

Decrypting the Effects of Starvation and Excess of Nitrogen and Phosphorus on *Nostoc calcicola*

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The physiological and biochemical behaviour of marine cyanobacterium (*Nostoc calcicola*) grown under starved and double the strength of nitrogen (N) and phosphorus (P) was studied. Culture grew in an Artificial Sea Nutrient III culture medium with five different concentrations of N and P, i.e., double the concentration of N (DN) or P (DP), starvation of nitrogen, (-)N and phosphorus, (-)P and combine deprivation of both nitrogen and phosphorus, (-)NP along with control. Growth rate and photosynthetic efficiency decreased under P starved condition alone, and combined starvation of N and P. Oxidative, and physiological stress was evidenced by increased ROS, MDA, and proline content level under starved conditions. Elevated activity of Ascorbic acid, SOD, CAT, and APX were also detected. In contrast, an increase in the growth rate and Fv/Fm ratio, along with low oxidative, osmotic stress, and antioxidant activity, was observed under double the strength of nitrogen and phosphorus. Our work demonstrated that P starvation alone and combine starvation of N and P affected the growth of *N. calcicola*, maybe due to oxidative damage (increased lipid peroxidation and carbonyl content). In contrast to nutrient-starved conditions, the growth rate of *N. calcicola* enhanced under DN and DP, followed by (-) N.

Key words: Antioxidant, Fv/Fm, lipid peroxidation, Proline, ROS

Abbreviations: DN, Double nitrogen; DP, Double phosphorus; (-)N, nitrogen starvation; (-)P, Phosphorus starvation; (-NP) Combine starvation of nitrogen and phosphorus; Fv/Fm, photosynthetic efficiency of PS II, Fv- variable fluorescence, Fm- maximal fluorescence, ROS, Reactive oxygen species; MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase; APX, Ascorbate peroxidase

Nitrogen (N) and phosphorus (P) are the vital elements required for the growth and development of photosynthetic organisms. Nitrogen is available in nitrate or ammonium ion; among the two forms, ammonium assimilation is energy effective than nitrate assimilation (Ruan and Giordano, 2017). However, most microorganisms find ammonium toxic for their growth and development (Drath *et al.*, 2008; Markou *et al.*, 2014), whereas phosphate is biologically available as orthophosphates in the aquatic environment (Shan *et al.*, 1994). Both elements are considered as an essential factor in determining diazotrophic cyanobacteria's spatial and temporal distribution (Niemi, 1979).

N and P play a essential role in synthesizing primary and secondary amino acids, proteins, nucleic acids, coenzymes, chlorophyll, and other photosynthetic pigments. Thus, indispensable in regulating the productivity and influencing species composition to sustain in any given habitats (Raven *et al.*, 1992). Cyanobacteria and Diatoms attain their optimum growth at an N:P ratio of 2-5:1 (Bulgakov and Levich, 1990). Whereas for other photosynthetic organisms, N and P comprise about 7-20% ($7.0E+15:1.0E+17-1:5$) and 0.2% (1:500), respectively, dry weight of their cells (Hu, 2004; Schachtman *et al.*, 1998).

N and P deficiency cause a reduction in the growth and photosynthesis of cyanobacteria. For instance, Liefer *et al.* (2018) reported a decline in the growth rate of diatoms and prasinophytes under N deficient conditions. In a similar type of work, Collen *et al.*, (2004), reported a decrease in the growth rate of *Gracilaria tenuistipitata* under N deficiency. In another study conducted by Degerholm *et al.*, (2006) and Xu *et al.*, (2010) showed a decline in the growth rate in *Nodularia*, *Aphanizomenon*, and *Gracilaria lemaneiformis*, (Rhodophyta) under low phosphorus and low CO₂ condition. Collen *et al.*, (2004) and Xu *et al.*, (2010) study further reported a reduction in the Fv/Fm ratio and photosynthetic rate under N and P limited condition.

Macroalgae, microalgae, and diatoms, when grown under N and P limited condition, display ROS generation in the form of hydrogen peroxide and hydroxyl radicle (Lin *et al.*, (2017; Hamid and Sibi, 2018). These ROS leads to oxidative damage to various biological

molecules such as oxidation of protein and nucleic acid and peroxidation of membrane lipids, causing high growth costs. To protect the organisms, certain metabolites such as non-enzymatic antioxidants and enzymatic antioxidants are produced to mitigate the organism's oxidative damage (Hamid and Sibi, 2018).

In contrast with deficiency, high N and P concentrations in the water bodies known to stimulate growth and photosynthesis of cyanobacteria. Elevated N and low P concentration (New Zealand rivers) and high P and Low N (Spain river) resulted in excess growth of different species of cyanobacteria (Wood and Young 2011, 2012; Sabater *et al.*, 2003; Vilalta *et al.*, 2003; Zou and Gao 2014). Felisberto *et al.*, (2011) study under four nutrient treatments (N; P; N plus P and control), reported that the growth densities of green algae and cyanobacteria prevailed under P and N plus P treatment. They also found that N and P's addition either individually or in combination, altered the algal growth densities. Vezie *et al.*, (2002) reported an increase in the growth of toxic *Microcystis* strain under a high concentration of N & P.

Further, Xu *et al.*, 2010, displayed an increase in the growth and photosynthesis of *Gracilaria lemaneiformis* under high P level and not recorded under low P condition and elevated CO₂ concentration in the medium.

The excess accumulation of N and P in aquatic ecosystems has also become the most extensive and challenging environmental problems nowadays (Dar *et al.*, 2011). Cyanobacteria can accumulate a substantial amount of lipids under nutrient-deficient state, especially under N limited condition, the most widely used strategy to induce lipid production. Whereas on the other hand, cyanobacteria are also well known for flourishing under high N & P conditions. Their cultures are being employed extensively for wastewater treatments over the last few decades (Chevalier *et al.*, 2000). Hence, excess N & P contained wastewater used as a culture medium for growth, making it useful for biofuel production while concurrently reducing the nutrient load from the water bodies (Sood *et al.*, 2015).

Only limited studies on the effect of nutrient deficiency on physiological and biochemical

mechanisms are known. In this study, we endeavored to investigate the effects of starvation and excess availability of N & P on the growth, physiology, and biochemistry of *N. calcicola*.

MATERIALS AND METHODS

Culture of *Nostoc calcicola*

The culture of *N. calcicola* Brébisson ex Bornet & Flahault procured from National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tamil Nadu, India. The cultures were grown in an Artificial sea nutrient (ASN) III sterile culture medium, as described by Rippka *et al.*, (1979) at 30°C under fluorescent white light (80 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) with a 16:8 h photoperiod and with 150 rpm. The growth of culture was monitored by measuring optical density (O.D) at 680 nm (Wang *et al.*, 2012) with a spectrophotometer (Shimadzu, UV-2450). The cells in the exponential growth phase (25th day) were harvested, lyophilized, and used for further experiments.

Nutrient treatment

Cyanobacterium culture was grown in the ASN III sterile medium containing a standard concentration of nitrogen (N) & phosphorus (P) according to Rippka *et al.*, (1979), double, triple, and four times the strength of nitrogen (N) and phosphorus (N) separately (supplied in the form of sodium nitrate (NaNO_3) or dipotassium phosphate (K_2HPO_4) respectively as well as under (-)P or (-)N starvation individually and in combination (-)NP. Along with the Nitrate study, a preliminary study was conducted with ammonium chloride on the growth of *N. calcicola*. Sodium nitrate of ASN III was substituted with ammonium chloride (single and double the strength of ammonium).

Measurement of photosynthetic efficiency of PSII (Fv/Fm)

Photosynthetic efficiency of PSII was measured using chlorophyll fluorometer (PAM, Walz, Germany) following Sharma *et al.*, (1998). Before measurement, *N. calcicola* was dark-adapted for 10 min. To measure initial fluorescence (F_0), the dark-adapted 1 ml cultures were exposed to a modulating light of intensity 4 $\mu\text{mol m}^{-2} \text{s}^{-1}$ followed by exposure to a saturating pulse of the white light of 4,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to obtained the

maximum fluorescence (F_m). Variable fluorescence (F_v), calculated by subtracting the F_0 from F_m ($F_v = F_m - F_0$), and the F_v/F_m ratio was determined.

Determination of Hydrogen peroxide (H_2O_2)

The total H_2O_2 content was estimated following Sagisaka *et al.*, (1979). The experiments were carried out with 10 mg lyophilized cyanobacterium tissue, homogenized in 5ml of 5% trichloroacetic acid (TCA), and centrifuged at 4°C for 10 min at 7000 rpm. Supernatant added with 50% TCA (40 μl), 2.5 M potassium thiocyanate (20 μl), and 10 mM ferrous ammonium sulfate (40 μl), and the reaction mixture was incubated for 30 s at RT. Absorbance was recorded at 480 nm using a UV-visible spectrophotometer (UV-2450, Shimadzu). H_2O_2 concentration was determined using a calibration curve with a 10 mM stock solution and expressed as $\mu\text{mol g}^{-1}$.

Determination of Hydroxyl radical ($\text{OH}\cdot$)

$\text{OH}\cdot$ (hydroxyl radical) content was determined following the protocol given by Liu *et al.*, (2009). $\text{OH}\cdot$ radical reduces the deoxyribose, which in turn forms thiobarbituric acid reactive substances (TBA-RS), detected spectrophotometrically. Lyophilized 10 mg tissue was homogenized in 2 ml of 5% TCA and centrifuged at 4°C for 10 min at 1000 rpm. The supernatant was added with 500 μl of 50 mM NaPO_4 buffer (pH 7), and 1 ml of 25 mM NaPO_4 buffer (pH 7) containing 2.5 mM, 2-deoxyribose and dark incubation was done for 1 h at 35°C. Soon after incubation, the reaction mixture was added with 1% TBA and glacial acetic acid (GAA), 1 ml each. Further, the reaction mixture was incubated at 95°C for 10 min in a dry bath, and immediately test tubes were cooled for 2 min on ice. Absorbance was recorded at 532 nm using a UV-Visible spectrophotometer (UV-2450, Shimadzu). An increase in the absorbance is related to the rise in $\text{OH}\cdot$ level. The concentration of $\text{OH}\cdot$ was expressed as Absorbance units (Absorbance x 1000)/1000.

Determination of lipid peroxidation

Malondialdehyde (MDA), is routinely used as a criterion to measure lipid peroxidation, determined by following Sankhalkar and Sharma (2002) protocol, where the level of lipid peroxidation products in the sample examined as 2-thiobarbituric acid-reactive

materials (aldehydes, mainly MDA and endoperoxides). Lyophilized 10 mg tissue was ground in 4 ml of 1% TCA and homogenate was centrifuged at 2000 rpm for 5 min. 1ml supernatant was added with 1.5 ml incubation buffer (50 mM Tris HCL, 175 mM NaCl), pH 8, 1.5 ml of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. Followed by incubation at 95°C for 30 min in a dry bath, cooled at RT and MDA content was determined by measuring absorbance at 600 nm and 532 nm using a UV-Visible spectrophotometer (UV-2450, Shimadzu). By subtracting absorbance measured at 600 nm from absorbance measured at 532 nm non-specific turbidity was corrected. MDA content is expressed as $\mu\text{mol g}^{-1}$ of tissue.

Protein carbonyl (R = O) estimation

Protein oxidation estimated following Vega-López *et al.*, (2013), lyophilized tissue (50 mg) was ground in 2 ml of 30% TCA and centrifuged, with a slight modification to the original protocol, at 7,000 rpm for 5 min and the supernatant was discarded. To the pellet, 0.5 ml of 2,4-Dinitrophenylhydrazine (DNPH) (10 mM in 2 N HCl) was added, followed by vortex for 30 s and dark-adapted for 1 h, vortexing after every 10 min. Three washing performed using ethanol: ethyl acetate (1:1 v/v) to eliminate excess DNPH and centrifuge between each wash at 5000 rpm. The precipitate was dissolved in 1ml of urea (6 M in 20 mM potassium phosphate at pH 2.5) and further incubated for 1 h at 37°C after mixing in a vortex. The absorbance was evaluated at 366 nm using a UV-Visible spectrophotometer (UV-2450, Shimadzu).

The protein carbonyl content was calculated with the molar extinction coefficient of $0.022 \mu\text{M}^{-1}\text{cm}^{-1}$ and reported in nmol ml^{-1} .

Measurement of proline content

Proline concentration estimated following the method of Bates *et al.*, (1973). Lyophilized tissue (10 mg) was homogenized with 5 ml of 3% sulfosalicylic acid, and centrifuged at 2,500 rpm for 5 min. The supernatant was added with glacial acetic acid and acid ninhydrin reagent (1 ml each) followed by incubation at 95°C for 1 h. After the test tubes were cooled, 10 ml of toluene was added to each test tubes and shook vigorously. The reaction mixture was allowed to

precipitate, and absorbance was read at 520 nm using a UV-Visible spectrophotometer (UV-2450, Shimadzu).

For quantification, L-Proline was used as a standard, and the standard graph plotted against concentration.

Ascorbic acid assay

Estimation of Ascorbate (ASA) was carried out following Kampfenkel *et al.*, (1995). Lyophilized tissue of 10 mg was homogenized with 2 ml of extraction buffer (0.1 M HCl + 0.1 mM EDTA). Tissue extract was centrifuged at 12,000 rpm for 2 min, and the supernatant was used for the ascorbate assay. The reaction mixture contained, 100 μl of sample, 100 μl of 0.4 M phosphate buffer (pH 7.4), 400 μl of color reagent (containing two solutions [A] TCA (4.6%), H_3PO_4 (15.3%), FeCl_3 (0.6%) and [B] 2, 2 dipyridyl (4%) in 70 % ethanol, in the ratio of 2.75:1) and 50 μl of 0.5% N-ethylmaleimide. The mixture incubated at 42°C for 45 min, and absorbance read at 520 nm UV-Visible spectrophotometer (UV-2450, Shimadzu). Ascorbic acid is used as a standard.

Superoxide dismutase (SOD)

Superoxide dismutase activity was measured following Dhindsa *et al.*, (1981). Lyophilized (10 mg) tissue was ground in 2 ml 0.5 M phosphate buffer (pH 7.5) and centrifuged at 4°C for 10 min, and the supernatant was used for the SOD activity assay. The entire enzyme extract preparation was carried out at 4°C. The SOD activity is determined by its ability to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT). The assay mixture contained reaction buffer of 1.5 ml (0.5 M phosphate buffer, pH 7.5 and 0.1 M phosphate buffer, pH 7.8), 200 μl of 200 mM methionine, 100 μl of 1M Na_2CO_3 , 100 μl of 2.25 mM NBT, 100 μl of 3mM EDTA, 100 μl of 60 μM Riboflavin and 100 μl of enzyme extract. Test tubes were shaken and incubated for 15 min under white fluorescent light (11 W) at RT. After incubation, the absorbance of the reaction mixture was read at 560 nm. The reaction mixtures, which were non-radiated, did not develop color and were considered as control. Activity of SOD was expressed as Unit mg^{-1} of protein 0.1 ml^{-1} of crude enzyme.

Catalase activity (CAT)

CAT was performed according to the method of

Aebi, (1984). Lyophilized tissue of 20 mg was extracted in 2 ml lysis buffer (0.1 M potassium phosphate buffer pH 7.8 and 1% PVP and 1 mM EDTA) and centrifuge at 8,000 rpm 4°C for 30 min, and the supernatant was used as crude enzyme extract for catalase assay. The reaction mixture had 1 ml of 50 mM sodium phosphate buffer (pH 7), 200 µl of H₂O₂, and 200 µl of enzyme extract. The absorbance was measured at 240 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Singapore).

Ascorbate peroxidase (APX)

APX activity is estimated according to the method of Nakano and Asada (1981). 20 mg of lyophilized tissue was ground in 2 ml of lysis buffer containing 0.1 M potassium phosphate buffer, pH 7.8, 1% PVP, and 1 mM EDTA. The homogenate was centrifuged at 4 °C at 8,000 rpm for 20 min. The reaction mixture contained 1 ml of reaction buffer (50mM sodium phosphate buffer, pH 7.2); 100 µl of 0.1 mM H₂O₂, 100 µl of 0.5 mM ascorbate, 100 µl of 0.5 mM EDTA, and 100 µl of crude enzyme extract. The reaction mixture was incubated for 30 s before taking the absorbance at 290 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Singapore). Estimation of APX activity was done using the average and extinction coefficient of 2.8 mM⁻¹ cm⁻¹ ($\epsilon=2.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

Statistical analysis

The results were studied statistically using one-way ANOVA. The test was conducted to find out the significant difference (*at a 95% confidence interval*) within different parameters across a different concentration of N and P, followed by Dunnett (two-sided) multiple range test using Microsoft excel XLSTAT (version 2020.1.3.65245). The data are the mean value \pm standard deviation in three replicates ($n=3$).

RESULTS

Growth

Analysis of the growth rate of *N. calcicola* under DN and DP conditions revealed a slight increase (5 & 4%) compared to control cultured in standard ASN III medium. The culture grown under N starvation condition registered an insignificant decline (4%) in the growth rate. However, *N. calcicola* cultured under P starvation

alone and combined deprivation of N&P condition exhibited a substantial reduction in the growth rate (61% & 71%), respectively, compared to control (Fig.1 and Table 1).

Photosynthetic efficiency of PS II (Fv/Fm)

The photochemical efficiency of PS II, measured in the form of the Fv/Fm ratio of *N. calcicola* grown under different P and N concentrations presented in Fig.2. There was a slight decline in Fv/Fm ratio when *N. calcicola* was grown in the medium containing DP (5%), no change under DN, and N starvation showed a 7% decline compared to control. However, under P starvation alone and combined N&P deprivation showed a significant decline of 25% and 44% significant respectively in the Fv/Fm ratio (Fig. 2 and Table 1).

Determination of Hydrogen peroxide (H₂O₂) and Hydroxyl radical (OH[•])

N. calcicola grown under nutrient stress showed a significant increment in both hydrogen peroxide and hydroxyl radical content (Fig. 3; Table 1). The increment in H₂O₂ production was more pronounced (~8 fold compared to the increase in the hydroxyl radical content (~2 fold), respectively, under P starvation and combined starvation of N&P as compared to their control (Fig. 3). Whereas *N. calcicola* grown under (-)N, DP and DN showed no significant increase in the H₂O₂ and OH[•] content than their controls.

Determination of lipid peroxidation and protein oxidation

Data on lipid peroxidation and protein oxidation are presented in Fig. 4; Table 1. Dunnett (two-sided) multiple range tests showed significant enhancement in both lipid peroxidation and protein oxidation in *N. calcicola* under P starvation alone and combined starvation of N&P; however, induction was more pronounced in protein oxidation (up to 3.7-fold) than in lipid peroxidation (up to 2.6-fold). Whereas *N. calcicola* grown under N starvation alone, DP and DN showed an insignificant increase in the lipid peroxidation and carbonyl content compared to their controls.

Estimation of proline and ascorbate content

The influence of nutrient stress (DP; DN; (-)N; (-)P & (-)NP) on proline and ascorbate content of *N. calcicola*

are represented in Fig. 5; Table 2. *N. caliccola* grown under nutrient stress showed a significant increment in both proline and ascorbate content. The increment was more pronounced in proline content (up to 4- fold) than in the ascorbate content (up to 2- fold), under P starvation and combined starvation of N&P as compared to their control. *N. caliccola* grown under N starvation alone or with DP and DN showed an insignificant increase in the proline and ascorbate content compared

to their controls.

SOD, CAT and APX activity

An insignificant difference in the SOD, CAT, and APX activity of algae grown with DP; DN and (-)N was observed compared to control. Among all the nutrient conditions studied, P starvation and combine starvation of N&P showed an increase of ~ up to 1.2, 2, and 4-fold in the SOD, CAT, and APX activity as compared to control. (Fig.6 and Table 2).

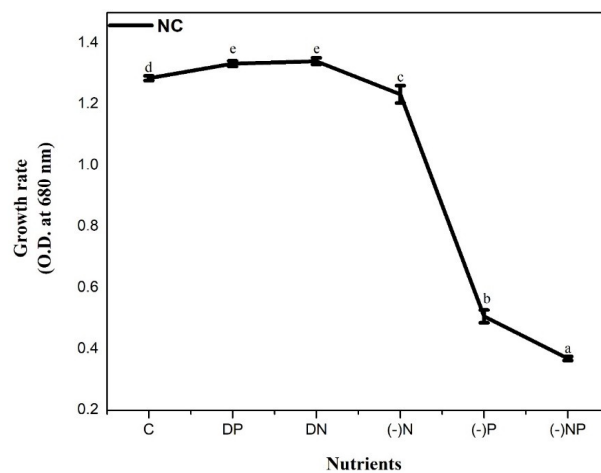


Figure 1. Effect of nutrient stress on the growth rate of *N. caliccola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus. Treatments with different letters are significantly different from each other values.

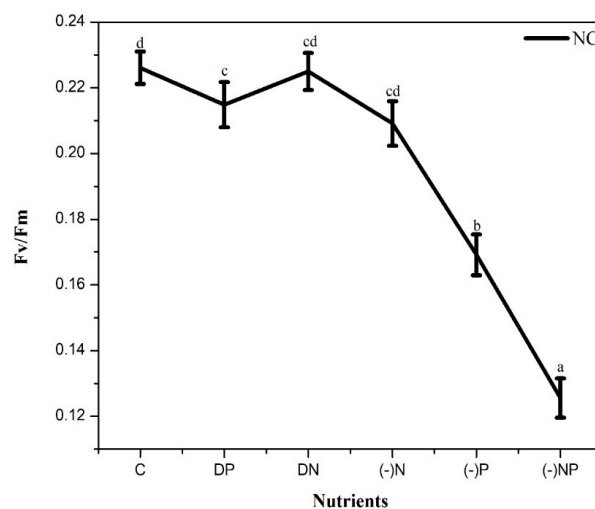


Figure 2. Effect of nutrient stress on photosynthetic efficiency of PS II (Fv/Fm) of *N. caliccola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments are as for figure 1.

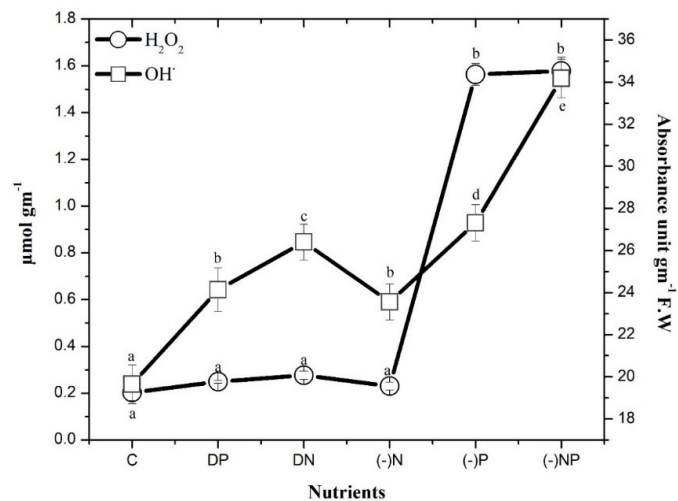


Figure 3. Effect of nutrient stress on hydrogen peroxide and hydroxyl radical content of *N. caliccola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus. Treatments with different letters are significantly different from each other values.

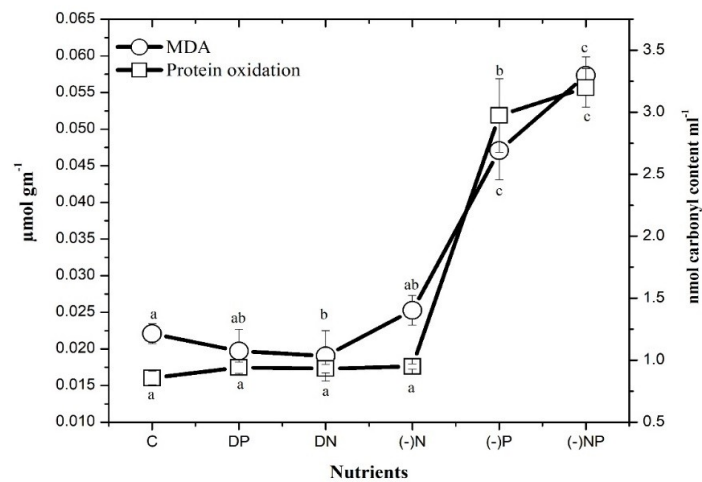


Figure 4. Effect of nutrient stress on lipid peroxidation (MDA) content and protein oxidation of *N. caliccola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus. Treatments with different letters are significantly different from each other values.

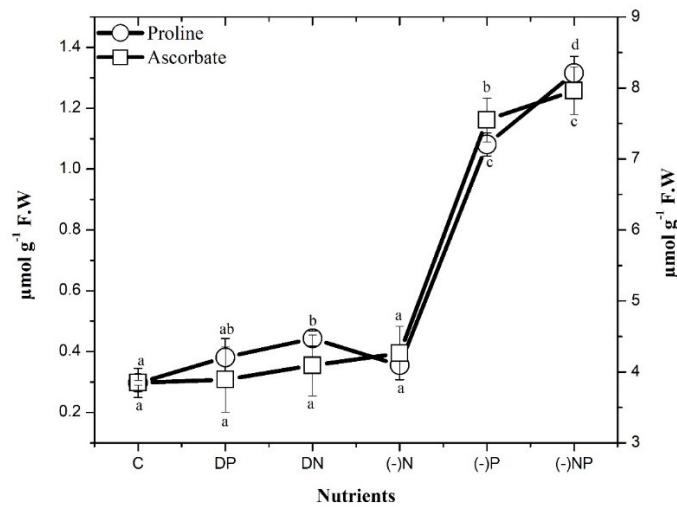


Figure 5. Effect of nutrient stress on the proline and ascorbate content of *N. calcicola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus. Treatments with different letters are significantly different from each other values.

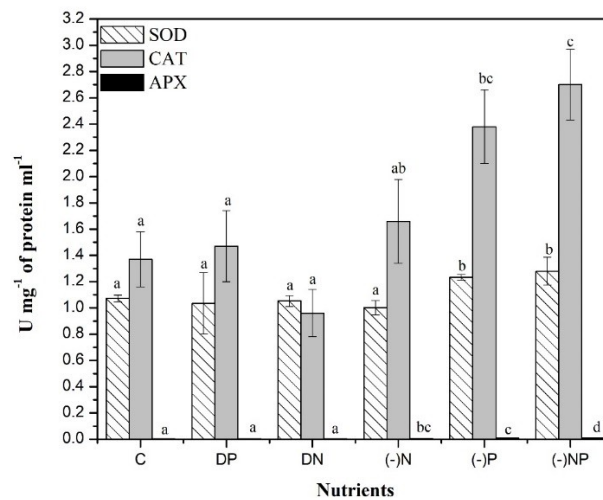


Figure 6. Effect of nutrient stress on the SOD, CAT, and APX activity of *N. calcicola*. The vertical bars represent the standard deviation (\pm SD, $n = 3$). Abbreviations for x-axis treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus. Treatments with different letters are significantly different from each other values.

Table 1. Growth rate, Photosynthetic efficiency of PS II (Fv/Fm), MDA content, protein oxidation, hydrogen peroxide and hydroxyl radical content of *N. calcicola* grown under different strength of N and P. The data represent the mean value \pm SD (n = 3). Treatments with different letters are significantly different from each other values.

Nutrients	Growth rate (O.D at 680 nm)	Fv/Fm	MDA ($\mu\text{mol gm}^{-1}$)	Protein oxidation (nmol carbonyl content ml^{-1})	H ₂ O ₂ Content ($\mu\text{mol gm}^{-1}$)	OH \cdot Content (Absorbance unit gm^{-1} F.W)
C	1.23 \pm 0.01 ^d	0.23 \pm 0.0049 ^d	0.022 \pm 0.001 ^a	0.856 \pm 0.055 ^a	0.203 \pm 0.03 ^a	19.64 \pm 0.92 ^a
DP	1.33 \pm 0.01 ^e	0.21 \pm 0.007 ^c	0.019 \pm 0.003 ^{ab}	0.941 \pm 0.04 ^a	0.249 \pm 0.006 ^a	24.13 \pm 1.03 ^b
DN	1.34 \pm 0.01 ^e	0.22 \pm 0.006 ^{cd}	0.019 \pm 0.003 ^b	0.932 \pm 0.03 ^a	0.277 \pm 0.018 ^a	26.40 \pm 0.84 ^c
(-)N	1.23 \pm 0.03 ^c	0.21 \pm 0.007 ^{cd}	0.025 \pm 0.002 ^{ab}	0.949 \pm 0.02 ^a	0.231 \pm 0.016 ^a	23.55 \pm 0.85 ^b
(-)P	0.507 \pm 0.02 ^b	0.17 \pm 0.006 ^b	0.047 \pm 0.004 ^c	2.975 \pm 0.03 ^b	1.56 \pm 0.046 ^b	27.31 \pm 0.86 ^d
(-)NP	0.369 \pm 0.01 ^a	0.13 \pm 0.006 ^a	0.057 \pm 0.003 ^c	3.197 \pm 0.2 ^c	1.57 \pm 0.059 ^b	34.17 \pm 0.91 ^e

Table 2. Proline, ascorbate, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activity of *N. calcicola* grown under different strength of N and P. The data represent the mean value \pm SD (n = 3). Treatments with different letters are significantly different from each other values.

Nutrients	Proline ($\mu\text{mol gm}^{-1}$)	Ascorbate ($\mu\text{mol gm}^{-1}$)	SOD (U mg^{-1} of protein)	CAT (U mg^{-1} protein ml^{-1})	APX (U mg^{-1} protein min^{-1})
C	0.297 \pm 0.048 ^a	3.48 \pm 0.03 ^a	1.07 \pm 0.02 ^a	1.37 \pm 0.21 ^a	0.003 \pm 8.92558E-4 ^a
DP	0.38 \pm 0.06 ^{ab}	3.89 \pm 0.46 ^a	1.03 \pm 0.2 ^a	1.47 \pm 0.27 ^a	0.003 \pm 7.63804E-4 ^a
DN	0.44 \pm 0.02 ^b	4.09 \pm 0.42 ^a	1.05 \pm 0.04 ^a	0.96 \pm 0.18 ^a	0.0035 \pm 4.44537E-4 ^{ab}
(-)N	0.355 \pm 0.05 ^a	4.26 \pm 0.37 ^a	1 \pm 0.05 ^a	1.66 \pm 32 ^{ab}	0.006 \pm 7.06454E-4 ^{bc}
(-)P	1.08 \pm 0.03 ^c	7.55 \pm 0.30 ^b	1.23 \pm 0.02 ^b	2.38 \pm 0.28 ^{bc}	0.008 \pm 0.00116 ^c
(-)NP	1.31 \pm 0.05 ^d	7.96 \pm 0.33 ^c	1.28 \pm 0.10 ^b	2.70 \pm 0.27 ^c	0.010 \pm 6.35994E-4 ^d

DISCUSSION

The inhibitory effect of P starvation and combined starvation of N&P on the growth and photosynthetic efficiency of PS II (Fv/Fm) in *N. calcicola*, highly emphasized through the present study (Table 1). The effect was mainly due to P starvation rather than N starvation, and algae grown under (-)N medium did not significantly affect the growth. Phosphorous is one of the vital elements of biomolecules, such as sugar phosphatase, nucleic acids, and phospholipids. It is expected to play a vital role in the growth and photosynthetic efficiency of cyanobacteria. Besides, P also participates in carbon metabolism, affecting various metabolic processes attributed to reduced growth of

cyanobacteria under P-starvation. Further, P is also an essential component of Adenosine triphosphate (ATP). Thus, ATP's regeneration is often limited under P starved conditions and might have attributed to the reduction in Fv/Fm ratio. Apart from the decrease in the Fv/Fm ratio, P starvation may also affect the RuBisCO activity by limiting the regeneration of Ribulose-Bis-Phosphate could be due to reduced supply of ATP and NADPH, which ultimately cause a reduction in the growth (Hammond and White, 2008; Qi *et al.*, 2013). Our results agree with Qi *et al.*, (2013), who also showed a decrease in the Fv/Fm in *P. subcordiformis* and *H. akashiwo* when grown under P deficient conditions. *N. calcicola* is a nitrogen-fixing

cyanobacterium, it did not show any pronounced effect when exposed to N starvation. A similar trend prevailed when grown under double the strength of N and P. However, Paerl *et al.*, (2001); Schindler *et al.*, (2008), studies reported an increase in growth of nitrogen-fixing cyanobacteria under excess phosphorus concentration.

In this study, an increase in MDA content and protein carbonyl content under P starvation and combined starvation of N&P indicated an increment in oxidative damage and impairment in membrane integrity and function (Table 1). According to our knowledge, not much work has been reported the effect of nutrient deficiency or excess on MDA in *N. calcicola*. However, several studies have reported the same on higher plants, e.g., Tewari *et al.*, (2004) on maize showed an upregulation in lipid peroxidation under N&P deficiency. Our results are similar to Tewari *et al.*, (2007) on mulberry and Singh *et al.*, (2018) on wheat, where N and P deficiency oxidized the cell membrane of root and shoot.

The observed increase in the oxidation of lipids and protein in our study is due to the higher production of ROS (H_2O_2 & $OH\cdot$). In addition to the ROS, lipid peroxidation also leads to $ROO\cdot$ Radicals resulted in lipid peroxidation, which starts a chain reaction and produces ketones, hydroxyl acids, and aldehydes as reactive products and is responsible for causing further oxidative damage by conversion of amino groups of protein to carbonyl moieties (Perry *et al.*, 2000). Romero-Puertas *et al.*, (2002) and Sandalio *et al.*, (2001) reported an increase in the carbonyl content in pea plant under cadmium stress.

In response to the oxidative damage, photosynthetic organisms possess a well-demarcated antioxidant defense system. Both non-enzymatic and enzymatic antioxidants scavenge the toxic ROS and protect the cyanobacteria from oxidative stress. Free proline content increases as a physiological response to these oxidative stress Sharma and Dietz (2006). Proline affects some cytoplasmic enzymes' stability whose functions and kinetics could be disturbed as consequences of the applied stress factors (Gibbon *et al.*, 1997). In the present work, proline content increased in P starvation and combined starvation of N&P (Table

2). It is suspected to be due to a decrease in the activity of the electron transport system (Venekemp, 1989; Sawhney *et al.*, 1990; Saradhi & Mohanty, 1993), leading to the accumulation of NADH and H^+ . Proline accumulation (presumably through synthesis from glutamic acid) might be an adaptive mechanism for reducing (a) the level of accumulated NADH and (b) the acidity; ($2NADH + 2H^+$) (Venekemp *et al.*, 1987). Our results showed conformity with Sarker and Karmokar (2011) who reported proline accumulation in P deficient Lentil (*Lens culinaris* Medik.); Ismail, (2005) in carrot and radish; Guo *et al.*, (2012) in rice seedlings; Yang and Chen, (2001) in Mungbean under Al toxicity and P deficiency.

A non-enzymatic antioxidant such as Ascorbate (AsA) also plays an imperative role in reducing ROS directly and indirectly (Noctor & Foyer, 1998). The increased level of Ascorbate in *N. calcicola* under nutrient stress conditions (P starvation alone and combine deprivation of N&P) may be due to its participation in reducing H_2O_2 to water (Table 2). The increase in ascorbate level correlated with increased APX activity observed in our result.

Among the enzymatic antioxidant SOD, CAT and APX were studied. Among these, SOD provides primary defense in catalyzing the dismutation of superoxide ion to H_2O_2 . Nutrient stress (excess as well as starvation) of P and N showed variation in the activity of SOD in *N. calcicola* (Table 2). However, SOD activity was more pronounced under the combined deprivation of N&P and P starvation. SOD is more effective in protecting the cyanobacteria against free ROS, which generated during nutrient stress. Kumar *et al.*, (2003) observed that *Oscillatoria willii* BDU 130511, which showed the presence of Fe-SOD under N starvation, could play a role in the dismutation of superoxide ion. Similarly, Billi & Caiola (1996) also reported an increase in the activity of SOD under N starvation in cyanobacteria. A study was done by Gulzar *et al.*, (2019) on mustard seedling showed that SOD activity was more under higher salinity, which further increased with P and Ca supply. The decrease observed in growth and biochemical parameters in our study under P, and (-)NP deprivation may be due to the sum of oxidative damage of lipids and

proteins due to generation of ROS and induction of enzymatic and non-enzymatic antioxidant system. Double the strength of N and P and N deprivation did not destroy the integrity of the cell membrane, giving more evidence for the hypothesis that *N. calcicola* being nitrogen-fixing cyanobacteria could survive in nitrogen starvation as well as double the concentration of N and P (Peng *et al.*, 2017).

Dismutation of superoxide anion by SOD produced H_2O_2 and accumulation of H_2O_2 if not removed, will result in severe membrane damage. However, photosynthetic organisms mitigate the level of H_2O_2 in the cells through catalase and ascorbate peroxidase and glutathione peroxidase (Gulzar *et al.*, 2019). In the present study, CAT and APX enhanced when the *N. calcicola* was grown under nutrient starvation conditions; however, the CAT and APX activity did not show any increment under excess nutrients and N starvation alone (Table 2). However, *N. calcicola* displayed much higher CAT activity than APX activity under nutrient-starved culture. CAT is known for having the highest turnover rates, as one molecule of it can convert nearly 6 million molecules of hydrogen peroxide to water and oxygen in 1 min (Gill and Tuteja, 2010). In comparison to APX activity, CAT showed remarkably higher activity in the nutrient-starved culture, thus reducing the availability of H_2O_2 for the APX. Reduction of APX activity in *N. calcicola* under nutrient starvation might be due to degradation of ascorbate, which is a principle electron donor for APX and gets oxidized to dehydroascorbate in the reaction of APX with H_2O_2 and gets re-reduced to ascorbate in the presence of glutathione reductase in water–water and ascorbic acid–glutathione cycles under nutrient starvation condition (Zhang and Kirkham, 1996; Gill and Tuteja, 2010). Hamid & Sibi (2018) also reported a similar result on green microalgae *Chlorococcopsis minuta*. Chokshi *et al.*, 2017 reported a similar type of work on *Acutodesmus dimorphus*, a green microalga under N starved condition. They reported an increase in CAT activity compared to APX activity. Gill and Tuteja, 2017 also said an increase in the CAT and APX activity under different abiotic stress.

CONCLUSION

The inhibitory effect of P starvation and combined starvation of N&P on the growth and photosynthetic efficiency of PS II (Fv/Fm) in *N. calcicola*, highly emphasized through the present study (Table 1). The effect was mainly due to P starvation rather than N starvation, and algae grown under (–)N medium did not significantly affect the growth. Phosphorous is one of the vital elements of biomolecules, such as sugar phosphatase, nucleic acids, and phospholipids. It is expected to play a vital role in the growth and photosynthetic efficiency of cyanobacteria. Besides, P also participates in carbon metabolism, affecting various metabolic processes attributed to reduced growth of cyanobacteria under P-starvation. Further, P is also an essential component of Adenosine triphosphate (ATP).

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CONFLICTS OF INTEREST

All authors have declared that they do not have any conflict of interest for publishing this research.

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