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Assessment of Salinity-Induced Antioxidative Defense System of Diazotrophic Cyanobacterium *Nostoc muscorum*

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The present study examined the salinity-induced oxidative damage and differential response of enzymatic and nonenzymatic antioxidants of *Nostoc muscorum*. As compared with carotenoid content that showed induction, the chlorophyll and phycocyanin contents were inhibited after salt stress. Acceleration of lipid peroxidation and peroxide production suggested the onset of oxidative damage. The activities of all studied enzymatic antioxidants were significantly increased by salt stress, with maximum induction occuring with superoxide dismutase (154.8% at 200 mM NaCl treatment). Interestingly, under severe stress condition (250 mM NaCl), ascorbate peroxidase seemed to be more crucial than catalase for peroxide scavenging. Among the studied nonenzymatic antioxidants, α-tocopherol was induced maximally (56.0%); however, ascorbate and reduced glutathione were increased by only 8.9% after 250 mM NaCl treatment as compared with control cells. Therefore, salinity was found to induce the antioxidative defense system of *N. muscorum*.

Keywords: Antioxidants, lipid peroxidation, *Nostoc muscorum*, oxidative damage, reactive oxygen species, salinity

The ever-increasing populations in developing countries including India are adversely affecting the agro-ecosystem, owing to unsustainable practices. The use of different chemical fertilizers and ill-managed irrigation practices year after year have resulted in different abiotic stresses, especially salinity, and thus reduced soil fertility and created stagnant crop productivity [25]. The widespread nature of salinity may affect about half of the irrigated lands like rice fields all over the world, mainly in tropical countries by 2050. Salinity not only alters the physicochemical properties of soil but is also harmful to microbial flora including cyanobacteria [24].

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Cyanobacteria are present in almost every ecological niche; however, they are the dominant primary producers in the paddy fields of tropical countries including India [24]. Being photosynthetic prokaryotes, cyanobacterial metabolism exploits the potential of interaction with oxygen during photosynthesis in general and electron transfer chain in particular [14]. Therefore, the generation of reactive oxygen species (ROS) during photosynthesis is a normal phenomenon, but any stress condition, including salinity, provokes increased production of toxic oxygen derivatives [11]. In addition to ROS production, salinity is also known to inhibit growth, and cause photosynthetic pigments *vis-à-vis* photosynthesis, as well as disturbance in ion homeostasis and osmoticum of the cell and protein synthesis [14, 27, 28].

The ROS generated by salt stress are detoxified by the antioxidative defense system of cyanobacteria composed of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), and by nonenzymatic antioxidants like carotenoid (CAR) ascorbate (ASA), reduced glutathione (GSH), and α-tocopherol (α-TOC). The antioxidative defense system of cyanobacteria has been explored under heavy metal, temperature, UV-B [25], and desiccation [17] stresses. Furthermore, the report of Srivastava *et al.* [27] provided a deep insight into the salinity-induced changes in the antioxidative defense system of *Anabaena doliolum*. Unfortunately, no report is available on the holistic view of salinity-induced changes in the physiology and antioxidative defense system of *Nostoc muscorum*, which is a dominant rice field cyanobacterium and relatively more salt tolerant than *Anabaena* sp. [25].

In view of the report of the Srivastava *et al.* [25] that *Nostoc* sp. has wide distribution across a range of salinity, due to its resilient ecophysiologies, it is hypothesized that the enzymatic antioxidants may play a major role in detoxification of salinity-induced ROS. It is further hypothesized that, under severe salt stress condition, nonenzymatic antioxidants will dominate over enzymatic antioxidants, owing to massive

change in cellular homeostasis. Therefore, this study is an attempt to examine the proposed hypotheses and to bridge the above-mentioned gap in our knowledge. The present study included tests of the effects of the salinity on photosynthetic pigments like chlorophyll (Chl), phycocyanin (PC), and carotenoid (CAR); cellular damage measured in terms of lipid peroxidation and peroxide production; activities of SOD, CAT, APX, and GR; and intracellular contents of ASA, GSH, and α-TOC of *N. muscorum*.

Materials and Methods

Organism and Growth Conditions

The test cyanobacterium *N. muscorum* Agardh was obtained from Professor L. C. Rai, Banaras Hindu University (Varanasi-221005, India) and grown axenically in Chu-10 medium [9] buffered with Tris-HCl at 24±2°C under 72 μmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) irradiance with a photoperiod of 14:10 h (light:dark) at pH 7.5. The cultures were shaken by hand two to four times daily. Separately autoclaved NaCl was added directly into the sterilized medium to achieve appropriate concentrations from 50 to 250 mM in different flasks. It is pertinent to mention that 150 and 250 mM NaCl were found to be the LC_{50} and lethal dose of NaCl to *N. muscorum*, respectively (data not shown). All experiments were done using exponentially grown cultures (OD_{663}-0.5) and conducted in triplicate as well as repeated at least twice to confirm the reproducibility of the results. For calculating the fresh weight, the cyanobacterial sample was centrifuged at 10,000 ×*g* in a preweighed centrifuge tube (W1) and blotted dry using kimwipe. This was again weighed (W2), and the difference between W2 and W1 was taken as the fresh weight of the cyanobacterial sample.

Protein Estimation

Total cell protein was estimated by the method of Bradford [4]. The cell extract (100 μl) was mixed with 1 ml of Bradford reagent prepared by adding 2 ml of 0.2% Coomassie brilliant blue G-250 and 4 ml of orthophosphoric acid in 14 ml of millipore water. Absorbance was recorded at 595 nm.

Photosynthetic Pigments Estimation

The photosynthetic pigments were analyzed as per the methods given in Thapar *et al.* [31]. Briefly, 15 ml of cyanobacterial culture was centrifuged and the pellet was suspended in 5 ml of acetone (80%). After overnight incubation at 4°C, the suspension was centrifuged and the supernatant was used for measuring the Chl and CAR contents. The residue so obtained was suspended in 30 mM Tris-HCl buffer (pH 7.5) and sonicated. The resulting suspension was recentrifuged and the supernatant was used for the measurement of PC pigment. Absorbance was recorded at 665, 480, and 610 nm for Chl, CAR, and PC, respectively, using a UV–Vis spectrophotometer (Systronic, India).

Measurement of Lipid Peroxidation

The total content of 2-thiobarbituric acid (TBA) reactive substances expressed as MDA (malondialdehyde) was used to measure the lipid peroxidation [5]. These reactive substances were extracted in 3 ml of 0.1% (w/v) trichloroacetic acid (TCA) at 4°C followed by centrifugation at 13,000 ×*g* for 2 min. An aliquot of 0.5 ml from the supernatant was added to 1.5 ml of TBA (0.5% in 20% TCA). This was followed by a 20 min incubation at 90°C, and the reaction was terminated under an ice bath. The reaction mixture was then subjected to centrifugation at 1,000 ×*g* for 5 min. The absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated at its extinction coefficient (155 mM^{-1} cm^{1}).

Peroxide Assay

The total peroxide was measured according to Sagisaka [19]. The cell pellets suspended in cell lysis buffer were subjected to sonication, followed by addition of 5% TCA and centrifugation. An aliquot of 1.6 ml of the resulting supernatant was mixed with 0.4 ml of 50% TCA, 0.4 ml of 10 mM ferrous ammonium sulfate, and 0.2 ml of 2.5 M potassium thiocyanate. The absorbance of the supernatant was measured at 480 nm after centrifugation. The peroxide content was quantitatively estimated using the standard curve.

Antioxidative Enzyme Assay

Cell pellets of *N. muscorum* exposed to different concentrations of NaCl suspended in cell lysis buffer (pH 7.0) were subjected to sonication in ice-cold condition. The cell lysis buffer for estimation of selected enzymes except APX contained 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). However, the above buffer additionally contained 1 mM ASA for APX assay. The sonicated sample was centrifuged at 15,000 ×*g* for 30 min at 4°C, and the resulting supernatant containing antioxidant enzymes was used for further assay. Total SOD activity was assayed by monitoring the inhibition of reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries [10]. A 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μl of enzyme extract. The reaction mixture was illuminated for 20 min at a light intensity of 5,000 μmol photon m^{-2} s^{-1}. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction monitored at 560 nm.

Catalase activity was estimated by measuring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min [1]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2, and 200 μl of the enzyme extract in a 3 ml volume. One unit of CAT activity was defined as the amount of enzyme utilized to decompose 1.0 μM of H_2O_2.

Ascorbate peroxidase activity was determined by measuring the reduction in absorbance at 290 nm (A_{290}) (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ASA, 0.1 mM H_2O_2, and 200 μl of enzyme extract [17]. The reaction was started by adding enzyme extract. Corrections were made for low, nonenzymatic oxidation of H_2O_2. One unit of APX was defined as the amount of enzyme that degrades 1 μM of ASA.

The activity of GR was examined using the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min in 1 ml of assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG (glutathione oxidized), and 200 μl of enzyme extract [21]. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm without NADPH. One unit of GR activity was defined as the enzyme required to produce 1.0 μM of $NADP^+$ from NADPH.

Estimation of Nonenzymatic Antioxidants

Glutathione was estimated by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)−glutathione reductase coupled assay using the method of Anderson [2]. Cells were harvested and resuspended in 5% sulfosalicylic acid and vortexed vigorously for 5 min. The extract was then centrifuged and the supernatant was assayed to determine the glutathione content.

Ascorbate was measured as per the method of Keller and Schwager [12]. Cells were sonicated in ice-cold extracting buffer (0.25 M oxalic acid and 1 mM EDTA) and centrifuged at 6,000 ×*g* for 15 min. The supernatant was mixed with 5 ml of 20 µg/ml DCPIP. The absorbance was measured at 520 nm. The ascorbate content was quantified with the help of a standard curve prepared using pure ascorbic acid.

α-Tocopherol was extracted using the method of Munné-Bosch *et al.* [16]. The pellet was sonicated in 5 ml of ice-cold methanol containing 1% ASA. Hexane was used to extract α-TOC by vigorous mixing for 2 min. After centrifuging the samples at 1,500 ×*g* for 20 min, the upper hexane layer was carefully removed and evaporated to dryness under vacuum. The dried hexane extract was dissolved in 2 ml of methanol and injected into a 10 µM HPLC column (300×3.9 mm, C-18 column; Waters Chromatography Division CAT No.27324, U.S.A.) and detected at 295 nm. Pure ± α-TOC was used as a standard.

Statistical Analysis

Results were statistically analyzed using a one-way ANOVA, followed by Duncan's new multiple range tests (DMRT) and correlation coefficients (r) using SPSS ver. 15.0. Three independent variables were considered for each experiment.

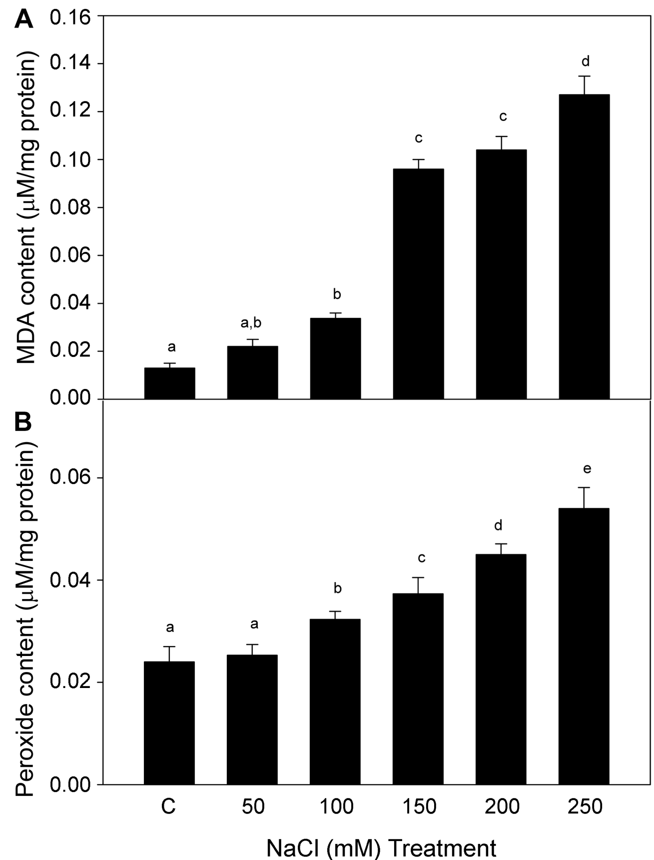

Fig. 1. Effects of NaCl on the (**A**) malondialdehyde content (µM/mg protein) and (**B**) peroxide content (µM/mg protein) of *Nostoc muscorum*.

All values are the mean ± SD of three replicates. A different analysis was done for each column (Duncan's new multiple range test). Bars having different letters are significantly different (P<0.01).

RESULTS

Photosynthetic Pigments

Chlorophyll and PC contents showed a dose-dependent decrease after NaCl treatment (Table 1). The chlorophyll content of *N. muscorum* showed 43.2% depletion after 250 mM NaCl treatment as compared with the control. Similarly, 41.0% PC content decreased under the similar condition. In contrast to the above results, a significant increase in CAR content was observed after salt treatment

Table 1. Chlorophyll, phycocyanin, and carotenoid contents of *Nostoc muscorum* exposed to different concentrations of NaCl.

S. No.	Treatments		Chlorophyll content (mg pigment/mg fresh weight)	Phycocyanin content (mg pigment/mg fresh weight)	Carotenoid content (mg pigment/mg fresh weight)
1.	Control		0.120±0.004^{f}	0.046±0.001^{e}	0.079±0.005^{a}
2.	NaCl (mM)	50	0.112±0.006^{e} (−6.2)	0.042±0.002^{d} (−9.1)	0.082±0.001^{b} (4.5)
3.		100	0.110±0.002^{d,e} (−7.8)	0.040±0.004^{c} (−12.5)	0.090±0.006^{c} (14.8)
4.		150	0.088±0.003^{c} (−25.9)	0.039±0.001^{c} (−13.4)	0.107±0.004^{d} (40.0)
5.		200	0.076±0.005^{b} (−36.6)	0.036±0.002^{b} (−19.1)	0.119±0.002^{e} (50.7)
6.		250	0.067±0.002^{a} (−43.2)	0.027±0.001^{a} (−41.0)	0.135±0.003^{f} (72.0)

All values are presented as the mean ± SD of three replicates. Values given in parentheses with "+" and "-" signs are percent increase and decrease, respectively, as compared with their respective controls. Values having different alphabets are significantly different (P<0.01; Duncan's new multiple range test).

(DMRT) with maximum induction (72.0%) in 250 mM NaCl-treated cells (Table 1). Chlorophyll and PC were positively correlated, but, CAR showed negative correlation with them ($P<0.01$).

Membrane Damage and Peroxide Production

Data pertaining to membrane damage and peroxide production are compiled in Fig. 1A and 1B. Duncan's new multiple range test showed significant enhancement in both lipid peroxidation and H_2O_2 production following NaCl treatment. However, induction was more pronounced in lipid peroxidation (9.8-fold) than in H_2O_2 production (2.3-fold) after 250 mM NaCl treatment, as compared with their respective controls. These were positively correlated with all studied parameters ($P<0.01$) except Chl and PC contents (negative correlation) as well as APX activity (no correlation).

Antioxidative Enzyme Activities

Among the different enzymes studied, SOD (154.8%) and CAT (65.6%) showed maximum induction after 200 mM NaCl treatment, as compared with their respective controls, followed by inhibited activity at 250 mM NaCl (Fig. 2A and 2B). However, the reduction was more prominent (approximately half) in CAT activity as compared with its activity at 200 mM NaCl. In contrast to this, APX and GR showed enhanced activities with increase in NaCl concentration (Fig. 2C and 2D). Ascorbate peroxidase (APX) and GR activities increased by 28.0% and 26.4%, respectively, after 250 mM NaCl, as compared with their respective controls. All studied enzymatic antioxidants, except APX, depicted significant positive correlation ($P<0.01$). Moreover, GR activity was also positively correlated with its product GSH content ($P<0.01$).

Nonenzymatic Antioxidants

Table 2 presents data of nonenzymatic antioxidants of *N. muscorum* exposed to different concentrations of NaCl. Of the different nonenzymatic antioxidants, α-TOC showed maximum induction (56.0%) after 250 mM NaCl treatment, as compared with control. Likewise, the ASA and GSH contents also increased after NaCl treatment, although the

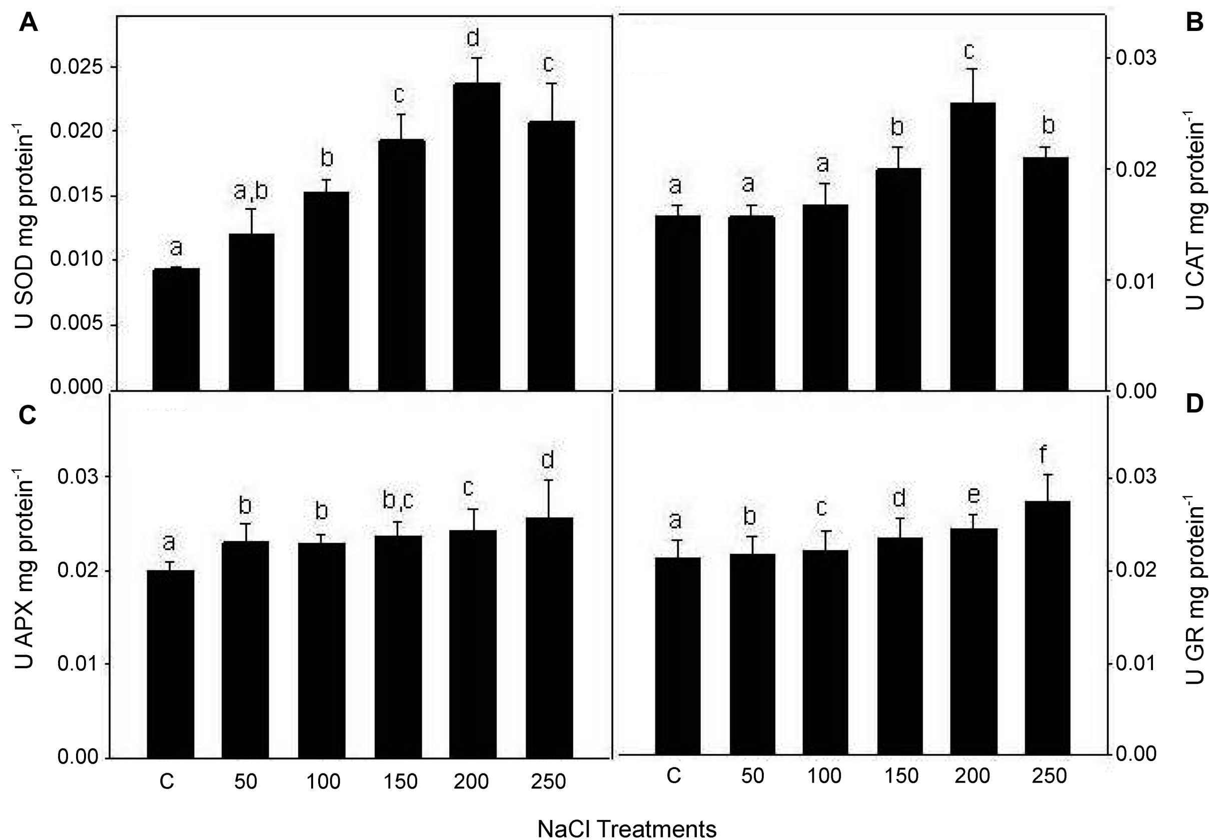

Fig. 2. Effects of NaCl on the (**A**) superoxide dismutase, (**B**) catalase, (**C**) ascorbate peroxidase, and (**D**) glutathione reductase activities of *Nostoc muscorum*.
One unit (U) of each studied enzyme is defined in the Materials and Methods section. All values are the mean ± SD of three replicates. A different analysis was done for each column (Duncan's new multiple range test). Bars having different letters are significantly different ($P<0.01$).

Table 2. Effects of different concentrations of NaCl on nonenzymatic antioxidants of *Nostoc muscorum*.

S. No.	Treatments		Ascorbate content (mg/mg protein)	Glutathione (reduced) content (mg/mg protein)	α-Tocopherol content (mg/mg protein)
1.	Control		0.933±0.05[a]	0.786±0.02[a]	0.525±0.03[a]
2.	NaCl (mM)	50	0.939±0.06[a] (0.6)	0.794±0.02[b] (1.1)	0.554±0.04[b] (5.5)
3.		100	0.937±0.03[a] (0.4)	0.799±0.05[b] (1.7)	0.610±0.02[c] (16.2)
4.		150	0.929±0.03[a] (−0.5)	0.815±0.03[c] (3.7)	0.684±0.04[d] (30.3)
5.		200	0.987±0.04[b] (5.7)	0.847±0.04[d] (7.8)	0.787±0.05[e] (49.7)
6.		250	1.016±0.07[c] (8.9)	0.856±0.05[e] (8.9)	0.819±0.06[f] (56.0)

All values are presented as the mean ± SD of three replicates. Values given in parentheses with "+" and "-" signs are percent increase and decrease, respectively, as compared with their respective controls. Values having different alphabets are significantly different ($P<0.01$; Duncan's new multiple range test).

induction was only 8.9% in both cases after exposure to 250 mM NaCl, as compared with their respective controls. Interestingly, the ASA content decreased insignificantly up to 150 mM NaCl (DMRT), followed by an increase in its content by 5.7% at 200 mM NaCl treatment. These antioxidants were found to be positively correlated with each other as well as to the parameters of oxidative damage ($P<0.01$). The pearson correlation coefficient revealed that APX and ASA were not correlated, although the latter is a substrate of APX.

DISCUSSION

Salinity-induced decrease in Chl content may be due to the fact that it is one of the prime targets of salinity, which hampers chlorophyll biosynthesis *vis-à-vis* its content [14, 23, 25]. Salinity-dependent decrease in PC content is also shown at the protein level in *A. doliolum* [28]. Similar results were also demonstrated in *Spirulina platensis* by Lu and Zhang [14]. The loss in two major light harvesting pigments (Chl and PC) may be the reason for salinity-induced inhibition of photosynthesis. In contrast to the above results, salinity was found to increase CAR content significantly (Table 1). This increase may be attributed to its role in protection of light-harvesting pigments in the antenna complex against photochemical damage, by facilitating the triplet−triplet energy transfer from Chl to CAR [13]. Moreover, CAR is one of the potent nonenzymatic antioxidants, which scavenges a variety of antioxidants produced in cyanobacteria exposed to different stresses [26, 31].

Because of the salinity-induced disturbance in light harvesting and cellular homeostasis, ROS production boosts over their scavenging, leading to oxidative stress as depicted by induced lipid peroxidation and H_2O_2 production. Salinity-induced lipid peroxidation in *N. muscorum* (Fig. 1A) finds support from the results of Srivastava *et al.* [27] and Tang *et al.* [29], who reported lipid peroxidation in *A. doliolum* and *Scytonema javanicum*, respectively, exposed to salt stress. The prime consequence of lipid peroxidation is a disturbance in membrane fluidity, and thus ion balance, resulting in altered metabolism and ROS production. Among the different ROS, H_2O_2 is of great significance owing to its enormous stability [32] and role as a secondary messenger to regulate the expressions of defense genes [35]. The result of salinity promoting H_2O_2 production in *N. muscorum* (Fig. 1B) finds support from observations of Yang *et al.* [35], who reported its hyper-accumulation in salt-exposed wheat. The foremost reason of ROS production in cyanobacteria is altered energy transfer from excited Chl to molecular oxygen during photosynthesis [8].

In order to survive under enhanced ROS, cyanobacteria induce a complex array of antioxidative defense system. Superoxide dismutase, which dismutates the superoxide radical, is known to be induced by different stresses in a variety of cyanobacteria [18, 36]. Increased SOD activity in *A. doliolum* exposed to salt and copper stresses was reported at the protein and transcript levels [3, 28]. The inhibition of SOD activity at 250 mM NaCl can be explained in light of the fact that Fe-SOD is sensitive to prolonged and severe oxidative stress [6]. The resulted peroxide radical is scavenged by the activities of CAT and enzymes of the Halliwell−Asada pathway (APX and GR) [11]. The increased CAT activity up to 200 mM NaCl is contrary to our previous finding of CAT sensitivity to salt stress in *A. doliolum* [27]. This can be explained in light of the fact that *Nostoc* sp. has better ecophysiologies under saline condition than does *Anabaena* sp. [25].

The other peroxide scavenging enzyme, APX, was also induced under salt stress (Fig. 2C). This increase may be attributed to a H_2O_2-mediated induction of the *apx* operon [34]. Therefore, it seems that H_2O_2 can be detoxified by a combined effort of both CAT and APX in *N. muscorum*.

However, under severe stress condition (in the present study, 250 mM NaCl), APX plays a more dominant role than CAT, as reported in *Synechococcus* PCC7942 [15] and in *A. doliolum* [27]. Increased GR activity is corroborated with induced APX activity owing to their direct relation in the Halliwell–Asada pathway. Salinity-induced GR activity may be due to its product GSH being required to maintain cellular homeostatsis, and a transient increase in intracellular Ca^{2+} [7], thus inducing NADPH level *vis-à-vis* dependent enzymes like GR under salt stress.

Likewise, all studied nonenzymatic antioxidants were found to be induced after salt stress, with maximum induction in α-TOC (Table 2), which is a most effective radial chain-breaking compound [22]. It is believed that among the photoautotrophs, α-TOC biosynthesis was started in cyanobacteria for the first time to protect them against ROS [20]. Protection of polyunsaturated fatty acids of the membrane and regulation of H_2O_2 production are other function attributed to α-TOC [22]. The other nonenzymatic antioxidants, ASA and GSH, work in coordination with other enzymatic antioxidants to detoxify a range of ROS [11]. Moreover, other functions of ASA include the synthesis of zeaxanthin from violaxanthin under light and the quenching of excess energy of PS II [33]. Glutathione was shown to mitigate UV-B toxicity in *N. muscorum* [33]. The protective roles of ASA and GSH in hydroperoxide scavenging in *N. muscorum* 7119 and *Synechococcus* 6311 have been characterized by Tel-Or *et al.* [30].

In view of the above results, it is concluded that the induction of enzymatic antioxidants was more pronounced than nonenzymatic counterparts, thus attesting to the hypothesis of the former having a more vital role than the latter in detoxification of salinity-induced ROS in *N. muscorum*. This is contrary to our pervious report of a salinity-induced coordinated activity of both counterparts in *A. doliolum*. This may be attributed to the more resilient ecophysiologies of *N. muscorum* compared with *A. doliolum*, and probably the greater inherent salt-tolerant nature of *N. muscorum*.

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