased by 1.88 folds, upon increasing KNO_3 from 2.5 to 7.4mM and it decreased by 13% at 9.9mM KNO_3. Then, at the later stage of growth (the 15th and 18th d), cell number was increased by 2.1%, upon increasing KNO_3 from 2.5 to 7.4mM but gradually decreased at 9.9mM KNO_3 (Fig. 2D).

Change in D. salina relative growth rate (RGR) in response to NaCl stress

RGR of *D. salina* was significantly better at lower (1.3 and 1.7M) than the higher NaCl levels (2.6, 3.4 and 4.3M) with divergent time course pattern. Whereas RGR of *D. salina* at 1.3 and 1.7M NaCl followed the same time course pattern across all growth periods 0–18 d, with a peak at 1.5/d and 1.2/d, respectively across the growth period 0–3 d (i.e., by the 1.5th day of growth), it remained steady from the period 3-6 d up to 12–15 d (i.e., the 4.5th to the 13.5th day of growth); afterwards, it was gradually declined with the progress of growth periods (Fig. 3A). On the other hand, RGR of *D. salina* at 2.6, 3.4 and 4.3M NaCl exhibited a progressive reduction with the progress of growth periods from 0-3 d up to 15–18 d (i.e., the 1.5th to the 16.5th day of growth), with a peak at 1/d, 0.7/d and 0.5/d, respectively across the same period 0–3 d (i.e., by the 1.5th day of growth).

RGR of *D. salina* was progressively decreased by increasing NaCl level from 1.3 to 4.3M with peaks at 1.3M NaCl across the period 0-3 d of growth, which their magnitudes differed according to the level of salinity. RGR at the early stage (0-3 d) was gradually diminished from 1.5/d at 1.3M to 0.49/d at 4.3M NaCl (Fig. 3B). Likewise, RGR was progressively reduced from 0.97/d, 0.98/d, and 0.89/d at 1.3M to 0.4/d, 0.17/d, and 0.08/d at 4.3M NaCl across the periods of growth 3-6 d, 6-9 d, and 9-12 d, respectively. RGR was progressively dropped from 0.85 to -0.12/d and 0.96 to -0.25/d, respectively, during the latest period of growth 12-15 d and15-18 d (i.e., by the 13.5th and 16.5th day of growth), respectively.

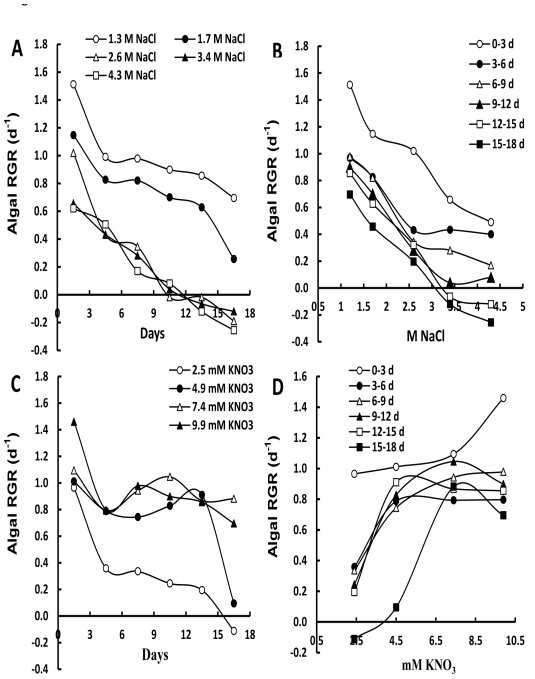


Fig. 3 . Effect of NaCl and N deficiency on RGR of *D. salina*: Time course of RGR at (A) NaCl stress and (C) KNO_3 deficiency. The response of RGR at (B) NaCl stress and (D) N deficiency at different growth stages [Each value is the mean of 3 replicates. Each point in Fig. 3B, D, and each series in Fig. B, D represent the response at the midpoint of two periods. Since RGR was calculated using the means of two periods, their values were not associated with SE]

Change in D. salina relative growth rate in response to N deficiency

RGR of *D. salina* was significantly better at higher than lower KNO_3 levels, with peaks at 1.5/d, 1.1/d, 1/d, and 0.96/d at 9.9, 7.4, 4.9, and 2.5mM KNO_3, respectively across the early growth period 0-3d (i.e., by the 1.5th day of growth) (Fig. 3C). Whereas RGR of *D. salina* at 4.9mM KNO_3 was more or less comparable to 7.4 and 9.9mM KNO_3 across the periods from 3-6 d up to 12–15 d, it was sharply declined by 8.6 folds across the period 15-18 d (i.e., the 16.5th day of growth). RGR of *D. salina* at 2.5mM KNO_3 exhibited a sharp decline from the early growth period 0–3 d (i.e., the 1.5th day of growth) to the period 3-6 d (the 4.5th day), this was followed by a steady value up to the period 12-15 d (i.e., the 13.5th day of growth); afterwards, it was sharply declined with the progress of growth period 15-18 d (i.e., the 16.5th day of growth).

RGR was better at higher than the lower KNO_3 level, with peaks at the 1.5th day of growth (Fig. 3D). RGR at the 1.5th day of growth was gradually increased from 0.97/d at 2.5mM to 1.5/d at 9.9 mM KNO_3. RGR at the 4.5th and 7.5th day was gradually increased from 0.36/d, 0.34/d at 2.5mM to 0.79/d, and 0.98/d at 9.9mM KNO_3, respectively. Whereas RGR at the 4.5th and 13.5th was gradually increased from 0.36/d and 0.2/d at 2.5mM to 0.79/d and 0.87/d at 4.9mM KNO_3, it remained unchanged up to 9.9mM KNO_3. However, RGR at the 7.5th day was gradually increased from 0.34/d at 2.5 to 0.98/d at 9.9mM KNO_3. Whereas RGR at the 10.5th day was progressively increased from 0.24/d at 2.5mM to 1.1/d at 7.4mM KNO_3, it decreased to 0.89/d at 9.9mM KNO_3. Likewise, RGR at the 16.5th was progressively increased from -0.11/d at 2.5mM to 0.88/d at 7.4mM and then gradually decreased to 0.69/d at 9.9mM KNO_3.

Change in β-carotene content in response to NaCl stress

The content of β-carotene in *D. salina* cells exhibited a bell-shaped time course at NaCl level, with peaks on the 9th day of growth, which magnitudes differed according to the level of salinity (Fig. 4A). Although β-carotene content at 3.4M NaCl was progressively and significantly increased by 15.4 folds at the 9th day of growth compared to zero time, there was a significant decrease at 18th day by 21% of that at 9th day. The effect of 1.3, 1.7, 2.6 and 4.3M NaCl is comparable in β-carotene production, except at the late stage of 4.3M NaCl ($P< 0.001$).

The response of β-carotene to salinity is similar across the growth periods (3d -18d); but varied according to salinity level, with peaks at 3.4M NaCl, except the early period 0-3 d, which didn't show any response to salinity level (Fig. 4B). β-carotene content was progressively increased as salinity level increased from the threshold of 2.6M reaching a peak at 3.4M NaCl at all growth periods; afterwards, it was sharply declined from that peak at 3.4 to 4.3M at all growth periods ($P< 0.001$), except the 3rd day.

Change in β-carotene content in response to N deficiency

The content of β-carotene exhibited a time course similar to that of NaCl, with peaks at the 9th day of growth, which magnitudes differed according to the KNO_3 level (Fig. 4C). The content of β-carotene at 2.5mM KNO_3 exhibited a significant progressive increase by 16 folds compared to zero time, with peaks at the 9th day of growth ($P< 0.001$), followed by a significant decrease from the 9th day to the 18th day. Despite the coincidence in the effect of time course on the lower (2.5mM) and the higher KNO_3 level (4.9, 7.4, and 9.9mM), the magnitude of response differed in the two levels.

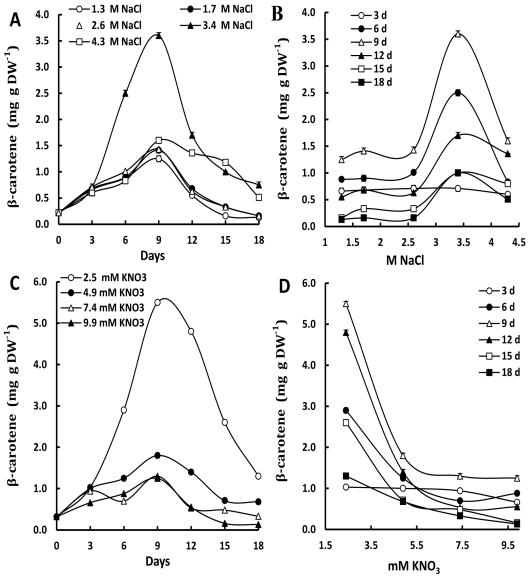


Fig. 4 . Effect of NaCl and N deficiency on β-carotene of *D. salina:* Time course of cell number at (A) NaCl stress and (C) KNO_3 deficiency. Cell number responses to (B) NaCl stress and (D) KNO_3 deficiency at different growth stages [Each value is the mean of 3 replicates ± SE]

The effect of KNO_3 level exhibited a strong reduction in β-carotene content at all growth periods from 2.5 up to 7.4mM KNO_3 ($P< 0.001$), except the 3rd day, which didn't show any change in β-carotene content. This reduction was followed by either a steady level (plateau) at the growth periods 6d, 9d, and 12d or a decline at the periods 3d, 15d, and 18d upon increasing KNO_3 from 7.4 up to 9.9mM. There is a reverse relationship between the β-carotene production and KNO_3 concentration (Fig. 4D).

GC–MS analysis of nitrogen deficiency- treated D. salina and untreated cells

Thirty-five compounds were encountered by GC–MS analysis in untreated *D. salina* cells. Among those thirty five compounds, five major chemical categories are 1H-purin-6-amine,(2-fluorophenyl) methyl (27.67%), followed by

9-hexadecenoic acid, methyl ester (Z) (24.10%), octacosane (AI3-52615) (4.89%), thieno (3,4-C) pyridine,1,3,4,7 tetraphenyl (4.86%) and 10,13-octadecadienoic acid, methyl ester (2.98%) (Suppl. Table 1).

With regard to N deficiency-treated *D. salina*, thirty compounds were investigated. Among those thirty compounds, six chief and unique compounds are phenol, 2,4-bis (1,1dimethyl ethyl) with MW 206, representing about 28.5% of total measured compounds, followed by indolo [2,3-a] quinolizin-4(12H)-one1,2,3,6,7,12b-hexahydro-3,12b-dimethyl (8.84%) and oleic acid (6.78%), carotenoid compounds such as (α,α-carotene-4,4›-dione,3,3›-dihydroxy-, (3S,3›S), psi.,.psi.-carotene and α-carotene) represented about 3.85%, followed by estrone (3.87%), geranyl-α-terpinene (3.8%), and (4-methoxyphenyl)-2-methyl-6 methoxy-+ benz imidazole (3.8%) (Suppl. Table 2). Other compounds existed with values ranged between 0.83% for dehydroisoandrosterone acetate and 2.15% for 3-phenanthrenol,4B, 5, 6, 7, 8, 8A, 9, 10 octahydro-4B,8,8-trimethyl-,(4Bs-trans).

By comparing the compounds investigated in N deficiency-treated *D. salina* with those of untreated *D. salina* cells, it was found that the number of compounds with molecular weight (MW) in between 200-300Da was 18 compounds (72.41%) in N deficiency-treated *D. salina* compared to nine compounds (70.85%) in untreated *D. salina* cells. Whereas the number of compounds with MW in between 300-400 Da was four compounds (6.11%) in N deficiency-treated *D. salina* compared to nine compounds (11.54%) in untreated *D. salina* cells, the number of compounds with MW in between 400-500 Da was three compounds (4.92%) in N deficiency-treated *D. salina* compared to nine compounds (12.24%) in untreated *D. salina* cells. Finally, the number of compounds with MW in between 500-700 Da was five compounds (16.66 %) in N deficiency-treated *D. salina* compared to eight compounds (5.36%) in untreated *D. salina* cells (Tables 2 and 3).

Discussion

Upon exposure *D. salina* to nitrogen deficiency and NaCl stress, β-carotene production was most obvious at the lower level of KNO_3 (2.5mM), followed by the higher salinity level (3.4M NaCl), the reverse was true for the *D. salina* growth (cell number and RGR). Therefore, N deficiency-treated *D. salina* received particular attention comparing to untreated *D. salina* cells and NaCl-treated *D. salina* in the discussion.

Growth of D. salina in response to NaCl stress or N deficiency

Although the opposite effect of NaCl stress and N availability on *D. salina* growth (cell number and RGR) and β-carotene production, yet their time course pattern was similar regardless of their levels, but the magnitude of response differed in the two treatments according to their levels. The growth curve of *D. salina* was sigmoidal–shaped with an exponential phase, a stationary phase, and ultimately a death phase in response to NaCl stress or N deficiency, indicating that *D. salina* as microalgae shares the growth phases of microbial populations as reported by El-Katony & El-Adl (2020). Although *D. salina* was growing and blooming in a hypersaline lagoon with a salt level of about 1.5M NaCl, yet it achieved maximal growth under the controlled laboratory conditions at a salt level (1.3M NaCl) far lower than that of the salt lagoon level. Therefore, the tolerance of this alga to salts level of hypersaline lagoon under natural conditions could be attributed to the uniqueness of saltwater mineral compositions, in which the macro- and micro-nutrients are found in a balanced formula which is hardly to be simulated under laboratory conditions, in addition to the absence of *D. salina* cell wall and/or high concentrations of some intracellular solutes and/or acidic protein, and/or buffer zones between the medium and cell compartments (Ben-Amotz, 1980, 1993). This signifies that *D. salina* could tolerate and adapt up to 1.3M NaCl as the optimum, beneficial concentration for flourishing and increasing *D. salina* growth under controlled conditions; but with further salinity, its growth markedly decreases, this could be attributed to the distortion of *D. salina* cells by higher salinity level (>1.3M NaCl). This correlates well with the results of Borowitzka et al. (1984) who demonstrated that a salinity level of more than 25% (4.3M NaCl) decreases the growth rate of *D. salina*.

The significant increase of N deficiency-treated *D. salina* growth at the higher KNO_3 level (7.4 or 9.9mM) was higher than that of NaCl-treated cells at the lower NaCl level (1.3M NaCl). This signifies that the higher level of KNO_3 (7.4mM or 9.9mM KNO_3) is the optimal concentration for *D. salina* growth. This might mean that this nitrogen

level (7.4mM KNO_3) enhances the photosynthetic rate, stimulating the growth of *D. salina* (Li et al., 2009). These results suggest the inverse relationship between NaCl stress and nitrogen availability on *D. salina* growth, whereas the high salinity levels exhibited a negative effect on cell number and RGR of *D. salina*, the sub-higher level of KNO_3 exhibited a stimulatory effect on them.

β-carotene accumulation in response to NaCl stress or nitrogen deficiency

The synthesis and accumulation of β-carotene stimulated by sub-higher level (3.4M NaCl) were better than those at both higher and lower salinity levels in *D. salina*. Whereas β-carotene accumulation did n't show any significant response in NaCl-treated *D. salina* at the lower salinity levels (1.3 to 2.6M NaCl), it sharply declined at the higher salinity level (4.3M NaCl) in addition to a negligible effect at the early stage (the 3rd day, Fig. 4A, B). This signifies that the effect of NaCl on β-carotene production and accumulation depends on the salt level and the age of algal culture. This agrees with the findings of Rabbani et al. (1998) who revealed that β-carotene content and accumulation vary according to several stress factors or their levels. Although *D. salina* can easily adapt, and properly produce and accumulate β-carotene at sub-higher NaCl level, yet it declined β-carotene productivity at the higher NaCl level. This agrees with the findings of Borowitzka et al. (1984) who demonstrated that the higher salinity level decreases the growth rate of *D. salina*.

The significant increase of β-carotene accumulation in N deficiency–treated *D. salina* at the lower level of KNO_3 was highly better than that in NaCl–treated cells at sub-higher level of NaCl. This could be attributed to the shortage of nitrogen where, the amounts of carbon and hydrogen contribute to the synthesis of non-nitrogen pigments and initiate β-carotene production and accumulation (Lamers et al., 2010, 2012). Furthermore, the algal cells can synthesize a series of chemicals under suitable concentrations of nitrogen and carbon in order to maintain normal metabolic development (Lamers et al., 2010). This signifies the stronger effect of nitrogen deficiency as an inducing factor for β-carotene accumulation in *D. Salina* as well as its metabolic development.

The accumulation of β-carotene was associated with the reduction in *D. salina* cell number and RGR in both KNO_3 and NaCl treatments. This may imply that the reduction in growth could be a result of inhibition of the photosynthetic rate (Paul & Foyer, 2001). The reduction in microalgal growth under salinity stress has been reported for *Scenedesmus* sp. CCNM 1077 (Pancha et al., 2015), and *Chlorella* sp. (Rai et al., 2015), and the slowing down of growth rate has been reported for *Chlorella vulgaris* (Church et al., 2017) and *Scenedesmus obliquus* (El-Katony & El-Adl, 2020).

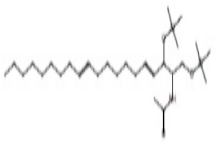
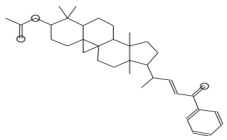
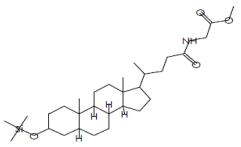
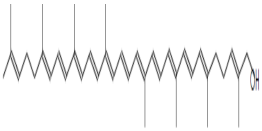
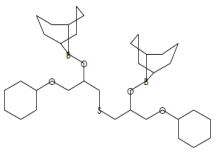
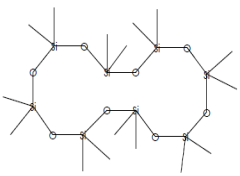
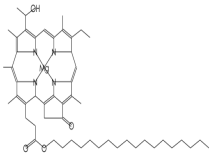
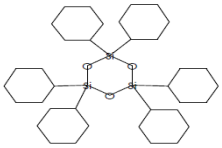
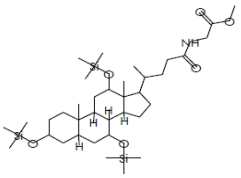
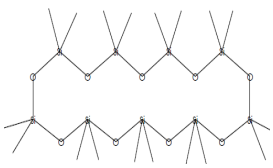
Change in GC–MS analysis of D. salina under nitrogen deficiency

GC-MS analysis demonstrated a substantial difference between the chemical composition of nitrogen deficiency-treated *D. salina* compared to that in untreated cells. Whereas nitrogen deficiency-treated *D. salina* produced five macromolecules (MW>500 Da) with 16.7%, untreated cells produced eight macromolecules (MW>500 Da) with 5.4% (Table 2 and Suppl. Tables 1, 2). The result suggests that macromolecules with MW >500 Da could be sources or derivatives of β-carotene. Where, β-carotene is a hydrocarbon with the formula of $C_{40}H_{56}$ and molecular weight of 536.9 Da, with eleven conjugated double bonds (Beutner et al., 2001).

TABLE 2. GC-MS analysis of N deficiency-treated *D. salina* V.S untreated *D. salina* cells

Untreated *D. salina* cells				N deficiency-treated *D. salina*			
MW (Da)	**No of compounds**	**(%)**	**Carbon skeleton**	**MW (Da)**	**No of compounds**	**(%)**	**Carbon skeleton**
200-300	9	70.85	C_{12}- C_{19}	200-300	18	72.41	C_{10}-C_{20}
300-400	9	11.54	C_{16}-C_{28}	300-400	4	6.11	C_{17}-C_{24}
400-500	9	12.24	C_{23} –C_{32}	400-500	3	4.92	C_{28}-C_{32}
500-700	8	5.36	C_{16}-C_{40}	500-700	5	16.66	C_{35}-C_{52}

TABLE 3. Chemical structure of suggested β-carotene macromolecule derivatives (>500 MW Da) of N deficiency-treated *D. salina* V.S untreated *D. salina* cells

No.	Untreated *D. salina* cells		N deficiency-treated *D. salina*	
	Compound	**Structure**	**Compound**	**Structure**
1	Bistrimethylsilyl N-acetyleicosasphinga -4,11-dienine		3A,6,6,12A-Tetramethyl-1-[1-Methyl-4-OXO-4-Phenyl-2-buteny] Tetracahydro-1H Cyclo-penta [A] cyclopropa [E] phenanthren-7-YL Acetate	
2	Glycine, N-[(3à,5á)-24-oxo-3-[(trimethylsilyl) oxy] cholan-24-yl]-, methyl ester		á carotene	
3	Psi.,.psi.-carotene-16-ol (Lycoxanthin)		á,á-Carotene-4,4'-Dione,3,3'-dihydroxy-, (3S,3'S)-	
4	1,9-dioxa-5-thianonane,3,7-bis (9 bor abicyclo [3.3.1] non-9-yloxy)-1,9-diphenyl-		Psi psi.-Carotene	
5	Cyclooctasiloxane, hexadecamethyl-		Bactroiochlorphyll-c-stea-ryl	
6	Cyclotrisiloxane, hexa-phenyl- (Diphenylsilox-ane cyclic trimer)			
7	Methyl glycocholate, 3TMS derivative , gly-cine, N-[(3à,5á,7à,12à)-24-oxo-3,7,12-tris [(trimethyl silyl)oxy] cholan-24-yl]-, methyl ester,			
8	Cyclononasiloxane, octadecamethyl-			

Although the two strains could able to produce β-carotene macromolecule derivatives (MW>500 Da), the quality and quantity of hydrocarbon derivatives that could be converted into β-carotene in N deficiency-treated *D. salina* clearly differed from those in untreated *D. salina* cells (Table 2). Therefore, the β-carotene macromolecule derivatives produced in nitrogen deficiency-treated *D. salina* (16.7%) could be highly quantified and qualified, regardless of the number of compounds, compared to that in untreated *D. salina* cells (5.4%). The positive effect of controlled growth conditions, particularly N deficiency, could be attributed to the strong influence of N deficiency (2.5mM KNO_3) on the hydrocarbon compositional profiles in nitrogen deficiency-treated *D. salina* compared to that in untreated *D. salina* cells. This was in agreement with the findings of Hu et al. (2008) who reported that the fatty acid compositional profiles of algal strains are influenced by specific growth conditions such as temperature, nutrient level, and light intensity. Furthermore, this partially agrees with Ben-Amotz & Avron (1983) who found that carotene production was increased by lowering the sulfate concentration of the medium to less than 5mM in cultures reaching the stationary state of growth.

Also, the formation of the monounsaturated fatty acid in nitrogen deficiency-treated *D. salina*, such as oleic acid (6.8%), α-carotene (6.2%) with molecular weight 536Da, and psi psi-carotene (3.12%) with molecular weight 600Da, could be of the main sources for increasing β-carotene accumulation, where oleic acid is a key component of the lipid-globule-localized triacylglycerols and thereby in β-carotene accumulation (Lamers et al., 2012). Moreover, the cellular oleic acid content was positively correlated with the cellular β-carotene content upon nitrogen deprivation (Mendoza et al., 1999). *D. salina* cells can synthetize a series of bioactive compounds under the suitable concentrations of nitrogen and carbon in order to maintain normal metabolic development (Lamers et al., 2010).

Among the main compounds produced in N deficiency-treated *D. salina*, phenol-2, 4-bis (1, 1 dimethyl ethyl) amounted to 28.5%, indolo [2,3-a] quinolizin-4 (12H) –one 1, 2,3,6,7,12b-hexahydro-3,12b-dimethyl amounted to 8.8%. Theses compounds exhibited various biological activities against fungal growth (Rangel-Sánchez et al., 2014), in addition, fatty acid synthesis is indispensable for β-carotene production and accumulation (Rabbani et al., 1998).

From the previous information, we conclude that there is a reverse relationship between *D. salina* growth and β-carotene production and accumulation under both NaCl stress and the availability of nitrogen. Although the nitrogen deficiency over than excessive salinity lead to accumulation of β-carotene, yet the β-carotene production under both was concomitant with reduced algal growth. There was a reverse relationship between NaCl stress and nitrogen availability on *D. salina* growth; where there was an adverse effect of higher salinity levels compared to the positive effect of the sub-higher level of KNO_3 on the growth (cell number and RGR) of *D. salina.* The present results suggest that N deficiency affects the hydrocarbon compositional profile in nitrogen deficiency-treated *D. salina* over than untreated *D. salina* cells in controlled conditions. The specific hydrocarbon could be converted into β-carotene macromolecule derivatives to increase β-carotene accumulation in nitrogen deficiency-treated *D. salina* over than untreated *D. salina* cells.

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Authors contribution: MFE: Designed the research, contributed to the experimental work, did data analysis and interpretation and wrote the manuscript. MAD: Designed the research, collected the algae and plant materials, contributed to data interpretation and revised the manuscript. MAG: Collected the algae and plant materials, performed the experimental work, contributed to data analysis and presentation. AYE: Contributed to the experimental work, data analysis, interpretation and writing of the manuscript.

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نقص النيتروجين يزيد من إنتاج وتراكم البيتا كاروتين عن طريق انتاج مشتقات كبيره الجزيئات في *Dunaliella salina* (Dunal) Teodoresco

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يدرس البحث آثار نقص النيتروجين و إجهاد NaCl على إنتاج البيتا كاروتين والنمو في *Dunaliella salina*. تعرضت *D. salina* لمستويات مختلفة من N: 2.5، 4.9، 7.4 ، 9.9 ملي مولار KNO_3 أو Na^+: 1.3، 1.7، 2.6، 3.4 ، 4.3 مولار NaCl. كان إنتاج البيتا كاروتين أفضل بكثير في *D. salina* المعالجة بنقص النيتروجين عنه في الخلايا المعالجة بكلوريد الصوديوم، بزيادة قصوى قدرها 5.5 مجم/ جم وزن جاف عند 2.5 ملي مولار نترات النتروحين. زاد نمو *D. salina* (المتمثل في عدد الخلايا ومعدل النموالنسبي) بتوافر النيتروجين ولكنه انخفض مع إجهاد كلوريد الصوديوم. وعلى الرغم من التأثير المعاكس لمستوى N و Na^+ على إنتاج البيتا كاروتين، إلا أن إنتاج البيتا كاروتين كان متزامنًا مع انخفاض نمو الطحالب ، مما يشير إلى العلاقة العكسية بين تراكم الكاروتين ونمو *D. salina*، بغض النظر عن نوع المؤثر ومستواه. أكد التحليل الكروموتوجرافي (GC) أن *D. salina* المعالجة بنقص النيتروجين تنتج بيتا كاروتين أفضل من تلك الخلايا الغيرمعالجة بنقص النتروجين (الخلايا الحاكمة)، مما يشير إلى الإمكانات الكبيرة *D. salina* المعالجة بنقص النيتروجين لإنتاج مشتقات مختلفة لجزيء البيتا كاروتين مقارنة بتلك الموجودة في الخلايا الغيرمعالجة بنقص النتروجين (الخلايا الحاكمة). يمكن تحويل هذه المشتقات الهيدروكربونية المختلفة إلى جزيئات البيتا كاروتين. ولذا يمكن استخدام *D. salina* كنظام بيولوجي واعد لإنتاج البيتا كاروتين. وتوصي الدراسة بزراعة *D. salina* تحت ملوحة منخفضة للحصول على نموات كثيفة وتحت ملوحة عالية للحصول علي البيتا كاروتين.