Biochemistry and Biophysics Reports 26 (2021) 100953

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

# Calcium mediated nitric oxide responses: Acquisition of nickel stress tolerance in cyanobacterium *Nostoc muscorum* ATCC 27893

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ARTICLE INFO

*Keywords:*
Antioxidant defense machinery
Calcium
Nitric oxide
Nitrogen metabolism
PS II photochemistry
Signaling molecules

ABSTRACT

Calcium ($Ca^{2+}$) and nitric oxide (NO) are potentially active and multitasking signaling molecules which are known to regulate abiotic stresses in plants, but their interactive role in the acquisition of metal stress tolerance in cyanobacteria remains elusive. In current study the signaling role of $Ca^{2+}$ (800 μM) and NO (10 μM SNP) on key physiological and biochemical attributes of the agriculturally and economically important cyanobacterium *Nostoc muscorum* ATCC 27893 subjected to Ni stress (2 μM) was examined. Results revealed that Ni at elevated level caused severe damages to the test organism but exogenous supplementation of $Ca^{2+}$ and NO efficiently mitigated its toxic effects and up-regulated the growth, pigment contents, rate of photosynthesis (whole cell oxygen evolution and Chl *a* fluorescence indices: Kinetic traits: $\Phi P_0$, $\Psi_0$, $\Phi E_0$ and $PI_{ABS}$, along with $Fv/F_0$), nitrogen metabolism ($NO_3^-$ and $NO_2^-$ uptake, nitrate:NR and NiR; and ammonia:GS and GOGAT; assimilating enzymes), and boosted the enzymatic (SOD, POD, CAT and GST) along with non-enzymatic (proline, cysteine and NP-SH) antioxidants. Whereas the increased values of energy flux traits: (ABS/RC, $TR_0$/RC, $DI_0$/RC and $ET_0$/RC) along with $F_0$/Fv, rate of respiration, oxidative stress biomarkers (SOR, $H_2O_2$ and MDA), and activity of GDH enzyme exhibited lowering trends with application of $Ca^{2+}$ and NO. Further, addition of EGTA ($Ca^{2+}$ scavenger) and PTIO (NO scavenger) reversed the positive impacts of $Ca^{2+}$ and NO and worsened the toxicity of Ni on test cyanobacterium, but the damages were more pronounced under PTIO application that demonstrated $Ca^{2+}$ mediated signaling role of NO in Ni toxicity alleviation.

## 1. Introduction

In recent years, the rise in development in every sphere of life has led to the enhancement in anthropogenic activities, usage of fertilizers and pesticides in agriculture, discharge of industrial wastes, emissions from vehicles, burning and smelting practices etc. have contributed to the addition of heavy metals in soil as well as agricultural fields [1]. Heavy metals have been released into the environment [2] and being toxic and persistent they are a major concern for agriculture fields and food chains [3]. Crops together with their associated beneficial micro-flora are exposed to several environmental threats and among them, heavy metal stress is becoming great concern for the survival of every living being [4]. One of the heavy metals, nickel is increasingly used in industries particularly in production of nickel-cadmium batteries, in electroplating, in production of stainless steel and food processing industries etc. [5]. Nickel at elevated level dangerously impacts physiological processes such as photosynthesis, mineral absorption and water relations in plants that lead to oxidative stress by hampering biochemical processes [1]. Nickel toxicity has also been reported to disturb the nutrient uptake, translocation from root to shoot, other physiological processes and cause oxidative damage in maize plants [6].

Cyanobacteria, an important component of paddy fields, are the most primitive group of oxygenic photosynthetic microorganisms [7] and also play significant role in fixing molecular nitrogen into ammonia [8]. Cyanobacterial growth gets hampered by excess amount of heavy metals such as Fe, Cr, Pb, etc. that induce ROS generation inside the cell which mainly initiates the membrane damage through lipid peroxidation resulting in the ferroptosis and ultimately preprogrammed cell death. Nickel has been found to be more toxic in cyanobacteria causing distorted growth, pigment deterioration and arresting the excitation energy transfer in phycobilisomes in *Nostoc muscorum* [9]. Recently, Prajapati et al. [10] have reported down regulation of several proteins along with of carbon metabolism and photosynthesis in Ni stressed *Anabaena* PCC 7120. The excess generation/accumulation of reactive oxygen species (ROS) causes damage to cellular membranes (lipids and proteins), enzymatic activities, reserve food (carbohydrates), genetic

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https://doi.org/10.1016/j.bbrep.2021.100953
Received 23 November 2020; Received in revised form 2 February 2021; Accepted 5 February 2021
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material (DNA) etc. that leads to decrease in survival rate and crop production [11–13]. Stress is perceived through the receptors by cells that execute signaling inside cell and ultimately activate different kinds of stress-responding genes [14]. Phytohormones such as abscisic acid (ABA), jasmonates (JA) and salicylic acid (SA), interact synergistically with polyamines, calcium ($Ca^{2+}$), nitric oxide (NO), sugars etc. under stress and regulate signaling through cell transporters, biochemical reactions, anti-oxidant defense system and gene expressions [11,12]. Exogenous application of NO in wheat seedlings has improved nitrogen assimilation by enhancing the gene expression and activities of enzymes [15]. In response to stress signals concentration of $Ca^{2+}$ increases inside the cell, thus $Ca^{2+}$ behaves as secondary messenger in cell signaling [16] and also activates other targets such as cAMP. Under salinity stress, effect of exogenous supplementation of $Ca^{2+}$ has caused improved electron transport rate from $Q_A^-$ causing protection of reaction center from being inactive. It has also caused improved expression of genes such as *ATP6E* and *CAMK2* under stressful conditions [17]. Ahad and Syiem [4] have shown the effect of calcium to improve photosynthetic pigments and nitrogen assimilating enzymes in the cyanobacterium *N. muscorum*. In addition, $Ca^{2+}$ signaling has been found to induce endogenous NO accumulation by inducing hydrogen peroxide ($H_2O_2$) generation during stomatal closure in guard cells [18]. Previous studies have reported about interaction of $Ca^{2+}$ and NO with each other in transmitting the signals under abiotic stress [13]. Nitric oxide and $Ca^{2+}$ together decreased membrane damage and reduced the generation of reactive oxygen species by enhancing anti-oxidant defense system enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) [19]. Studies have shown that nitric oxide mediated lateral root formation in rice through involvement of $Ca^{2+}$ and also activated defense gene expression [20]. These studies further proved by the application of $Ca^{2+}$chelators and calmodulin antagonists that $Ca^{2+}$/calmodulin are involved in NO induced root formation under osmotic stress [19]. The interactive role of NO and $Ca^{2+}$ has also been observed in tall fescue leaves under high light intensity [21]. Thus, exogenous application of signaling molecules such as NO, $H_2S$, 24-Epibrassinolide, $Ca^{2+}$ are being employed to protect the crops against stress [12,22] but further studies are still required for their wider applications.

Much is explored about the role of NO and $Ca^{2+}$ independently in the stress mitigation in plants but their interacting role in heavy metal stress regulation, a most prevalent stress in paddy fields is not yet reported in case of cyanobacteria, an important microflora commonly known as biofertilizers enriching the paddy for better yield. In such context it is necessary to protect them against stress therefore the present study was carried out to come up with strategies. The objective of the study is to understand about independent or interdependent mode of signaling action of $Ca^{2+}$ and NO under the Ni stress regulation in cyanobacterium *Nostoc muscorum*.

## 2. Materials and methods

### 2.1. Test organism and culture conditions

The homogenous, filamentous and heterocystous cyanobacterium *Nostoc muscorum* ATCC 27893 was cultured in BG-11 medium (pH 7.5) in a temperature controlled room having 25 ± 2 °C under 75 μmol photons $m^{-2}$ $s^{-1}$photosynthetically active radiation (PAR, 400–700 nm) provided by white fluorescent tubes (Osram L 40 W/25–1) with a 14:10 h regime of light: dark. The experiments were carried out with exponential phase cultures of *Nostoc muscorum* ATCC 27893.

### 2.2. Experimental design and treatments

Cultures of *N. muscorum* growing exponentially were harvested and centrifuged at 4,000g for 10 min and then collected cells were washed thrice with sterile distilled water. The healthy cells were used for treatments and re-suspended in BG-11 medium containing Ni ($NiCl_2.6H_2O$; 2 μM), $Ca^{2+}$ ($CaCl_2$;800 μM), sodium nitroprusside (SNP; 10 μM), ethyleneglycol-bis (β-aminoethyl)-N,N,N,N-tetraacetic acid (EGTA; 1 mM), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 20 μM) in different combinations. Thus considering this, the experiments were set with different combinations such as; control (untreated cells), Ni, Ni+$Ca^{2+}$, Ni+SNP, Ni+$Ca^{2+}$+SNP, Ni+EGTA+SNP, Ni+$Ca^{2+}$+PTIO. At the end different parameters were analyzed after 72 h of treatment.

### 2.3. Measurement of growth

Growth of cyanobacterium was taken as a dry mass. The cyanobacterial cells were centrifuged at 4,000g for 10 min and then cells were washed thrice with the distilled water. At 80 °C cells were oven dried for 48 h and finally, weighed through a digital balance (Contech- CA 223, India).

### 2.4. Estimation of exopolysaccharides

The content of exopolysaccharides (EPS) was estimated by Sharma et al. [23]. For this cell free supernatant containing exopolysaccharides were concentrated by evaporating at 40 °C. Dried samples were washed with isopropanol thrice, and hydrosylates were estimated for glucose [24], and content was calculated with the help of standard curve of prepared with graded solution of glucose.

### 2.5. Estimation of Ni accumulation

To estimate the intracellular Ni accumulation, 80 ml treated and untreated cyanobacterial cells were centrifuged and pellets were harvested and washed with EDTA (1 mM). Further, these cells were re-suspended in chilled phosphate buffer for 15 min to remove excess apoplastic Ni. Thereafter, obtained pellets were oven dried at 65–75 °C for 48 h. Completely dried cyanobacterial cells were digested by adding 5 ml of a tric-acid mixture containing $HNO_3$, $H_2SO_4$ and $HClO_3$ in the respective ratio of 5:1:1 and warmed at 80 °C until obtained a transparent solution. After cooling, the sample was maintained up to 20 ml by adding double distilled water, and Ni was estimated by using atomic absorption spectrophotometer (iCE 3000 series, Model 3500 AAS, Thermo Scientific, UK). The instrument was calibrated by applying standard solution of Ni.

### 2.6. Estimation of the photosynthetic pigments

Method of Porra et al. [25] and Goodwin [26] were used to measure the chlorophyll *a* (Chl *a*) and carotenoids (Car) contents, respectively. For the measurement of both the pigments, a definite volume of cyanobacterial cultures were centrifuged to get pellets. Cells were kept at 4 °C treated with 100% methanol. Absorbance of clear supernatant was taken at 665 nm and 450 nm, respectively for Chl *a* and Car. Amounts of Chl *a* and Car are expressed in terms of μg $mg^{-1}$ dry weight.

Method of Benett and Bogorad [27] was followed to estimate phycobiliproteins i.e phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) of test cyanoabacterial cells. As per the method, cells were treated with toluene overnight and absorbance was taken at 562, 615, and 652 nm for PE, PC, and APC, respectively. The contents of phycobiliproteins was calculated according to following equations and their amounts are expressed as μg $mg^{-1}$ dry weight.

$$PC = A_{615} - (0.474 * A_{652})/5.34$$

$$APC = A_{652} - (0.208 * A_{615})/5.09$$

$$PE = A_{562} - 2.41(PC) - 0.849\ (APC)\ /\ 9.62$$

### 2.7. Measurement of rate of photosynthesis and respiration

Oxygen electrode (Digital Oxygen System, Model-10, Rank Brothers, UK) under the saturating light intensity of 400 μmol photons $m^{-2}s^{-1}$ PAR was used to measure net photosynthetic $O_2$ evolution of the cyanobacterial cells. Further, the same system was used to read the rate of respiration in terms of $O_2$ consumption under dark condition.

### 2.8. Measurement of PSII photochemistry (JIP-Test)

As per the Strasser et al. [28], dark adapted cyanobacterial cells of treated and untreated samples were taken and chlorophyll *a* fluorescence parameters were determined using fluorometer (Aqua Pen AP 100, Photon System Instruments, Czech Republic).

### 2.9. Biochemical analysis of oxidative stress biomarkers

Method of Elstner and Heupel [29] was followed to determine the superoxide radical (SOR; $O_2^{\bullet -}$). The 40 ml of cyanobacterial cultures were centrifuged and pellets were washed four times in 1 mM EDTA and thoroughly crushed in 65 mM phosphate buffer (pH 7.8) then centrifuged for 10 min at 10,000g to get a clear supernatant by using refreegerated high speed centrifuge (CPR-30, Remi, India). Reaction of hydroxylamine with superoxide radical present in each sample formed nitrite which gave pink colour with sulfanilamide and NEED whose absorbance was recorded at 530 nm. Rate of SOR formation was calculated by the standard curve prepared with graded concentration of $NaNO_2$.

For $H_2O_2$ estimation method of Velikova et al. [30] was followed. Equal amount of cyanobacterial cultures were centrifuged to get pellets, which were crushed in 0.1% (w/v) trichloroacetic acid (TCA). The 1 M KI solution, 10 mM potassium phosphate buffer (pH 7.0) and 0.5 ml of the extract were mixed in 2 ml, and absorbance was read at 390 nm. By using the standard curve prepared with graded concentration of $H_2O_2$, the rate of formation of $H_2O_2$ was estimated.

Method of Heath and Packer [31] was used to estimate lipid peroxidation in terms of malondialdehyde equivalents content (MDA equivalent contents), which were formed by the oxidation of unsaturated fatty acid and a product known as 2-thiobarbituric acid (TBA) reactive metabolite were produced. For this, cyanobacterial cultures were centrifuged and pellets were washed with 1 mM EDTA and 50 mM phosphate buffer (pH 7.0) and thereafter samples were crushed with 5% (w/v) TCA. Further, homogenates were centrifuged at 10,000g for 10 min. From this, 0.5 ml of supernatant was taken and mixed with 2 ml of 20% TCA (containing 0.5% TBA). Finally, samples were heated at 90 °C for 20 min and quickly cooled in ice-bath, then centrifuged and absorbance was recorded at 532 nm and 600 nm. For estimation of MDA equivalent contents extinction coefficient of 155 mM $cm^{-1}$ was used.

### 2.10. In-vivo analysis of oxidative stress biomarkers

*In-vivo* staining of $O_2^{\bullet -}$ and $H_2O_2$ was done by adopting the method of Förster et al. [32] by using nitrobluetetrazolium (NBT) and 3, 3 diaminobenzidine (DAB) as respective staining dyes. For the staining of MDA and membrane damage in the form of electrolyte leakage (EL), Schiff's reagent and Evan's blue were respectively used as staining dyes by following the respective methods of Pompella et al. [33] and Yamamoto et al. [34].

### 2.11. Biochemical analysis of activity of enzymatic antioxidants

Superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.3.6), peroxidase (POD; EC 1.11.1.7) and Glutathione-*S*- transferase (GST; EC 2.5.1.18) activities were calculated as per the methods of Giannopolitis and Reis [35], Aebi [36], Gahagan et al. [37] and Habig et al. [38], respectively. One unit of SOD activity is defined as the amount of enzyme which causes 50% inhibition in the reduction of NBT. The absorbance of purple formazone formed due to reduction of NBT was measured at 560 nm. One unit of CAT activity is defined by 1 nmol $H_2O_2$ dissociated $min^{-1}$. In this case as $H_2O_2$ is dissociated so decrease in absorbance was measured at 240 nm to calculate the enzyme activity, and for this extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ was used. One unit of POD activity was defined as 1 nmol pyrogallol oxidized $min^{-1}$ and extinction coefficient of 25.5 $mM^{-1}cm^{-1}$ was used to calculate it. Pyrogallol undergoes oxidation therefore, the increase in absorbance was measured at 430 nm. One unit of GST activity is defined as 1 nmol of CDNB-conjugates formed $min^{-1}$, and for this extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used. Here, conjugates develop between GSH and CDNB, thus increase in absorbance was measured at 340 nm.

### 2.12. Measurement of non-enzymatic antioxidants activity

Proline, cysteine and non-protein thiols (NP-SHs) were estimated as per the methods of Bates et al. [39], Gaitonde [40] and Ellman [41], respectively. For proline, pellets were crushed in sulphosalicylic acid and supernatant were obtained after centrifugation, mixed with glacial acetic acid and acid ninhydrin solution, kept at 95 °C for 1 h. Reaction mixture was extracted into toluene by vortexing it for 5 min and toluene layer was used to record absorbance at 520 nm. For cysteine, pellets were crushed in perchloric acid and supernatant was obtained after centrifugation. Reaction mixture consisted of glacial acetic acid, acid ninhydrin and the supernatant, which were exposed to 95 °C and absorbance was read after the cooling at 560 nm. For NP-SHs pellets were crushed in sulphosalicylic acid, supernatant was reacted with Ellman's reagent and then absorbance was recorded at 412 nm. The amounts of proline, cysteine and non-protein thiols were calculated with the help of standard calibration curves.

### 2.13. Estimation of inorganic nitrogen contents: nitrate ($NO_3^-$) and nitrite uptake ($NO_2^-$)

Cyanobacterial cultures were pre-incubated with 100 μM $KNO_3$/$KNO_2$ for 24 h to study the nitrate and nitrite uptake, respectively. The amount of $NO_3^-$ in cell free medium was estimated by the method of Cawse [42] by recording absorbance at 210 nm and similarly, $NO_2^-$ was measured by the method of Snell and Snell [43] by recording absorbance at 540 nm.

### 2.14. Estimation of nitrate assimilating enzymes: nitrate reductase (NR) and nitrite reductase activity (NiR)

Cyanobacterial cells were pre-treated with $KNO_3$ and $NaNO_2$ to induce NR and NiR enzymes, respectively. The addition of alkyltrimethyl ammonium bromide (MTA) as reductant and dithionite reduced methyl viologen to cell suspension made cells permeable. The estimation of NR and NiR activities was done by the methods of Herrero et al. [44,45] and Herrero and Guerrero [46], respectively. One unit of NR activity is demarcated as 1 nmol $NO_2^-$ formed $min^{-1}$ and one unit of NiR activity is demarcated as 1 nmol $NO_2^-$consumed $min^{-1}$

### 2.15. Estimation of ammonium assimilating enzymes: glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (NADH-GDH) activity

Glutamine synthetase (GS; EC 6.3.1.2) activity was assayed by method of Mérida et al. [47] by measuring the formation of gamma-glutamylhydroxamate at 500 nm. One unit of GS activity is defined as 1 nmol $\gamma$-glutamylhydroxamate formed $min^{-1}$. GOGAT (EC 1.4.1.14) activity was measured by following the methods of Meers et al. [48]. Enzyme activity was estimated by recording the oxidation of NADH by recording absorbance at 340 nm. One unit of GOGAT activity is defined as 1 nmol NADH oxidized $min^{-1}$.GDH (EC 1.4.1.2) activity

was estimated by method of Chávez and Candau [49]. In this case, activity of NADH-GDH was measured by recording the oxidation of NADH at 340 nm, but reaction was initiated after the addition of $NH_4Cl$. One unit of GDH activity is defined as 1 nmol NADH oxidized $min^{-1}$.

### 2.16. Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of results. The Duncan's multiple range test (DMRT) was used for mean separation for significant differences among the treatments at $P < 0.05$ significance levels. Presented results are the means ± standard error of three independent experiments with three replicates in each experiment (n = 9).

## 3. Results

### 3.1. $Ca^{2+}$ and NO enhanced the growth

Nickel stress imposition in the growing medium caused radical generation in the tested cyanobacterium *Nostoc muscorum* ATCC 27893 which created a great disturbance in the functioning of the organism. In the current study; Fig.1a shows that, 2 μM Ni caused about 30% reduction in growth of organism in comparison to control. But, this reduction was eliminated by either $Ca^{2+}$ or SNP (donor of NO) supplementation to Ni stressed test cyanobacterium, thereby reduction was found only 15 and 12% respectively in comparison to the control. Here, the role of SNP appeared better because recovery was found greater. Further, combined exposure of $Ca^{2+}$ and SNP reduced the impact of Ni stress maximally and only 10% reduction was noticed as compare to control. In order to clarify the interaction between the $Ca^{2+}$ and NO; the scavengers of $Ca^{2+}$ (EGTA) and NO (PTIO) were added to the growth medium. Accordingly, in the treatment Ni + EGTA + SNP (showed the dependency of NO on $Ca^{2+}$); the reduction in growth was increased up to 18% in comparison to control, hence showing that $Ca^{2+}$ is not necessarily needed for working of NO in toxicity alleviation. While in the next treatment; Ni + $Ca^{2+}$+PTIO (showed the dependency of NO for the functioning of $Ca^{2+}$) severely reduced (i.e. about 39%) the growth, thereby suggesting $Ca^{2+}$ cannot work without NO.

### 3.2. $Ca^{2+}$ and NO promoted the secretion of EPS layer to prevent endogenous Ni accumulation

Nickel stress significantly dropped the content of EPS by 16% in comparison to control; contrary to this, the individual supplementation of $Ca^{2+}$/SNP with Ni exposure reversed the degradation, and EPS content was enhanced by 14 and 16% respectively (Fig. 1b). Parallel to this, endogenous accumulation of Ni was recorded as 102.0 ± 3.1 μg Ni $g^{-1}$ dry weight under the exposure of 2 μM Ni. However, the accumulation of Ni was lowered to 85.1 ± 2.6 μg Ni $g^{-1}$dry weight and 78.0 ± 2.3 μg Ni $g^{-1}$ dry weight after the exposure of $Ca^{2+}$ and SNP (NO) respectively (Fig. 1c). But the effect of SNP was found more pronounced than that of $Ca^{2+}$. Afterward, under same stress a considerable improvement in EPS secretion i.e. about 28% concomitantly significantly reduced intracellular Ni content i.e. 72.2 ± 2.2 μg Ni $g^{-1}$ dry weight was noticed on the combined exposure of $Ca^{2+}$ and SNP. Further, 10% reduction in EPS content and 93.0 ± 2.8 μg Ni $g^{-1}$ dry weight content were detected under the exposure of SNP even in the presence of EGTA; showing the independent role of NO in synthesis of defensive layer of EPS. On contrary to this, a critical reduction in EPS content (i.e. about 22%) and excessive Ni accumulation (i.e. 106.1 ± 3.2 μg Ni $g^{-1}$ dry weight) were noticed in case of Ni + $Ca^{2+}$+PTIO combinations thereby indicating $Ca^{2+}$itself is not responsible alone, but it regulates NO to secrete EPS for Ni toxicity alleviation in the cells of cyanobacterium *Nostoc muscorum* ATCC 27893 (Fig. 1b and c).

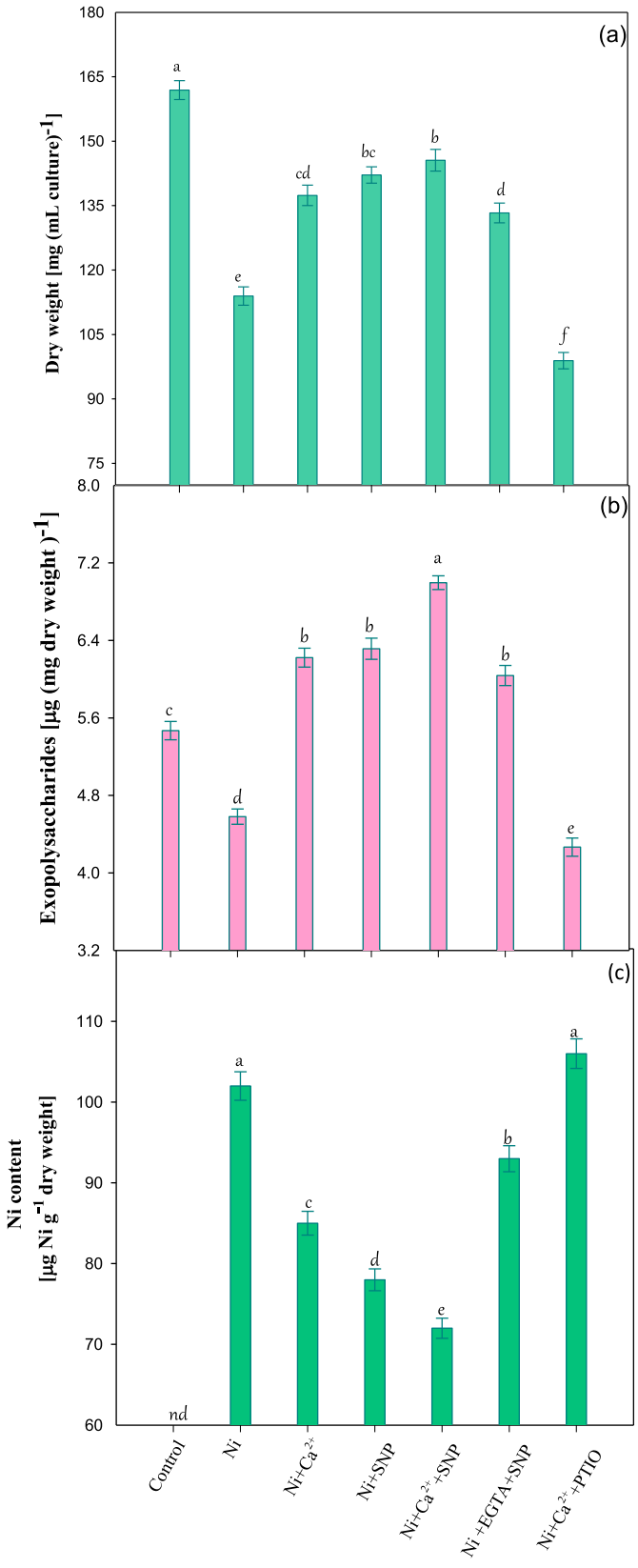


**Fig. 1.** Effect of $Ca^{2+}$ and SNP on growth **(a),** exopolysaccharides (EPS) contents **(b),** intracellular nickel accumulation **(c)** of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

### 3.3. $Ca^{2+}$ and NO up-regulated the pigment contents

Prolonged exposure of *Nostoc* to Ni stress resulted in the marked decrease of about 29% in Chl *a* content in comparison to control. Unlikely, $Ca^{2+}$ and NO (SNP) recovered the Chl *a* content and reduction was observed only 13 and 9% respectively (Table 1). Further, a greater improvement in Chl *a* content was detected under combined supplementation of $Ca^{2+}$ and SNP (NO) during same stress condition. The content of Chl *a* was found less affected and reduced by only 15% by Ni toxicity when $Ca^{2+}$ was scavenged and SNP (NO) was supplied exogenously. Comparatively, an adverse outcome was noticed when endogenous NO was blocked by applying PTIO and reduction was found about 38% and here, it is depicted (Table 1) that $Ca^{2+}$was unable to recover the pigment content without NO. Similar results were observed for Car contents.

Similarly, light harvesting antenna complexes (phycobiliproteins) have also been investigated and results are depicted in Table 1. Nickel stress adversely affected the antenna pigments and a greater reduction of 23, 22 and 24% was found for phycocyanin (PC), allophycocyanin (APC) and phycoerythrins (PE) respectively over the values of respective controls. In contrast to this, exposure of $Ca^{2+}$ reduced the toxic effect of Ni and about 4% increase was noticed for all the phycobiliproteins. Similarly, exposure of SNP also enhanced their levels by 8, 7 and 7% respectively. Their contents were noticed tremendously enhanced under combined exposure of $Ca^{2+}$ and SNP. In contrast to this, under same stress on the exposure of EGTA with SNP the reduction was about 7, 6 and 14% respectively for PC, APC and PE. But the addition of PTIO with $Ca^{2+}$, worsened the Ni toxicity and a critical reduction was noticed i.e. 27, 34 and 38% respectively (Table 1).

### 3.4. $Ca^{2+}$ and NO up regulated the photosynthetic activity and down regulated the respiratory activity –

In order to explain the impact of two signaling agents ($Ca^{2+}$ and NO) on tested organism exposed to Ni stress on photosynthetic and respiratory activity, the overall consumption and release of $O_2$ was analyzed and results are framed in Fig. 2a and b. The results showed that tested dose of Ni declined the photosynthetic $O_2$ evolution rate by 32%; contrastingly the respiratory $O_2$ uptake was enhanced by 31% in comparison to respective controls. However, under the tested stress exogenous application of $Ca^{2+}$, SNP and $Ca^{2+}$+SNP significantly ameliorated the damaging effect on photosynthetic rate and at the same time declining trend was noticed in respiratory rate. On EGTA supplementation, the photosynthetic $O_2$ evolution rate was found to suppress by 19% and respiratory rate was enhanced by 11%. But, the decline in photosynthetic rate was noticed more substantial by 37% and respiratory activity was enhanced by 42% under PTIO exposure.

### 3.5. Effects of $Ca^{2+}$ and NO on the chlorophyll a fluorescence

Chlorophyll *a* fluorescence analysis is frequently used to detect the photosynthetic capacity and vitality of photosynthetic organisms. Results of present study clearly show that maximum photochemical efficiency of PSII (Fv/Fm or $\Phi P_0$), size and number of active reaction centers ($Fv/F_0$) and the other kinetic parameters i.e., $\Psi_0$, $\Phi E_0$ and $PI_{ABS}$ were negatively affected by Ni stress while specific energy fluxes were found to enhance significantly. Under $Ca^{2+}$, SNP and $Ca^{2+}$+SNP the addition of EGTA and PTIO again worsened their ratios which indicated the role of $Ca^{2+}$ and NO in overall photosynthetic electron transport machinery. But the role of NO was more pronounced than $Ca^{2+}$ (Fig. 2c). On the other hand Fig. 2c also shows that energy flux parameters i.e., ABS/RC, $TR_0$/RC, $DI_0$/RC and $ET_0$/RC along with the efficiency of oxygen evolving complexes ($F_0$/Fv) were found significantly enhanced under Ni exposure and they were normalized under the treatments of $Ca^{2+}$, SNP and $Ca^{2+}$+SNP but their values were noticed differently increased under EGTA and PTIO exposures (increase was more prominent under PTIO treatment).

### 3.6. Effects of $Ca^{2+}$ and NO on oxidative stress biomarkers

Results pertaining to the oxidative stress biomarkers (SOR, $H_2O_2$ and MDA) in test organisms have been portrayed in Fig. 3. The results pointed that under Ni exposure the contents of SOR, $H_2O_2$ and MDA equivalents significantly ($P < 0.05$) were enhanced by 33, 37 and 30% respectively over the values of respective control. Their contents in cells were normalized under exogenous supplementation of $Ca^{2+}$, SNP and $Ca^{2+}$+SNP, and a more pronounced effect was noticed under $Ca^{2+}$+SNP treatments; the contents were found only 2, 4 and 6% more respectively. Further, this positive effect on reducing the oxidative stress was reversed under EGTA supplementation, and contents were increased by 11, 16 and 19% respectively. Moreover a critical hindrance in normalizing the levels of biomarkers was noticed on the exposure of PTIO hence, enhanced levels i.e. 43, 43 and 46% were observed respectively.

Above results were strongly supported by histochemical analysis as depicted in Fig. 4. The intense colors of SOR dependent blue formazan, $H_2O_2$ dependent brown patches, pink patches for MDA equivalents and sky blue staining for EL were observed inside the cells of test organism under Ni stress. These intense patches were considerably eliminated under combined exposure of $Ca^{2+}$+SNP under applied stress condition. Further, under EGTA supplementation slight intense patches were noticed in comparison to control. However, comparatively more intense staining inside the cells was observed under PTIO exposure.

### 3.7. Effects of $Ca^{2+}$ and NO on antioxidant defense machinery

Enzymatic antioxidants i.e. SOD, POD, CAT and GST activities were also analyzed to clarify the relation between $Ca^{2+}$ and NO for which results are presented in Fig. 5. Nickel slightly enhanced the levels of SOD, POD, CAT and GST but combined external supplementation of $Ca^{2+}$+SNP upregulated their activities rapidly by 38, 43, 34 and 41% respectively comparatively over the respective control values. Again under the same stress on exposure of EGTA + SNP the activities of these enzymes i.e. SOD, POD, CAT and GST were also positively affected and increased by only 20, 21, 17 and 13% respectively. Contrastingly a

**Table 1**
Effect of $Ca^{2+}$ and SNP on chlorophyll *a*, carotenoids, phycocyanin, allophycocyanin and phycoerythrin contents of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

| Treatments | Photosynthetic pigments μg (mg dry weight)$^{-1}$ | | Phycobiliproteins μg (mg dry weight)$^{-1}$ | | |
|---|---|---|---|---|---|
| | Chlorophyll *a* | Carotenoids | Phycocyanin (PC) | Allophycocyanin (APC) | Phycoerythrin (PE) |
| Control | $13.56 \pm 0.23^{a}$ | $6.40 \pm 0.11^{a}$ | $52.98 \pm 0.91^{c}$ | $8.13 \pm 0.14^{b}$ | $8.16 \pm 0.14^{b}$ |
| Ni | $9.57 \pm 0.16^{d}$ | $4.43 \pm 0.07^{e}$ | $40.77 \pm 0.70^{e}$ | $6.33 \pm 0.10^{d}$ | $6.18 \pm 0.10^{d}$ |
| Ni + $Ca^{2+}$ | $11.87 \pm 0.20^{bc}$ | $5.56 \pm 0.09^{c}$ | $55.27 \pm 0.95^{bc}$ | $8.47 \pm 0.14^{ab}$ | $8.47 \pm 0.14^{ab}$ |
| Ni + SNP | $12.40 \pm 0.21^{b}$ | $5.78 \pm 0.10^{bc}$ | $57.02 \pm 0.98^{b}$ | $8.72 \pm 0.15^{a}$ | $8.75 \pm 0.15^{a}$ |
| Ni + $Ca^{2+}$+SNP | $13.01 \pm 0.22^{a}$ | $5.96 \pm 0.10^{b}$ | $59.86 \pm 1.03^{a}$ | $8.83 \pm 0.15^{a}$ | $8.86 \pm 0.15^{a}$ |
| Ni + EGTA + SNP | $11.47 \pm 0.19^{c}$ | $5.26 \pm 0.09^{d}$ | $49.22 \pm 0.85^{d}$ | $7.61 \pm 0.13^{c}$ | $7.02 \pm 0.12^{c}$ |
| Ni + PTIO + $Ca^{2+}$ | $8.40 \pm 0.14^{e}$ | $3.80 \pm 0.06^{f}$ | $38.64 \pm 0.66^{e}$ | $5.38 \pm 0.09^{e}$ | $5.08 \pm 0.08^{e}$ |

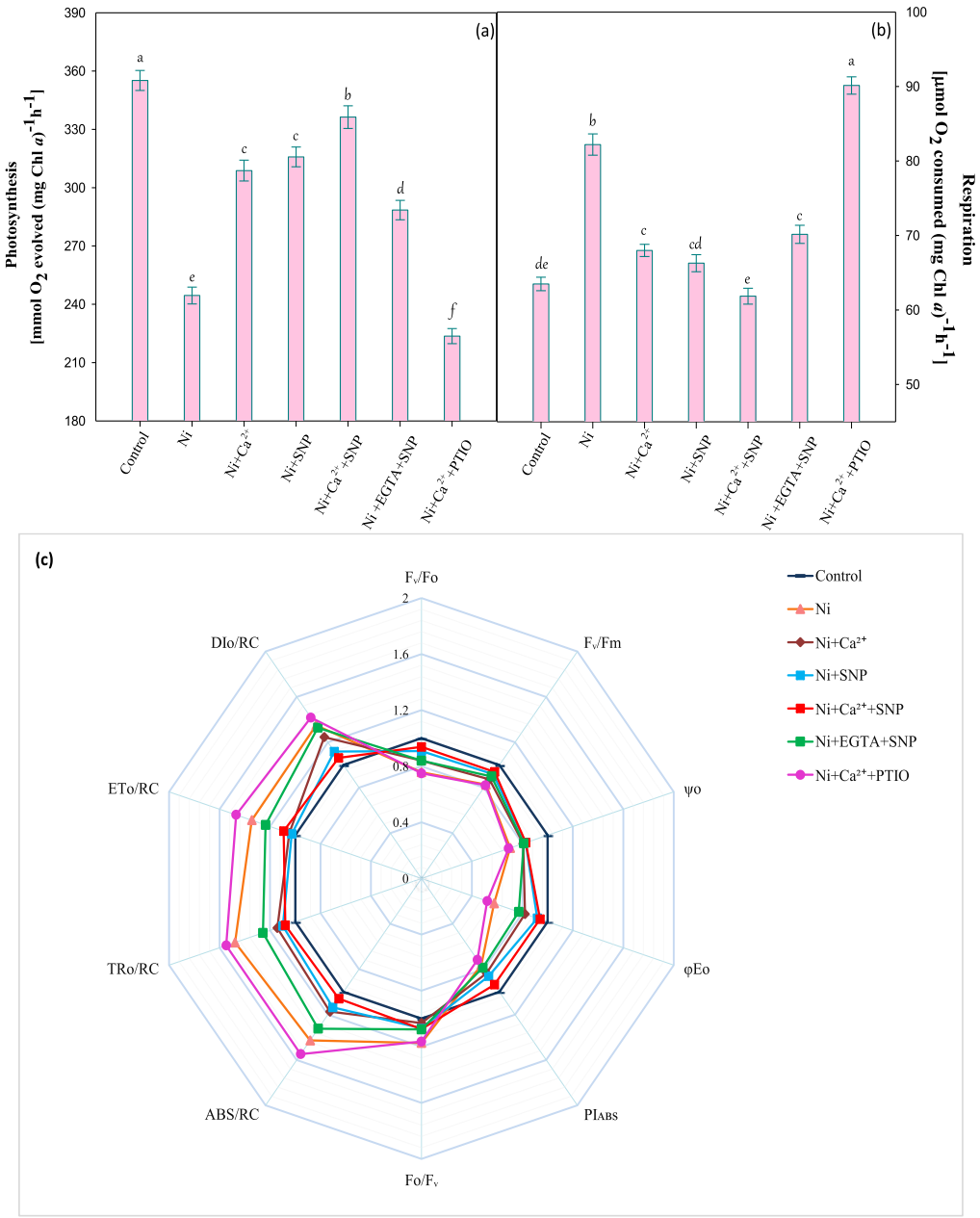


**Fig. 2.** Effect of $Ca^{2+}$ and SNP on rate of photosynthesis **(a)** and rate of respiration **(b)** chlorophyll *a* fluorescence **(c)** of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments.

critical damage in the respective values of above enzymatic antioxidants i.e. 18, 15, 16 and 17% were noticed respectively under PTIO treatment.

Unlike to enzymatic antioxidants, the levels of the non-enzymatic antioxidants; proline, cysteine and NP-SHs as a result of Ni stress declined by 15, 14 and 10% respectively in comparison to their respective control. However, the levels were slightly enhanced under individual treatment of $Ca^{2+}$ and SNP (NO), and a more pronounced effect of combined treatment of $Ca^{2+}$+SNP under the similar stress was noticed showing tremendous rise i.e. 31, 35 and 35% in the levels of proline, cysteine and NP-SHs respectively. Further, after scavenging the $Ca^{2+}$ by EGTA their levels were not much adversely affected due to the presence of NO (SNP) but scavenging of NO by addition of PTIO under the tested stress harmed to the levels of proline, cysteine and NP-SHs in cells, and their levels were found critically reduced by 21, 27 and 17% respectively (Fig. 5).

### 3.8. *Effects of $Ca^{2+}$ and NO on nitrate ($NO_3^-$) and nitrite ($NO_2^-$) uptake*

Results pertaining to $NO_3^-$ and $NO_2^-$ uptake in cyanobacterial cells have been depicted in Table 2. The results suggested that Ni at tested dose reduced the uptake of $NO_3^-$ by 38% and $NO_2^-$ by 39%. When Ni stressed organism was subjected to the $Ca^{2+}$, SNP and $Ca^{2+}$+SNP treatments, the levels of $NO_3^-$ and $NO_2^-$ uptake were found to be improve and under $Ca^{2+}$+SNP treatment the effect was more pronounced. Supplementation of EGTA along with the SNP, reduced their uptake level by 18 and 21% whereas PTIO along with the $Ca^{2+}$under Ni stress critically reduced the uptake levels by 44 and 59% respectively.

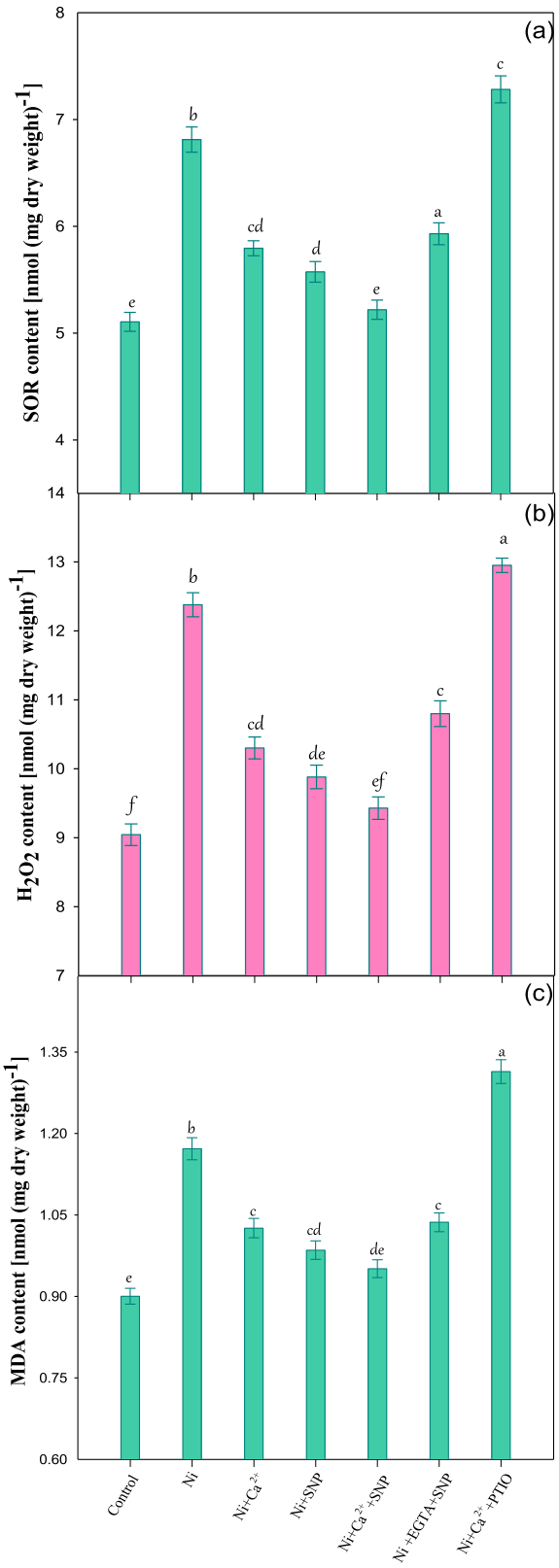


**Fig. 3.** Effect of $Ca^{2+}$ and SNP on SOR **(a),** $H_2O_2$ **(b)** and MDA **(c)** contents of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

### *3.9. Effect of $Ca^{2+}$ and NO on activities of nitrate assimilating enzymes*

The results related to the activities of NR and NiR in test organism have been portrayed in Table 2. Under Ni stress activities of NR and NiR were declined significantly by 44 and 38% respectively over the values of respective control. Furthermore, upon $Ca^{2+}$ or SNP supplementation, the negative effect on NR and NiR activities caused by Ni stress was alleviated, and with $Ca^{2+}$+SNP treatment to Ni stressed organism was found to be more effective in alleviating the negative effect on NR and NiR activities. Contrary to this, treatment of EGTA+SNP under the same stress, reduced their activities by 22 and 26% whereas PTIO along with $Ca^{2+}$ under Ni stress, critically reduced their activities by 57 and 59% respectively.

### *3.10. Effects of $Ca^{2+}$ and NO on activities of ammonia assimilating enzymes*

The results showing the activities of GS, GOGAT and GDH of test organism have been depicted in Table 2. Nickel suppressed the activities of GS and GOGAT by 35 and 37% respectively in comparison to control. Exogenous supplementation of $Ca^{2+}$, SNP and $Ca^{2+}$+SNP separately to Ni stressed cells caused significant improvement in the activities of both enzymes, and more efficient effect was found under $Ca^{2+}$+SNP combination. Notwithstanding to this, exposure with EGTA+SNP combination under the same stress reduced their activities by 24 and 28% while cells subjected to PTIO+$Ca^{2+}$ treatment exhibited sharp decline in activities of these enzymes under Ni stress as reduction was 54 and 53% respectively.

Contrary to GS and GOGAT, a reversed trend was noticed as GDH activity under Ni stress exhibited significant enhancement showing a rise of 33% and it was further accelerated under PTIO+$Ca^{2+}$ exposure as it was raised by 42% in comparison to control. Further, on exogenous supplementation of $Ca^{2+}$/SNP or $Ca^{2+}$+SNP to the Ni stressed cultures, a declining trend was observed, however the activity was still substantially greater than that of control value.

## 4. Discussion

The present study mainly revealed the regulatory strategies of NO and $Ca^{2+}$ in Ni toxicity alleviation in *N. muscorum* ATCC 27893. The 2 μM Ni showed the damaging effect on tested cyanobacterium (Fig. 1a). Ni-induced decline in growth of cyanobacterium might be due to 1) decreased EPS content, 2) excess Ni accumulation inside the cell, 3) declined pigment contents and 4) interrupted PS II activity which ultimately hindered the photosynthetic activity and provoked the accumulation of ROS inside the cells 5) disturbed overall N-metabolizing machinery. Aziz et al. [50] reported reduced growth of rice plant by accumulating Ni in root and shoot region which triggered the loss of chlorophyll contents by ROS generation. Nitric oxide and $Ca^{2+}$ are known for regulating several abiotic stresses in plants as well as in cyanobacteria [51–53]. In this study, application of SNP and $Ca^{2+}$ alone and together significantly ameliorated the Ni-mediated toxicity in tested cyanobacterium which might be due to obstruction in the pathway of Ni entry inside the cell governed by enhanced EPS contents (Fig. 1b). Recent study of Tiwari et al. [54] supports the present study by justifying the restricted movements of Al inside the *Anabaena* PCC 7120 in the presence of SNP by enhanced EPS content which acts as a protective layer of cyanobacteria. Similarly, Ahad and Syiem [4] reported that $Ca^{2+}$ restricts the Cd movement inside the *N. muscorum* Meg 1 and hence, improved the physiological responses. But, NO-scavenger lessened the $Ca^{2+}$ guided effects suggesting its dependency on NO.

Cyanobacterial cells possess a protective layer known as exopolysaccharides (EPS) which protect the cells from several external stressors and increase the survival of the organism. They are mainly attached with the external surface of cell and/or secreted in the surrounding medium as described by Mezhoud et al. [55]. Contents of EPS were declined

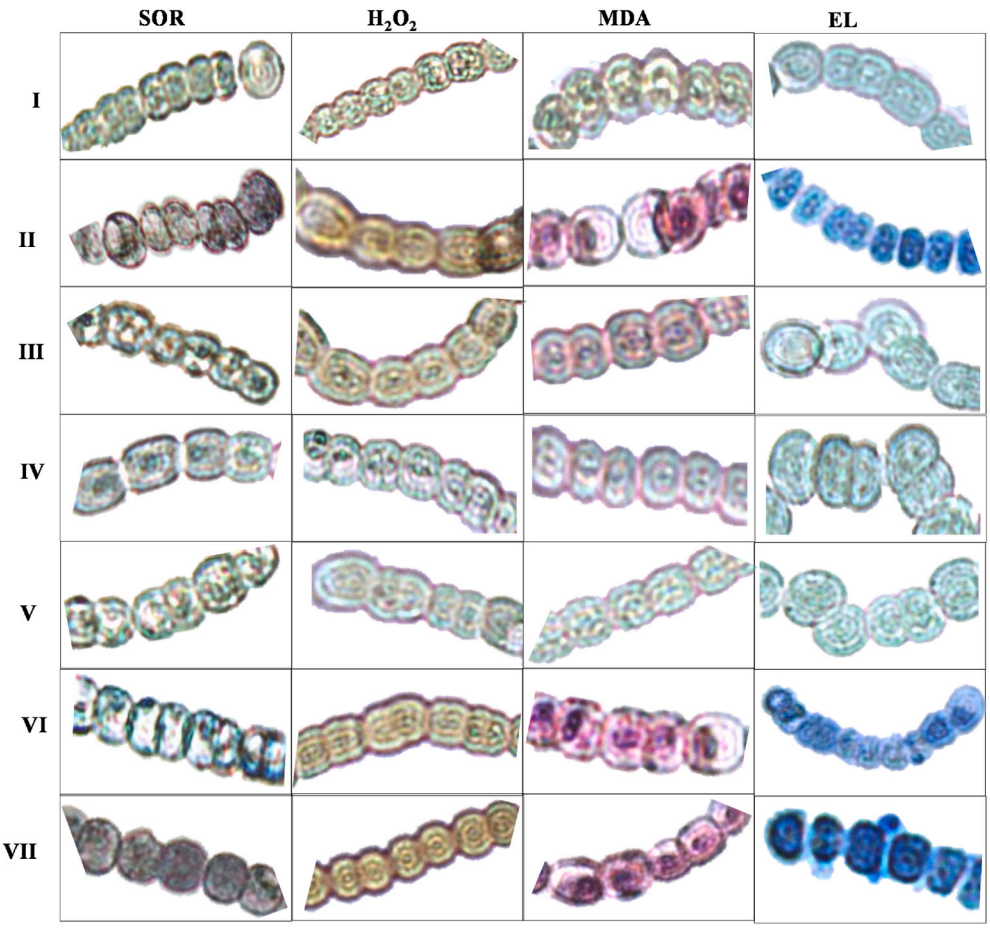


**Fig. 4.** *In-vivo* visualization of SOR, $H_2O_2$, MDA and EL of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments.

under Ni treatment that enhance the endogenous Ni accumulation while $Ca^{2+}$ or/and SNP favoured its secretion thus increase in content of EPS was recorded (Fig. 1b) which might have hindered the passage of Ni inside the cell (Fig. 1c), reduced cellular toxicity and prevailed the growth of organism. But, PTIO application further enhanced the Ni content and worsened the Ni toxicity inside the cell by diminishing the role of $Ca^{2+}$ (Fig. 1c).

Cyanobacteria are mainly acknowledged for occurrence of different photosynthetic pigments executing several peculiar functions. Any disturbance in the structure of these pigments creates anomalies regarding their stability and function. It is known that heavy metals disrupt the structure of photosynthetic pigments in plants [56–58] as well as in cyanobacteria [54,59]. In congruence with these studies, similar results were observed for contents of Chl *a*, carotenoids and phycobilisomes (Table 1). Nickel declined the pigment contents drastically that may be due to activation of chlorophyllase enzyme (chlorophyll degrading enzyme) or displacement of Mg and Fe that are considered as essential components for chlorophyll synthesis [50], degradation of thylakoid membrane [60] and enhanced production of reactive oxygen species (ROS) [54]. Carotenoids are the protective jacket of Chl *a* molecules and save these molecules by light induced photo oxidation. Therefore, significant reduction in carotenoids content by the action of Ni results in serious consequences on chlorophyll molecules leading to reduced photosynthetic activity which is in consonance with Tiwari et al. [54]. Phycobilisomes are major antenna pigments of PS II which are vulnerable to heavy metals due to their external localization on thylakoid membrane [54] and direct or indirect interaction of Ni caused (via oxidative radicals) its degradation. Exogenous application of $Ca^{2+}$and SNP significantly enhanced the pigment content in *N. muscorum* which could be due to expression of ELIPs-like proteins (early light induced proteins) and chlorophyll synthesis which is supported by the study of Riquelme et al. [61]. Nitric oxide also plays its role by up regulating cytokinin function inside the cell and restored the pigments content [57,62,63]. Likewise, role of $Ca^{2+}$ is also reported for boosting up the chlorophyll synthesis in plant *Cucumis sativus* L [53]. and *Solanum lycopersicum* [64]. Findings of Singh et al. [65] elaborated the mechanism of $Ca^{2+}$-mediated enhancement in the activity of enzymes involved in pigment synthesis and minimized their degradation. Additionally, carotenoids induction could also guide the survival of organism by lessening the oxidative damage by the action of $Ca^{2+}$and SNP. In accordance with this study Singh et al. [66], reported the positive role of $Ca^{2+}$ and NO for synthesis of Chl *a* and carotenoids in Cr(VI) stressed *Solanun lycopersicum* and *Solanum melongena* plants. On the other hand, all the positive effects of $Ca^{2+}$ on *N. muscorum* were reversed by PTIO, pointing towards the dominant role of NO in the regulation of Ni toxicity.

Any alteration in pigment biosynthesis disturbed the biological process of photosynthesis, and thereafter affects the growth of cyanobacterium. In the current finding, Ni stress reduced the rate of photosynthesis (Fig. 2a) which might be due to the structural and functional modifications in thylakoid membrane, created by direct interference with light reaction. Simultaneously, due to possible reduction in photophosphorylation (ATP), high rate of respiration was also noticed under Ni stress [67]. Calcium and SNP effectively improved the rate of photosynthesis and normalization of the rate of respiration which were again reversed by supplementation of PTIO even in the presence of $Ca^{2+}$. Thus, it shows the important role of $Ca^{2+}$ mediated function of NO in regulation of photosynthetic machinery. These findings are firmly supported by the study of Singh and Prasad [68], suggesting $Ca^{2+}$facilitated positive role of NO in chromium toxicity alleviation in tomato and

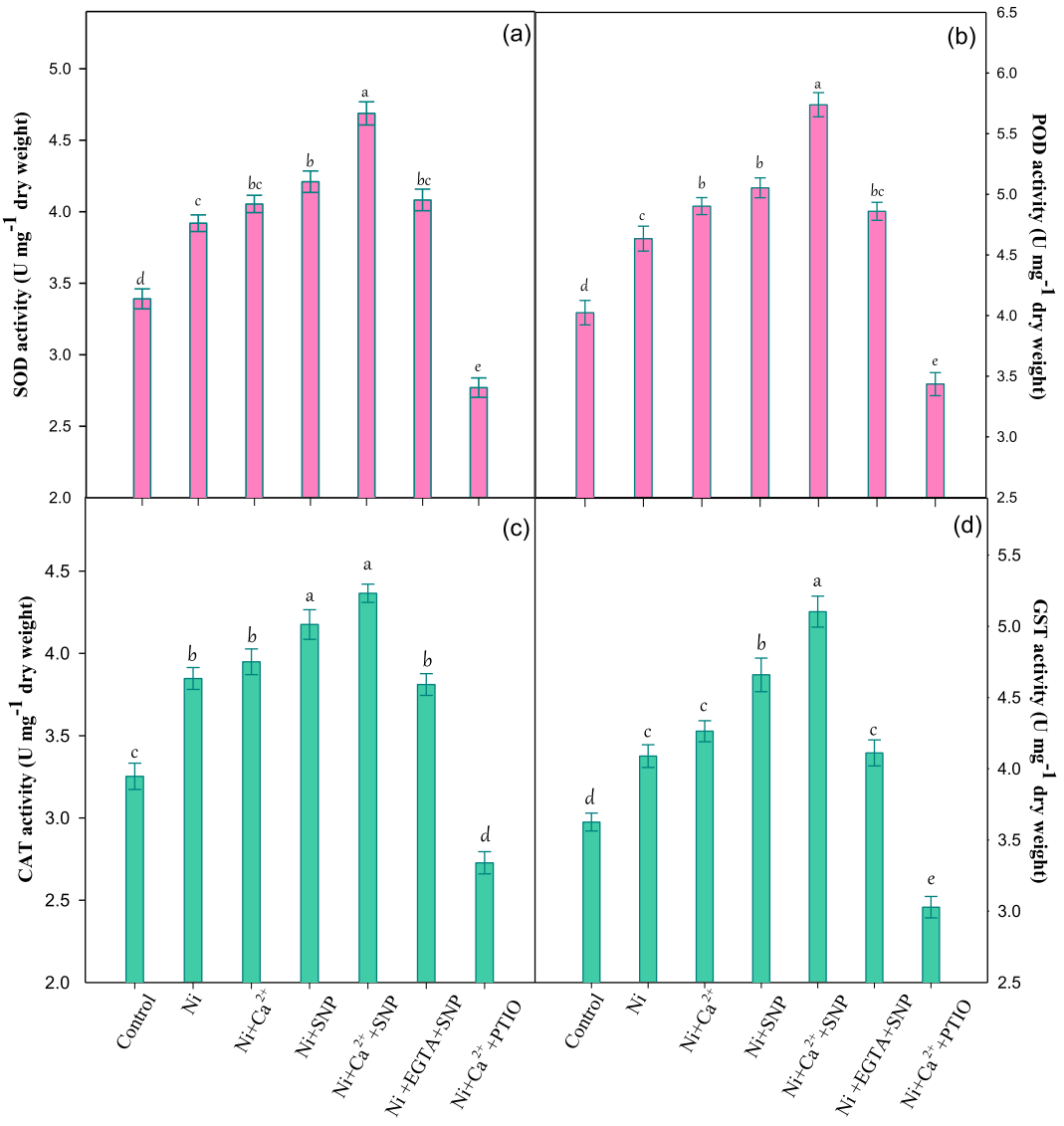


**Fig. 5.** Effect of $Ca^{2+}$ and SNP on activity of enzymatic antioxidant machinery; SOD **(a),** POD **(b),** CAT **(c)** and GST **(d)** of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

**Table 2**
Effect of $Ca^{2+}$ and SNP on nitrate ($NO_3^-$) and nitrite ($NO_2^-$) uptake rate, and the enzymatic activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase and glutamate dehydrogenase (GDH) of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

| Treatments | Nutrient uptake (μ mole $g^{-1}$ dry weight $h^{-1}$) | | Nitrateassimilating enzymes activity (U $mg^{-1}$ dry weight) | | Ammonium assimilating enzymes activity (U $mg^{-1}$ dry weight) | | |
|---|---|---|---|---|---|---|---|
| | Nitrate ($NO_3^-$) uptake | Nitrite ($NO_2^-$) uptake | Nitrate reductase (NR) | Nitrite reductase (NiR) | Glutamine synthetase (GS) | Glutamate synthase (GOGAT) | Glutamate dehydrogenase (GDH) |
| Control | $110.20 \pm 1.90^{a}$ | $80.15 \pm 1.38^{a}$ | $47.20 \pm 0.81^{a}$ | $28.80 \pm 0.49^{a}$ | $30.10 \pm 0.52^{a}$ | $35.20 \pm 0.60^{a}$ | $11.50 \pm 0.19^{e}$ |
| Ni | $68.00 \pm 1.17^{e}$ | $48.70 \pm 0.84^{e}$ | $26.30 \pm 0.45^{e}$ | $17.60 \pm 0.30^{e}$ | $19.50 \pm 0.33^{e}$ | $21.90 \pm 0.37^{e}$ | $15.40 \pm 0.26^{b}$ |
| Ni + $Ca^{2+}$ | $93.70 \pm 1.62^{cd}$ | $67.30 \pm 1.16^{c}$ | $39.90 \pm 0.69^{c}$ | $23.80 \pm 0.41^{c}$ | $25.10 \pm 0.43^{c}$ | $28.80 \pm 0.49^{c}$ | $12.50 \pm 0.21^{d}$ |
| Ni + SNP | $96.20 \pm 1.66^{c}$ | $70.80 \pm 1.22^{b}$ | $42.10 \pm 0.72^{b}$ | $24.80 \pm 0.42^{bc}$ | $26.40 \pm 0.45^{b}$ | $30.20 \pm 0.52^{bc}$ | $12.10 \pm 0.20^{de}$ |
| Ni + $Ca^{2+}$+SNP | $101.10 \pm 1.75^{b}$ | $72.40 \pm 1.25^{b}$ | $43.50 \pm 0.75^{b}$ | $25.60 \pm 0.44^{b}$ | $26.90 \pm 0.46^{b}$ | $31.20 \pm 0.54^{b}$ | $11.90 \pm 0.20^{de}$ |
| Ni + EGTA + SNP | $89.60 \pm 1.55^{d}$ | $62.90 \pm 1.08^{d}$ | $36.40 \pm 0.63^{d}$ | $21.10 \pm 0.36^{d}$ | $22.70 \pm 0.39^{d}$ | $25.30 \pm 0.43^{d}$ | $13.60 \pm 0.23^{c}$ |
| Ni + PTIO + $Ca^{2+}$ | $61.50 \pm 1.06^{f}$ | $32.50 \pm 0.56^{f}$ | $20.10 \pm 0.34^{f}$ | $11.70 \pm 0.20^{f}$ | $13.80 \pm 0.23^{f}$ | $16.30 \pm 0.28^{f}$ | $16.40 \pm 0.28^{a}$ |

brinjal plants.

An analysis of Chl *a* fluorescence was undertaken to pinpoint the target site of high concentration of Ni on photosynthesis. The study demonstrated that Ni at tested dose diminished the size and number of active reaction centers ($Fv/F_0$) along with the kinetic parameters of Chl *a* fluorescence, i.e. $\Phi P_0$, $\Psi_0$, $\Phi E_0$ and $PI_{ABS}$ that suggests towards the damage in antenna pigments and disturbance in electron flow between PS II to PS I across the thylakoid membrane [69]. However, exogenous supplementation of $Ca^{2+}$ and SNP restored the size and numbers of active reaction centers and also improved the kinetic parameters. In consonance of this, the improved $Fv/F_0$ along with kinetic parameters ($\Phi P_0$, $\Psi_0$, $\Phi E_0$ and $PI_{ABS}$) were noticed under exposure of calcium in arsenic stressed *Brassica* seedlings [70] and under exposure of NO in Al stressed *Anabaena* [54]. On the other hand, the values of energy flux parameters i.e., ABS/RC, $TR_0$/RC, $DI_0$/RC and $ET_0$/RC together with the efficiency of oxygen evolving complexes ($F_0/Fv$) were significantly raised under Ni stress. Ni at tested dose decreased the size and numbers of active reaction centers which resulted into the increase in energy flux parameters. But supplementation of $Ca^{2+}$ and SNP recovered the size and numbers of active reaction centers (Fig. 2c) by improving antenna pigments (Table 1) which ultimately normalized the energy fluxes i.e., ABS/RC, $TR_0$/RC, $DI_0$/RC and $ET_0$/RC. These results are firmly supported by Singh and Prasad [71], suggesting the positive role of $Ca^{2+}$ and NO in balancing overall PS II activity.

Reactive oxygen species (ROS) are highly reactive and toxic components responsible for direct damage to lipids, proteins, carbohydrates and DNA, hence cause oxidative stress and diminished growth of organisms [13,54,63]. Oxidative stress can be defined as the exposure of molecules, cells or tissues to the excess level of oxidants (ROS), particularly to the free radicals [72]. Always there should be existence of an equilibrium between the production and the scavenging of ROS. Any disturbance in the equilibrium leads to increase in intracellular levels of ROS which can cause significant damage to cell structures [13,63]. All stress factors generally follow common mechanisms for inducing ROS formation and antioxidant defense system to combat ROS in plants [73] as well as in cyanobacteria [63,74] In this study, Ni at elevated concentration provoked the production of ROS resulted into membrane damage (*in vivo* and *in vitro*) (Figs. 3 and 4) which might be due to disturbance in ferredoxin pool which determines the Calvin cycle regulation [75]. The rise in respiration rate (Fig. 2b) as well as decrease in PS II activity (Fig. 2c) in test cyanobacterium under Ni stress might have favoured the occurrence of oxidative stress. In the presence of $Ca^{2+}$ and SNP lower rate of ROS accumulation concluded their alleviatory role in Ni mediated toxic condition in test organism. Rizwan et al. [76] and Ahad and Syiem [4] testified the ameliorative role of NO and $Ca^{2+}$ under Ni and Cd toxicity in rice plant and *N. muscorum* Meg 1. Apart from the above result, NO scavenger markedly elevated the ROS accumulation by diminishing the SNP mediated positive effect even in the presence of $Ca^{2+}$.

To minimize the toxic effect of ROS, cyanobacterial cells possess self-regulatory defense system that includes enzymatic and non-enzymatic defense system. Our study revealed the negative impact imposed on the activities of SOD, POD, CAT and GST under Ni treatment (Fig. 5). In spite of this, proline, cysteine and NP-SH accumulation were also found to be declined (Fig. 5) but SNP and $Ca^{2+}$ proficiently enhanced the antioxidant defense system (Figs. 5 and 6) to minimize the effect of ROS which was clearly visualized in *in-vivo* image (Fig. 4). While PTIO reversed the $Ca^{2+}$ mediated betterment of defensive process even in the presence of SNP. Similar to this, Singh et al. [77] have also demonstrated the interactive signaling role of $Ca^{2+}$ and NO in up-regulation of antioxidant defense machinery and thereby reduction of ROS accumulation inside the cells of mustard plant, leading to arsenic toxicity alleviation (see Fig. 7).

*Nostoc muscorum* is a nitrogen fixing cyanobacteria, however it may efficiently assimilate nitrate and nitrite in natural field conditions thus, it becomes an integral part of metabolic activities. In the present study,

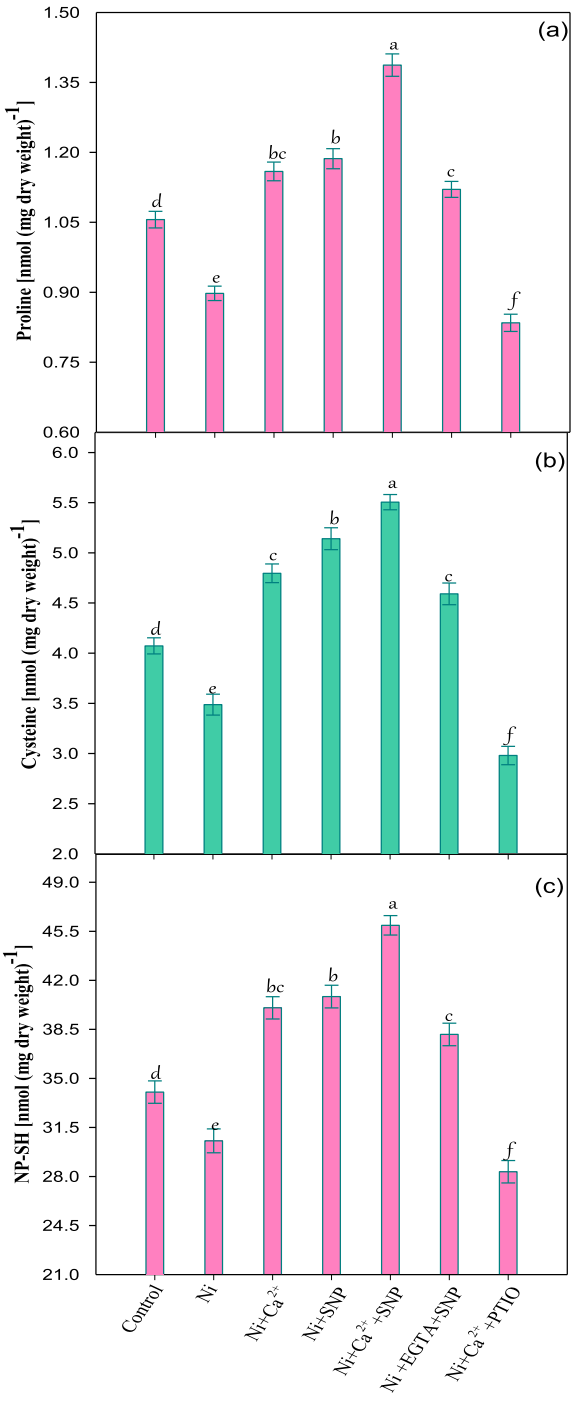


**Fig. 6.** Effect of $Ca^{2+}$ and SNP on activity of non-enzymatic antioxidant machinery; proline **(a),** cysteine **(b),** and non-protein thiols (NP-SHs) **(c)** of *Nostoc muscorum* ATCC 27893 treated with Ni after 72 h of experiments. Data are means ± standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters show significant difference at $P < 0.05$ significance level according to the Duncan's multiple range test.

the decrease of $NO_3^-$ and $NO_2^-$ uptake was observed (Table 2) that may probably be due to damage in their respective membrane bound transporters [78]. Damage in transporters may be correlated with the increased ROS contents (Fig. 3a and b) and MDA equivalents contents (Fig. 3c). The added SNP and $Ca^{2+}$to the cells enhanced the $NO_3^-$ and $NO_2^-$ uptake in *N. muscorum* under test condition reflecting their protecting nature for membrane stability which was also supported by

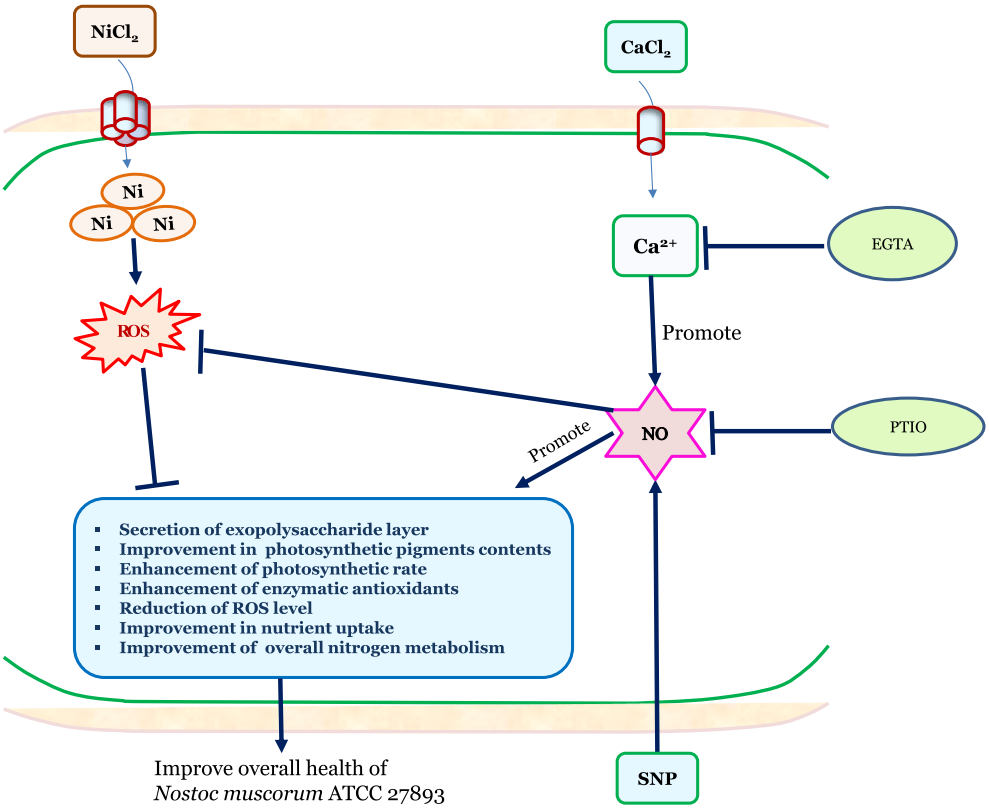


**Fig. 7.** Intracellular mechanism and interrelation between $Ca^{2+}$ and NO in regulation of Ni stress toxicity from the cells of cyanobacterium *Nostoc muscorum* ATCC 27893.

decreased MDA equivalents formation (Fig. 3c) and increased PSII activity and photosynthesis (whole cell oxygen evolution) but PTIO application further enhanced the Ni-mediated decline in uptake of $NO_3^-$ and $NO_2^-$ (Table 2).

The $NO_3^-$is utilized in two successive steps: firstly, $NO_3^-$ is reduced into $NO_2^-$ by the action of nitrate reductase (NR) and secondly, $NO_2^-$ is converted into ammonium ions ($NH_4^+$) by nitrite reductase (NiR) in cyanobacteria [59,79]. Activities of NR and NiR were diminished significantly by the action of Ni (2 μM) in *N. muscorum* (Table 2). Furthermore, NR and NiR enzymes are known as $NO_3^-$ and $NO_2^-$ inducible enzymes, respectively [80]. Therefore, Ni at this dose declined the activities of both NR and NiR enzymes possibly due to reduced photosynthetic activity which might be crucially responsible for the reduced activity of NR and NiR in the test organism. In consonance with our study, Singh et al. [59] also concluded that copper directly interfered with the photosynthesis process which ultimately decreased the activities of NR and NiR in *N. muscorum* and *P. foveolarum*. The activities of both the enzymes were found to increase under SNP and $Ca^{2+}$ treatments (Table 2). These results might be dependent on enhanced photosynthesis and improved rate of nitrate and nitrite uptake (Table 2) and unlike this, NO scavenger reversed this result reflecting the failure of $Ca^{2+}$ alone mediated amelioration in the test organism.

Under normal condition ammonia is assimilated during nitrogen metabolism process by GS-GOGAT pathway thus, it incorporates $NH_4^+$ into carbon skeletons in cyanobacteria. In the present study, Ni considerably declined the activities of GS and GOGAT enzymes while SNP and $Ca^{2+}$ application maintained their activities and recovered the ammonium assimilation process in the test organism. Enhanced photosynthetic rate under SNP and $Ca^{2+}$ treatment might be a reason to up regulate the activities of GS and GOGAT because these enzymes utilize photosynthetic generated ATP and photosynthates in cyanobacteria [59, 79]. Ahad and Syiem [4], noticed the positive role of calcium on GS activity in *N. muscorum* Meg 1 under cadmium stressed condition. On the other hand, GDH activity was increased under Ni treatment that might have compensated the GS and GOGAT activities. Skopelitis et al. [81] have described that when GS/GOGAT system is not fully effective under stress conditions, increase in GDH activity may release pressure exerted by accumulating amounts of $NH_4^+$ and provides glutamate for the biosynthesis of several protective compounds. While, comparatively weaken activity of GDH was noticed under SNP and $Ca^{2+}$ application showing the decrease in toxicity induced by Ni. But, the positive effects of SNP and $Ca^{2+}$were deteriorated under PTIO (a NO scavenger), clarifying the NO mediated induction of $Ca^{2+}$ in the activity in *N. muscorum* under Ni stress condition.

## 5. Conclusion

Present study concludes that Ni at elevated concentration reduced the growth of paddy field cyanobacterium *N. muscorum* and caused a greater damage to growth promoting biological processes of organism. The two effective signaling molecules NO (SNP) and $Ca^{2+}$ were found to be potentially efficient to overcome the Ni toxicity, and possible mechanism and interdependency of these molecules was explored by applying chelators of NO (PTIO) and $Ca^{2+}$ (EGTA). Growth and its supporting processes i.e. photosynthesis and nitrogen metabolism under Ni stress were improved under combined exposure of NO (SNP) and $Ca^{2+}$ but positive effects were reversed significantly by arresting endogenous NO even in the presence of $Ca^{2+}$. Nonetheless, when $Ca^{2+}$was chelated, NO (SNP) maintained the growth by reducing ROS levels in cells through boosting antioxidant defense machinery, PS II photochemistry and $N_2$-metabolism. Thus, overall findings suggest that $Ca^{2+}$ and NO (SNP) together efficiently regulate Ni toxicity where NO played vital role in alleviating toxicity. The outcome of study also points that SNP, a cheaper chemical at very low level can be applied together

with $Ca^{2+}$ to maintain the growth of cyanobacterium *Nostoc muscorum* which as biofertilizer can support the paddy for better yield even in Ni contaminated field.

## CRediT authorship contribution statement

**Nidhi Verma:** Conceptualization, Investigation, Formal analysis, Writing - original draft. **Aparna Pandey:** Investigation, Writing - original draft. **Santwana Tiwari:** Writing - original draft. **Sheo Mohan Prasad:** Conceptualization, Methodology, Data curation, Supervision.

## Declaration of competing interest

Authors declare that they have no any conflict of interest.

## Acknowledgments

Authors are very thankful to Department of Botany, University of Allahabad, for providing essential laboratory facilities. University Grant Commission, New Delhi is also acknowledged for providing the financial support to Nidhi Verma as UGC-AU research scholar to carry out the present work. Aparna Pandey is also thankful to University Grant Commission, New Delhi for providing the financial support as Junior Research Fellow UGC-Ref. No.: 627 (CSIR-UGC NET DEC. 2018).

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