Regulatory role of Ca2+ and NO in salicylic acid (SA) induced signaling against Ni2+-induced toxicity in Anabaena sp. PCC 7120

Nidhi Verma[1], Parul Parihar[1], Rachana Singh[1], Sheo Mohan Prasad[2], and Aparna Pandey[1]

[1]University of Allahabad Faculty of Science
[2]Affiliation not available

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Abstract

Present study deals with the regulation of SA induced Ca2+-mediated NO signaling in cyanobacterium Anabaena PCC 7120. Nickel (Ni2+)-toxicity strongly inhibited growth (DW), exopolysaccharides, photosynthetic pigments, PSII photochemistry (OJIP transient parameters: Fm, Fv, Fm/Fo, Fv/Fo, Ψo, ΦPo, ΦEo, Area, N, and PIABS were suppressed; while Fo, Fo/Fm, Fo/Fv, Sm, ΦDo, ABS/RC, TRo/RC, ETo/RC and DIo/RC were raised), nitrogen metabolism status and non-enzymatic antioxidant system by increasing intracellular Ni2+ accumulation and excessive ROS production. However, salicylic acid (SA), calcium (CaCl2) and sodium nitroprusside (SNP) addition to the culture medium counteracted on negative impact of Ni2+ thereby improving the growth. Further, to investigate the relation between Ca2+ and NO, when c-PTIO was supplemented to Ni+SA+Ca and EGTA to Ni+SA+NO treated culture medium, the recovery on above studied traits caused due to Ca2+ and NO was arrested even in presence of SA to Ni2+-stressed Anabaena cells thereby suggesting the signaling behavior of Ca2+ and NO in SA induced pathway; however, the impact was worsened under c-PTIO supplemented Ni+SA+Ca than EGTA supplemented Ni+SA+NO treatment thereby suggesting key role of NO in SA-induced Ca2+-mediated signaling. Additionally, NO accumulation, intracellular Ni2+ accumulation, ROS and indices, and non-enzymatic antioxidant system were also examined that showed varied results.

Title: Regulatory role of Ca^{2+} and NO in salicylic acid (SA) induced signaling against Ni^{2+}-induced toxicity in *Anabaena* sp. PCC 7120

Running Head: Ca^{2+} and NO in SA signaling against Ni^{2+}- toxicity

Authors: Nidhi Verma^{+}, Parul Parihar^{+}, Rachana Singh*, Sheo Mohan Prasad*, Aparna Pandey

Affiliation: Ranjan Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Allahabad, Prayagraj- 211002 India.

Emailing addresses:nidhi111verma@gmail.com (N. Verma), parulprhr336@gmail.com (P. Parihar)

***Corresponding authors' email:**rachanaranjansingh@gmail.com (R. Singh), profsmprasad@gmail.com (S.M. Prasad)

$^{+}$**Contributed equally**

Abstract: Present study deals with the regulation of SA induced Ca^{2+}-mediated NO signaling in cyanobacterium*Anabaena* PCC 7120. Nickel (Ni^{2+})-toxicity strongly inhibited growth (DW), exopolysaccharides, photosynthetic pigments, PSII photochemistry (OJIP transient parameters:F_m , F_v ,F_m/F_o ,F_v/F_o , Ψ_o ,ΦP_o , ΦE_o , $Area$,N , and PI_{ABS} were suppressed; whileF_o , F_o/F_m ,F_o/F_v , S_m

,ΦD_o , ABS/RC ,TR_o/RC , ET_o/RC andDI_o/RC were raised), nitrogen metabolism status and non-enzymatic antioxidant system by increasing intracellular Ni^{2+} accumulation and excessive ROS production. However, salicylic acid (SA), calcium ($CaCl_2$) and sodium nitroprusside (SNP) addition to the culture medium counteracted on negative impact of Ni^{2+} thereby improving the growth. Further, to investigate the relation between Ca^{2+} and NO, when c-PTIO was supplemented to Ni+SA+Ca and EGTA to Ni+SA+NO treated culture medium, the recovery on above studied traits caused due to Ca^{2+} and NO was arrested even in presence of SA to Ni^{2+}-stressed *Anabaena* cells thereby suggesting the signaling behavior of Ca^{2+} and NO in SA induced pathway; however, the impact was worsened under c-PTIO supplemented Ni+SA+Ca than EGTA supplemented Ni+SA+NO treatment thereby suggesting key role of NO in SA-induced Ca^{2+}-mediated signaling. Additionally, NO accumulation, intracellular Ni^{2+}accumulation, ROS and indices, and non-enzymatic antioxidant system were also examined that showed varied results.

Capsule of main finding

This is the first study that demonstrates, NO plays key role in SA-induced Ca^{2+}-mediated signaling in Ni^{2+}-stressed *Anabaena* sp. PCC 7120 by regulating photosynthesis, nitrogen metabolism status and antioxidant system.

Keywords: Calcium; Nickel; Nitric oxide; Nitrogen metabolism status; PSII photochemistry; Salicylic acid.

1. Introduction

By 2050, the human population is projected to reach 9.7 billion, which is concomitant with increasing food demand in future. To overcome this challenge, the World Health Organization has suggested doubling the global food production. Thus, to make the balance between production and demand, suitable farming should be practiced for intensive agricultural production. Great pressure mounts on agricultural land to grow high-yielding varieties of crops on the account of continuous inputs of chemical fertilizers that leaches into the ground water reaching the water bodies. These chemical fertilizers are huge source for several heavy metals, apart from this, release of industrial wastes in aquatic systems, and several natural and anthropogenic activities are also some sources. Nickel (Ni^{2+}) is one of the heavy metal released into agro-ecosystems such as rice field, through effluents, from Ni-Cd batteries, iron, steel, electroplating industries, sewage sludge, burning of fossil fuels etc. (Nnorom and Osibanjo, 2009), which disturbs the soil equilibrium. Nickel is an essential metal; therefore, its lower concentrations is essential for the activities of some enzymes (Muyssen et al., 2004); however, at higher concentration this metal is fatal for the growth of plants/ cyanobacteria where it reduces photosynthetic pigments and lipid contents, interferes with electron transport chain, photosynthetic activity and antioxidant defense system (Muyssen et al., 2004; Boisvert et al., 2007; Martínez-Ruiz and Martínez-Jerónimo, 2015; Jahan et al., 2020). It also produces reactive oxygen species (ROS), which damages macromolecules like carbohydrate, lipids, DNA, proteins and causes cell death (Prasad et al., 2005; Boisvert et al., 2007; Balaji et al., 2013; Jahan et al., 2020).

Since few decades, the focus is being shifted to utilize the protectants like signaling molecules, nutrient management, metabolites, phytohormones to manage the negative impact of toxicants like Ni^{2+} to improve the plant/ cyanobacterial growth (Chen et al., 2013; Tiwari et al., 2018; Batista et al., 2019; Tiwari et al., 2019a,b; Singh et al., 2018a,b; Singh et al., 2020a,b). Plant hormones are necessary for plant-environment communication; salicylic acid (SA) is one of them which are naturally produced by plants (Akula and Ravishankar, 2011). The SA ameliorates toxicity symptoms by adjusting enzymatic and non-enzymatic defense systems, promotes important physiological processes like enzymes of defense compounds, nitrogen metabolism, osmolytes by stimulating glycinebetaine and proline, under stressful situations (Belkadhi et al., 2014; Ma et al., 2017; Batista et al., 2019; Rai et al., 2020). SA reduces toxicity by modulating carbohydrate metabolism, leaf gas exchange, improving photosynthetic rate, RubisCO activity, ATP synthesis and maintains Na^+/K^+ ratio in plants (Lee et al., 2014; Ghassemi-Golezani and Lotfi, 2015; Batista et al., 2019). However, the impact of SA in cyanobacteria is rarely studied.

Calcium (Ca^{2+}) and nitric oxide (NO), on the other hand are the backbone of plant signaling events in unstressed and stressed conditions. From earlier studies it has been cleared that Ca^{2+} plays essential role

in regulation of growth, and different physiological processes of plants like stabilizes membrane structures by bonding with phospholipid bilayer, declines ROS levels, enhances key antioxidant enzymes, improves RubisCO activity and CO_2 fixation (Singh et al., 2018a,b; Tiwari et al., 2019a; Verma et al., 2018; Singh et al., 2020a,b), being an essential part of catalytic site of water oxidation ($Mn_4Ca_1O_xCl_y$) of PSII, it helps in the assemblage of water oxidizing complex (OEC) and directly regulates the photosynthetic activity (Andréasson et al., 1995; Bartlett et al. 2008; Najafpour et al., 2012; Kalaji et al., 2014; Singh et al., 2018b; Singh et al., 2020a) under stressful environment. NO is also known for improvement in growth by regulating different physiological events like growth, photosynthetic pigments, photosynthetic activities, antioxidants defence system and reduces ROS levels produced by metals/ metalloids either by gene expression through signaling in a molecular cascade or by neutralizing excessive ROS through its free radical functioning (Zhang et al., 2008; Peto 2011; Chen et al., 2013; Xu et al., 2013; Sun et al., 2018; Tiwari et al., 2018; Singh et al., 2020a; Verma et al., 2020). According to a report, NO alleviate salinity stress by promoting photosynthetic rate in eggplant either by improving PSII quantum yield or by splitting excess energy via improving antenna molecules and carotenoid content (Wu et al., 2013).

In paddy fields, about 20-30 kg N $ha^{-1}season^{-1}$ is being fixed by *Anabaena* , which reveals its importance in improving the paddy yield (Chaurasia and Apte, 2011). *Anabaena* is the microscopic photosynthesizing organism thus, are the primary producers that sustain aquatic food web (Martinez-Ruiz and Martinez-Jeronimo, 2015); therefore, the toxicity produced at this level is estimated to affect the consumers and higher trophic levels. Recently, due to increasing involvement of human activities in the aquatic ecosystem, these nitrogen fixing cyanobacteria are facing enormous challenges especially in terms of metal toxicity, which leads to decline in soil fertility and consequently the paddy productivity. From the above literature, it is clear that SA, Ca^{2+} and NO plays important role in regulating different processes in plants and cyanobacteria under fluctuating environmental condition; however, their cumulative effect in orchestrating cyanobacterial responses against different environmental cues is not established. Therefore, in order to understand the interaction and interrelation of SA, Ca^{2+} and NO in the regulation of Ni^{2+}-induced toxicity in*Anabaena* PCC 7120, various physiological, and biochemical approaches in *Anabaena* cells suffering from Ni^{2+}-toxicity, has been investigated. Further, to find out the probable mechanism(s) in regulating SA induced signaling (Ca^{2+} and NO mediated) in *Anabaena* PCC 7120 suffering from Ni^{2+}-toxicity the key components i.e. growth and growth regulating processes: intracellular Ni^{2+} accumulation, exopolysaccharaides, photosynthetic pigments, PSII photochemistry, nitrogen metabolism status, ROS level and antioxidants defense system were assessed in the present investigation.

2. Material and methods

2.1. Test organism and culture conditions

The homogenous, filamentous and hetrerocystous cyanobacterium*Anabaena* sp. PCC 7120 was cultured in BG-11 medium (pH 7.5) in a temperature (25 +- 2 degC) controlled room 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 in early log phase culture of *Anabaena* PCC 7120.

2.2. Experimental design and treatments

The cultures of *Anabaena* PCC 7120 were harvested at their exponential phase by centrifugation at 4,000g for 10 min and gently washed thrice with distilled water. After this, cells were resuspended in BG-11 medium (pH 7.5) having different combinations of treatments viz., Ni ($NiCl_2$; 1.5 µM), salicylic acid (SA; 40 µM), calcium ($CaCl_2$; 800 µM), sodium nitroprusside (SNP; 10 µM), ethyleneglycol-*bis* (β- aminoethyl)-N,N,N,N- tetraacetic acid (EGTA; 1mM) and 2-4-carboxyphenyl- 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; 20 µM). The following combinations were made: control (only BG-11 medium), Ni, Ni+SA, Ni+Ca, Ni+NO, Ni+SA+Ca+c-PTIO, Ni+SA+NO+EGTA and Ni+SA+c-PTIO+EGTA. After 72 h of treatment, cultures were harvested and various parameters were analyzed.

2.3. Measurement of growth

Growth of *Anabaena* PCC 7120 was analysed in terms of dry weight. The 100 ml of untreated and treated cultures were centrifuged (4000 g) for 10 min and the pellets obtained were washed 2-3 times with double distilled water. Thereafter, filamentous pellets of each set were oven dried at 80 °C for 48 h and further after cooling weighed in a digital balance (Contech- CA 223, India).

2.4. Estimation of exopolysaccharides (EPS)

The content of EPS was determined according to Sharma et al. (2008). The 100 ml culture from each set was centrifuged (3000 g) for 15 min and cell free supernatant (containing EPS) was concentrated ten folds by evaporating at 40 °C. The obtained precipitate was washed thrice with isopropanol and dried at 37 °C. Lastly, hydrolysate was analyzed for glucose (Seifter et al., 1959) and EPS content was calculated with the help of a standard curve prepared with the help of glucose.

2.5. Estimation of the photosynthetic pigments

The photosynthetic pigments i.e. chlorophyll *a* (Chl *a*) and carotenoids (Car), in each culture of *Anabaena* PCC 7120 was extracted following the methods of Porra et al. (1989) and Goodwin (1954), thereby recording the absorbance spectrophotometrically (Systronics, India) at 665 nm and 450 nm, respectively. The contents of phycobiliproteins i.e. allophycocyanin (APC), phycocyanin (PC) and phycoerythrin (PE) were determined spectrophotometrically by recording the absorbance of the supernatant at 652, 615 and 562 nm, respectively following the method of Bennett and Bogorad (1973). Following equation was used to determine the phycobiliproteins content (µg mg^{-1} dry weight):

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

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

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

2.6. Measurement of whole cell O_2 evolution and respiration

The rate of photosynthesis in treated and untreated cells of*Anabaena* PCC 7120 were measured in terms of photosynthetic oxygen evolution using Clark type O_2 electrode (Digital oxygen system model-10, Rank Brothers, U.K). After 72 h of growth, cyanobacterial cells were harvested and the cell suspension were illuminated under the saturating light intensity of 360 µmol photons m^{-2} s^{-1} PAR in a temperature controlled air tight reaction vessel, at 25 °C for 3 min. Respiration rate was measured in the darkness with the help of same instrument in terms of O_2 consumption. Photosynthetic oxygen evolution rate was expressed as µmol O_2 evolved (mg^{-1} Chl *a*) h^{-1} and respiratory rate was expressed as µmol O_2 consumed (mg^{-1} Chl *a*) h^{-1}.

2.6.1. Measurement of PSII activity (JIP-Test)

To check the PSII performance, Chl *a* fluorescence measurements [**Structural and functional heterogeneity:** (i) **antenna heterogeneity** is related with Q_A reduction when electron flow to PQ pool or (ii) **reducing side** (functioning of reduction side *i.e.* Q_B- reducing and non-reducing centres): (a) oxidation/ reduction rate of PQ (F_o/F_m), (b) efficiency of water splitting complex (F_o/F_v), (c) fluorescence yields ratio for open and closed states (F_m/F_o) and size and number of active reaction centres (F_v/F_o);**Technical parameters:** (i) initial fluorescence (*F[?]*), (ii) variable fluorescence (*F?*), (iii) maximum fluorescence (F_m), (iv) Q_A redox turnover number until F_m is reached (N) and (v) size of plastoquinone (PQ) pool i.e. area beyond the fluorescence induction curve between F_o andF_m (*Area*); **Quantum yields and efficiencies:** (i) probability of an electron movement by trapped exciton into electron transport chain beyond Q_A- (yield of electron transport per trapped exciton; Psi_oor Ψ_o), (ii) quantum yield of primary photochemistry (Phi_P_o orΦP_o), (iii) probability of dissipation of an absorbed photon (ΦD_o) and (iv) quantum yield of electron transport (Phi_E_o orΦE_o); **Specific energy fluxes:** (i) absorption of PSII antenna Chls shared by number of active RC (Q_A reduction) (*ABS/RC;* denotes antenna size), (ii) trapped energy flux per active RC [denotes only active RCs (Q_A-Q_A-);TR_o/RC], (iii) electron transport flux per active RC (from Q_A- to PQ;ET_o/RC), (iv) dissipation amount of active RCs (DI_o/RC) and (v) energy needed to close all the RCs (S_m); **Overall performance index**(PI_{ABS})] were carried out in the dark adapted cyanobacterial samples

by using hand held fluorometer (AquaPen AP 100, Photon System Instruments, Czech Republic) following the method of Strasser et al. (2000).

2.7. Determination of nitrogen metabolism

2.7.1. Nitrate and Nitrite uptake

Nitrate (NO_3^-) uptake in untreated and treated cyanobacterial cells was estimated spectrophotometrically at 210 nm by measuring the depletion of $NO_3^-$from the external medium following the method of Cawse (1967).

Nitrite (NO_2^-) uptake in each sample was estimated spectrophotometrically at 540 nm by measuring the depletion of NO_2^- from the external medium following the method of Snell and Snell (1949).

2.7.2. Assay of nitrogen metabolizing enzymes

2.7.2.1. Nitrate reductase (NR; EC 1.6.6.1) and nitrite reductase (NiR; EC 1.7.7.1) activities

The activities of Nitrate reductase (NR; EC 1.6.6.1) and nitrite reductase (NiR; EC 1.7.7.1) were performed with dithionite-reduced methyl viologen as reductant in cyanobacterial cells made permeable by alkyltrimethylammonium bromide (MTA) in the reaction mixture following the method of Herrero et al. (1981, 1984) and Herrero and Guerrero (1986), respectively. The NO_2^- ions in cell free media were estimated according to Snell and Snell (1949). One unit (U) NR activity is defined as 1 nmol NO_2^- formed min^{-1}. One unit (U) NiR activity is defined as 1 nmol NO_2^- consumed min^{-1}.

2.7.2.2. Glutamine synthetase (GS; EC 6.3.1.2) activity

Glutamine synthetase activity was determined by the formation ofγ -glutamylhydroxamate (transferase assay) following the method of Mérida et al. (1991). One unit (U) GS activity is defined as 1 nmolγ -glutamylhydroxamate formed min^{-1}.

2.7.2.3. Glutamate synthase (Fd-GOGAT; EC1.4.1.13) activity

Glutamine 2-oxoglutarate aminotransferase (Fd-GOGAT) activity was estimated by following the method of Navarro et al. (1995). One unit of GOGAT activity is defined as 1 nmol glutamate formed min^{-1}.

2.7.2.4. Glutamate dehydrogenase (NADH-GDH; EC 1.4.1.2) activity

Glutamate dehydrogenase activity (aminating) was determined spectrophotometrically at 340 nm by measuring oxidation of NADH by the method of Chávez and Candau (1991). One unit (U) GDH activity is defined as 1 nmol NADH oxidized min^{-1}.

2.8. Determination of reactive oxygen species (ROS: O_2^{*-} and H_2O_2) and stress biomarker (MDA equivalents content)

2.8.1. Biochemical analysis

The content of superoxide radicals (SOR; O_2^{*-}) in all the treatments was determined by recording the absorbance of colored aqueous solution at 530 nm following the method of Elstner and Heupel (1976), and the amount of formed O_2^{*-} was quantified using the standard curve prepared with the help of $NaNO_2$.

Likewise, the content of hydrogen peroxide (H_2O_2) in each sample was determined by recording the absorbance of cleared solution at 390 nm following the method of Velikova et al. (2000), and the amount of formed H_2O_2 was quantified using the standard curve prepared with the help of H_2O_2.

Lipid peroxidation in each sample was quantified in terms of malondialdehyde (MDA) equivalents by recording the absorbance at 532 and 600 nm following the method of Heath and Packer (1968) and the content of MDA equivalents was quantified using an extinction coefficient 155 mM^{-1} cm^{-1}.

2.8.2. Histochemical staining (in-vivo visualization)

For the quantification of O_2^{*-} and H_2O_2 production in cyanobacterial cells, the *in-vivo* staining with the help of nitrobluetetrazolium (NBT) and 3, 3 diaminobenzidine (DAB)-HCl, respectively was achieved following the method of Forster et al. (2005). Similarly, for the visualization of MDA equivalents and membrane integrity, cyanobacterial cells were stained with Schiff's reagent and Evan's blue following the method of Pompella et al. (1987) and Yamamoto et al. (2001), respectively and the photographs were captured with the help of microscope (Leica, model-DM 2500).

2.9. Estimation of activities of antioxidants

2.9.1. Enzymatic antioxidants

Superoxide dismutase (SOD; EC 1.15.1.1) activity in each sample was monitored spectrophotometrically by measuring the formation of purple formazan compound formed due to photoreduction of NBT at 560nm, using the method of Giannopolitis and Reis (1977). One unit (U) of SOD activity is the amount of enzyme necessary to inhibit (50%) the NBT reduction.

Peroxidase (POD, EC 1.11.1.7) activity in each sample was determined spectrophotometrically by measuring the increase in absorbance due to pyrogallol oxidation at 430 nm using the extinction coefficient of 25.5 mM^{-1} cm^{-1}, as suggested by Gahagan et al. (1968). One unit (U) of POD activity is 1 nmol pyrogallol oxidized min^{-1}.

Catalase (CAT; EC 1.11.3.6) activity in each sample was monitored spectrophotometrically by measuring the decrease in absorbance due to H_2O_2 dissociation at 240 nm using the extinction coefficient of 39.4 $mM^{-1}cm^{-1}$, as suggested by Aebi (1984). One unit (U) of CAT activity is 1 nmol H_2O_2 dissociated min^{-1}.

Likewise, glutathione-*S* -transferase (GST, EC 2.5.1.18) activity in each sample was monitored spectrophotometrically by measuring the increase in absorbance due to conjugate formation between GSH and CDNB at 340 nm using the extinction coefficient of 39.4 mM^{-1} cm^{-1}, as suggested by Habig et al. (1974). One unit (U) of GST activity is 1 nmol CDNB-conjugates formed min^{-1}.

2.9.2. Non-enzymatic antioxidants

The content of cysteine (Cys) in each sample was determined by recording the absorbance of reaction mixture at 560 nm following the method of Gaitonde (1967), and the amount of formed Cys was calculated by the help of standard curve.

Similarly, the content of proline (Pro) in each sample was determined by recording the absorbance of toluene layer of the reaction mixture at 520 nm following the method of Bates et al. (1973), and the amount of formed Pro was calculated by the help of standard curve.

The content of non-protein thiols (NPTs) in each sample was determined by recording the absorbance of reaction mixture at 412 nm following the method of Ellman et al. (1959) and the amount of formed NPTs was calculated by the help of standard curve.

2.10. 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 replicates in each experiment (n=3). Principal component analysis (PCA) was also done using the XLSTAT-2016 to study the correlation of the biomass and other parameters as well as with the treatments.

3. Results and discussion

3.1. SA, Ca^{2+} and NO recover Ni^{2+}-induced damage in growth

To examine the SA induced Ca^{2+} and NO signaling in easing Ni^{2+}-induced toxicity, *Anabaena* PCC 7120 cells were exposed to different donors, scavengers and inhibitors of SA, Ca^{2+} and NO. Nickel at its tested dose significantly declined the growth (dry weight) of *Anabaena* by 31%, which is attributed to: (i) higher

Ni^{2+} accumulation in the cell (Fig. 1D), (ii) reduction in photosynthetic pigments content (Table 1) and PSII performance (Fig. 2C), which subsequently decreased photosynthetic rate (Fig. 2A), (iii) overproduction of ROS (Fig. 4), (iv) reduction in NO_3^- and NO_2^- uptake rate (Fig. 3A,B), and (v) decrease in EPS content (Fig. 1B). However, under similar condition, addition of SA, $CaCl_2$ and SNP in the growing medium counteracted Ni^{2+}-induced negative impact on DW (12, 11 and 7% respectively) of test cyanobacteria, which is in agreement with the higher NO accumulation than in the Ni^{2+}- stressed cyanobacterial cells alone (Fig. 1C). The SA, Ca^{2+} and NO induced progressive response on growth have also been reported by Tiwari et al. (2018, 2019a) and Singh et al. (2020a,b) in *Anabaena* PCC 7120 and mustard, respectively. Further, in order to study the possible link between SA, Ca^{2+} and NO; when Ni+SA+Ca and Ni+SA+NO treated cyanobacteria cells were supplemented with c-PTIO (a NO scavenger) and EGTA (a Ca^{2+} scavenger), respectively; a significant reduction in DW was observed, thereby satisfying the fact of involvement of Ca^{2+} and NO in signaling event. Interestingly, the growth of c-PTIO supplemented Ni+SA+Ca treated cyanobacterial cells was comparatively more affected even in presence of Ca^{2+}(Fig. 1A), suggesting that NO is required for the maximal and constant Ca^{2+} signaling, which corresponds to reduce NO content in presence of c-PTIO (Fig. 1C). The report of Xu et al. (2013) have strengthened our findings that by promoting NO release, ABA hormone protected tall fescue plant from oxidative stress, and Singh et al. (2020a,b) have suggested that NO is essential in Ca^{2+}mediated signaling event in arsenic-stressed mustard seedlings.

Results for intracellular Ni^{2+} accumulation showed higher Ni^{2+} accumulation under Ni^{2+}-stressed condition, which was neutralized upon SA, $CaCl_2$ and SNP treatment to Ni^{2+}-stressed cyanobacterial cells; however, in presence of c-PTIO and EGTA, even SA was unable to limiting Ni^{2+}-accumulation; therefore, highest Ni^{2+}-accumulation was found in Ni+SA+c-PTIO+EGTA even than that of Ni^{2+}-stressed cyanobacterial cells alone (Fig. 1D).

3.2. SA, Ca^{2+} and NO induces exopolysaccharides (EPS) secretion to protect cells from Ni^{2+}-induced damage

The EPS secretion by cyanobacterial cells is a defense strategy to combat from toxicity; which was found to decreased by 23% under Ni^{2+}-stress while upon SA, $CaCl_2$ and SNP addition, a sharp increment in EPS content was noticed with respect to control (Fig.1B). The EPS synthesized by cyanobacteria either are secreted in surrounding medium or attached with cell wall, which mainly functions to protect the cell from environmental stresses like heavy metals and pesticides (Tchounwou et al., 2012; Tiwari et al., 2020). Due to its strong anionic nature (bearing sulphate groups and uronic acids), it is suitable candidate in blocking the entry of toxic heavy metals like cadmium (Jittawuttipoka et al., 2013). In addition, EPS also provides microenvironment to maintain the level of essential elements, which are in limited concentration in surroundings. Therefore, our findings suggests that SA, Ca^{2+} and NO might have induced the EPS level to restrict/ limit the Ni^{2+}availability to the test organism as is also evident from biochemical detection for intracellular Ni^{2+} accumulation (Fig. 1D). But this increment in EPS content was further arrested upon c-PTIO and EGTA application thereby suggesting that in absence of Ca^{2+} or/ and NO, SA was unable to protect the cell from Ni^{2+}-induced toxicity.

3.3. SA, Ca^{2+} and NO revive Ni^{2+}-induced losses of photosynthetic pigments, up-regulate photosynthetic rate and PSII photochemistry and down-regulate respiratory rate

To further interpret the SA induced Ca^{2+} and NO signaling on photosynthetic activity, photosynthetic pigments: chlorophyll *a* (Chl *a*), carotenoids (Car), and phycobiliproteins [phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE)] were studied (Table 1). Nickel at its tested dose caused a significant reduction in the photosynthetic pigments content probably due to degradation of precursors or pigments' biosynthesis itself as argued by Prasad et al. (2005); that consecutively decreased the photosynthetic capacity of tested cyanobacteria, which was in conformity with the reduced DW of tested organism (Fig. 1A). SA, $CaCl_2$ and SNP treatment, on the other hand recovered the Ni^{2+}-induced damage in photosynthetic pigments content which could partly attributed to improvement either: (i) in the gene expression encoding protochlorophyllide oxidoreductase (Kusnetsov et al., 1998; Verma et al., 2018), or (ii) in the phytol isomers, necessary for pigment biosynthesis (Tiwari et al., 2019b). However, the Ni^{2+}-induced reduction in photosynthetic pigments content was worsened when Ni+SA treated *Anabaena* cells were supplemented with

c-PTIO+EGTA, which decreased the photosynthetic rate (Fig. 2A) and consequently the growth (Fig. 1A). The reduction in water soluble PC pigment content under Ni^{2+}-toxicity could be its external location on thylakoid membrane and thus easily bleached by ROS produced due to heavy metal (Tiwari et al., 2019a). However, SA, $CaCl_2$ and SNP treatment under similar condition significantly declined the ROS levels, which indirectly improved the PC content that ultimately favored photosynthetic activity than that of stressed condition alone (Fig. 2A). Ni^{2+} significantly reduced the photosynthetic rate which could be due to the reason that after entering into cell this divalent cation depolarizes the membrane by binding to it, thereby acidifying the cytoplasm and interferes with photosynthesis and respiration both as suggested by Balaji et al. (2013) in *Spirulina* strains.

Further, to deeply understand the SA induced Ca^{2+} and NO signaling in structural and functional properties of PSII, the biophysical traits deduced from OJIP transient curves were studied (Fig. 2C). Ni^{2+} exposure, strongly inhibited the oxygen evolution rate by 31% in comparison to control of *Anabaena* cells (Fig. 2A), indicate that photosynthetic apparatus especially the oxygen-evolving complex (OEC) was the main target of Ni^{2+}, which could be due to interaction of Ni^{2+} with 16 and 24 kDa extrinsic polypeptides causing conformational changes and provoked the Ca^{2+} release from OEC that ultimately inhibited electron transport activity as argued by Boisvert et al. (2007) or might have inactivated the OEC (decreased F_m). Further, upon c-PTIO or/ and EGTA supplementation to Ni^{2+}-stressed cyanobacteria cells, the increment in F_o/F_v(efficiency of water splitting complex/ OEC) values indicate the injurious effect on OEC, which could be due to obstruction in mineral uptake (like Ca^{2+} and Mn^{2+}) necessary for OEC functioning (Najafpour et al., 2012), that ultimately blocked electron transfer rate from donor side (OEC) to PSII (Kalaji et al., 2014; 2016; 2017).

In the present study, a drastic change in JIP-test parameters under Ni^{2+}-toxicity suggests that PSII was the primary target of Ni^{2+}. The radar plot reveals that Ni^{2+} addition in culture medium sharply increased the initial fluorescence (F_o) values, which could results from (i) dissociation of LHCII from RC complexes, (ii) back flow of electrons from plastoquinone (PQ) pool (Yamane et al., 2000), and (iii) decrease in size and number of active photosynthetic reaction centers (RCs) (F_v/F_o); which might have blocked the electron flow beyond Q_A- thus, higher F_o was measured in presence of Ca^{2+} and NO scavenger as was reported in amoxicillin-stressed *Synechocystis* sp. (Pan et al., 2008). While decrease in the variable fluorescence (F_v) and maximum fluorescence (F_m) values could be due to (i) decrease in size of plastoquinone (PQ) pool (F_v $/F_o$) (Fig. 2C) and (ii) hindrance in the movement of electron from PSII to the PQ pool thereby resulting into accumulation of a strong fluorescence quencher: $P_{680}{}^+$ (Govindjee, 1995), as revealed by decline in the *Area* (area over the fluorescence curve), which ultimately reduced maximum quantum yield for primary photochemistry (ΦP_o) (Fig. 2C). Therefore, reduced ΦE_o or Phi_E_o (yield of electron transport) and Ψ_o or Psi_o (efficiency to move the electrons of trapped exciton into ETC beyond Q_A-) were obtained, that leads to decreased Q_A- pool size on PSII acceptor side, thereby signifying the obstruction in electron flow from PSII to PSI and limitation in Q_A- reoxidation (Q_A–Q_A) rate, which is confirmed by the increased F_o/F_m values in the present study that actually reveals that Q_A- reoxidation (Q_A–Q_A) rate was much lower than its reduction rate by Q_B. Ni^{2+}-stressed *Anabaena* cells, showed little changes in the values of Ψ_o ; however, yield of primary electron transport (ΦP_o) and its product yield of electron transport (designated by ΦE_o) were sharply decreased in comparison to control thereby indicating that hindrance in electron transport was higher due to light dependent reaction (designated by ΦP_o) than dark reaction beyond Q_A- (Ψ_o), which collectively suggests that Ni^{2+} might have hampered the primary charge separation by the replacement of Mg^{2+} in the antennas' chlorophyll and PSII RCs as discussed by Kupper et al. (2003). Furthermore, the drop in above parameters was extreme when Ni+SA treated cyanobacterial cells were supplemented either with c-PTIO (Ni+SA+Ca+c-PTIO), EGTA (Ni+SA+NO+EGTA) or both (Ni+SA+c-PTIO+EGTA), suggesting that SA in absence of Ca^{2+} or/ and NO was unable to counteract Ni^{2+}-toxicity. Ni^{2+}-induced reduction in fluorescence yields ratio for open and closed states (F_m/F_o) signifies the destructive effect of the Ni on the structural integrity of PSII RCs. Further, the significant increase in S_m values upon treatments of Ca^{2+} and NO scavengers denotes that PQ heterogeneity fasten the electron transfer capacity as well as Q_A reduction on PSII acceptor side, thereby explaining that these treatments hampered the total electron accepting capacity

(Ghassemi-Golezani and Lotfi, 2015).

Further, upon c-PTIO or/ and EGTA treatment to Ni^{2+}-stressed *Anabaena* cells, the balance between active/ inactive RCs was disturbed, and the greater load might have been experienced by active RCs (F_v/F_o); therefore, increased values for energy flux parameters i.e. ABS/RC, TR_o/RC, ET_o/RC andDI_o/RC were noticed in the present study (Fig. 2C). These active RCs, absorbed the available light but were not able to collect the huge excitation energy and convert into 'energy sink' thereby dissipating most of the energy in the form of fluorescence/ heat as advocated by $\Phi P_o(F_v/F_m$) values; therefore, under similar conditions, the ABS/RC values were increased as limited number of active RCs increased the RC turnover rate to meet the complete reduction of PQ pool (N). The increment inTR_o/RC (Q_A-Q_A-) values upon c-PTIO or/ and EGTA treatment to Ni^{2+}-stressed test organism, might be due to inhibition in Q_A- reoxidation (Q_A–Q_A), which hampered the electron transfer efficiency to Q_B. The increasing values for dissipated energy flux (DI_o/RC) and the quantum yield of energy dissipation (ΦD_o) upon Ca^{2+} or NO scavenger supplementation to Ni^{2+}-stressed cells, justified that in order to maintain the equilibrium between absorption and consumption of energy, excess excitation energy was transformed into thermal dissipation as argued earlier by Wang et al. (2012); which drastically decreased the overall performance of PSII (PI_{ABS}) (Fig. 2C).

On the other hand, SA, $CaCl_2$ and SNP supplementation to Ni^{2+}-stressed culture medium repaired the structural integrity of PSII by decliningF_o/F_v and improvingF_v/F_o ,F_m/F_o andΦP_o values. The F_o values got lowered due to which increment in F_v was observed which suggested towards betterment of PSII acceptor side (Ghassemi-Golezani and Lotfi, 2015). Upon SA, $CaCl_2$ and SNP application, the recovery in F_m values elaborated that either they might have bring the conformational changes in D1 protein of PSII by modifying the properties of electron acceptors (Andréasson et al., 1995) or might improve the $Mn^{2+}$and OEC extrinsic proteins, which impeded electron donation from H_2O to PSII (Najafpour et al., 2012; Kalaji et al., 2016) and ultimately the PSII activity. While working on heat-stressed tall fescue plants, Chen et al. (2013) have suggested that NO either might have improved the gene expression of PSII complex like$psbA$, $psbB$ and $psbC$ or speeded electron transportation from OEC to D1 protein. The possibility behind the Ca^{2+}-induced recovery in Ni^{2+}-stressed *Anabaena* cells could be: (i) improvement in the pigment contents, which actively participates in the assembly of PSII to support the proper functioning (Zakar et al., 2016), and (ii) improvement in intracellular Ca^{2+}-accumulation, which is essential cofactor for water oxidation, the Ca binding sites in PSII, and the transition of all S-state, as was assumed by Najafpour et al. (2012). In addition, a steep decline in ABS/RC , TR_o/RC ,ET_o/RC and DI_o/RC values under similar conditions, justified that PSII complex was enough capable to handle the energy equilibrium between absorption and utilization of light energy by active PSII RCs thereby minimizing the potential for photo-oxidative damage; which finally improved the overall performance of PSII as higher PI_{ABS} values were observed in the present study (Fig. 2C).

The significant increment in respiratory rate upon c-PTIO or/ and EGTA application to Ni^{2+}-stressed *Anabaena* cells (Fig. 2B) was probably to meet out the basic demand of ATP to carry out basic metabolism of cell (Tiwari et al., 2019a), as photosynthetic electron transport activities were hampered by reducing ATP supply under similar conditions (Fig. 2A,C).

3.4. SA, Ca^{2+} and NO recover Ni^{2+}-induced damage in nitrogen metabolism status

Nitrogen metabolism is an important event which directly affects growth of the organisms. The inorganic nitrogen contents and related enzyme activities were differentially affected by Ni^{2+}, SA, Ca^{2+}, NO, and c-PTIO or/ and EGTA. Upon Ni^{2+} exposure, *Anabaena* cells exhibited a decrease upto 34 and 29% in NO_3- and NO_2- uptake rate, respectively; however these were recovered after SA, $CaCl_2$ and SNP supplementation. Interestingly, this recovery in NO_3- and NO_2- uptake rate was further arrested when c-PTIO or/ and EGTA were added in culture medium (Fig. 3A,B). This inhibition in NO_3- and NO_2- uptake rate could be due to: (i) alteration in cellular homeostasis and membrane permeability as increased ROS levels, lipid peroxidation and electrolyte leakage was observed in the present study (Fig. 4), and (ii) impaired photosynthetic ETC decreased the photosynthetic rate (Fig. 2) and consequently the ATP pool, which is used as energy source by ABC-type transporter proteins that are essential for facilitating the entry of NO_3- and NO_2- in cyanobacterial

cells (Flores and Herrero, 1994); therefore, Ni induced reduction in NO_3- and NO_2- uptake rate could be credited to decreased ATP pool, which is an outcome of impaired ETC as advocated by Rai et al. (1995) in Cu and UV-B stressed *Anabaena doliolum* . The two important enzyme of N-metabolism i.e. NR and NiR by successive reactions convert NO_3- into NO_2- and then NO_2- into highly toxic NH_4^+, respectively. Results revealed that both NR and NiR enzyme activities were sharply decreased upon Ni^{2+} exposure which was aggravated when Ni+SA treated cells were supplemented with c-PTIO or c-PTIO+EGTA (Fig. 3C,D). Ohmori and Hattori (1970) have proposed that NO_3-induces NR activity while NO_2- induces NiR activity; therefore decline in NR and NiR activities could be directly interrelated with decreased NO_3^- and NO_2- uptake rate, (Fig. 3A,B). Another possibility behind this could be that NR needed sulfhydryl (-SH) groups for its catalytic action as suggested by Sharma and Dubey (2005); therefore Ni^{2+} by binding with -SH groups of the active sites of the enzyme might have altered the NR activity (Fig. 3C). Besides this, the limited availability of NO_2- ions which mainly arises from NR-catalyzed NO_3- reduction, might have decreased the NiR activity (Fig. 3D). Further, upon SA, $CaCl_2$ and SNP application to the culture medium, a significant alleviation in Ni^{2+}-induced decrease in enzyme activities of cyanobacterial cells was noticed thereby suggesting the improvement in the NO_3- and NO_2- uptake rate; while the sharp decrease in enzyme activities upon c-PTIO or c-PTIO+EGTA supplementation to Ni+SA treated cells suggest that in absence of Ca^{2+} and/ or NO, SA was unable to cope up with the Ni^{2+}-induced toxic effect. Due to reduced NR and NiR activities, toxic NH_4^+accumulate in the cells, which rapidly is assimilated into organic compounds by the activity of glutamine synthetase (GS) enzyme, a primary route of NH_4^+ assimilation in cyanobacteria. Ni^{2+}-stress significantly declined the GS activity by 33% in respect to control value (Fig. 3E), which could be due to the oxidative modifications of the enzyme. The GS along with GOGAT enzyme produces glutamate to synthesize numerous amino acids; therefore, decreased GS and GOGAT activities under Ni^{2+}, Ni+SA+Ca+c-PTIO and Ni+SA+c-PTIO+EGTA, may carbon/ nitrogen homeostasis and consequently the growth of the cyanobacteria (Fig. 1A). Our results are in corroboration with the findings of Rai et al. (1998). The GS and GOGAT activity needed photosynthesis products ATP and NADPH (Muro-Pastor et al., 2003), for its functioning; therefore, decreased photosynthetic rate (Fig. 2A) might have decreased the ATP pool and NADPH and hence the GS activity. However, Ni^{2+}-induced reduction in GS and GOGAT activity was appreciably alleviated by SA, $CaCl_2$ and SNP treatment (Fig. 3E,F) indicating the betterment in NH_4^+-assimilation and its incorporation into glutamate compared to Ni^{2+} as well as Ca^{2+} and NO scavenger treated cells. In contrast to GS and GOGAT, aminating activity of GDH was sharply increased under Ni^{2+}-stress (62%) as well as Ni+SA+c-PTIO+EGTA (68%) treated cells in respect to control (Fig. 3G), which might have occurred to improve the NH_4^+-assimilation, that was inhibited due to decreased GS and GOGAT activity. Indeed Skopelitis et al. (2006) have also reported that during stress when GS/GOGAT cycle for NH_4^+-assimilation doesn't operate efficiently, then GDH activity increases to relieve the pressure of NH_4^+-accumulation and also to provide the glutamate for the biosynthesis of Pro like protective compounds. On the other hand, SA, $CaCl_2$ and SNP addition to Ni^{2+}-stressed culture medium showed decrease in GDH activity while improvement in the activities of GS/GOGAT enzymes, which may be due to decreased NH_4^+ content that shifted the cycle towards GS/GOGAT pathway.

3.5. SA, Ca^{2+} and NO recover antioxidant system to neutralize Ni^{2+}-induced oxidative stress

To define the interaction between SA, Ca^{2+} and NO in alleviating the detrimental effect of Ni^{2+} in*Anabaena* cells, the oxidative stress status of cyanobacteria was investigated. In the present study, excessive Ni^{2+}accumulation revealed severe oxidative stress as evident by increased ROS (O_2^{*-} and H_2O_2: 33 and 37%, respectively) levels, which consequently lead to oxidative injuries credited by lipid peroxidation and membrane damage as increased levels of MDA equivalents and electrolyte leakage were observed in the present study (Fig. 4) and hence decreased the growth of cyanobacteria. The possibility behind this could be the blockage in ETC of PSII, due to which most of the electrons slipped out and reacts with available molecular oxygen to produce O_2^{*-}. Prasad et al. (2005) and Jahan et al. (2020) have also reported the increased levels of ROS, MDA formation and electrolyte leakage in Ni^{2+} or/ UV-B stressed *Glycine max* L. and tomato seedlings, respectively due to higher Ni^{2+} accumulation. Further, SA, Ca^{2+} and NO addition to the culture medium counteracted the Ni^{2+}-induced loss in cell structure and function by decreasing ROS

levels and the indices of damage as manifested by decreased Ni^{2+} accumulation and increased NO content (Fig. 1) as was also reported by Tiwari et al. (2019a) and Singh et al. (2020a,b) in aluminium and As-stressed*Anabaena* and mustard seedlings, respectively. The increased SA, Ca^{2+} and NO might be involved in inducing several ROS-scavenging enzyme activities such as SOD, CAT, POD and GST (Fig. 5) as was discussed by Tiwari et al. (2019a,b) or increased NO content might have been involved in the formation of less toxic peroxynitrite (ONOO-) as was discussed by Peto (2011) in Cu-stressed auxin supplemented *Arabidopsis* seedlings. Interestingly, c-PTIO or/ and EGTA application to Ni+SA stressed cyanobacteria arrested the Ca^{2+} and NO induced effect in Ni^{2+}-stressed cells, which was further confirmed by histochemical visualization by staining SOR, H_2O_2, lipid peroxidation and injury of plasma membrane integrity (more dense color under $Ni^{2+}$and c-PTIO or/ and EGTA supplemented cells; however, upon SA, $CaCl_2$ and SNP supplementation to Ni^{2+}-stressed cells showed comparatively less intense color) (Fig. 4). While working on chromium-stressed *Nostoc muscorum* , Tiwari et al. (2018) have reported that kinetin recovered the growth of Cr-stressed cyanobacteria by up-regulating the activities of enzymatic antioxidants. Indeed in the present study, an increasing trend in the activities of enzymatic antioxidants: SOD, POD, CAT and GST was noticed that could have helped to overcome the damaging effect of Ni^{2+}, and the activity of these enzymes was further increased upon SA, $CaCl_2$ and SNP addition but this increment in the enzyme activities was arrested after c-PTIO or/ and EGTA treatment, most importantly under Ni+SA+Ca+c-PTIO and Ni+SA+c-PTIO+EGTA, and it was even less than that of control (Fig. 5). The SOD, POD and CAT act first fence against stress, which dismutate O_2^{*-} into H_2O_2 and consecutively into H_2O; therefore, increment in these enzyme activities might have speeded the reduction in ROS accumulation, but it was inadequate to overcome the huge Ni^{2+}-induced c-PTIO and EGTA mediated ROS accumulation; thus still high levels of ROS were noticed (Fig. 4), especially under c-PTIO or/ and EGTA. Similarly, glutathione-*S* -transferases (GST) enzyme regulates Ni^{2+}-toxicity by eliminating xenobiotics through conjugation with GSH; therefore, increase in GST activity (Fig. 5D) might have reduced the availability of Ni^{2+} through its conjugation, hence might helped the cyanobacteria cells to overcome the stress situation. These findings suggest the regulatory role of Ca^{2+} and NO in SA induced signaling. Our results are in accordance with the earlier findings of Tiwari et al. (2018; 2019a,b) and Singh et al. (2018a, 2020a) in *Nostoc muscorum* and mustard seedlings, respectively.

3.6. SA, Ca^{2+} and NO rescue Ni^{2+}-induced damage by up-regulating non-enzymatic antioxidants

The non-enzymatic antioxidants: Cys, NPTs and Pro act as barrier against metal toxicity; which were negatively regulated by Ni^{2+}-stress (Fig. 6), while upon SA, $CaCl_2$ and SNP addition, a sharp increment in the these antioxidants was noticed, which might be due to their increased activation after perceiving NO signal or de-novo synthesis (Sun et al., 2018). This increment in antioxidants was arrested after c-PTIO or/ and EGTA treatment which was even less than that of untreated control except Ni+SA+NO+EGTA treatment (Fig. 6), which could be due to their greater demand to (i) stabilize the tertiary structures of protein, (ii) synthesize proteins, (iii) synthesize GSH, (iv) Fe-S cluster of photosynthetic apparatus, and (v) synthesize phytochelatins (PCs), which decreases metal availability by binding with them (Foyer and Noctor, 2005) to counteract the Ni^{2+}-stressed situation as was also reported by Singh et al. (2012) in Cu-stressed cyanobacteria. The increment in Pro accumulation under stress is an adaptive mechanism either to detoxify ROS by chelation or as an antioxidant (Zhang et al, 2008). The arresting of these antioxidants after c-PTIO or/ and EGTA addition, confirmed the role of Ca^{2+} and NO in regulating the SA induced antioxidant defense system to combat the Ni^{2+}-toxicity.

Figure 7(A) reveals the correlation of growth with that of different physiological and biochemical parameters. DW is positively correlated with the biomass and the biomass had positive correlation with that of parameters related to photosynthesis, nitrogen metabolism (except GDH) and antioxidants, while negatively correlated with the oxidative stress markers, respiration and GDH. Among different extracted components that determined the significant variance among studied parameters, the maximum contribution is of F1 (87.48) followed by F2 (9.72) with cumulative contribution (97.21) of both factors. If we analyze the impact on the biomass with respect to treatments (Fig. 7B), Ni, Ni+SA+Ca+c-PTIO, and Ni+SA+c-PTIO+EGTA were found to be negatively correlated with the biomass, while remaining treatments were positively correlated

or showed some varied results.

Conclusion

From the present study, the conclusion could be drawn that Ni^{2+} inhibits overall growth of nitrogen fixing*Anabaena* cells; while SA, $CaCl_2$ and SNP addition significantly recovered the growth and growth regulating processes (Fig. 8). However, upon supplementation of NO (c-PTIO) or/ and Ca^{2+} (EGTA) scavenger to Ni+SA+Ca, Ni+SA+NO or Ni+SA stressed *Anabaena* cells, the improvement in growth caused by SA, Ca^{2+} and NO was further arrested, which suggests that Ca^{2+} and NO both are the part of SA induced signaling event. But the steep decline in growth and growth regulating attributes: EPS secretion, pigments, photosynthetic rate, nitrogen metabolism status and antioxidants system was mainly observed under Ni+SA+Ca+c-PTIO than that of Ni+SA+NO+EGTA treatment even in presence of SA, thereby suggesting that SA induced positive impact on physiological and biochemical traits of *Anabaena* cells are mostly regulated by Ca^{2+} mediated NO signaling (Fig. 8).

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Author contributions

SP, NV and RS designed the experiments. NV and AP performed the experiment. SP and NV finalized the data. RS and PP wrote and finalized the manuscript.

Competing interests

The authors declare no competing interests.

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Fig. 1. Effect of SA, $CaCl_2$ and SNP on**(A)** growth, **(B)** exopolysaccharides (EPS) content,**(C)** NO content, and **(D)** Ni accumulation in Ni-stressed*Anabaena* Sp. PCC 7120 subjected to c-PTIO (a NO scavenger; 2-4-carboxyphenyl-*4,4,5,5* -tetramethylimidazoline-1-oxyl-3-oxide) and EGTA (a Ca scavenger; ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'* -tetraacetic acid), after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at$p<0.05$ level according to DMRT (Duncan's multiple range test).

Fig. 2. Effect of SA, $CaCl_2$ and SNP on**(A)** photosynthesis and **(B)** respiration rate, and**(C)** the JIP-kinetics obtained from polyphasic fast chlorophyll*a* fluorescence in Ni-stressed *Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at $p<0.05$ level according to DMRT.

Fig. 3. Effect of SA, $CaCl_2$ and SNP on**(A-B)** nitrate (NO_3^-) and nitrite (NO_2^-) uptake rate, and the enzyme activities of: **(C)** nitrate reductase (NR), **(D)**nitrite reductase (NiR), **(E)** glutamine synthetase (GS),**(F)** glutamate synthase, and **(G)** glutamate dehydrogenase (GDH) in Ni-stressed *Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at $p<0.05$ level according to DMRT.

Fig. 4(a). Effect of SA, $CaCl_2$ and SNP on the contents of **(A)** superoxide radical (SOR: O_2^{*-}), **(B)** hydrogen peroxide (H_2O_2), and **(C)** malondialdehyde (MDA) equivalents in Ni-stressed *Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at $p<0.05$ level according to DMRT.

Fig. 4(b). *In-vivo* visualization of SOR, H_2O_2, lipid peroxidation (MDA), and electrolyte leakage (EL) in Ni-stressed *Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments.

Fig. 5. Effect of SA, $CaCl_2$ and SNP on the activities of enzymatic antioxidants: **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), and**(D)** glutathione-*S* -transferase (GST) in Ni-stressed*Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at $p<0.05$level according to DMRT.

Fig. 6. Effect of SA, $CaCl_2$ and SNP on the activities of non-enzymatic antioxidants: **(A)** cysteine (Cys),**(B)** proline (Pro), and **(C)** non-protein thiols (NPTs) in Ni-stressed *Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Bars followed by different letters have significant difference at$p<0.05$ level according to DMRT.

Fig. 7. Principal component analysis **(A)** of different growth, physiological as well as biochemical parameters of Ni-stressed*Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments, and **(B)** Biplot of treatments with that of studied parameters. DW= dry weight, PS= photosynthesis, RS= respiration, CAR= carotenoids, CHLA= chlorophyll *a* , EPS= exopolysaccharides, NO= nitric oxide, NR= nitrate reductase, NiR= nitrite reductase, NO_3= nitrate uptake rate, NO_2= nitrite uptake rate, GS= glutamine synthetase, GDH= glutamate dehydrogenase, SOR= superoxide radical, H_2O_2= hydrogen peroxide, MDA= malondialdehyde equivalents, SOD= superoxide dismutase, POD= peroxidase, GST= glutathione-s-trasnferase, CAT= catalase, Pro=proline, Cys= cysteine, and NPT= non-protein thiol.

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Fig. 1

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Fig. 2

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Fig. 3

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Fig. 4(a)

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Fig. 4(b)

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Fig. 5

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Fig. 6

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Fig. 7(A)

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Fig. 7(B)

Table 1: Effect of SA, $CaCl_2$ and SNP on the photosynthetic pigments: chlorophyll *a* (Chl *a*) and carotenoids (Car), and phycobiliproteins: phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) in Ni-stressed*Anabaena* Sp. PCC 7120 subjected to c-PTIO and EGTA, after 72 h of experiments. Data signifies the mean+-standard error of three replicates, each with three independent experiments (n=3). Values within same column followed by different subscripts have significant difference at$p<0.05$ level according to DMRT.

Treatments	Photosynthetic pigments µg (mg dry weight)$^{-1}$	Photosynthetic pigments µg (mg dry weight)$^{-1}$	Phycobiliproteins µg (mg dry weight)$^{-1}$	Phycobiliproteins µg (mg dry weight)$^{-1}$	Phycobiliprote µg (mg dry weight)$^{-1}$
	Chlorophyll *a* (Chl *a*)	**Carotenoid (Car)**	**Phycocyanin (PC)**	**Allophycocyan (APC)**	**Phycoerythr (PE)**
Control	12.27 ± 0.21^{a}	6.01 ± 0.10^{a}	50.03 ± 0.86^{c}	7.40 ± 0.12^{d}	7.41 ± 0.12^{d}
Ni	8.62 ± 0.15^{e}	4.30 ± 0.07^{e}	40.71 ± 0.70^{d}	5.33 ± 0.09^{f}	6.56 ± 0.11^{e}
Ni+SA	10.82 ± 0.19^{cd}	5.41 ± 0.09^{c}	54.68 ± 0.94^{b}	8.44 ± 0.14^{bc}	8.44 ± 0.14^{bc}
Ni+Ca	10.99 ± 0.19^{c}	5.59 ± 0.09^{bc}	55.31 ± 0.95^{ab}	8.68 ± 0.15^{ab}	8.80 ± 0.15^{ab}
Ni+NO	11.57 ± 0.20^{b}	5.70 ± 0.09^{b}	57.65 ± 0.99^{a}	9.00 ± 0.15^{a}	9.04 ± 0.15^{a}
Ni+SA+Ca+c-PTIO	8.46 ± 0.15^{e}	4.15 ± 0.07^{e}	47.81 ± 0.82^{c}	6.77 ± 0.11^{e}	7.52 ± 0.13^{d}
Ni+SA+NO+EGTA	10.32 ± 0.18^{d}	5.12 ± 0.08^{d}	57.15 ± 0.98^{ab}	8.24 ± 0.14^{c}	8.19 ± 0.14^{c}
Ni+SA+c-PTIO+EGTA	7.32 ± 0.13^{f}	3.45 ± 0.05^{f}	37.72 ± 0.65^{e}	4.72 ± 0.08^{g}	5.62 ± 0.09^{f}

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