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Regulation of redox homeostasis in cadmium stressed rice field cyanobacteria by exogenous hydrogen peroxide and nitric oxide

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In the present study, defensive strategies of H₂O₂ mediated NO signaling were analyzed in Cd stressed *Nostoc muscorum* and *Anabaena* sp. Exogenously supplied SNP (10 μM) and H₂O₂ (1 μM) lessen the toxicity of Cd (6 μM) but without NO; H₂O₂ was unable to release the stress from cyanobacterial cells potentially. The reduced contents of exopolysaccharide, protein content, endogenous NO and enzymatic antioxidants (SOD, POD, CAT, and GST) due to Cd toxicity, were found increased significantly after exogenous application of H₂O₂ and SNP thereafter, cyanobacterial calls flourished much better after releasing toxic level of Cd. Moreover, increased level of ROS due to Cd stress also normalized under exogenous application of H₂O₂ and SNP. However, chelation of NO hindered the signaling mechanism of H₂O₂ that diminished its potential against Cd stress while signaling of NO has not been hindered by chelation of H₂O₂ and NO potentially released the Cd stress from cyanobacterial cells. In conclusion, current findings demonstrated the synergistic signaling between H₂O₂ and NO towards the improvement of cyanobacterial tolerance to Cd stress, thereby enhancing the growth and antioxidant defense system of test cyanobacteria that improved fertility and productivity of soil even under the situation of metal contamination.

Cadmium (Cd) contamination in agricultural fields like rice crop fields has become a serious environmental issue. Potentially active Cd ions in the soil can accumulate in rice plants and ultimately reduce the uptake and translocation of vital minerals and nutrients for plants^{1–3}. Cd ions in agricultural soil can also negatively affect the beneficial microbes present in the rice field⁴. Cyanobacteria are pioneer phototrophic prokaryotic microorganisms that are known to display their potential role in sustainable agricultural development especially for paddy fields. *Nostoc muscorum* and *Anabaena* sp. are categorized as heterocystous cyanobacteria and known for their nitrogen-fixing ability. They are mainly present on soil and water bodies and fix about 20–25 kg/ha atmospheric nitrogen. More efficiently, *Anabaena* sp. can fix about 60 kg/ha of nitrogen in a season⁵. Unfortunately, Cd ions present in rice soil are major threats to these nitrogen fixers. Previous studies demonstrated that some photosynthetic bacteria can tolerate up to 1 mg Cd kg⁻¹ soil in the association with Hay bacillus (*Bacillus subtilis*) and some other lactic acid bacteria⁶. But the release of Cd is much higher (200 mg Cd kg⁻¹ soil) in the soils near the leather and electro-plating factories⁷ that creates a hazardous environment for paddy field cyanobacteria which ultimately results to the poor quality of rice crop. Cd interrupts the cell functioning and hampers the regular metabolic processes of cyanobacteria. Thus, Cd ions can enter into the cell through P-type Cd²⁺ ATPases (e.g. *cadA1* and *cadA2*) and interrupt electron transport chain and produce excessive ROS (reactive oxygen species) thereby disturbing the balance between antioxidants and ROS which tend to the cell death⁸. Study of Qiao et al.⁹ shows that there are three possible ways through which Cd imposes ROS accumulation in cells: (1) excess level of Cd²⁺ enhances the expression of miR398 that ultimately inhibits the functioning of Cu/Zn/SOD and finally, a drastic rise of ROS; (2) excess Cd²⁺ in cells can inhibit the regulatory role of second messengers like Ca²⁺, or other signaling molecules like NO and H₂O₂; (3) excess accumulation of Cd²⁺ enhances the activity of NADPH oxidase, that is a major producer of ROS. So to enhance the tolerance against different environmental stresses the external applications of different signaling molecules like H₂O₂, NO, and H₂S, are considered as one of the most beneficial methods¹⁰.

NO and H₂O₂ are the potentially active and key biological signaling molecules known for the plant defense. At a low and very specific concentration, H₂O₂ and NO work as signal messengers for cell communication during

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stress situations. Tiwari et al.¹¹ reported that exogenous supplementation of SNP (a donor of NO) reduced the level of ROS which is produced by aluminium stress in *Anabaena* PCC 7120. Verma et al.¹² reported that in stress situation the increased ROS ($O_2^{\cdot-}$, H_2O_2 , $\cdot OH$, 1O_2) level promotes the production of reactive nitrogen species (RNS: NO, ONOO⁻, N_2O_3 , NO_2) that finally control the balancing of antioxidants to protect the plants from severe damage. Several recent findings show the existence of several signaling topologies between NO and H_2O_2 .¹³ It has been reported that H_2O_2 mediates some other signaling molecules like NO or H_2S to regulate oxidative stress.¹⁴ Hasanuzzaman et al.¹⁵ suggested that Cd toxicity has been removed expressively by pretreatment of H_2O_2 in the seedlings of *Brassica napus*. Pretreatment of H_2O_2 possibly empowers the internal defense system of the organism, but its downstream signal transduction mechanisms and pathways are not effusively clear in cyanobacteria. The defense strategies in organisms are a much complex process in which different signaling molecules are linked together as reported in the study of Li et al.¹⁶; it is proven that there is a multifaceted interaction among NO, H_2S , and H_2O_2 promoting thermotolerance in maize seedlings. Christou et al.¹⁷ also noticed a faster and stronger response against salt stress by the priming of H_2O_2 and SNP (donor of NO) in strawberry plants. Gonzalez et al.¹⁸ have also revealed the cross linking mechanism of calcium, H_2O_2 , and NO in copper treated *Ulva compressa*. However, in the case of cyanobacteria to the best of our knowledge the interacting role of signaling molecules H_2O_2 and NO in Cd stress alleviation is lacking. Therefore, we have hypothesized that synergistically H_2O_2 and NO could initiate certain protective mechanisms that may be associated with metal stress particularly Cd tolerance in cyanobacteria.

Hence, the prime focus of this study was to evaluate the potential role of H_2O_2 and NO as defense signaling molecules to cadmium stress tolerance in cyanobacteria *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120. Also a key objective was to explore the probable mechanism and connective pathway between H_2O_2 and NO.

Results

Effect of H_2O_2 and NO on growth of Cd stressed cyanobacteria. In the present study growth of both the tested cyanobacteria was measured in terms of dry weight as shown in Fig. 1a,b which depicted that 6 μM Cd inhibited the growth by 28% in *Nostoc muscorum* ATCC 27893 and 30% in *Anabaena* sp. PCC 7120 (hereafter referred as *Nostoc muscorum* and *Anabaena* sp.) in comparison to control. However, on exogenous supplementation of H_2O_2 , growth was recovered and inhibition was noticed only 9 and 11% respectively for *Nostoc muscorum* and *Anabaena* sp. Similar to this, exogenous SNP significantly reduced ($P < 0.05$) the inhibition exerted by Cd, and found only 11 and 7%, respectively as compared to control. Further, to know the regulatory role of endogenous ROS and NO on growth, Cd treated cells were subjected to NAC, DPI, PTIO and l -NAME; therefore, a greater reduction in dry weight was noticed under PTIO and l -NAME treatment in comparison to control (Fig. 1b). Furthermore, to clarify the cross-talk of H_2O_2 and NO, cells were treated with scavengers (NAC and PTIO, respective scavengers of H_2O_2 and NO) and inhibitors (DPI and l -NAME, respective inhibitors of NADPH oxidase and nitric oxide synthase (NOS) enzymes) with Cd stress. In this case, our results showed a critical decline in growth under treatments of PTIO or l -NAME i.e. 41 and 44% respectively in *Nostoc muscorum* and 44 and 46% respectively in *Anabaena* sp. even in the presence of H_2O_2 . Contrastingly, growth was found improved moderately under SNP treatment even in the presence of NAC and DPI (Fig. 1a). Moreover, in a combined treatment of both the signaling molecules, H_2O_2 and SNP along with both PTIO and l -NAME under the same stress condition, inhibition in growth again found crucial i.e. 38 and 39% in *Nostoc muscorum* and *Anabaena* sp. respectively. Whereas, under similar stress, combined treatment of H_2O_2 and SNP along with NAC and DPI inhibition in growth was reduced and found only 12 and 14% in *Nostoc muscorum* and *Anabaena* sp. respectively on comparison to control.

Effect of H_2O_2 and NO on the secretion of exopolysaccharide (EPS) layer during Cd stress. Impact of exogenous supplementation of H_2O_2 and SNP on secretion of defensive layer of EPS in test cyanobacteria has been portrayed in Fig. 1c. In both the test cyanobacteria *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 secretion of the EPS layer was declined significantly ($P < 0.05$) by 23 and 25% respectively under Cd treatment in contrast to control values (Fig. 1c). Under the treatment of Cd + H_2O_2 and Cd + SNP, the EPS secretion was enhanced significantly ($P < 0.05$) by 20 and 24% respectively in *Nostoc muscorum* and 17 and 19% respectively in *Anabaena* sp. comparative to control values. Contrastingly, under the same stress, the decline in EPS secretion was found more critical under the treatments of PTIO or l -NAME i.e. 28 and 43% respectively in *Nostoc muscorum* and 33 and 51% respectively in *Anabaena* sp. even in the presence of H_2O_2 . Whereas, treatments of NAC or DPI along with SNP do not hinder the signaling mechanism of NO and an increased EPS content was noticed i.e. 11 and 8% respectively in *Nostoc muscorum* and 10 and 7% respectively in *Anabaena* sp. Moreover, combined treatments of H_2O_2 and SNP along with PTIO and l -NAME under the same stress crucially declined the EPS content that showed the incapability of H_2O_2 without NO. On the other hand, enhanced EPS content was noticed on the combined treatment of H_2O_2 and SNP along with NAC and DPI under the same stress showed the potential of NO without H_2O_2 in the removal of the toxic impact of Cd on EPS secretion.

Effect of H_2O_2 and NO on protein content in Cd stressed cyanobacteria. The results pertaining to the effect of H_2O_2 and SNP supplementation on the protein content in Cd challenged *N. muscorum* and *Anabaena* sp. have been depicted in Fig. 1d. Cd at 6 μM doses significantly decreased the protein content by 32% in *N. muscorum*, and by 37% in *Anabaena* sp. respectively, in comparison to control values. However, exogenous supplementation of H_2O_2 and SNP to Cd stressed cyanobacteria, considerably lowered the inhibitory effect of Cd on protein content but values were still less than control. Contrastingly, all the positive effects of H_2O_2 and

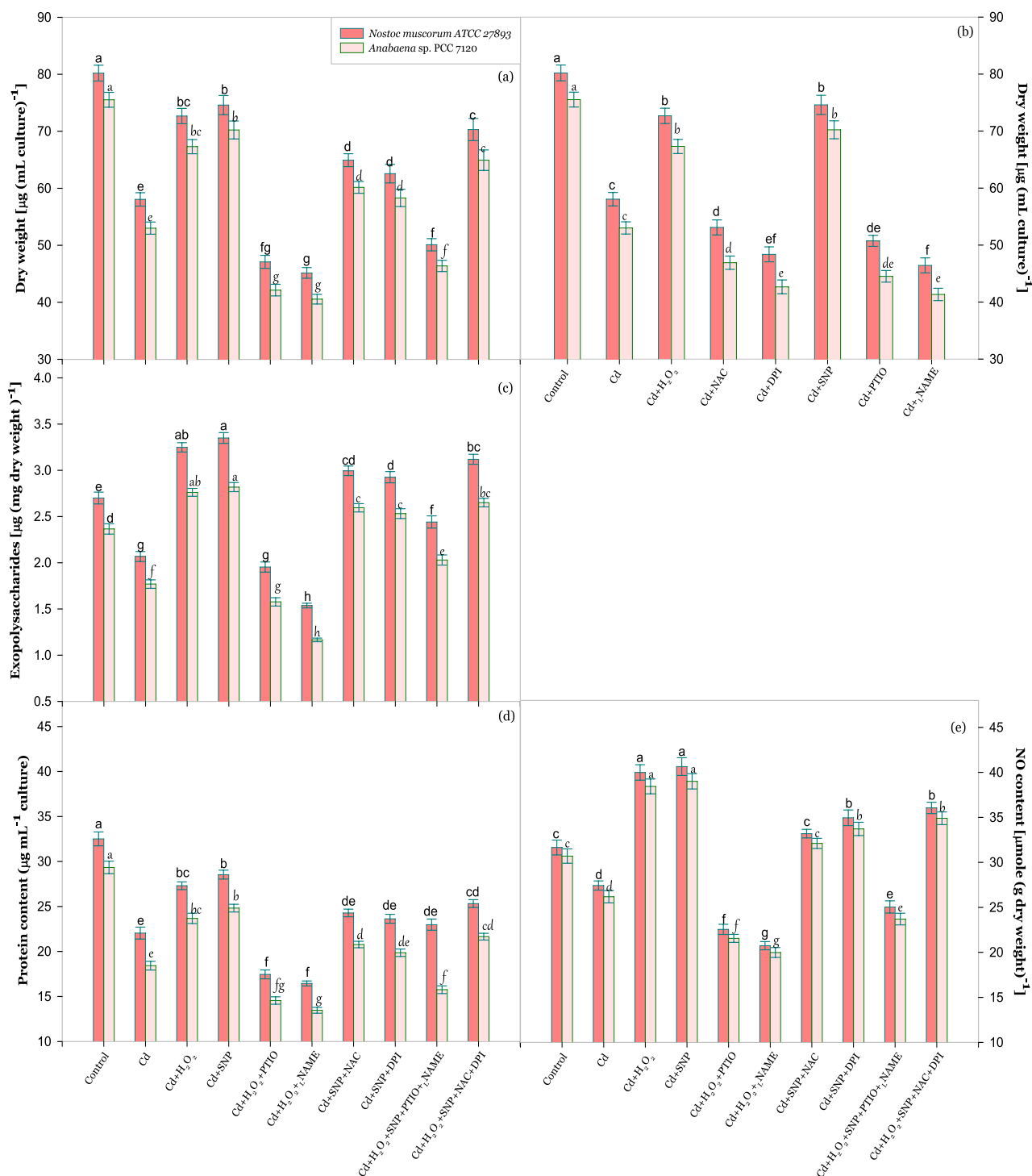


Figure 1. Effect of H₂O₂ and NO (SNP) on growth (a,b) and contents of exopolysaccharides (c), protein (d) and endogenous NO (e) of *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 exposed to Cd after 24 h of treatment. Data presented are means \pm standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters represent significant difference at P < 0.05 significance level according to the DMRT.

SNP on protein content were reversed on the application of PTIO and L-NAMF. Whereas application of NAC and DPI does not alter the role of SNP on protein content.

Effect of exogenous H₂O₂ and NO on the content of endogenous NO during Cd stress. The impact of exogenously supplied H₂O₂ and SNP on the endogenous level of NO have been depicted in Fig. 1e. The 6 μM Cd reduced the NO accumulation significantly (P < 0.05) by 13 and 15% in *Nostoc muscorum* and *Ana-*

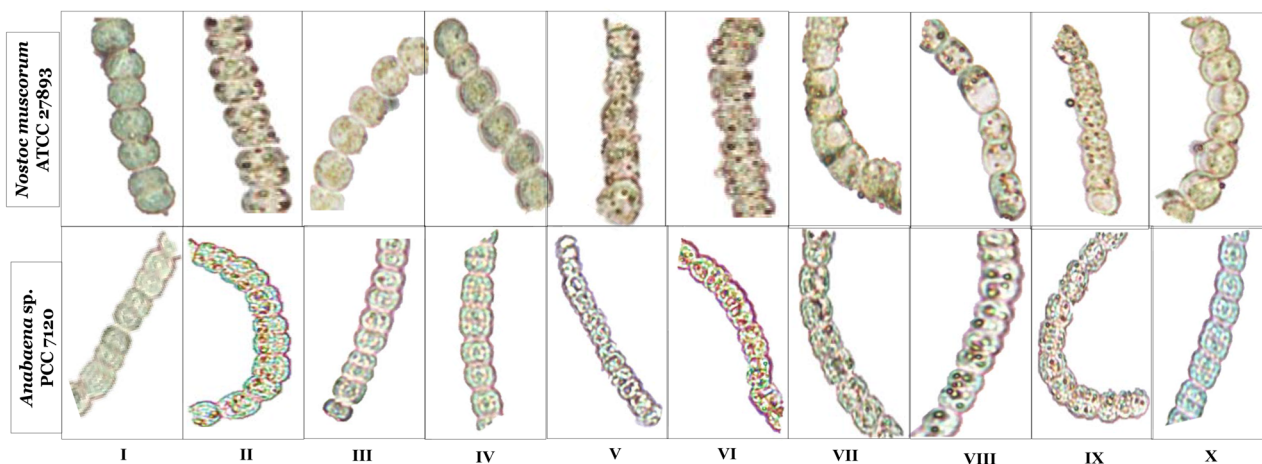


Figure 2. Histochemical analysis of Cd accumulation; red patches inside the cells of Cd stressed *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 exposed to H_2O_2 and NO (SNP). Where lane I: Control, lane II: Cd, lane III: Cd + H_2O_2 , lane IV: Cd + SNP, lane V: Cd + H_2O_2 + PTIO, lane VI: Cd + H_2O_2 + l_1 NAME, lane VII: Cd + SNP + NAC, lane VIII: Cd + SNP + DPI, lane IX: Cd + H_2O_2 + SNP + PTIO + l_1 NAME, lane X: Cd + H_2O_2 + SNP + NAC + DPI.

baena sp. respectively. Contrastingly, exogenously supplied H_2O_2 significantly ($P < 0.05$) enhanced the content of endogenous NO by 26 and 25% and more pronouncedly SNP enhanced ($P < 0.05$) the NO content inside the cell by 28 and 27% in *Nostoc muscorum* and *Anabaena* sp. respectively. Under similar stress, a very severe reduction in NO content was noticed on the separate treatments of PTIO and l_1 NAME even in the presence of H_2O_2 . In contrary to this exogenous supplementation of SNP slightly improved NO content even in the presence of NAC and DPI (Fig. 1e). Moreover, under the same stress, combined treatment of H_2O_2 and SNP along with PTIO and l_1 NAME critically declined the NO content by 21 and 23% in *Nostoc muscorum* and *Anabaena* sp. respectively while endogenous NO content was found enhanced by 14 and 13% in *Nostoc muscorum* and *Anabaena* sp. respectively on combined exposure of H_2O_2 and SNP along with NAC and DPI under the same stress.

Effect of H_2O_2 and NO on intracellular Cd accumulation under Cd stress. Histochemical analysis of Cd inside the cells was observed in the form of red patches which were the insoluble red salt that appeared due to the complex of dithizone with Cd. Results pertaining to the in vivo visualization of Cd accumulation in both the tested organisms have been portrayed in Fig. 2. The appearance of intense red patches inside the vegetative cells of cyanobacteria showed the accumulation of Cd inside the cells. In Cd treated cells, red patches are found more intense than H_2O_2 and SNP treated cells. Whereas in control, no red spots have appeared. Critically intense red patches appeared in the cells under treatments of PTIO or l_1 NAME even in the presence of H_2O_2 . Treatment of SNP along with NAC or DPI; reduced the appearance of red spots inside the cell. The patches were found more intense in *Anabaena* sp. than *Nostoc muscorum* which showed that Cd easily entered into the cells of *Anabaena* sp. than *Nostoc muscorum*.

Effect of H_2O_2 and NO on endogenous level of ROS and indices of damage under Cd stress. Results pertaining to the contents of SOR, H_2O_2 and MDA equivalents showed a sharp increase of 18, 4 and 34% respectively in *Nostoc muscorum* and 19, 7 and 37% respectively in *Anabaena* sp. (Table 1). When H_2O_2 and SNP were supplied to Cd stressed cyanobacteria the contents of SOR, H_2O_2 and MDA equivalents were declined significantly ($P < 0.05$). However, exposure of PTIO enhanced the level of SOR, H_2O_2 and MDA even in the presence of exogenous H_2O_2 and this increasing trend was continued when l_1 NAME applied together with exogenous H_2O_2 . While exposure of SNP along with NAC or DPI was significantly able to lessen the toxic levels of SOR, H_2O_2 and MDA. Furthermore, the combined addition of H_2O_2 and SNP along with PTIO and l_1 NAME vigorously enhanced the levels of SOR, H_2O_2 and MDA while combined exposure of H_2O_2 and SNP along with NAC and DPI under the same stress declined the levels of SOR, H_2O_2 and MDA in both Cd stressed cyanobacteria.

The biochemical results of SOR, H_2O_2 and MDA were supported more strongly by in-vivo analysis inside the cells of both tested organisms *Anabaena* sp. and *Nostoc muscorum* (Figs. 3, 4). Blue patches were appeared by the staining with NBT for SOR, brown colored patches were appeared by DAB staining for H_2O_2 while pink patches were the result of staining with Schiff's reagent for MDA, and overall effects of oxidative stress were represented as electrolyte leakage (EL) shown by the sky blue spots (Figs. 3, 4). In the figure blue, brown, pink, and sky blue patches were appeared more intense under the exposure of Cd but spots were normalized under exogenous addition of H_2O_2 or SNP along with Cd stress. But in the absence of NO inside the cell means under the exposure of PTIO and l_1 NAME exogenously supplied H_2O_2 cannot limit the ROS production and intense patches were noticed. While in lack of H_2O_2 means under the exposure of NAC and DPI exogenously added SNP can able to limit the ROS production and faded patches have appeared inside the cells of both the organisms. This finding clearly showed the efficiency of NO towards controlling the ROS inside the cells and also clarified that

Treatments	<i>Nostoc muscorum</i> ATCC 27893			<i>Anabaena</i> sp. PCC 7120		
	SOR	H ₂ O ₂	MDA	SOR	H ₂ O ₂	MDA
	[nmol (mg dry weight) ⁻¹]			[nmol (mg dry weight) ⁻¹]		
Control	3.25 ± 0.05 ^e	3.57 ± 0.06 ^d	0.32 ± 0.01 ^c	3.57 ± 0.06 ^c	4.03 ± 0.07 ^c	0.41 ± 0.01 ^c
Cd	3.83 ± 0.06 ^d	3.71 ± 0.09 ^d	0.44 ± 0.01 ^b	4.24 ± 0.07 ^d	4.32 ± 0.07 ^d	0.55 ± 0.01 ^b
Cd + H ₂ O ₂	1.76 ± 0.08 ^h	2.51 ± 0.04 ^h	0.19 ± 0.00 ^f	2.04 ± 0.04 ^h	2.98 ± 0.09 ⁱ	0.24 ± 0.00 ^f
Cd + SNP	1.69 ± 0.02 ^h	2.46 ± 0.04 ^h	0.18 ± 0.00 ^f	1.92 ± 0.05 ^h	2.85 ± 0.05 ⁱ	0.23 ± 0.00 ^f
Cd + H ₂ O ₂ + PTIO	4.62 ± 0.08 ^b	4.92 ± 0.09 ^b	0.45 ± 0.01 ^{ab}	5.16 ± 0.08 ^b	5.65 ± 0.10 ^b	0.56 ± 0.01 ^b
Cd + H ₂ O ₂ + ₁ NAME	4.82 ± 0.08 ^a	5.27 ± 0.09 ^a	0.47 ± 0.01 ^a	5.36 ± 0.09 ^a	6.03 ± 0.10 ^a	0.59 ± 0.01 ^a
Cd + SNP + NAC	2.61 ± 0.07 ^f	3.01 ± 0.05 ^f	0.26 ± 0.01 ^{de}	2.82 ± 0.05 ^{fg}	3.55 ± 0.06 ^g	0.32 ± 0.01 ^d
Cd + SNP + DPI	2.71 ± 0.04 ^f	3.22 ± 0.06 ^e	0.27 ± 0.01 ^d	2.91 ± 0.05 ^f	3.78 ± 0.07 ^f	0.33 ± 0.01 ^d
Cd + H ₂ O ₂ + SNP + PTIO + ₁ NAME	4.12 ± 0.07 ^c	4.27 ± 0.10 ^c	0.46 ± 0.01 ^a	4.64 ± 0.08 ^c	4.87 ± 0.08 ^c	0.59 ± 0.01 ^a
Cd + H ₂ O ₂ + SNP + NAC + DPI	2.35 ± 0.04 ^g	2.78 ± 0.05 ^g	0.25 ± 0.00 ^c	2.68 ± 0.04 ^g	3.28 ± 0.06 ^h	0.29 ± 0.01 ^e

Table 1. Effect of H₂O₂ and NO (SNP) on the contents of SOR, H₂O₂ and MDA equivalents in *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 exposed to Cd after 24 h of treatment. 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 treatment of H₂O₂ enhanced the level of NO to normalize the endogenous level of H₂O₂, showed a positive relation between NO and H₂O₂.

Effect of H₂O₂ and NO on the activity of enzymatic antioxidants under Cd stress. Figure 5a–d show the effect of signaling molecules on activities of enzymatic antioxidants (SOD, POD, CAT and GST) under Cd stress. Results represented that the activities of SOD, POD, CAT and GST got barely enhanced (P < 0.05) by 15, 15, 14 and 27% respectively in Cd stressed *Nostoc muscorum* and 13, 14, 14 and 20% respectively in Cd stressed *Anabaena* sp. Under similar stress activities of these antioxidants were found extremely enhanced under the exposure of H₂O₂ and SNP with a more pronounced effect of SNP. Contrary to this, activities of these antioxidants were got arrested under the exposure of PTIO or ₁NAME even in the presence of H₂O₂ while enhanced activities of these enzymatic antioxidants were found on the exposure of SNP even in the presence of NAC or DPI in both the test organisms.

The expressions of isoenzymes of SOD, POD, CAT and GST were more strongly supported the biochemical analysis of antioxidant enzymes clearly depicted in Fig. 6. where the intensity of bands of SOD, POD, CAT and GST were found nominally intense under Cd stress and band intensity was found extremely intense under the supplementation of H₂O₂ and SNP with Cd. Unlikely, the bands were found negligibly appeared when NO is blocked while band slightly appeared where H₂O₂ is blocked and SNP is provided. In *Nostoc muscorum* the activities of antioxidants were found more intense than *Anabaena* sp. showed the resistive behavior of *Nostoc muscorum*.

Results finally clarify the signaling role of H₂O₂ and NO. They can detoxify the Cd stress and as it is evident from both the tested organisms by enhancing their defensive layer exopolysaccharides secretion as well as by boosting their antioxidant machinery and also by reducing the excessive level of ROS formed inside the cell. On the other hand, improvement was found more pronounced in the case of *Nostoc muscorum* showing its resistive behavior in comparison to *Anabaena* sp.

Discussion

H₂O₂ up regulates NO to enhance the growth of cyanobacteria under Cd stress. In the present study, both H₂O₂ and NO successfully cope up with the Cd stress and enhance the growth of cyanobacteria which is similar to the findings of Uchida et al.¹⁹, Liu et al.²⁰ and Nahar et al.²¹. The possibility of reasons behind this improved growth can be summarized into the following points. (1) H₂O₂ and NO can reduce the intracellular accumulation of Cd (Fig. 2) by enhancing the secretion of EPS (Fig. 1c); (2) protein content can also be enhanced by exogenous exposure of H₂O₂ and NO (Fig. 1d); (3) H₂O₂ and NO can enhance endogenous NO content (Fig. 1e) and (4) H₂O₂ and NO can reduce the excessive ROS contents (Table 1) by enhancing enzymatic antioxidant activities (Fig. 5a–d). Similar to this, Christou et al.¹⁷ reported that H₂O₂ and NO reduced the salt stress in the strawberry plants. Several studies reported that a high dose of H₂O₂ is used for eradication of algal bloom in ponds²². But against this, in the present study, a very low dose (1 μM) of H₂O₂ (provided for a very short time; only for 3 h) is used and very satisfactory results have been obtained against the Cd toxicity and enhanced growth of test cyanobacteria have been found under H₂O₂ treatment (Fig. 1a,b). Basal level of endogenous ROS and NO are very important to the cell functioning under stress condition which was proven by individual treatments of NAC, DPI, PTIO and ₁NAME along with Cd (Fig. 1b). Further experiments of the present study show the interlinked process of internal signaling of H₂O₂ and NO. In this way, the application of inhibitor and scavenger of NO reversed the enhanced growth of cyanobacteria by worsening the Cd toxicity inside the cells that confirms the dependency of H₂O₂ on NO. Our results are in consonance with the finding of

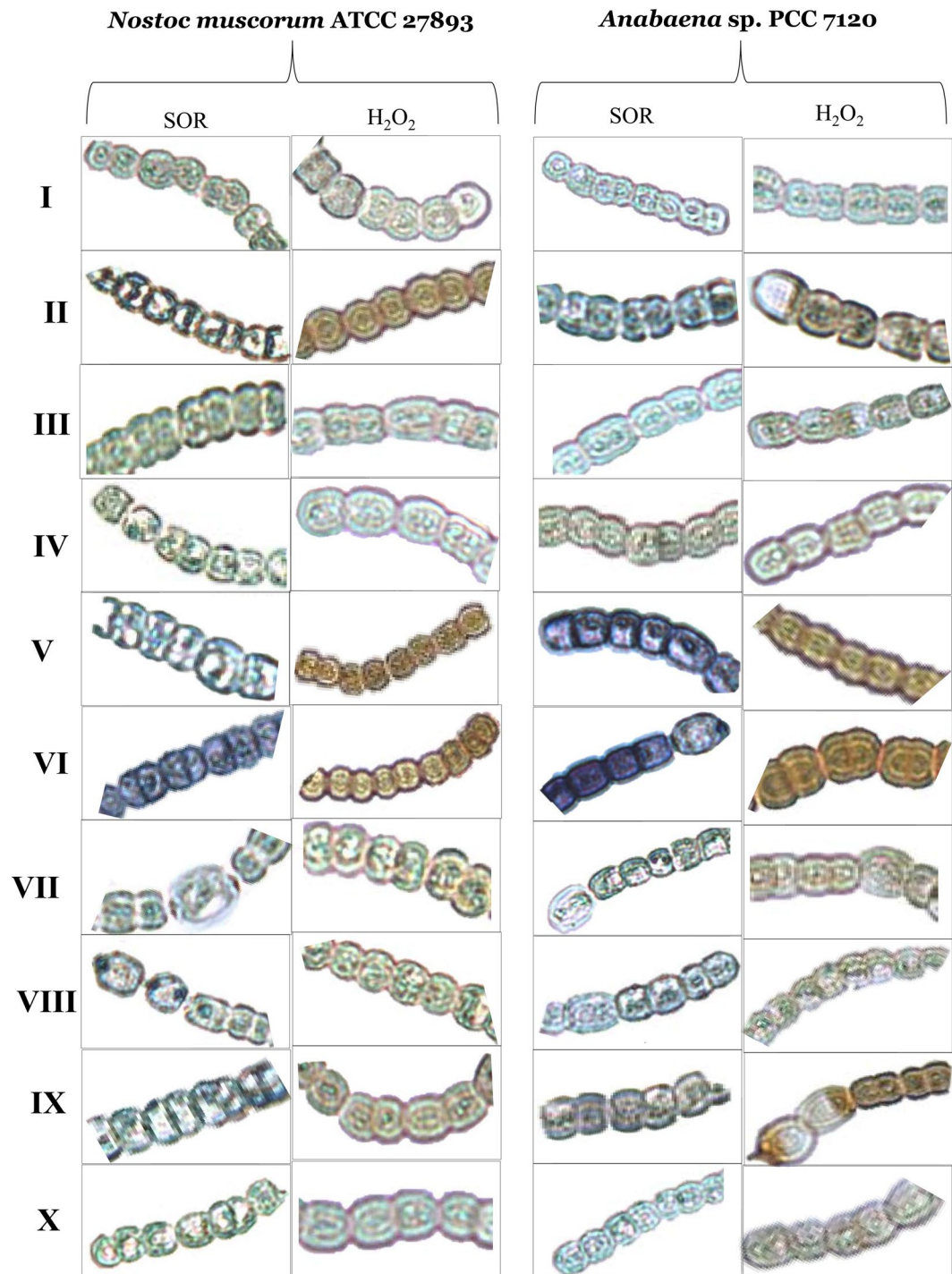


Figure 3. In vivo visualization of SOR; blue staining and H₂O₂ accumulation; brown staining inside the cells of Cd stressed *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 when treated with H₂O₂ and NO (SNP). Where lane I: Control, lane II: Cd, lane III: Cd + H₂O₂, lane IV: Cd + SNP, lane V: Cd + H₂O₂ + PTIO, lane VI: Cd + H₂O₂ + LNAME, lane VII: Cd + SNP + NAC, lane VIII: Cd + SNP + DPI, lane IX: Cd + H₂O₂ + SNP + PTIO + LNAME, lane X: Cd + H₂O₂ + SNP + NAC + DPI.

Li et al.¹⁶; they revealed that under heat stress, the combined treatment of H₂O₂ and NO enhanced the survival percentage of maize seedlings and the stress was found worsened under the application of cPTIO. Furthermore, from the present results, it is found that NAC and DPI have not worsened the Cd toxicity in the presence of endogenous or exogenously supplied NO (because exogenously supplied SNP might fulfill the cellular level of NO that is regulated by H₂O₂), that showed a direct relationship between H₂O₂ and NO (Fig. 7) and it is cleared that H₂O₂ regulates NO to cope up with Cd toxicity.

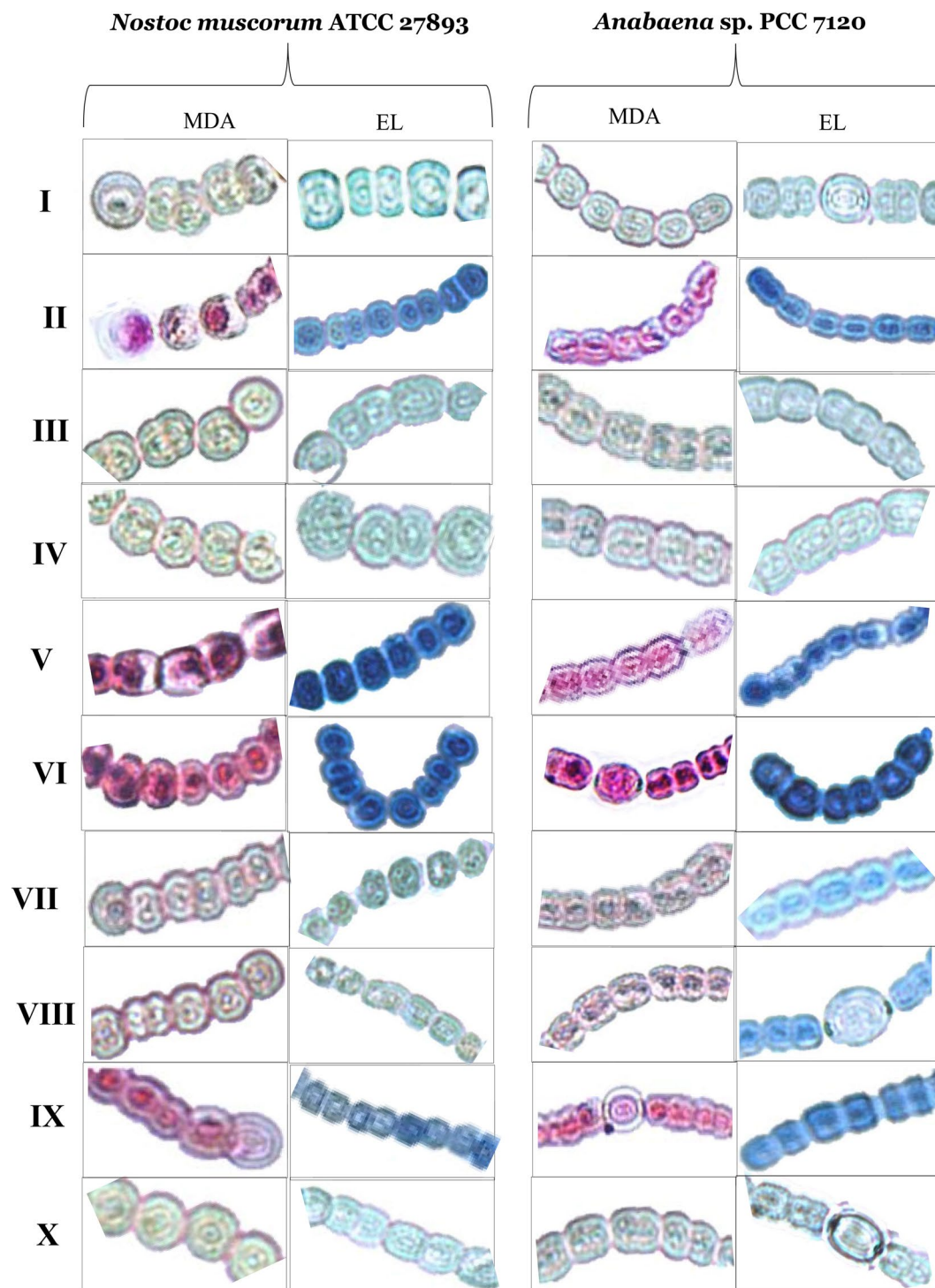


Figure 4. In vivo visualization of MDA; pink staining and EL; sky blue staining inside the cells of Cd stressed *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 when treated with H_2O_2 and NO (SNP). Where lane I: Control, lane II: Cd, lane III: Cd + H_2O_2 , lane IV: Cd + SNP, lane V: Cd + H_2O_2 + PTIO, lane VI: Cd + H_2O_2 + l -NAME, lane VII: Cd + SNP + NAC, lane VIII: Cd + SNP + DPI, lane IX: Cd + H_2O_2 + SNP + PTIO + l -NAME, lane X: Cd + H_2O_2 + SNP + NAC + DPI.

H_2O_2 promotes NO to enhance the EPS secretion which reduces the intracellular cd accumulation. The protective layer of EPS mainly comprises a group of biopolymers with high molecular weight which are secreted in response to any environmental stress that make a barrier against the stress²³. Cadmium is a highly

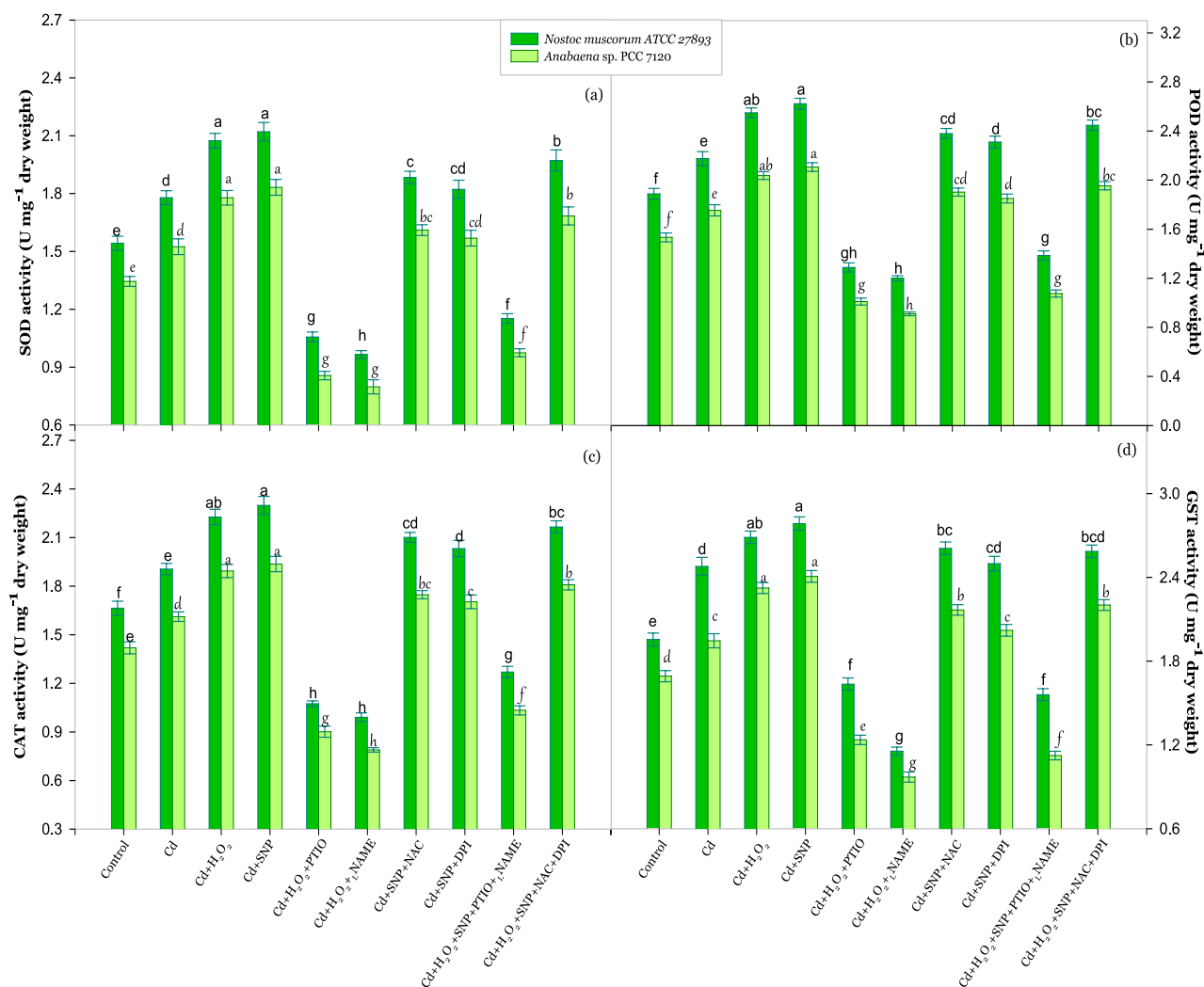


Figure 5. Effect of H₂O₂ and NO (SNP) on the activity of enzymatic antioxidants; SOD (a), POD (b), CAT (c) and GST (d) of *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 exposed to Cd after 24 h of treatment. Data presented are means \pm standard error of three independent experiments with three replicates in each experiment (n = 9). Bars with different letters represent significant difference at P < 0.05 significance level according to the DMRT.

toxic metal found on Earth that disrupts the protective layer of EPS in tested cyanobacteria (Fig. 1c) which can be related with the more Cd accumulation inside the cells (i.e. displayed by red patches) demonstrated in Fig. 2. Similar to this Patel et al.²⁴ also found the reduced EPS content along with increased As accumulation in *Nostoc muscorum* and *Anabaena* sp. Such disruptions in EPS content have been eliminated by the application of H₂O₂ and SNP and red patches inside the cells are less appeared (Fig. 2). However, when NO is completely arrested and H₂O₂ is supplied, the content of EPS has been reduced vigorously and intracellular accumulation of Cd enhanced critically. From this result, it is clear that H₂O₂ promotes NO to produce more EPS to chunk the intracellular metal accumulation. Similarly, Tewari et al.¹¹ demonstrated that in *Anabaena* sp. the application of PTIO and l-NAME reduced the EPS content and enhanced the Al accumulation inside the cells.

H₂O₂ up-regulates NO to enhance the protein content of Cd stressed cyanobacteria. During adverse conditions, protein content of any organism is directly associated with the growth. In the present study, decreased protein content (Fig. 1d) subjected to Cd stress might be due to the direct impact of Cd on protein biosynthesis²⁵. Contrary to this, exogenous H₂O₂ and NO reversed the negative impact of Cd on protein content (Fig. 1d). H₂O₂ and NO might be inducing the protein biosynthesis by controlling the ROS metabolism inside the cell²⁶. Interestingly, ROS production was further induced (Table 1) by supplementation of PTIO and l-NAME that resulted to the damage in protein content (Fig. 1d) which collectively reduced the growth of Cd stressed cyanobacteria.

Exogenously supplied H₂O₂ and SNP promote the endogenous production of NO for balancing of ROS and electrolyte leakage induced by Cd stress. As endogenous NO is known to play an important role in all the plant developmental activities and metal stress badly reduces the NO content by

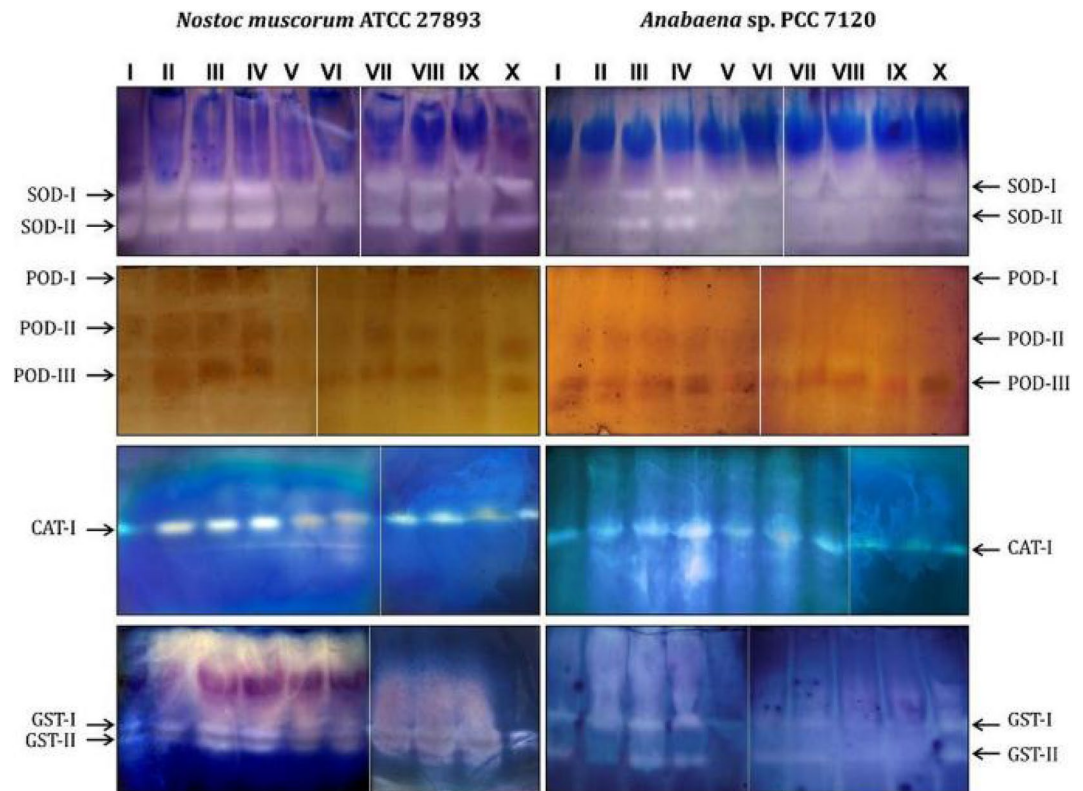


Figure 6. Isoenzymes profiling of SOD, POD, CAT and GST in H_2O_2 and NO (SNP) treated *Nostoc muscorum* ATCC 27893 and *Anabaena* sp PCC 7120 exposed to Cd stress. For the determination of isoenzyme activity, 300 μ g proteins from cell extracts were loaded into the wells of native PAGE; where lane I: Control, lane II: Cd, lane III: Cd + H_2O_2 , lane IV: Cd + SNP, lane V: Cd + H_2O_2 + PTIO, lane VI: Cd + H_2O_2 + l -NAME, lane VII: Cd + SNP + NAC, lane VIII: Cd + SNP + DPI, lane IX: Cd + H_2O_2 + SNP + PTIO + l -NAME, lane X: Cd + H_2O_2 + SNP + NAC + DPI; full length gels are presented in supplementary Fig. S1.

interfering with nitric oxide synthase enzyme²⁷. Our present study revealed that endogenous NO content was reduced under Cd stress which was further improved by treatment of H_2O_2 and SNP (Fig. 1e) and such increase was eliminated by adding PTIO and l -NAME, which are in the same line with previous findings of Xuan et al.²⁸ and Li et al.¹⁶. Elimination of NO content by l -NAME clearly shows that nitric oxide synthase enzyme involves in NO biosynthesis inside the tested organisms. Correspondingly, Bouchard and Yamasaki²⁹ have also clarified that microalgal NO is produced in a time-dependent manner under heat shock exposure which was further eliminated by adding cPTIO. Christou et al.¹⁷ have also demonstrated that SNP and H_2O_2 enhanced the NO content under NaCl stress in strawberry plants. Furthermore, exogenous SNP also dose a positive regulation of NO accumulation in the presence of NAC and DPI under Cd stress (Fig. 1e), suggesting that Cd tolerance induced by H_2O_2 may be achieved by increasing endogenous NO level, and NO is a downstream signal molecule of H_2O_2 .

Moreover, Cd can block ETS (electron transport chain) or displace the iron (Fe) molecule with other protein molecules that cause the abundant production of ROS inside the cell³⁰. This excessive ROS can cause direct damage to lipids, proteins, carbohydrates as well as to cellular genetic materials³¹. So the balancing of ROS inside the cell is very necessary. The current study showed that SOR, H_2O_2 and MDA contents were significantly increased under Cd stress (Table 1; Figs. 3, 4) which might be due to hindrance in ferredoxin pool thereby disturbance in Calvin cycle regulation³². Such increases in ROS were abolished by the addition of H_2O_2 and SNP which was in consistent with the studies of Koningshofer et al.³³, Hu et al.³⁴ and Li et al.³⁵. A previous study also shows that at very low concentration H_2O_2 works as a signaling mediator and modulates deferent stress managing genes. H_2O_2 up-regulate the NO facilitated ABA-induced mitogen-activated protein (MAP) kinase cascade pathway to empower the defensive mechanism of maize leaves. During stress situation H_2O_2 , can regulate NO, and NO itself works as a ROS scavenger that is the point of interaction between these two incredible signaling molecules¹⁵.

Our results showed that H_2O_2 and SNP alleviated electrolyte leakage (Fig. 4; sky blue spots) which is supported by the previous finding of Song et al.³⁶. Similarly, Li et al.¹⁶ suggested that pretreatment of H_2O_2 in heat-stressed maize seedlings reduced the electrolyte leakage and endogenous MDA content to improve the overall photosynthesis and growth. Ali et al.³⁷ also showed that the application of SNP reduced the level of H_2O_2 in the *Triticum aestivum* L. plant. Again the contents of these ROS species have been more pronouncedly increased under the treatment of PTIO and l -NAME. However, in the current study, a reduced level of endogenous H_2O_2 content have been found under the exposure of exogenous H_2O_2 showed a reversible pathway in which H_2O_2

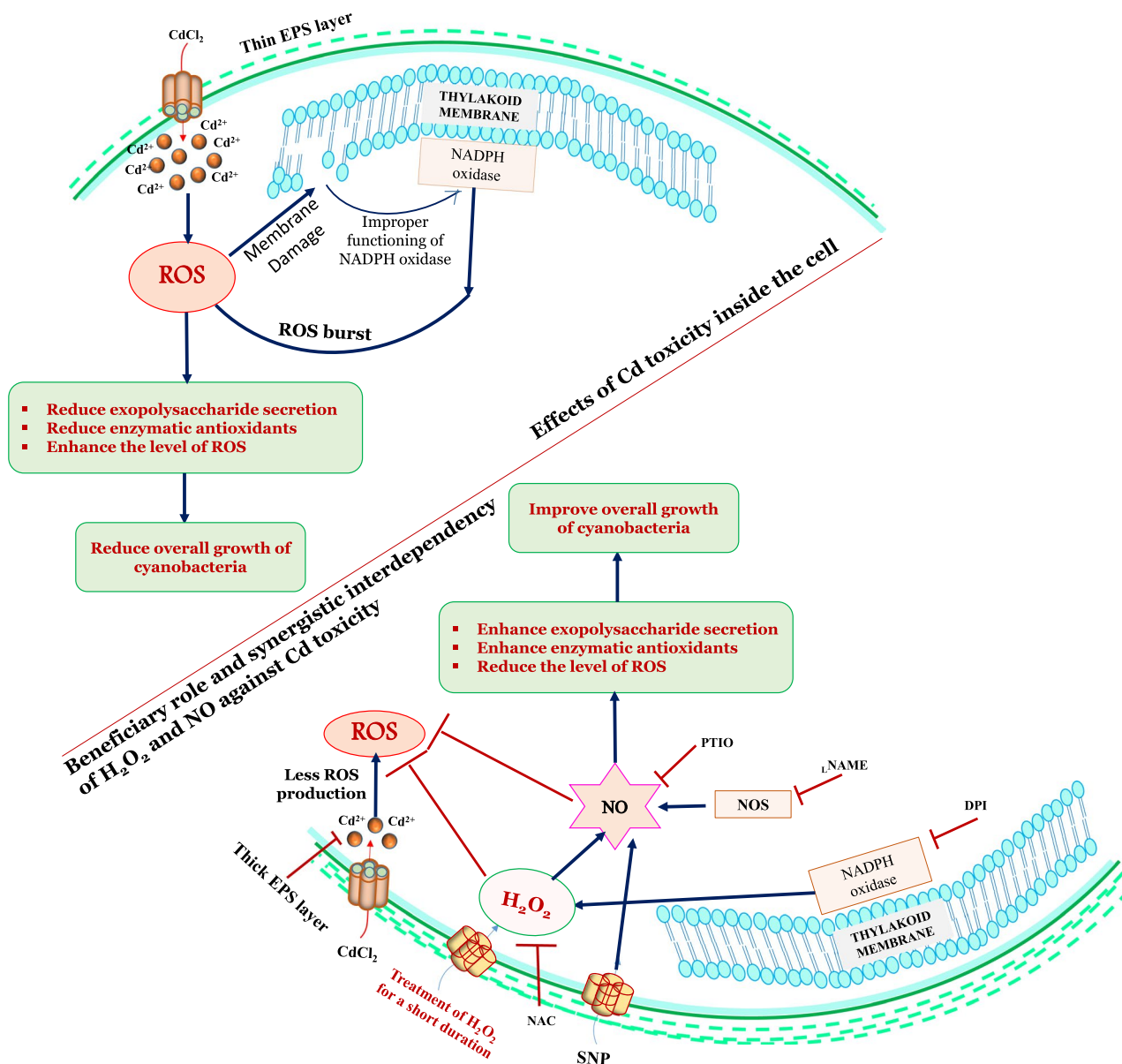


Figure 7. Schematic representation of negative impacts of Cd toxicity and positive synergistic mechanism of H_2O_2 and NO in acquisition of Cd stress tolerance in cyanobacteria.

signals may promote endogenous NO to enhance the activity of enzymatic antioxidants that ultimately expels out the overall ROS species from the cell as demonstrated by Verma et al.¹²

H_2O_2 and NO enhanced the activity of enzymatic antioxidants under Cd stress. The previous study of Farooqui et al.³⁸ in *Nostoc muscorum* disclosed that SOD, CAT and POD antioxidants were found to decrease under high concentrations of Cd. In accordance with previous studies, the current results showed that Cd reduced the level of enzymatic antioxidants (SOD, POD, CAT and GST) and bands of their isoenzymes were also found less enhanced (Figs. 5, 6). The possible reason behind this disturbance in activities of enzymatic antioxidants might be due to the alterations in basal level of ROS and thereby increased oxidative stress²⁷. Cd has a strong affinity to bind with -SH groups or proteins to disturb the activity and synthesis of various enzymes³⁹. In present work increased activities of SOD, POD, CAT and GST (Fig. 5a–d) were recorded on exogenous exposure of H_2O_2 and SNP which played a vital role in the reduction of ROS from the cells which is supported by the studies of Hasanuzzaman et al.¹⁵ and Xu et al.⁴⁰. Also, the study of Qian et al.⁴¹ revealed the role of NO in positive regulation of several enzymatic antioxidants under herbicide stress in *Chlorella vulgaris*. SOD, POD, CAT and GST are known to directly catalyze the ROS scavenging reaction. A mild dose of H_2O_2 supplied to cyanobacteria in the present study, enhanced the activities of these enzymatic antioxidants that may regulate the AsA-GSH cycle in Cd affected cyanobacteria which is directly related to ROS scavenging process and that is why the low amount of H_2O_2 decreased the intracellular ROS levels^{42–44}. The whole above mentioned results implied that the

acquisition of Cd stress tolerance in cyanobacteria may be involved in cross-talk between H_2O_2 and NO and a very complex feedback regulation is found between both signaling molecules for metabolic adaptation against abiotic stress.

Materials and methods

Experimental organisms and culture condition. *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120, heterocyst containing filamentous cyanobacteria were grown in BG-11 medium (pH 7.5). Their homogenous cultures were grown in controlled growth conditions; at a temp of 25 ± 2 °C under the illumination of $75 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR, 400–700 nm) with a controlled regime of 14:10 h light/dark cycle.

Experimental design and chemical treatment. Experimental design used for the present study was completely randomized design (CRD). To perform the experiments, cultures of both tested cyanobacteria were collected at their exponential phase by centrifuging them at 3000g for 15 min and washed with sterilized distilled water (DW). Henceforth, cyanobacterial cells were subjected to the growth medium containing concentrations of Cd (6 μM), sodium nitroprusside (SNP: a donor of NO) (10 μM), PTIO (2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide); scavenger of NO (20 μM), L -NAME (*N* ω -Nitro-*L*-arginine methyl ester hydrochloride); NOS enzyme's inhibitor (100 μM), H_2O_2 (1 μM), NAC (*N*-acetyl-*L*-cysteine); scavenger of H_2O_2 (1 mM), DPI (diphenyleneiodonium chloride); an inhibitor of NADPH oxidase enzyme (1 μM). Accordingly, succeeding treatments were designed- Control; C (only in BG-11 medium), Cd (cadmium), Cd + H_2O_2 , Cd + NAC, Cd + DPI, Cd + SNP, Cd + PTIO, Cd + L -NAME, Cd + H_2O_2 + PTIO, Cd + H_2O_2 + L -NAME, Cd + SNP + NAC, Cd + SNP + DPI, Cd + H_2O_2 + SNP + PTIO + L -NAME, Cd + H_2O_2 + SNP + NAC + DPI.

As H_2O_2 is a short-lived photodegradable component so for the experiments of the present study, H_2O_2 was prepared very carefully in dark and added to the experimental set up at the end wherever it was required. Finally, all cultures were incubated 3 h in dark and after that they were placed for cycle 14:10 h light and dark. All the parameters were analyzed after 24 h of treatment.

Measurement of growth. The growth of test cyanobacteria was measured in terms of dry weight. The 100 ml cultures of both the organisms were collected and centrifuged at 4000g for 10 min and then washed twice with distilled water. Further, pellets were dried at 80 °C and weighed gravimetrically (Contech-CA 223, India).

Determination of exopolysaccharides content. Exopolysaccharides (EPS) content was determined according to Sharma et al.⁴⁵ For EPS extraction, 100 ml samples were taken from each treatment, centrifuged at 3000g for 15 min supernatants were collected and then dried separately at 40 °C. The obtained precipitates were washed thrice with isopropanol and dried again at 37 °C. Further, hydrolysate was analyzed for glucose by Seifter et al.⁴⁶ and calculation was done by standard curve of glucose.

Estimation of protein content. For the extraction of protein, the method of Bradford⁴⁷ was adopted. Cells were centrifuged and homogenized with 50 mM potassium phosphate buffer (PPB) (pH 7.8) containing 1 mM EDTA and 2% polyvinyl pyrrolidone at 4 °C. Further, 0.1 ml of obtained homogenates was mixed with 2.5 ml of Coomassie brilliant blue G-250 reagent, then kept for 2 min in dark at 25 °C and absorbance was read at 595 nm. Total soluble protein content was determined by using bovine serum albumin as standard protein solution.

Detection and quantification of NO. The NO content was determined by using Griess reagent (Sigma-Aldrich) by following the procedure of Zhou et al.⁴⁸ with some modifications. For NO estimation desired volume of cultures was centrifuged and pellets were crushed in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). Further, homogenates were centrifuged and supernatant was mixed with 50 mg of charcoal. After vortex and filtration, the filtrate was leached and collected. The 1 ml of filtrate and 1 ml of Griess reagent was mixed and incubated at room temperature for 30 min. Absorbance was taken at 540 nm. NO content was calculated by the standard curve prepared by graded solution of NaNO_2 .

Histochemical detection of Cd. Histochemical analysis of Cd was conducted through the method of Seregin and Kozhevnikova⁴⁹ with some minor changes. To detect the intracellular Cd accumulation; cells were collected by centrifugation then gently washed twice or thrice with double distilled water (DDW) to remove excess Cd. Obtained cells were mixed with 1 ml of dithiozone stock solution containing 6 gm of dithiozone and DW (3:1). Further 1–2 drops of acetic acid glacial were added and after 4 h of incubation; cells were observed under microscope (Leica, model- DM 2500).

Estimation of oxidative biomarkers and indices of damage. Superoxide radical (SOR; $O_2^{\cdot-}$), H_2O_2 contents, and lipid peroxidation were quantified by following the procedure of Elstner and Heupel⁵⁰, Velikova et al.⁵¹ and Heath and Packer⁵² respectively. For SOR content the absorbance was recorded at 530 nm. The determination of SOR content was based on the formation of NO_2 from hydroxylamine in presence of $O_2^{\cdot-}$ and the content was quantified by the standard curve of nitrite (NaNO_2). Absorbance for H_2O_2 was recorded at 390 nm and content was calculated by the standard curve prepared with graded solution of H_2O_2 . SOR and H_2O_2 induced oxidative damage to the lipids i.e. lipid peroxidation was calculated in the form of malondialdehyde

(MDA) equivalents contents. Absorbance for MDA was recorded at 532 and 600 nm and content was calculated by using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

In-vivo imaging of ROS ($\text{O}_2^{\cdot-}$ and H_2O_2), membrane damage and electrolyte leakage. The Histochemical analysis of the accumulation of $\text{O}_2^{\cdot-}$ and H_2O_2 was performed by following the method of Forster et al.⁵³ in which cells were suspended into nitrobluetetrazolium (NBT; Sigma) and 3, 3 diaminobenzidine (DAB) for in-vivo staining respectively. MDA equivalents contents and intensity of membrane damage were visualized by using Schiff's reagent and Evan's blue by following the methods of Pompella et al.⁵⁴ and Yamamoto et al.⁵⁵ respectively. Images were taken in a high-quality microscope (Leica, model-DM 2500).

Enzymatic antioxidant assay. The activities of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT; EC 1.11.3.6) and glutathione-S-transferase (GST, EC 2.5.1.18) were analyzed by following the methods of Giannopolitis and Ries⁵⁶, Gahagan et al.⁵⁷, Aebi⁵⁸ and Habig et al.⁵⁹ respectively. For SOD activity, the photoinhibition of NBT was recorded at 560 nm after 20 min illumination of light ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). One unit of SOD activity is demarcated as the required amount of enzyme to cause 50% inhibition in the reduction of NBT. For POD, absorbance of the reaction mixture was recorded at 430 nm for 3 min. The activity of the enzyme was calculated by using an extinction coefficient of $25.5 \text{ mM}^{-1} \text{ cm}^{-1}$ and one unit of enzyme activity is defined as 1 nmol pyrogallol oxidized min^{-1} . Activity of CAT was determined by monitoring the decrease in absorbance at 240 nm in the reaction mixture⁵⁸. The activity of enzyme was calculated by using an extinction coefficient of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$. Here 1 nmol H_2O_2 dissociated min^{-1} was equivalent to one unit of enzyme activity. For GST, absorbance of reaction mixture was recorded at 340 nm. Enzyme activity was calculated by an extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and one unit of enzyme activity is equivalent to 1 nmol of CDNB conjugates formed min^{-1} .

Native polyacrylamide gel electrophoresis for isoenzyme profiling. Native-PAGE analysis was carried out on discontinuous polyacrylamide gels (PAGE) with 4.5% polyacrylamide in stacking and with its varying concentrations (10% for SOD and GST, 6% for CAT and 8% for POD). The separation of individual isoenzyme was performed in a vertical gel electrophoretic unit (GeNei, India) by considering the method of Laemmli⁶⁰. A uniform amount (300 mg) of proteins mixed with sample buffer (0.5 M Tris-HCl, pH 6.8) was loaded in each well and proteins were electrophoretically separated at 80 V through stacking gel followed by 120 V in the separating gel at 4 °C. For visualizing SOD isoenzymes, the gels were immersed in PPB (50 mM; pH 7.8) containing NBT (1.125 mM) in darkness for 20 min and followed by incubation in PPB containing TEMED (28 mM) and riboflavin (28 mM) in dark for 15 min. The gels were then placed in PPB containing mM EDTA (0.1 ml) and exposed to light for 20 min at 25 °C⁶¹. For POD, gels were immersed in 1 mg ml^{-1} benzidine and 1 mM H_2O_2 in 0.1 M Tris-acetate buffer (pH 5.0) at 25 °C till the brown colored bands appeared⁶². For CAT isoenzymes gels were incubated with H_2O_2 (0.01%) for 15 min, rinsed with water, and shaken in freshly prepared solutions each of 0.1% FeCl_3 and $\text{K}_3\text{Fe}(\text{CN})_6$ at 25 °C until the achromatic bands appeared⁶³. For GST isoenzyme staining a reaction mixture containing 0.1 M PPB (pH 6.5), GSH (5.0 mM), CDNB (1.0 mM) and NBT (1 mM) was used and bands were developed by illuminating the gel under $25 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ of photon flux density⁶⁴. Photographs of isoenzymes were captured with digital camera, photo-plate was prepared in CorelDRAW and image quality was enhanced in Photoshop 7.0.

Statistical analysis. Statistical analysis of variance (ANOVA) was performed to test the significance at probability level $P < 0.05$. Duncan's multiple range test was applied to compare significant differences among the mean values. Graphical representations are the means of three independent experiments with three replicates in each experiment ($n = 9$). Lower case letter (a, b, c, d, e, f, g, h, i, j) shows statistical significance.

Conclusions

From the aforementioned results, it can be concluded that there is an interlinked pathway between NO and H_2O_2 that exists. In Fig. 7, it is clear that Cd create oxidative stress inside the cell and produces more ROS and creates membrane damage by increasing MDA equivalents contents^{15,21,65}. But exogenously supplied NO and H_2O_2 promote the EPS secretion and check the entrance of Cd inside the cells after that they also enhance the antioxidant system and endogenous NO content that's why the level of ROS get minimized. Here, the low amount of exogenous H_2O_2 works as a signal transmitter to enhance the beneficial amount of RNS species that indirectly respond to the balancing of antioxidants to cope up with cd stress. Afterward PTIO and lNAME with H_2O_2 block the internal NO that creates a disturbance in the working of signaling mechanism of H_2O_2 and NO here it is clear that exogenously supplied H_2O_2 cannot work without NO. However, application of NAC and DPI cannot disturb the signaling of NO. Hence, from this it is clear that NO is a down-stream regulator of H_2O_2 but the pathway of signaling between both the molecules is very complex because of their multidimensional roles in a stressful environment.

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Author contributions

S.M.P. designed the experiments. N.V. performed the experiments. S.M.P. and N.V. wrote and finalized the manuscripts.

Competing interests

The authors declare no competing interests.

Additional information

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