El-Sheekh M *et al*. (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 1, Article number 13426
DOI:10.15835/nbha52113426
Research Article

Effect of salinity, nitrogen and phosphorus stresses on growth and photosynthetic activity of the marine microalga *Dunaliella parva*

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Abstract

The growth of the marine green alga *Dunaliella parva* was studied and optimized under different salinity levels of NaCl (0.5, 1, 2, 2.5, and 3.5 M). The growth was monitored by cell number pigment content (Chl. a, Chl. b, and carotenoids). The grown alga, under the optimal conditions, was exposed to different stresses (nitrogen, phosphorus starvation, and salinity either singly or combined. Under nitrogen and phosphorus starvation, either singly or combined, the growth rate and the metabolic activities were decreased. Under salt stress (2.5 M NaCl) combined with N starvation and heavy metals stress, glycerol production increased, while glycerol synthesis decreased under salt stress of 1 M NaCl and P starvation. Also, free radicals (total antioxidant, reducing power, DPPH, and Lipid peroxidation), pigment content, and activity of antioxidant enzymes were recorded. *D. parva* grown under salinity level (2.5 M NaCl) combined with nutrient starvation correlated with more efficient enzymatic antioxidant activity accumulation. This study strongly suggested that the induction of antioxidant defense was one component of the tolerance mechanism of *D. parva* to salinity, as evidenced by its growth behavior.

Keywords: antioxidant enzymes; *Dunaliella parva*; photosynthesis; salinity

Introduction

Species in the genus *Dunaliella* are found in aquatic marine habitats such as the sea and island salt lakes with a wide range of chemical compositions and salt concentrations ranging from 0.5% to saturation (around 35%), and this makes this alga the most halotolerant eukaryote known (Shariati and Lilley, 1994). The mechanism by which *Dunaliella* cells adapt to this wide range of salt concentrations was shown to be based on

Received: 25 Sep 2022. Received in revised form: 21 Oct 2023. Accepted: 21 Dec 2023. Published online: 11 Mar 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

the ability of the alga to change its intracellular concentration of glycerol (Mofeed, 2015; Taha *et al.*, 2012; Tammam *et al.*, 2011). Pick (2002) stated that *Dunaliella* responded to salt stress by its massive accumulation of glycerol, enhanced elimination of Na^+ ions, and accumulation of specific proteins (Pick, 2002). Hadi *et al.* (2008) reported that glycerol's physiological role and function might differ in each *Dunaliella* species and that glycerol seemed to serve as an osmotic regulator (Hadi *et al.*, 2008; Shariati and Hadi, 2011). From an ecological standpoint, *Dunaliella*, specifically *D. salina* and *D. bardawil*, is important due to its remarkable capacity for β-carotenoid accumulation (Shariati and Hadi, 2011). The pigment is utilized for a diverse array of purposes, including in cosmetics, as a natural food-coloring agent, as a nutritional supplement, and as animal feed (Mofeed, 2015; Taha *et al.*, 2012; Tammam *et al.*, 2011). It is additionally employed in the treatment of noxious wastewater plants by means of the adsorption, sequestration, and metabolism of heavy metal ions. The biotechnological potential of *Dunaliella* has been extensively utilized since the discovery was made that specific species can accumulate up to 16% of their dry weight in β-carotenoids (Mofeed, 2015; Taha *et al.*, 2012; Tammam *et al.*, 2011). Additionally, lakes and lagoons exhibiting pink or red hues are indicative of abundant populations of *D. salina*, which can constitute as much as 13.8% of the dry organic matter. An example of such a location is Pink Lake in Victoria, Australia (Mofeed, 2015; Taha *et al.*, 2012; Tammam *et al.*, 2011).

Salinity stress conditions lead to a series of changes in the photosynthesis, photorespiration, osmotic adjustment, compartmentation, and amino acid and carbohydrate synthesis of *Dunaliella* species (Mofeed, 2015). Variations in the salinity and nitrate concentrations of the growth medium are responsible for changes in the growth rate, cell volume, pigment concentration, light-harvesting efficiency, and cell carbon and nitrogen content in *Dunaliella viridis* (Reshma *et al.*, 2021; Tammam *et al.*, 2011). Researchers found that the cell volume and the ratios of chlorophyll a to chlorophyll b, Car to chlorophyll a, and carbon (C) to nitrogen (N) increased in cells grown in conditions of high salt and low N, while the opposite occurred in terms of the growth rate and package effect of pigments.

Tammam *et al.* (2011) demonstrated that the growth of *Dunaliella* under salinity stress correlated with the accumulation of more efficient non-enzymatic and enzymatic antioxidants(Tammam *et al.*, 2011). The alga increased the production of antioxidants or elevated the activities of protective enzymes to detoxify and eliminate the high numbers of reactive oxygen species (ROS). Under stress conditions, molecules of ROS are not controlled by protective systems and destroy membrane lipids, pigments, and proteins (Reshma *et al.*, 2021).

Plants possess several low-molecular-weight antioxidants (e.g., ascorbate, glutathione, phenolic compounds, tocopherols) and various antioxidative enzymes such as superoxide dismutase, ascorbate peroxidase, and catalase, which are involved in the detoxification of ROS and the avoidance of damage under salt stress (Bamary and Einali, 2022; Gill & Tuteja, 2010). Moreover, compatible solutes such as glycine betaine, proline, mannitol, trehalose, and myoinositol may also play regulatory roles in mitigating the damaging effects of oxidative stress (Cuin and Shabala, 2007).

Nitrogen is one of the primary requirements of growth media for any algal cell. The source, type, and quantity of the N in the culture medium can influence the growth and/or composition of the algal biomass (Converti *et al.*, 2006). Fabregas *et al.* (1989) studied the effect of N starvation on *D. tertiolecta* (Fabregas *et al.*, 1989). The alga was grown in ammonium chloride, sulfate, nitrate, and carbonate at a concentration range of 0.25 to 26 mg N/L. The researchers found that the biomass production at the end of the stationary phase, expressed as the cell density, was affected by the concentration of ammonium–N in the medium rather than the ammonium compound used.

Tillberg and Rowley (1989) studied the effect of P starvation on the morphology, intracellular structure, and reactions related to the energy and metabolism of the unicellular green alga *Scenedesmus obtusiusculus* (Tillberg and Rowley, 1989). The investigators reported that an increase in cell size and shape and cell wall thickness and disorganization in the internal structure were the dominating features of P starvation. El-Sheekh

and Rady field also obtained similar results for the effect of P starvation on the growth and photosynthesis and some of the metabolic processes of the unicellular green alga *Chlorella kessleri* (El-Sheekh and Rady, 1995).

This work aimed at studying the optimal growth conditions of *D. parva* under different salinity levels. These conditions were monitored by determining the numbers of algal cells in the growth medium; the cells' optical density, pigment content, and photosynthetic efficiency (Fv/Fm); the activity of the enzymatic antioxidants' catalase, peroxidase, and ascorbic acid oxidase in the cells; and the content of the non-enzymatic antioxidant ascorbate in the cells. Investigations were also done of some metabolic activities and total antioxidant levels under some stress conditions, either singly or combined (the stresses were N starvation, P starvation, and salinity).

Materials and Methods

Tested alga and growth conditions

A pure sample of the unicellular marine alga *Dunaliella parva* was obtained from the Tsukuba University culture collection Japan. According to Loeblich Field (Loeblich, 1982), the alga was grown in the MH nutrient medium. The alga was incubated at room temperature in a culture chamber having fluorescent light providing a light intensity of 400 µ mol m^{-2} s^{-1} (4000 lux).

Factors affecting the growth of Dunaliella parva

Effect of salinity stress

To determine the optimal salinity level, different concentrations of NaCl (0.5, 1, 2, 2.5, and 3.5 M) beside the control (1.25 M NaCl) were prepared in Erlenmeyer Pyrex - glass flasks (500 ml) containing 300 ml of MH medium. These flasks were autoclaved, cooled, inoculated, and incubated under controlled laboratory conditions (temp. 25 ±3 °C and light intensity of 400 µ mol m^{-2} s^{-1}).

Effect of nitrogen starvation

In Erlenmeyer flasks (500 ml), 300 ml of MH medium containing KNO_3 (0.1 g/ 100 ml) were added and named control. In another Erlenmeyer flask (500 ml), 300 ml of MH medium having no KNO_3 were added and named N-starved medium. The flasks, after being autoclaved, were inoculated with an initial volume (100 ml) of pre-culture under controlled conditions (temp. 25 ±3 °C and light intensity 400 µ mol m^{-2} s^{-1}).

Effect of phosphorus starvation

In Erlenmeyer flasks (500 ml), 300 ml of MH medium containing K_2HPO_4 (0.0035 g/ 100 ml) were added and named control. Other Erlenmeyer flasks having 300 ml of MH medium without K_2HPO_4 were named starved medium. As mentioned in the experiment, the flasks were inoculated and incubated for 10 days.

Effect of combined nitrogen and phosphorus starvations

Erlenmeyer flasks 500 ml contained 300 ml of MH medium without KNO_3 and K_2HPO_4 under conditions (temp. 25 ±3 °C and light intensity 400 µ mol m^{-2} s^{-1}). The flasks were inoculated and incubated under the growth conditions of the previous experiment.

Effect of salinity under nitrogen or phosphorus starved conditions

Erlenmeyer flasks (500 ml) containing 300 ml of MH medium with two concentrations of NaCl (1 and 2.5M) were subjected to conditions of either N starvation or P starvation, or both. In all experiments, the flasks were inoculated and incubated for 10 days.

Determination of growth

Optical density

The growth of *Dunaliella parva* was measured by determining the optical density of the algal suspension at 560 nm as Wetherell Field (Walter *et al*., 2011) recommended using a Unico UV-2000 spectrophotometer. The growth rate was calculated as follows:

Growth rate = $(OD_t - OD_{t0})/t$,

Where OD_t = optical density at time t.

OD_{t0} = initial optical density; t = incubation period by days.

Cell count

This was carried out using the 0.1 mm^3 deep haemocytometer slide. Counts were estimated as a number of cells/ml culture, and counts were made with at least 5 replicates.

Relative growth rate – the relative growth rate (K') was calculated according to Guillard (Guillard, 1973) from the following formula:

$$K' - [Log\ N - LogN0]/t$$

N = Number of cells/ml after time (t) (days).

N_0 = Number of cells/ml at the initial time.

Generation time: Generation time (G) is the time needed for doubling the number of cells. It was calculated according to the following formula proposed by Fogg (1975):

G.T = 0.301/K'

Where: K'= the relative growth rate.

A number of recycling: The Number of recycling for doubling the cells within a definite time could be calculated as follows:

No. of recycling = T/G

Where:

T= time from the beginning to the end of the experiment and G= mean doubling time during that period.

Growth rate: The growth rate (R) (Number of divisions/ day) was calculated by using the equation proposed by Guillard (1973):

$R = [3.322/ (t_2\text{-}t_1)] \times \log (N_2/N_1)$.

Where:

3.322 = growth constant, t_1= time at the beginning of the experiments, t_2= time at the end of the experiment, N_1= Number of cells/ml culture at t_1, and N_2= number of cells/ml culture at t_2.

Mean growth rate: the mean growth rate (R') (Number of divisions / definite time "days") was calculated from the formula proposed by Guillard (Guillard, 1973):

$R = [3.322/ (t\text{-}t_0)]$ x $\log (N/N_0)$.

Where:

3.322 = growth constant, t_0= time at the beginning of the experiments (days), t = time at the end of the experiment (days), N_0= Number of cells/ml culture at t_0, and N= Number of cells/ml culture at t.

Estimation of photosynthetic pigments

Chlorophyll a, b, and carotenoids were determined spectrophotometrically using the method recommended by Mackinney (1941).

Determination of total soluble carbohydrates

After pigment extraction, the algal cells were extracted with 1 N NaOH in a boiling water bath for 2 hours, as described by Payne and Stewart (1988). Total carbohydrates were quantitatively determined by the method of phenol-sulphuric acid described by Kochert (1973).

Estimation of total soluble proteins

Total soluble protein content in the cell-free medium of *Dunaliella parva* was estimated using the method of Bradford (Bradford, 1976).

Determination of glycerol

Glycerol was determined according to the method recommended by Chitlaru and Pick (1989).

Measurement of chlorophyll a fluorescence

The chlorophyll a fluorescence was measured at room temperature. Using a modulated light MINI-PAM portable fluorometer (Walz, Germany), the initial fluorescence (F_0), maximum fluorescence (F_m) were analyzed, and quantum efficiency of open photosystem II centres-quantum yield (F_v/ F_m) was calculated. The fluorescence variable (Fv) was calculated from the difference between F_m and F_0. The F_v and F_m values were used to obtain the maximum photochemical efficiency of PSII (Fv/Fm) ratio.

Enzymes assay

Catalase (EC-1.11.1.6) was assayed according to Kato and Shimizu (1987)by measuring the initial rate of disappearance of H_2O_2. Peroxidase (EC 1.11.1.7) activity was measured according to Kato and Shimizu (1987). The activity of ascorbic oxidase [EC1.10.3.3] was assayed according to Oberbacher and Vines (1963). The activity was calculated using the extinction coefficient (14 mM^{-1} cm^{-1} at 265 nm) as described by Nakano and Asada (1981). Enzyme activity was expressed in units of µM of the substrate converted per min. per gram f.wt. Non-enzymatic antioxidant, ascorbic acid, was estimated according to Oser (1965).

DPPH (2, 2- dipheny l-picrylhydrazyl) radical scavenging activity of Dunaliella parva

The capacity of the treated *Dunaliella parva* to reduce the 2, 2-diphenyl-picrylhydrazyl (DPPH) stable free radical was assessed using the method of Blois (Blois, 1958).

Ferric Reducing Antioxidant Potency (FRAP)

The reducing ability was assayed as described by Tsai *et al.* (2006).

Determination of total antioxidant activity

The total antioxidant activity of algal cells was determined according to the method of Prieto *et al*. (1999).

Determination of lipid peroxidation

Lipid peroxidation was measured by the amount of malondialdehyde (MDA), as a product of peroxidation of unsaturated fatty acids (Linolenic 18:3). The concentration of MDA was estimated by the method of Heath and Packer (Heath and Packer, 1968).

Statistical analysis

Results are presented as mean ± SD (standard deviation) from three readings. The statistical analyses were carried out using SPSS version 29.0 for Mac OS. Data obtained were checked for normality using the Shapiro-Wilk test to check whether data was parametric or nonparametric, accordingly, parametric data analysis recommended. One- and two-way analysis of variance (ANOVA) was applied to check the difference between treatment groups at $p \leq 0.05$. Additionally, Duncan's Multiple Range Test (DMRTs) post hoc test was applied to further check the difference between groups at 0.05 level.

Results

Effect of salt stress on numbers of Dunaliella parva cells after 10 days of incubation

It was clear from the results that the numbers of *D. parva* cells decreased at all NaCl concentrations compared with the control (Table 1). The magnitude of decrease was 6.62% at 3.5 M NaCl and 25.17% at 0.5 NaCl. This reduction in numbers at high salt concentrations was accompanied by a decrease in the relative growth rate of algal cells (K'), number of algal cells recycled, and mean growth rate of algal cells (R').

Table 1. Effect of different salt concentrations on growth parameters of *Dunaliella parva* after 10 days of incubation

NaCl concentration (M)	cell no * 10^6/ml	K'	G	No of recycling	R	R'
0.5	1.13±0.03	0.067	4.473	2.235	0.141	0.224
1	1.21±0.05	0.070	4.284	2.334	0.138	0.233
1.25 (control)	1.51±0.01	0.099	3.014	2.653	0.811	0.331
2	1.32±0.07	0.074	4.066	2.460	0.165	0.246
2.5	1.37±0.02	0.076	3.979	2.513	0.172	0.251
3.5	1.41±0.03	0.077	3.914	2.555	0.192	0.255

Each value is the mean of five readings ± standard deviation. K'= the relative growth, G= generation time, R= rate of growth and R'= mean growth rate.

Effect of different salt concentrations on pigment content and photosynthetic activity (Fv/Fm) of Dunaliella parva after 10 days of incubation

Table 2 shows that conditions of hypo- and hyper salinity led to a highly significant decrease in chlorophyll a content. The percentage of reduction reached 14.67% and 10.12% at 0.5 and 1 M NaCl, respectively, below the control. However, the percentage of decrease at 2, 2.5, and 3.5 M NaCl was 24.34%, 44.67%, and 44.55%, respectively, relative to the control. The percentage of chlorophyll b decreased by 8.06% and 7.08%, respectively, under hypo salinity, below the control. Hyper salinity significantly decreased the chlorophyll b content by 9%, 9.59%, and 20.25%, respectively, compared with the control. The decrease in carotenoids was 19.58% and 11.24%, respectively, at low salinity, while at high salinity levels, it was 3.05% and 3.57%, respectively, below the control (Figures 1 and 2).

The content of total chlorophyll was significantly decreased at all salinity levels, whether low or high, except at 2 M NaCl, where the decrease was significant relative to the control. Also, results revealed a highly significant decrease in the ratio of chlorophyll a to chlorophyll b at all salt concentrations except for 1 M NaCl, where there was a significant decrease in comparison with the control. Thus, the decrease at a low level of salt, or 0.5 M NaCl, amounted to 7.20%, while it was 16.84%, 28.27%, and 30.63%, respectively, at high levels of salt (2, 2.5, and 3.5 M NaCl), compared with the control. Concerning the total chlorophyll/carotenoids ratio at hypo salinity, there was a highly significant increase at 0.5 M NaCl, while the ratio was also significantly increased at 1 M with respect to the control. On the contrary, at hyper salinity, the results showed that there was a highly significant decrease in the total chlorophyll/carotenoids ratio at hyper salinity. The magnitude of decline reached 17.78%, 24.77%, and 34.61% at 2, 2.5, and 3.5 M NaCl, respectively, compared with the control. The data also showed that there was a highly significant decrease in the photosynthetic efficiency of PSII (F_V/F_m) at low and high salt concentrations with respect to the control. The magnitude of the reduction at the low salt levels of 0.5 and 1 M NaCl amounted to 25.30% and 16.87%, with respect to the control, whereas it was 27.71%, 30.39%, and 32.53% at the high salt levels of 2, 2.5, and 3.5 M NaCl, respectively (Figures 1 and 2). The interrelationships between the salts and various photosynthetic parameters are presented in the heatmap in Figure 3. The correlation matrix shows the effect of different levels of NaCl on various photosynthesis parameters. The blue color indicates a positive correlation, the red color indicates a negative correlation, and the boxed colors indicate a significantly positive or negative correlation (see Figure 3).

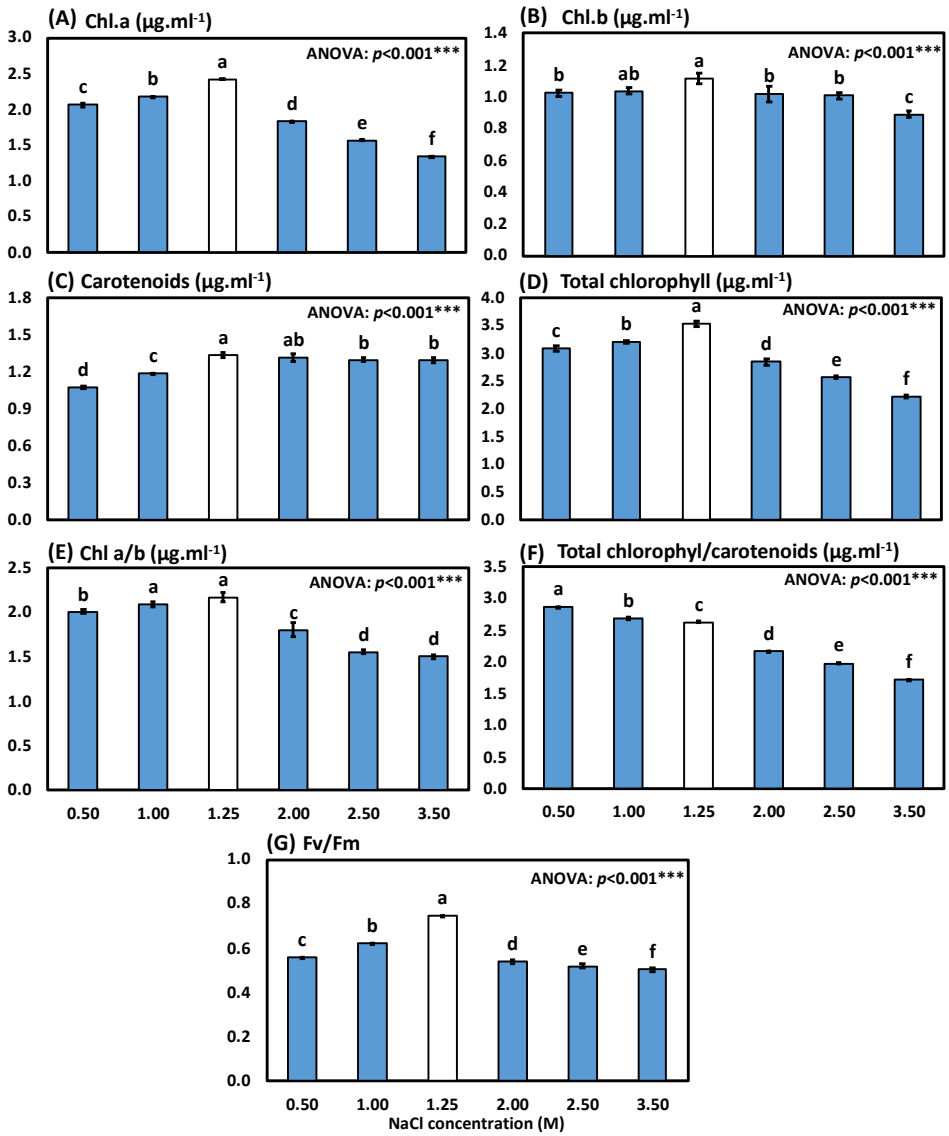

Figure 1. Effect of different salt concentrations on pigments content and photosynthetic activity (FV / Fm) of *Dunaliella parva*

Bars presented as mean ±standard deviation (SD). Bars followed by different letters are significantly different according to Duncan's Multiple Range

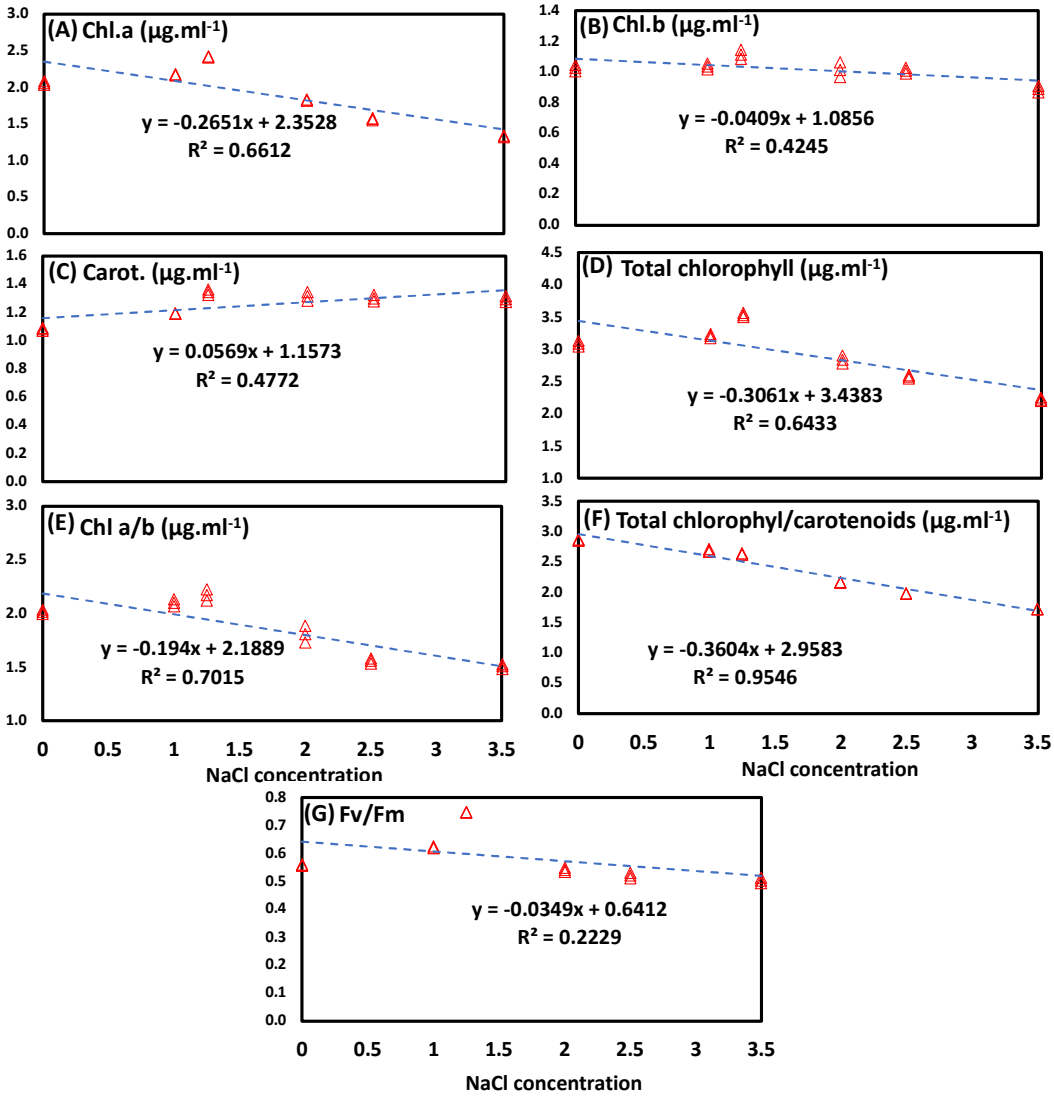

Figure 2. Regression trendlines presenting the effect of different salt concentrations on pigments content and photosynthetic activity (FV/Fm) of *Dunaliella parva*

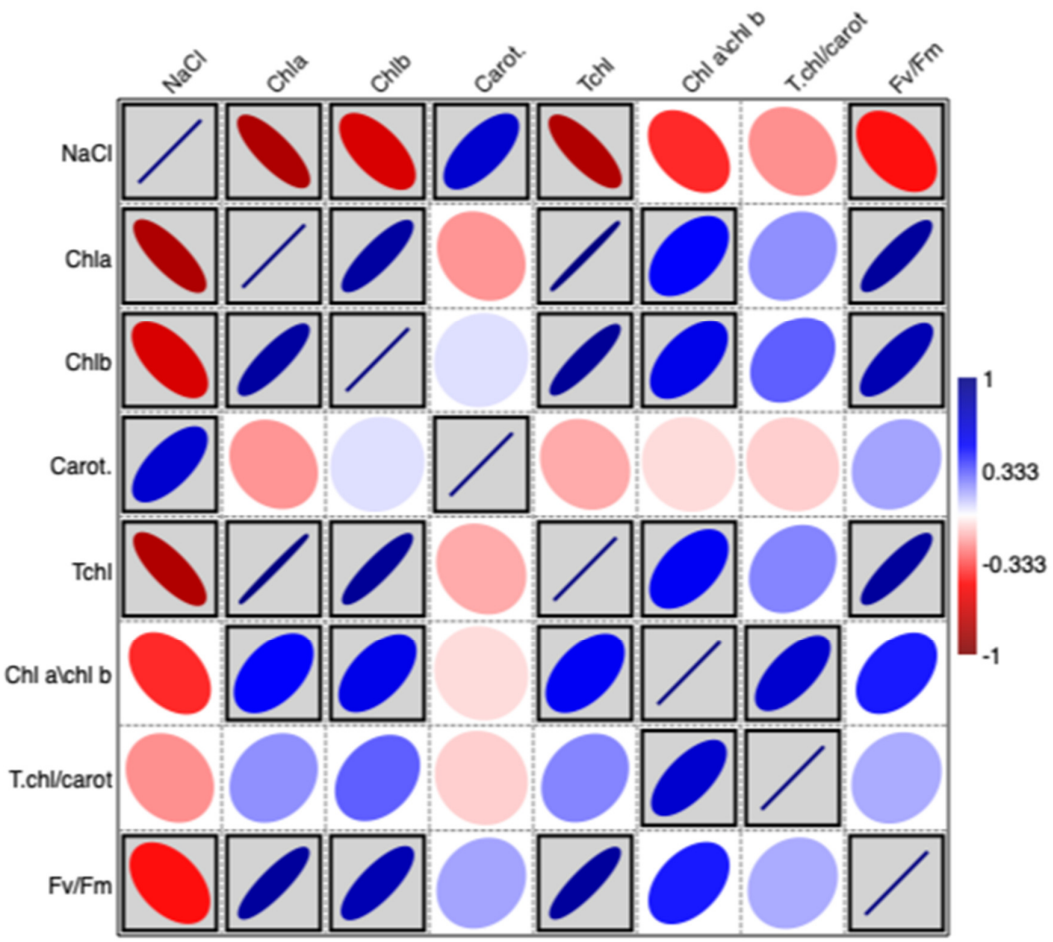

Figure 3. Heatmap presenting the correlation matrix of the effect of different NaCl on various photosynthesis parameters

Blue colour indicate positive correlation, red colour indicate negative correlation, boxed colors indicate significant either positive or negative correlation.

Effect of different salt concentrations on the antioxidant enzymes and non-enzymatic antioxidant of Dunaliella parva

The changes in the enzymatic antioxidants catalase, peroxidase, and ascorbic acid oxidase (AO) and the non-enzymatic antioxidant ascorbate of the *D. parva* cells as influenced by salt concentrations are presented in Figure 4a to d. It is clear from the results that there was no significant change in the activity of catalase at either low or high salinity levels. On the other hand, the peroxidase activity was highly significantly stimulated at hyper salinity levels; there was no significant change at the hypo salinity levels of 0.5 and 1 M NaCl. The percentages of stimulation at the hyper salinity levels of 2, 2.5, and 3.5 M NaCl were 19.21%, 16.10%, and 15.54%, respectively, compared with the control. Meanwhile, the AO was significantly increased at both low and high salinity levels compared with the control. However, the magnitude of increase was great at hypo salinity levels. The percentages of increase in AO activity at the low salinity levels of 0.5 and 1 M NaCl were 37.14% and 35.36%, respectively, compared with the control. At the high salinity levels of 2, 2.5, and 3.5 M NaCl, the percentages of increase were 68.43%, 88.94%, and 94.90%, respectively.

Regarding the non-enzymatic antioxidant ascorbate, the data indicated a highly significant decrease parallel to the increase in AO activity. The percentages of reduction in ascorbate content at a hypo salinity level

of 0.5 or 1 M NaCl were 40.03% and 38.39%, respectively, compared with the control, while they were was 56.48%, 60.83%, and 61.60%, respectively, at hyper salinity levels 2, 2.5, and 3.5 M NaCl.

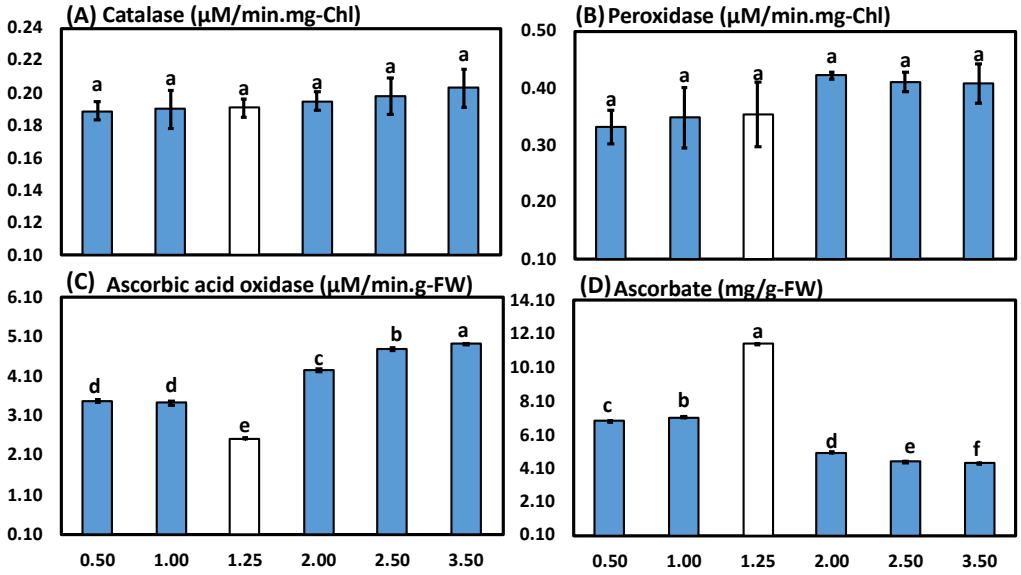

Figure 4. Effect of different salt concentrations on the activity of antioxidant enzymes (catalase, peroxidase, and ascorbic acid oxidase) and the content of non-enzymatic antioxidant (ascorbate) of *Dunaliella parva* Bars presented as mean ±standard deviation (SD).

Effect of nitrogen and phosphorus starvation, either singly or combined, on the growth parameters of Dunaliella parva

The changes in growth parameters of *D. parva* under the influence of N and P starvation are presented in Table 2. The data revealed that the number of cells of *D. parva* was decreased by 50.70% under N starvation, 29.58% under P starvation, and 57.75% under starvation of both N and P below the control value. This decrease in cell number was accompanied by a decrease in the relative growth of cells (K'), numbers of cells recycled, and mean growth rate of cells (R').

Table 2. Effect of nitrogen and phosphorus starvation either singly or combined on *Dunaliella parva* after 10 days of growth

Treatment	cell no *10^6/ml	K'	G	No. of recycling	R	R'
Control	2.13±0.001	0.094	3.174	3.150	0.248	0.314
-N	1.05±0.005	0.064	4.695	2.129	0.233	0.212
-P	1.5±0.01	0.080	3.782	2.644	0.322	0.264
-N, -P	0.9±0.001	0.057	5.244	1.907	0.181	0.191

Each value is the mean of five readings ± standard deviation. K'= the relative growth, G= generation time, R= rate of growth and R'= mean growth rate.

Effect of nitrogen and phosphorus starvation, either singly or combined, on the content of pigments, carbohydrates, glycerol, and proteins and on photosynthetic efficiency (F_v/F_m)

Figures 5 and 6 show the changes in the content of pigments (Fv/Fm), carbohydrates, glycerol, and proteins of *D. parva* under the influence of N starvation, P starvation, and combined N and P starvation. The data indicated that the chlorophyll a content was highly significantly decreased by 30.79%, 25.77%, and 40.51%, respectively, during N starvation, P starvation, and combined N and P starvation, below the control.

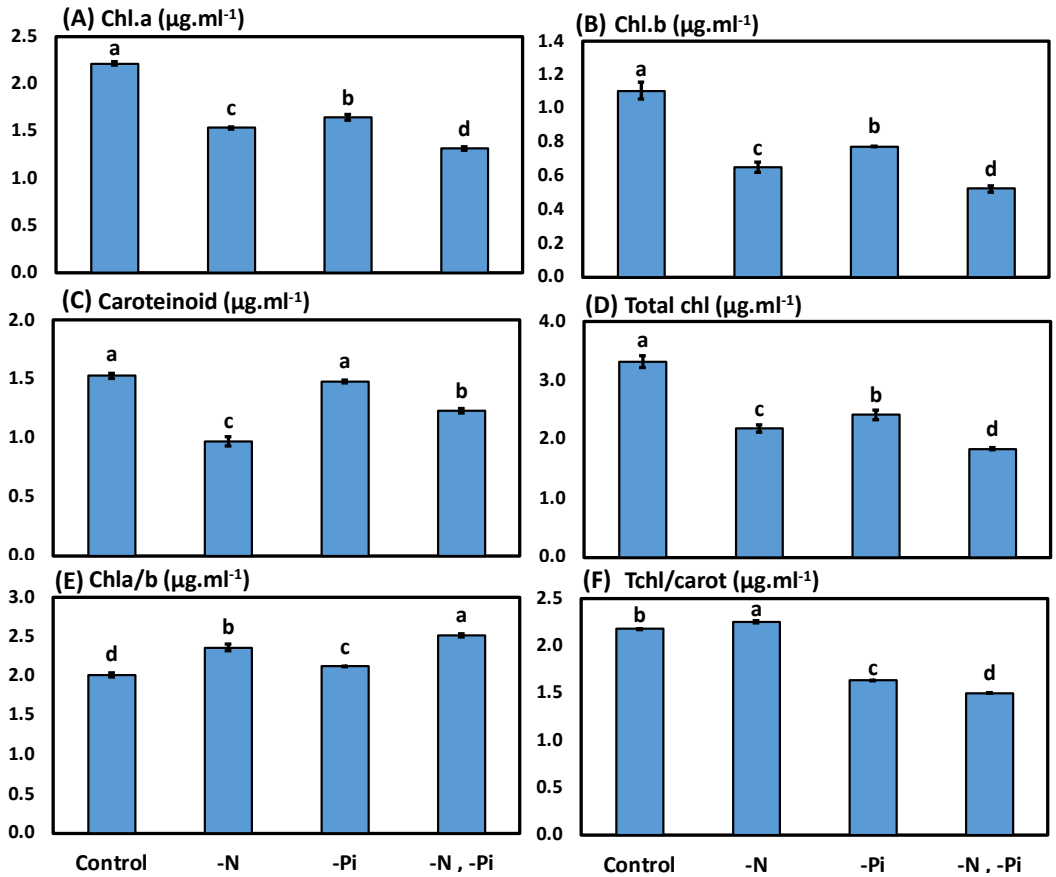

Figure 5. Effect of Nitrogen and Phosphorus starvation either singly or combined on (A) chlorophyll-a, (B) chlorophyll-b, (C) Carotenoids, (D) Total chlorophyll, (E) Chla/b, and (F) Total chlorophyll/carotenoid

Bars presented as mean ±standard deviation (SD). Bars followed by different letters are significantly different according to Duncan's Multiple Range

Meanwhile, the three types of starvation induced a highly significant decrease in chlorophyll b content, reaching 41.12% during N starvation, 29.89% during P starvation, and 52.63% during combined N and P starvation, below the control value. Regarding carotenoids, it was evident from the results that the three types of starvation led to a highly significant decrease, but there was no significant change in carotenoid content under treatment with P. The data also revealed that the total chlorophyll was high and significantly decreased below the control value under all three starvation treatments. However, the lowest value of total chlorophyll was recorded for the combined N and P treatment. Concerning the ratio of chlorophyll a to chlorophyll b, it was clear that the exposure of the alga to N starvation or combined N and P starvation resulted in a highly significant increase of 17.44% and 25.36%, respectively, compared with the control. However, there was no significant change in the ratio of chlorophyll a to chlorophyll b under P starvation. Furthermore, there was a highly significant decrease in the ratio of total chlorophyll to carotenoids under all starvation treatments, except for a non-significant change under N treatment. The results in Figures 5 and 6 also indicate that the photosynthetic efficiency (F_V/F_m) was high and significantly reduced below the control value under all starvation treatments. The magnitude of reduction reached 66.07%, 56.56%, and 67.54% under N starvation, P starvation, and combined N plus P starvation, respectively.

Concerning the change in carbohydrates and proteins, there was a highly significant decrease in their content below the control value under all starvation treatments. The percentages of decrease in the content of

carbohydrates amounted to 51.03%, 59.49%, and 54.45% under N starvation, P starvation, and N plus P starvation, respectively.

Regarding glycerol, the results showed a highly significant increase in its content only under N starvation. However, the glycerol content significantly decreased by 50% under P starvation and 18.39% under combined N and P starvation below the control value. On the other hand, the percentage of decrease in protein content reached 63.02%, 40%, and 69.66% under N starvation, P starvation, and N plus P starvation, respectively.

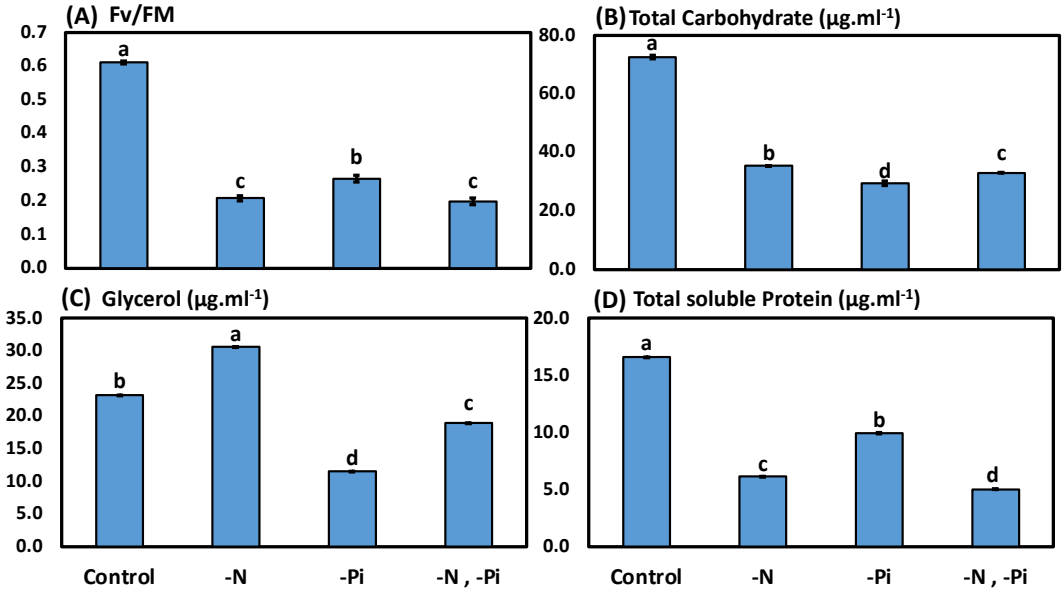

Figure 6. Effect of Nitrogen and Phosphorus starvation either singly or combined on (A) photosynthetic efficiency (F_V/F_m)., (B) total carbohydrate, (C) glycerol and (D) total soluble protein contents
Bars followed by different letters are significantly different according to Duncan's Multiple Range. Bars presented as mean ±standard deviation (SD).

Effect of nitrogen and phosphorus starvation, either singly or combined, on the antioxidant enzymes and non-enzymatic antioxidants in Dunaliella parva

The changes in the activity of the antioxidant enzymes catalase, peroxidase, and AO and of the non-enzymatic antioxidant are presented in Figure 7a to d. It is evident from the results that the activity of catalase and peroxidase was high and significantly stimulated above the control value under all starvation treatments. The magnitude of the rise in catalase activity was 123.68%, 85.79%, and 137.89% at N starvation, P starvation, and combined N plus P starvation, respectively. Meanwhile, the peroxidase activity was enhanced by 85.51%, 38.35%, and 95.17% above the control value at N starvation, P starvation, and combined N plus P starvation, respectively. Regarding the activity of AO, it was evident from the results that N starvation, P starvation, and combined N and P starvation led to a highly significant reduction, reaching 29.93%, 34.85%, and 39.44%, respectively, below the control value. Moreover, the content of ascorbate was highly significantly increased by 56.39%, 34.97%, and 78.73% under N starvation, P starvation, and combined N and P starvation, respectively, above the control value.

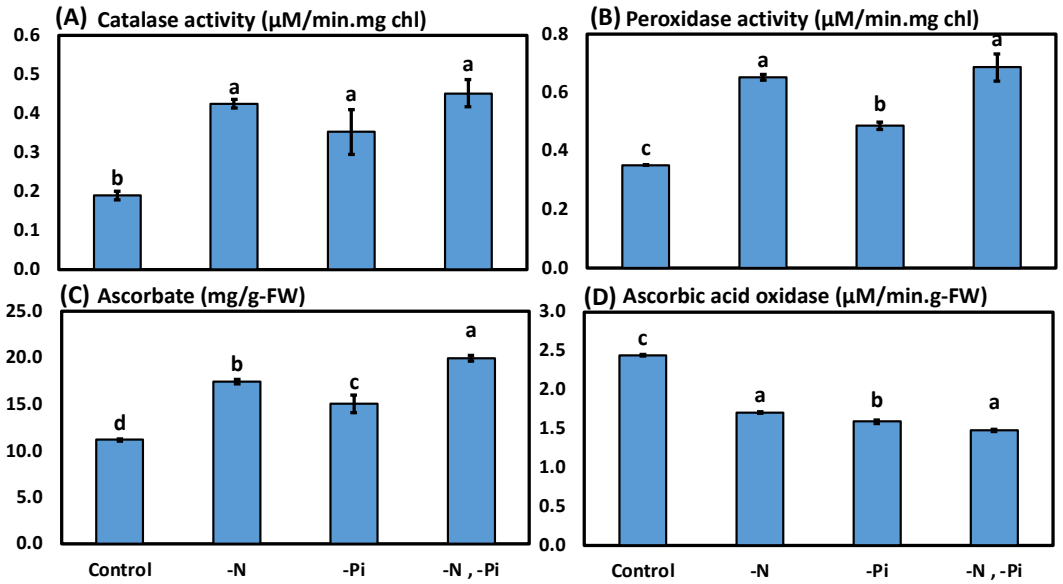

Figure 7. Effect of Nitrogen (N), Phosphorus (P) starvation either singly or combined on the antioxidant enzymes (catalase, peroxidase, and ascorbic acid oxidase) and non-enzymatic antioxidant (ascorbate) in *Dunaliella parva*

Bars presented as mean ±standard deviation (SD).

Effect of nitrogen and phosphorus starvation, either singly or combined, on total antioxidants, reducing power, radical scavenging, and lipid peroxidation

The changes in the total antioxidant content and the reducing power (ferric reducing antioxidant potency [FRAP]), radical scavenging assay (DPPH), and lipid peroxidation (malondialdehyde [MDA]) in *D. parva* under the influence of N starvation, P starvation, and combined N and P starvation are presented in Figure 8a to d. The total antioxidants were significantly increased at all starvation treatments. The percentages of increase were 95.51%, 43.40%, and 106.14% at N starvation, P starvation, and combined N and P starvation, respectively, above the control value. Concerning the reducing power (FRAP), it was significantly increased at N starvation and at combined N and P starvation, while the increase was insignificant at P starvation. The increase was 87.10%, 36.13%, and 83.87% at N starvation, P starvation, and combined N plus P starvation, respectively, compared with the control value. Furthermore, the DPPH percentage significantly increased during all starvation treatments. The percentage of increase reached 25.13% above the control. The maximum value was recorded for N starvation, reaching 95.77%, while the DPPH percentage at N was 25.13% and at P was 48.10% compared with the control. Regarding the MDA, there was a highly significant increase in its content at all starvation treatments, indicating that there was lipid peroxidation at all starvation treatments. The magnitude of increase was 9.15-fold, 6.23-fold, and 9.38-fold at N starvation, P starvation, and combined N plus P starvation, respectively, above the control.

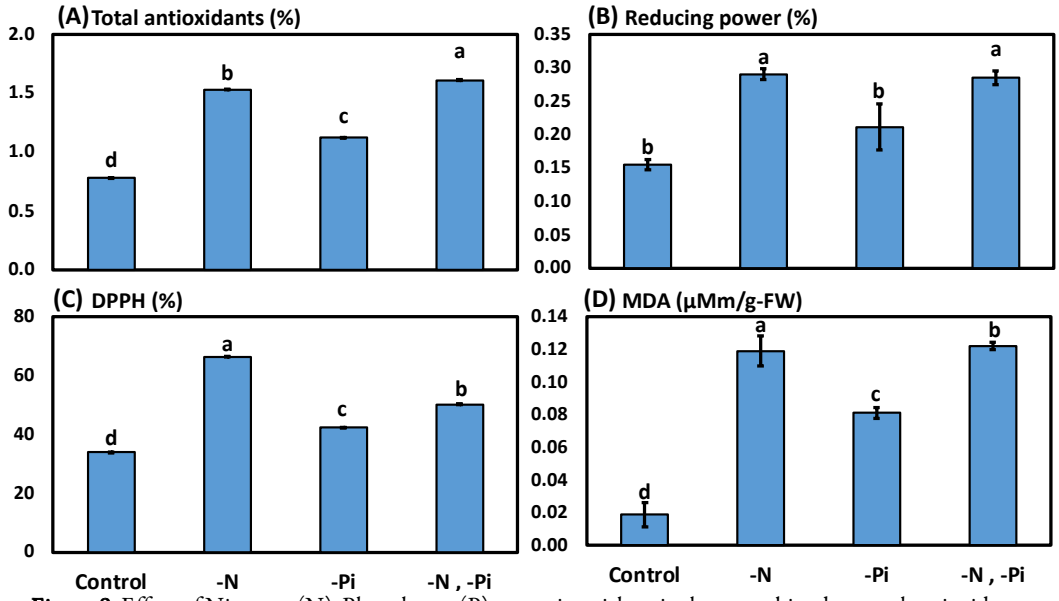

Figure 8. Effect of Nitrogen (N), Phosphorus (P) starvation either singly or combined on total antioxidant, reducing power FRAP (Ferric reducing antioxidant potency), radicals scavenging assay (DPPH), and malondialdehyde content (MDA)
Bars presented as mean ±standard deviation (SD).

Effect of salinity (at 1 and 2.5 M NaCl) combined with starvation (of nitrogen, phosphorus, or both nitrogen and phosphorus) on the growth of Dunaliella parva

The changes in growth parameters of *D. parva* under the combined effect of salinity and starvation stress (from N starvation, P starvation, and combined N and P starvation) are illustrated in Table 3. The number of cells grown under 1 M NaCl and the three types of starvation stress was decreased relative to the control. The percentage of decrease reached 68.87%, 51.66%, and 60.26% compared with the control under N starvation, P starvation, and combined N and P starvation, respectively, combined with salinity stress of 1 M NaCl. On the other hand, the number of cells was also decreased under the combined effect of 2.5 M NaCl and each of the three types of starvation. However, the magnitude of decrease at 2.5 M NaCl was less than that at 1 M NaCl. The percentages of decrease were 57.62%, 47.02%, and 50.99%, relative to the control, under N starvation, P starvation, and combined N and P starvation, respectively, combined with 2.5 M NaCl.

Table 3. Effect of salinity level (1 and 2.5 M NaCl) and starvation (nitrogen, phosphorus and nitrogen with phosphorus) on cell number of *Dunaliella parva*

Treatment	Control	1 M NaCl			2.5 M NaCl		
		-N	-P	-N, -P	-N	-P	-N, -P
cell no * 10^6/ml	1.51 ±0.01	0.47 ±0.05	0.73 ±0.02	0.60 ±0.02	0.64 ±0.01	0.80 ±0.03	0.74 ±0.02
K'	0.099	0.029	0.048	0.039	0.042	0.052	0.048
G	3.014	10.312	6.230	7.563	7.066	5.756	6.155
No. of recycling	2.653	0.969	1.605	1.322	1.415	1.737	1.624
R	0.812	0.081	0.302	0.292	-0.033	0.037	0.029
R'	0.332	0.096	0.160	0.132	0.141	0.173	0.162

Each value is the mean of five readings ±standard deviation. K'= the relative growth, G= generation time, R= rate of growth and R'= mean growth rate.

Effect of salinity (at 1 or 2.5 M NaCl) combined with nutrient starvation on pigment content, photosynthetic efficiency F_v/F_m, and the contents of carbohydrate, glycerol, and proteins of Dunaliella parva

The changes in pigment content, photosynthetic efficiency (F_v/F_m), and contents of carbohydrate, glycerol, and proteins under the influence of salinity (1 M and 2.5 M NaCl) and the three types of starvation stress are illustrated in Figures 9 to 11. The present results indicated that the content of chlorophyll a was high and significantly decreased under both salinity levels combined with all three starvation stresses. However, at all starvation stresses, the decrease at 2.5 M NaCl was higher than at 1 M NaCl. Chlorophyll b followed the same trend as that of chlorophyll a at both salinity levels combined with all starvation treatments (see Figure 9).

The carotenoid content was also significantly decreased at both salinity levels combined with the tested starvation stresses. However, the percentage of decrease at 2.5 M NaCl was greater than that at 1 M NaCl. The percentage of decrease in carotenoids at 1 M NaCl was 16.44%, 10.09%, and 21.10% at N starvation, P starvation, and combined N and P starvation, respectively. At 2.5 M NaCl plus N starvation, P starvation, or combined N and P starvation, the decrease in percentage reached 29.08%, 20.75%, and 35.36 %, respectively (see Figure 9c).

The total chlorophyll content was significantly decreased at both salinity levels (1 M and 2.5 M NaCl) combined with the studied starvation stresses. However, at all starvation stresses, the magnitude of the decrease at 2.5 M NaCl was greater than that at 1 M NaCl. The percentages of decrease in total chlorophyll at 1 M NaCl were 30.75%, 17.20%, and 37.16% at N starvation, P starvation, and combined N plus P starvation, respectively, relative to the control, while at 2.5 M NaCl, the percentages of decrease were 37.40%, 25.30%, and 44.54%, respectively. Regarding the ratio of chlorophyll, a to chlorophyll b, the results revealed no significant changes in this ratio at both salinity levels combined with all starvation treatments (see Figure 9d).

The results revealed that the total chlorophyll/carotenoids ratio was high and significantly decreased at 1 M NaCl combined with starvation stress and also at 2.5 M NaCl combined with N starvation or with combined N and P starvation, while there was no significant change in this ratio at 2.5 M combined with P starvation.

It is clear from the data that there was a highly significant reduction in F_V/F_m at both salinity levels (1 M and 2.5 M NaCl) combined with all starvation treatments. However, the magnitude of reduction in F_V/F_m at the higher salinity level (2.5 M NaCl) combined with all starvation treatments was greater than that at the lower salinity level (1 M NaCl) compared with the control. Furthermore, the present results revealed that at 1 M NaCl, the content of total carbohydrates was high and that it significantly increased at all starvation treatments, except for a non-significant change at the P starvation treatment relative to the control. On the contrary, at 2.5 M NaCl, there was a highly significant and sharp decrease in total carbohydrate content at all starvation treatments compared with the control. The magnitude of the decrease reached 59.52%, 72.36%, and 78.54%, respectively, relative to the control.

The results presented in Figure 10C indicated that the glycerol content was significantly decreased at 1 M NaCl combined with all starvation treatments. On the other hand, at 2.5 M NaCl, there was a highly significant and remarkable increase in glycerol content at all starvation treatments relative to the control. The magnitude of the increase reached 50.94%, 24.55%, and 60.60%, respectively, relative to the control. Concerning the total soluble protein, the results indicated that its content significantly decreased at both salinity levels combined with all starvation treatments. The percentages of decrease at 1 M NaCl combined with N starvation, P starvation, and combined N and P starvation amounted to 67.71%, 36.04%, and 74.53%, respectively, relative to the control, while at 2.5 M NaCl, the percentages of decrease were 73.89%, 48.30%, and 80.73%, respectively, compared with the control.

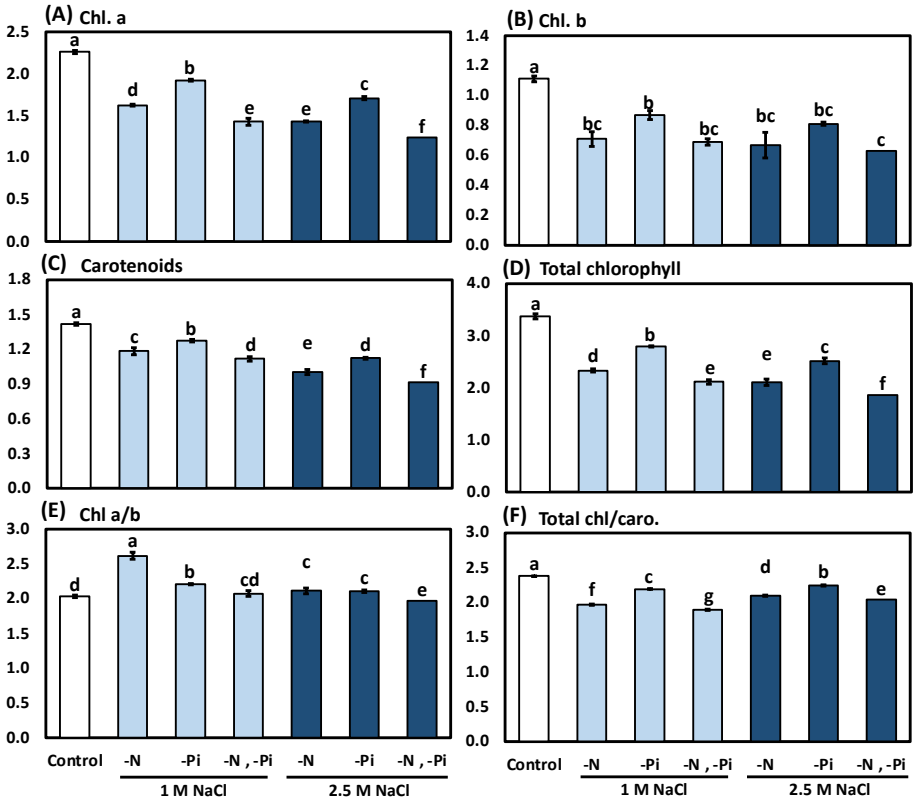

Figure 9. The effect of salinity level (1 and 2.5 M NaCl) and starvation (nitrogen, phosphorus, and nitrogen with phosphorus) on pigments content, photosynthetic efficiency of PSII F_v/F_m, carbohydrates, glycerol and protein, contents of *Dunaliella parva*

Bars presented as mean ±standard deviation (SD).

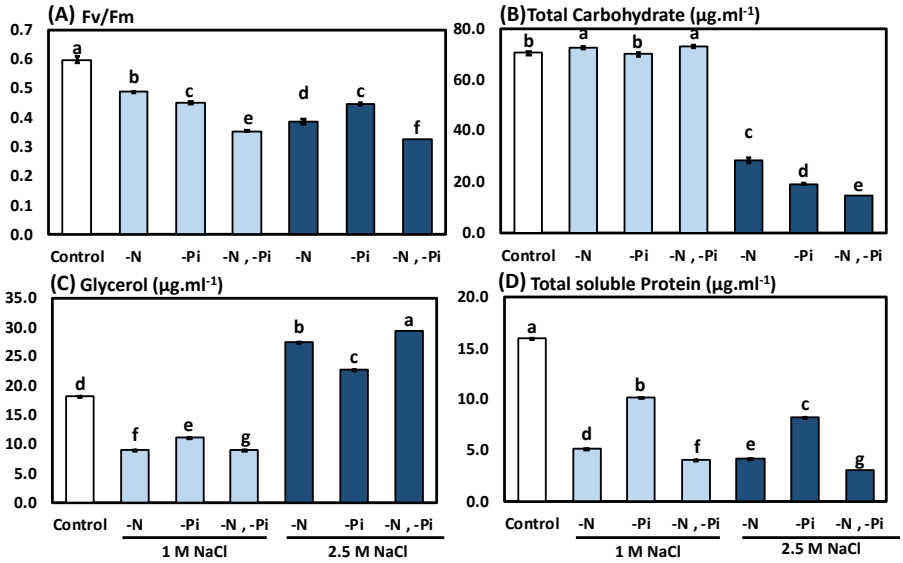

Figure 10. The effect of salinity level (1 and 2.5 M NaCl) and starvation (nitrogen, phosphorus, and nitrogen with phosphorus) on pigments content, photosynthetic efficiency of PSII F_v/F_m, carbohydrates, glycerol and protein, contents of *Dunaliella parva*. Bars presented as mean ±standard deviation (SD).

Effect of salinity (at 1 and 2.5 M NaCl) combined with nutrient starvation on the antioxidant enzymes and ascorbate content in Dunaliella parva

The changes in the activity of the antioxidant enzymes catalase, peroxidase, and AO and the ascorbate content under the combined effect of salinity (1 M and 2.5 M NaCl) and the three types of starvation stress are illustrated in Figure 11. The present results revealed that the catalase and peroxidase activity was highly significantly stimulated at both salinity levels (1 and 2.5 M NaCl) combined with all three starvation stresses. However, the stimulation magnitude was higher at 2.5 M NaCl than at 1 M NaCl combined with all starvation treatments, especially with N starvation. At 1 M NaCl, the percentages of increase in catalase activity were 149.73%, 87.38%, and 156.76% relative to the control when combined with N starvation, P starvation, and combined N and P starvation, respectively. Meanwhile, at 2.5 M NaCl combined with the three starvation treatments, the percentages of stimulation amounted to 225.95%, 192.97%, and 244.32%, respectively, compared with the control.

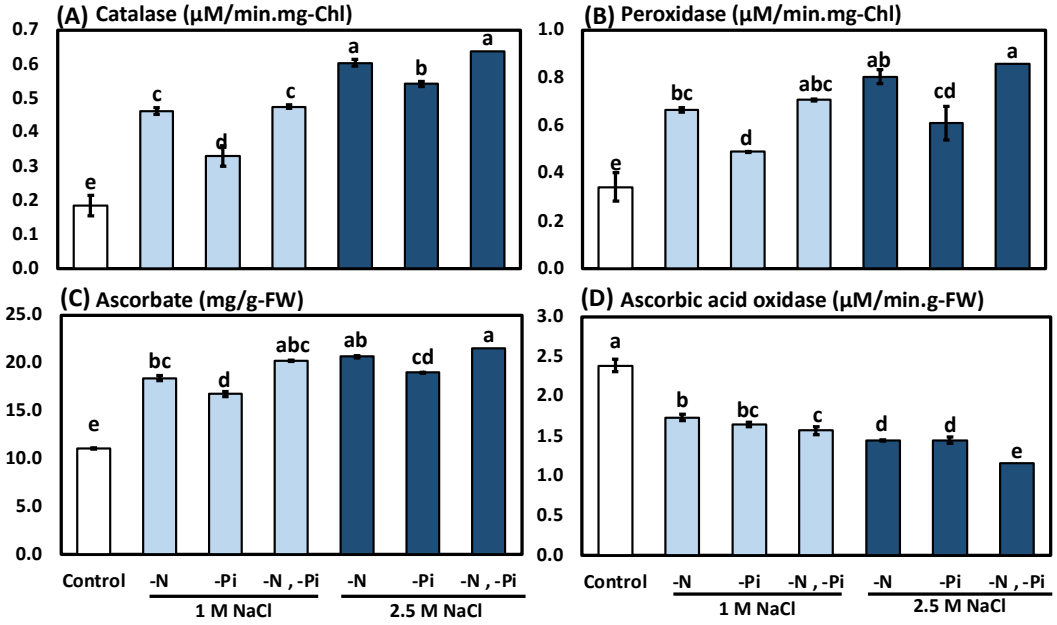

Figure 11. The combined effect of salinity levels (1 and 2.5 M NaCl) and starvation (nitrogen, phosphorus and nitrogen with phosphorus) on the antioxidant enzymes (catalase, peroxidase and ascorbic acid oxidase) and ascorbate content in *Dunaliella parva*
Bars presented as mean ±standard deviation (SD).

The activity of peroxidase followed the same trend as that of catalase, and the percentages of stimulation at 1 M NaCl combined with the N starvation, P starvation, and combined N and P starvation treatments reached 94.74%, 43.27%, and 106.73%, respectively, relative to the control. At 2.5 M NaCl, the percentages of stimulation reached 135.09%, 78.65%, and 150.88%, respectively. On the contrary, the results indicated a reduction in AO activity at both salinity levels (1 M and 2.5 M NaCl) combined with all starvation treatments. This reduction in AO activity was parallel to a highly significant increase in ascorbic acid content. The magnitude of increase in ascorbic acid content at 1 M NaCl combined with the N starvation, P starvation, and combined N plus P starvation treatments was 66.21%, 51.10%, and 82.36%, respectively, relative to the control. At 2.5 M NaCl, the percentages of increase reached 86.16%, 71.10%, and 94.18%, respectively, compared with the control.

Discussion

Algae adapt differently to salinity and are classified according to the extent of their tolerance as halophytic (salt required for optimal growth) and halotolerant (having response mechanisms that permit their existence in a saline medium) (Rao *et al.*, 2007). *Dunaliella* responds to salt stress by accumulating a significant amount of vitamins, proteins (Ghoshal *et al.*, 2002), and other important nutritional compounds (Rad *et al.*, 2015).

Data from the present work revealed that *D. parva* had a remarkable tolerance to hypo- and hypersaline conditions and survived in nearly all tested concentrations from 0.5 to 3.5 M NaCl. These findings agreed with those of Rad *et al*. (2015), who reported that *Dunaliella* responded to any changes in osmotic pressure by changing the volume of its cell.

The decreased cell numbers of the studied organism may be attributed to the recorded decrease in photosynthetic pigments associated with reduced photosynthetic efficiency (F_v/Fm). This is a response of the alga to oxidative stress imposed by salinity, as was shown later. These results were in accordance with those of Tammam *et al*. (2011), who speculated that a reduction in photosynthetic pigments during halo adaptation of *D. parva*, *D. salina,* and *D. tertiolecta*, , was probably due to the non-adaptability of the organism to salinity stress and a reduction in the photosynthetic rate. Furthermore, the present study revealed that the contents of chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll were decreased at both hypo- and hyper salinity levels.

Also, the present study showed a highly significant reduction in Fv/Fm at levels of hypo- and hyper salinity. However, the reduction in hyper salinity was higher than that in hypo salinity. This finding was in agreement with the results of Liu and Shen (2004), who found that NaCl and several other salts induced LHCII phosphorylation in the thylakoid membrane of *D. salina* under both dark and light conditions via the stimulation of kinase activity.

The present data indicated that catalase activity was insignificantly changed at all NaCl concentrations. Concerning peroxidase, hyper salinity induced a highly significant increase in peroxidase activity of the tested alga, but hypo salinity levels were associated with a non-significant change in its activity.

Meanwhile, the activity of AO was highly significantly increased at both salinity levels. However, the magnitude of increase was higher at hyper salinity levels than at low ones. The content of the non-enzymatic antioxidant ascorbate was highly significantly decreased, with a higher magnitude of decrease at hyper salinity levels than at hypo salinity levels.

Catalase responses to salinity stress can be variable, from large increases in activity (Shalata *et al.*, 2001) to no changes (Fadzilla *et al.*, 1997). In agreement with the present results, Tammam *et al*. (2011) found no significant difference in the levels of catalase activity of *D. salina* by increasing the salinity levels.

The stimulation in peroxidase activity of the studied alga at hyper salinity was in harmony with the results of Tammam *et al*. (2011). They found that the peroxidase activity in *D. salina* at 4 M NaCl was double that at 0.05 M NaCl.

An increase in antioxidant activity is the key to preventing salt damage, while sensitive species typically exhibit either no change or decreased activity (Shalata *et al.*, 2001). In the present study, the stimulation in ascorbic acid oxidase (AO) activity under hyper salinity levels was associated with decreased ascorbic acid (AA) content. AO oxidizes AA to the unstable radical monodehydroascorbate (MDHA), which rapidly converts to yield dehydroascorbic (DHA) and AA (Smirnoff, 2000). The MDHA radical can be recycled back to AA by the activity of NAD(P)-dependent monodehydroascorbate reductase (MDHAR). AO expression is modulated by a complex transcriptional and translational control (Esaka *et al.*, 1992), so salinity might stimulate the genes responsible for AO synthesis of the tested alga. In this respect, other stresses such as cadmium (Cd) increased the activity of AO in *Raphanus sativus* (Anuradha and Rao, 2007).

Moreover, the findings on the decrease in the content of AA under high salinity levels in the present work was in accordance with the results of Tammam *et al*. (2011). They reported a higher magnitude of decrease in the AA content of *D. tertiolecta* at 4 M NaCl than at 0.5 and 1 M NaCl.

Nitrogen is one of the primary requirements of growth media for any cell. The absence of N or the starvation condition is considered a form of stress (Pisal and Lele, 2005). Phosphate is absent in nature and is regarded as a limiting factor in many environments, since it is mainly found in forms not readily available, such as insoluble salts (Eberl *et al.*, 1996).

It is clear from the present work that the number of cells was decreased by growing the alga either under N or P or under their combined effect. This was associated with reduced growth parameters. These findings agreed with those of Pisal and Lele (2005), who found that the growth rate declined under N starvation. Also, they reported that the growth rate in P-starved cells was reduced in *C. kessleri*, *D. tertiolecta,* and *D. parva* (El-Sheekh and Rady, 1995; Said, 2009).

Concerning the pigments, the results showed that the content of all pigments (chlorophyll a, chlorophyll b, and carotenoids) was highly significantly decreased at all starvation treatments, except for a non-significant decrease in carotenoid content at P starvation. The reduction in the carotenoid content of *D. parva* in this study may be attributed firstly to the fact that the alga does not accumulate carotenoids under stress. Secondly, the recorded enhancement of glycerol production (at the expense of carbohydrates) as an osmoprotectant may have decreased the requirement of the cell for carotenoids that could serve as antioxidants that scavenged ROS. The increase in the chlorophyll a/chlorophyll b ratio in this study was in harmony with the results of Jiménez and Niell (2003), who found that an increase in the chlorophyll a/chlorophyll b ratio was associated with a reduction in the growth rate and an increase in the cell volume of *D. viridis*.

This work indicated that reducing the Fv/Fm of PSII was correlated with reduced carbohydrate production at all starvation treatments. This reduction at N starvation may be attributed to the inability of the organism to synthesize chlorophyll efficiently, as N is the main constituent. In addition, chlorophyll degradation may occur due to impairment of the thylakoid membrane via ROS, as shown later in the results.

The data also showed a 50% reduction in carbohydrate production, while glycerol synthesis was stimulated by 32%. This finding indicated that glycerol synthesis suppressed carbohydrate production and was consistent with Field's results (Taha *et al.*, 2012). It is generally believed that under stress conditions, the unicellular alga *D. bardawil* overproduces and accumulates β-carotene in newly formed triacylglycerol droplets. When triacylglycerol synthesis is blocked, the overproduction of β-carotene is also inhibited. Thus, the formation of these structures and β-carotene are interdependent (Rabbani *et al.*, 1998; Riahi, 2022).

Furthermore, the results indicated that glycerol, total soluble carbohydrates, and total soluble proteins were the metabolites that steeply decreased under conditions of P starvation. The recorded decrease in total carbohydrates and total protein content in P-starved cultures may be due to the fact that most newly fixed carbon appears to be directed toward respiratory metabolism and other pathways (Dietz and Heilos, 1990).

The results showed that the catalase, peroxidase, and ascorbic acid contents were significantly increased at all starvation treatments. On the contrary, the activity of AO was significantly decreased. In the present work, the increase in catalase and peroxidase activities may be due to the recorded oxidative stress monitored by the MDA content and membrane leakage (Bamary and Einali, 2022). Catalase is an enzyme responsible for the levels of peroxides occurring during acute oxidative stress (Bamary and Einali, 2022). Catalase, a key antioxidant enzyme in the cell, responds against the overproduction of H_2O_2 (Tammam *et al.*, 2011). The present results were consistent with those of Abd-El Baky *et al*. (2004), who reported a remarkable stimulation in the activities of catalase, peroxidase, and superoxide dismutase under the combined effect of N limitation and salinity.

The data showed a significant rise in AO activity, correlated with increased AA content, at all starvation treatments. This indicated that the alga responded to nutrient starvation (N starvation, P starvation, and combined N plus P starvation) by stimulating the activities of the antioxidant enzymes catalase and peroxidase

and the non-enzymatic antioxidant ascorbate as a defense mechanism. These findings were in accordance with the results of Abd El-Baky *et al.* (2004) on *D. salina* under the combined effect of salinity and N starvation.

The content of all photosynthetic pigments was significantly decreased at both salinity levels (1 and 2.5 M NaCl) combined with nutrient starvation. However, the decrease was more pronounced at hyper salinity stress than at hypo salinity stress combined with starvation. Moreover, the results indicated that the Fv/Fm and total soluble protein content were reduced at both salinity levels combined with all starvation treatments. This reduction was more pronounced at a high salinity level than at a low salinity level combined with all starvation treatments. The recorded high protein content associated with the low growth at the hypo salinity level (1 M NaCl) combined with starvation may be due to the ability of the organism to divert some protein from its state of growth to its state of osmoregulation. The concurrent results agreed with those of Tammam *et al*. (2011), who found that protein content was increased by about 2-fold and 2.6-fold for *D. salina* and *D. tertiolecta*, respectively, at conditions of hypo salinity.

Furthermore, the results indicated that under hypo salinity, there was a highly significant increase in carbohydrate content, which was associated with a highly significant decrease in glycerol content, at N starvation and combined N and P starvation. On the contrary, the highly significant reduction in carbohydrate content at the hyper salinity level was correlated with a highly significant increase in glycerol content at all starvation treatments. These findings were in harmony with the findings of others (Mofeed, 2015), who reported an increase in glycerol content at hyper salinity.

When the tested organism was grown under conditions of high salinity (2.5 M NaCl) combined with either N starvation or combined N plus P starvation, the intracellular concentration of glycerol was increased over 50%. This increase may be sufficient to account for all the osmotic pressure required to balance the extracellular osmolarity. This was consistent with the report of Tafreshi and Shariati (2009).

Data from the present study showed that the activities of catalase, peroxidase, and ascorbate were highly significantly increased under both salinity levels combined with all starvation treatments. However, the magnitude of the increase was great in conditions of hyper salinity. This is probably due to the combined stresses imposed on the studied alga, which led to the increased production of ROS, as monitored by the recorded increase in MDA and membrane leakage. The results agreed with those of Abd El-Baky *et al*. (2004), who found that a high salt concentration (16%) combined with N limitation enhanced the activity of catalase, peroxidase, and superoxide dismutase in *D. salina*.

Furthermore, the data demonstrated that the total antioxidants, FRAP and MDA, significantly increased at both salinity levels (1 and 2.5 M NaCl) combined with all starvation treatments. On the other hand, DPPH at 1 M NaCl showed a non-significant change at N starvation and combined N and P starvation, but it decreased with P starvation. Moreover, the increase in those parameters was higher at 2.5 M NaCl at N starvation and combined N and P starvation, except for a decrease at P starvation. The difference in the magnitude of the increase in FRAP and the total antioxidant capacity, in the present work, at high salinity indicated that the alga responded to the high salinity (Annegowda *et al.*, 2010; Bamary and Einali, 2022).

Regarding MDA, the present results indicated that its content was significantly increased under all treatments, indicating high ROS production leading to lipid peroxidation. The recorded increase in MDA content was associated with a rise in the activity of catalase and peroxidase. The concurrent results agreed with those of (Abd El-Baky *et al.*, 2004), who found that the content of MDA, an indicator of lipid peroxidation, was increased when a *Dunaliella* culture was exposed to N limitation and salt stress conditions.

Conclusions

Finally, *D. parva* grown under the combined effect of nutrient starvation plus high levels of salt (2.5 M NaCl) exhibited the following characteristics: (1) high antioxidant potency as monitored by the total

antioxidants and FRAP assays; (2) enhanced capability of free radical scavenging as monitored by the DPPH percentage; and (3) increased glycerol production, which reached 32.53% and 60.76%, respectively, at N starvation and at P starvation combined with 2.5 M NaCl. Therefore, it is recommended that *D. parva* be used as a natural pharmaceutical product for commercial purposes, and it may be promising for biofuel production.

Authors' Contributions

Conceptualization M.E, S.D,.; methodology, A.H, A.A, M.A, A.E.; validation, M.E, S.D, A.H, A.A, M.A, A.E.; formal analysis, M.E, S.D, A.H, A.A, M.A, A.E.; investigation, M.E, S.D, A.H, A.A, M.A, A.E.; resources, M.E, S.D, A.H, A.A, M.A, A.E.; data curation, M.E, S.D, A.H, A.A, M.A, A.E.; writing—original draft preparation, M.E, S.D, A.E,.; writing—review and editing, M.E, S.D, A.H, A.A, M.A, A.E. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors also thank the Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R182), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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