Exogenous Phytohormones Modulates Cypermethrin Stress in *Anabaena* sp. and *Nostoc Muscorum*: Toxicity Alleviation by Up-regulation of Ascorbate-glutathione Cycle

Santwana Tiwari

University of Allahabad

Sheo Mohan Prasad (✉ profsmprasad@gmail.com)

University of Allahabad

Research Article

Keywords: Ascorbate-glutathione cycle, cypermethrin, kinetin, oxidative stress

Posted Date: April 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-415081/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Present study demonstrated the effect of phytohormones KN and IAA under cypermethrin (Cyp₁; 2 µg ml⁻¹ and Cyp₂; 4 µg ml⁻¹) toxicity in two nitrogen fixing cyanobacteria *Nostoc muscorum* ATCC 27893 and *Anabaena* sp. PCC 7120 by investigating growth, exopolysaccharides (EPS) contents, protein content, oxidative stress visualization inside the cell and ascorbate- glutathione cycle. Decline in growth were noticed under both the doses of cypermethrin but the decline was more (30%) at higher dose in *Anabaena* sp. PCC 7120 as compare to *N. muscorum*. This decrease was due to increased production of oxidative biomarkers (i.e. SOR and H₂O₂) subsequently membrane got damaged which was noticed by measuring MDA equivalents content (*in vivo* visualization). Kinetin and IAA alleviated the SOR and H₂O₂ content resulting in recovery of cellular membrane and the growth was optimized up to control level. Detoxification of H₂O₂ is guided by enzymes/metabolites of AsA-GSH cycle like ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) activity were found to be stimulated at lower dose of cypermethrin as compare to higher dose. while the amount of metabolites: total ascorbate (AsA), total glutathione (GSH) and ratios of reduced/oxidized AsA (AsA/DHA) and GSH (GSH/GSSG) showed significant reduction at both the doses but the reduction was more at higher dose of cypermethrin. Kinetin and IAA positively regulate the AsA-GSH cycle by enhancing the activity of APX, GR, MDHAR and DHAR activity and raising the metabolites content and their reduced/oxidized ratio. This study suggests the increased enzymatic activity and AsA/DHA ratio leads to reduced production of H₂O₂ in the presence of both the phytohormones which further leads to enhanced growth in both the organism but the effect of KN and IAA was more pronounced in *N. muscorum* suggesting its resistivity against stress.

Introduction

Indiscriminate application of pesticide in agriculture badly affects the growth of plants and cyanobacteria by generating reactive oxygen species (ROS)¹⁻³. Excessive production of ROS, such as the superoxide radical (O₂^{•-}) and H₂O₂ results a toxic state called oxidative stress⁴. Their generation are known by various electron transport systems and thus increased the chance of damage of large biomolecules like lipids, proteins and DNA^{3,5,6}. On the other hand, ROS in cell is now being confirmed as secondary messenger and guide several physiological processes via redox signalling and regulate the protein and gene expression^{7,8}. Defence machinery having numerous antioxidant systems evolved in cells to control the amount of ROS and minimizes the damage caused to macromolecules and involve them in signalling pathway. Defence system comprises both enzymatic and non-enzymatic antioxidants and among antioxidants, ascorbate-glutathione (AsA-GSH) cycle play a prominent role to control/detoxify the ROS⁹⁻¹¹. It enclosed three independent redox couples: reduced and oxidized ascorbate ratio (AsA/DHA), reduced and oxidized glutathione ratio (GSH/GSSG), and NADPH/NADP⁺^{6,12-14} and enzymes i.e., ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR)^{12,15-17}. These enzymes are responsible for redox cycling of AsA

and GSH. By the involvement of these enzymes, the reduced and active forms of ascorbate and glutathione are maintained at optimal level and consequence of this AsA-GSH cycle operated well inside the cell to minimize the toxicity caused by excess ROS^{3,17,18}.

Cyanobacteria are essential constituents of the fresh water ecosystem and paddy field soil where moist and nutrient rich environment regulate their growth, physiology, abundance and habitat selection^{19,20}. The indirect effect of pesticide also recorded on cyanobacteria by notifying the oxidative stress caused by overproduction of free radical^{17,21,22}. Apart from having several defensive processes, they are known to regulate cellular oxidative stress by operating AsA-GSH cycle including few enzymes under pesticide as well as metal stress condition^{23,24}.

Phytohormones are known to regulate the growth of plants as well as cyanobacteria under normal as well as stress condition^{25,26}. They provide signals against several stress conditions and allow plants to survive by adopting crucial strategies to counteract the adverse effects of environmental stresses²⁷⁻³⁰. Cyanobacteria were also reported to produce several phytohormones³¹⁻³⁵. In plants, effect of phytohormones on regulating the oxidative stress were highly discussed by AsA-GSH cycle³⁶⁻³⁹ but in cyanobacteria, it is still a matter to unrevealing the mechanism. Therefore, present study focused on the potential role of KN on regulating the oxidative stress generated by pesticide via AsA-GSH cycle in *Anabaena* PCC 7120 and *Nostoc muscorum*.

Result

Phytohormones regulated growth of cyanobacteria

In this study, role of KN and IAA on growth of both the organisms was depicted in figure 1. Both the doses of cypermethrin (Cyp) decreased the biomass accumulation of tested organisms in dose dependent manner (i.e. 10% and 30% at 2 and 4 $\mu\text{g mL}^{-1}$) as compared to control but both the phytohormones significantly alleviated the decrease and increase in biomass was noticed in both the cyanobacteria.

Phytohormones regulated protein content in cyanobacteria

Data pertaining to protein content are exhibited in figure 1. Under Cyp₁ stress *Anabaena* sp. and *N. muscorum* showed a rise of 10% and 18% in protein content whereas under Cyp₂ stress, there was significant decrease: 25% and 17% in protein content, respectively.

Phytohormone KN addition to Cyp₁ stressed cultures of both the cyanobacteria *Anabaena* sp. and *N. muscorum* further enhanced: 35% and 42% the protein content, respectively. At higher dose i.e. Cyp₂ KN also showed appreciable increase (3% in *Anabaena* sp. and 12% in *N. muscorum*) as compare to control. With IAA in both the cyanobacteria exposed to Cyp₁ stress, similar enhancement in protein content was recorded. Though IAA application in Cyp₂ stressed *Anabaena* sp. exhibited increasing trend in protein

content but it was still less than that of untreated control. Under similar condition the level of protein content in *N. muscorum* was almost parallel to the control sample (Fig. 1).

Phytohormones regulated EPS content in cyanobacteria

Results pertaining to exopolysaccharides (EPS) content are represented in figure 2. Upon cypermethrin treatment (Cyp₁ and Cyp₂) the content of exopolysaccharides was found to decline by 14% and 33% in *Anabaena* sp., and by 10% and 28% in *N. muscorum*, respectively over the value of controls.

Exogenous phytohormones KN/IAA enhanced the content of EPS appreciably in both the cyanobacteria exposed to Cyp₁ stress over the values recorded in controls. Both the phytohormones in Cyp₂ stressed tested cyanobacteria, though showed increasing trend in EPS content but it remained less than that obtained in untreated controls.

Phytohormones regulated ROS content in cyanobacteria

Histochemical analysis of ROS content ($O_2^{\bullet-}$, H_2O_2 , MDA equivalents content) inside the cyanobacterial cell was in accordance with biochemical analysis (Fig. 3). Intensity of blue, brown and pink colors determined the accumulation of $O_2^{\bullet-}$, H_2O_2 and MDA equivalents content in both the tested organism which is found to be in dose dependent manner of cypermethrin. Kinetin efficiently lowered their formation inside the cell which was visible in less intense color formation for the respective ROS content.

Phytohormones modulated enzymatic activities of AsA-GSH cycle: APX, GR, DHAR and MDHAR

Results pertaining to the enzymes of ascorbate-glutathione cycle are represented in figures 4 and 5. In cultures of *Anabaena* sp. and *N. muscorum* exposed to Cyp₁ stress, the activity of APX was found to accelerate by 5% and 11% while after Cyp₂ treatment the activity of this enzyme suppressed by 8% and 5%, respectively as compare to respective controls. Addition of KN to cypermethrin stressed cultures exposed to Cyp₁ dose caused further enhancement (16% and 25%) in the activity of APX in *Anabaena* sp. and *N. muscorum*, respectively. Kinetin supplementation to Cyp₂ stressed cultures improved the activity of APX which was parallel to the activity of control in both the cyanobacteria (Fig. 4). Almost similar pattern in the activity of APX enzyme in both the cyanobacteria exposed to cypermethrin (Cyp₁ and Cyp₂) was recorded with the addition of phytohormone IAA (Fig. 5).

Activity of GR, DHAR and MDHAR (Figs 4 and 5) showed similar trend in both the treated cyanobacteria but at Cyp₂ dose their activity increased as compare to control. Above result mentioned that both the hormone efficiently expressed its role in *Nostoc muscorum*.

Phytohormones optimized ascorbate (AsA) and glutathione (GSH) level

Total ascorbate (AsA+DHA) and AsA/DHA ratio were also quantified under Cyp and KN and IAA treatment (Table 1 and 2). There was a decrease in total ascorbate (T-ASA) content both the doses of cypermethrin in both the tested organisms. Under similar condition, reduction was more pronounced in AsA, so that AsA/DHA ratio also decreased. However, under the treatment of KN and IAA, AsA-GSH pool restored as compared to the samples treated with cypermethrin.

Likewise ascorbate, total glutathione (T-GSH) and GSH/GSSG ratio showed similar trend in the presence of KN and IAA (Table 3 and 4). However, GSH content declined more as compared to AsA content at both the doses in both the cyanobacteria. In the above mentioned result, *N. muscorum* is more expressive for KN and IAA.

Discussion

In current study decline in growth at both the doses of cypermethrin (Cyp₁: 2 µg ml⁻¹ and Cyp₂: 4 µg ml⁻¹) (Figs. 1 and 2) was noticed in both the cyanobacteria which might be due to enhanced production of ROS causing membrane damage (Fig. 3). Tiwari and Prasad⁴⁰ noticed the decline in growth due to decline in physiological activity and defense mechanism in cyanobacteria (*Anabaena* PCC 7120 and *N. muscorum*). This decline was mitigated by the phytohormones (KN and IAA). Similar results were noticed by Tiwari et al.⁴¹ in the organism *N. muscorum* in the presence of KN. Protein is also one of the important indicators to assess the growth of organism under stress conditions. In this study protein content increased at lower dose of cypermethrin (Fig. 1) representing its defensive strategy which is concurred with increased activity of enzymatic antioxidants (Tables 1 and 2) which is rather unable to minimize the oxidative stress cause by cypermethrin. At Cyp 2 dose, severe decline in protein content (Figs. 1) was noticed which might be due to enhanced production of ROS inside the cell leading to membrane damage visible in figure 3. Kinetin and IAA significantly enhanced the protein content (Figs. 1) at both doses of cypermethrin in both the organisms leading to better performance of enzymes involved in AsA-GSH cycle hence minimizing the oxidative damage. Cyanobacteria are known for synthesizing exopolysaccharides (EPS) layer on external surface and they may remain attached to the cell or released into the immediate external environment^{24,42,43}. The EPS are the complex mixture of polysaccharides, proteins, nucleic acids and lipids with various functional groups such as carboxylic, phosphoric, amino and hydroxyl groups³. These are believed to have protective role against several adverse conditions including penetration of xenobiotic by producing biofilms and prevent membrane fusion^{44,43}. In the current study decline in EPS content at both the doses of cypermethrin (Figs. 2 and 3) is directly correlated with pesticide membrane interaction leading to destruction of outer protective polysaccharide layer which favored the entry of cypermethrin and caused oxidative stress to the cell. Application of KN and IAA promoted the EPS content (Figs. 2 and 3) which might have hindered the entry of cypermethrin inside the cell and curtailed the oxidative stress condition which ultimately promoted the growth of both the cyanobacteria. Our study

is concurrent with the findings of Tiwari et al.⁴³ in which increase in EPS content due to application of signaling molecule i.e nitric oxide, protects the *Anabaena* sp. against Al-induced toxicity and favors the growth of organisms. Thus, IAA and KN induced increment in EPS may further be explored in light of any role of nitric oxide in present experimental conditions.

Cyanobacteria adopted several strategies to cope up ROS mediated toxicity either by minimizing its production or to scavenge them. Metabolism of ascorbate and glutathione by ascorbate–glutathione (AsA-GSH) cycle is a key process which crucially plays role in scavenging ROS, especially H₂O₂. The APX is the first enzyme which involves in the breakdown H₂O₂ into H₂O and O₂ and detoxifies the cellular oxidative environment¹⁷. As a result, monodehydroascorbate (MDHA) obtained which is further reduced into ASA or DHA by the action of MDHAR or non-enzymatically, respectively¹⁷. Next to this, DHA is reduced into AsA by the action of DHAR which utilizes GSH as reductant. Thus, DHAR crucially maintains the AsA pool in cells³⁸. Likewise, cellular GSH content also has to be maintained and this was done by GR enzyme which utilizes NADPH for electrons⁶. In this study, differential result was noticed for the activity of enzymes of AsA-GSH cycle. At Cyp₁ dose increase in the activity of enzymes of AsA-GSH cycle (Figs. 4 and 5) represents cellular defensive strategy to minimize the ROS content inside the cell but at higher dose of pesticide (Cyp₂), activity of enzymes was decreased (Figs. 4 and 5) leading to increased H₂O₂ concentration inside the cell thereby causing membrane damage (Figs. 3). Similar results were observed by Srivastava et al.²⁴ in *N. muscorum* and *P. foveolarum* under chlorpyrifos stress and by Prasad et al.⁴⁵ in two species of *Azolla* under pretilachlor stress. Similar to this study, increased activity of AsA-GSH cycle enzymes were noticed in *Amaranthous tricolor* under drought condition to detoxify the ROS (Sarkar and Oba, 2018). Lou et al.¹⁸ reported the enhanced expression of genes (*APX1*, *GRC1*, *DHAR*, *MDHAR*, *GPX1*, *GS3*) of AsA-GSH cycle enzymes in *Triticum aestivum* L. under drought stress. Concurrent with this, at Cyp₁ dose, there might be over expression of such genes in the tested organisms resulting in elevation in the activity of APX, GR, MDHAR and DHAR. In the presence of both the phytohormones (KN and IAA), activities of these enzymes were further enhanced (Figs. 4 and 5) and resulting in minimized cellular ROS contents which ultimately favored growth performance (Fig. 1). Similar results were recorded by Singh et al.³⁸ in tomato seedlings where KN ameliorated the Cd-induced toxicity by enhancing the activity of AsA-GSH cycle enzymes. Likewise, Ahanger et al.⁴⁶ revealed the potential role of KN under NaCl-induced stress condition for promoting the activity of enzymes involved in AsA-GSH cycle in *Solanum lycopersicum*. Bashri and Prasad⁴⁷ also pointed out the positive regulation governed by IAA under Cd mediated inhibition of AsA-GSH cycle in *Trigonella foenum-graecum* L.

Effect of oxidative stress was also correlated with the status of metabolites: ascorbate (AsA and DHA, AsA/DHA) and glutathione (GSH and GSSG, GSH/GSSG) involved in AsA-GSH cycle. These metabolites play integrative role in scavenging the ROS and prevent the cell from oxidative stress. Ascorbate (AsA) is known to quench ROS either by direct or indirect way via generating other non-enzymatic antioxidants like alpha-tocopherol¹². Dolatabadian and Jouneghani,⁴⁸ showed the effective role of exogenously supplied ascorbate on activity of enzymatic antioxidants of NaCl-treated common beans. In this study, total

ascorbate (AsA+DHA) and total glutathione (GSH+GSSG) contents decreased under cypermethrin (Cyp₁ and Cyp₂) treatment (Tables 1-4) which might have took place due to its inhibitory effect on their regeneration and/or excessive utilization of these compounds during the process of pesticide detoxification. Similarly, AsA/DHA and GSH/GSSG ratio decreased at both the doses of cypermethrin justifying the decreased AsA-GSH pool leading to oxidized environment inside the cyanobacterial cells. Decline in AsA and GSH content might be due to pesticide mediated hindrances in their biosynthesis. After KN and IAA treatment to the tested organisms, considerable enhancement in AsA and GSH pool supported the cells in combating the cypermethrin induced toxicity which further tends to reduction in H₂O₂ content (Fig. 3). In this study, we found that KN and IAA controlled the ascorbate-glutathione metabolism by promoting the activities of APX, GR, DHAR and MDHAR (Figs. 4 and 5) and increasing the contents of T-AsA, T-GSH, AsA/DHA and GSH/GSSG contents (Tables 1-4) under cypermethrin stress conditions.

Materials And Methods

Organisms and culture conditions

The homogenous cultures of *Anabaena* sp. PCC 7120 and *Nostoc muscorum* ATCC 27893 were grown in BG-11 medium in a temperature controlled culture room at $25 \pm 2^\circ\text{C}$ under illumination of $75 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR, 400-700 nm), provided by white fluorescent tubes (Osram L 40 W/25-1) with a 14:10 h of light/dark cycle. Exponential phase cultures were used to perform each experiment.

Selection of insecticide and kinetin doses

The two effective doses of tested insecticide cypermethrin (Cyp₁: $2 \mu\text{g ml}^{-1}$ and Cyp₂: $4 \mu\text{g ml}^{-1}$) correspond to LC₁₀ and LC₃₀ and kinetin (KN) dose (20 nM) and IAA (240 nM) were selected after screening experiment. This screening study was performed on *Anabaena* sp. PCC 7120.

Experimental design

For treatment of cypermethrin along with kinetin IAA, cyanobacterial cells at exponential phase were harvested and treated with two concentrations of cypermethrin (Cyp₁; $2 \mu\text{g ml}^{-1}$ and Cyp₂; $4 \mu\text{g ml}^{-1}$) alone and along with both the hormone with control sample which lacks cypermethrin and phytohormones. Further, treated and untreated samples were placed in culture room and after 96 h of treatment, different parameters were analyzed.

Measurement of growth

The cell culture (100 ml) was harvested, centrifuged at 4000g for 10 min, then washed with distilled water thrice and oven dried at 80° C for 48 h and weighed using digital weighing balance (Contech-CA 223, India).

Measurement of EPS

Extraction and estimation of EPS content were done by the method of Sharma et al.⁴². For extraction of the cyanobacterial EPS, 100 ml of treated and untreated cyanobacterial cells was centrifuged at 3000 g and after separating the settled biomass, cell-free culture containing the EPS was taken. It was then concentrated tenfold by evaporation at 40 °C, washed with isopropanol two–three times to remove any contaminants, and then finally dried at 37 °C and then analyzed for glucose.

Measurement of protein

Protein content was estimated by the method of Lowry et al.⁴⁹ modified by Herbert et al.⁵⁰. The amount of protein was expressed as Protein ($\mu\text{g mL}^{-1}$ culture). The amount of protein was assessed by using standard curve set with bovine serum albumin (BSA).

In vivo assessment of ROS production and lipid peroxidation

The histochemical visualization of $\text{O}_2^{\bullet-}$, H_2O_2 and MDA equivalents content was performed by *in vivo* staining of treated and untreated cyanobacterial cells with nitrobluetetrazolium (NBT; Sigma) and 3, 3' diaminobenzidine (DAB) (Sigma)⁵¹ and Schiff's reagent (Sigma)⁵², respectively. NBT stained deep blue formazan precipitate, DAB forming a reddish-brown polymerization product and MDA equivalents reaction product stained by Schiff's reagent. Thereafter, cells were imaged with Leica (Model: DM 2500).

Measurement of activities of APX, GR, MDHAR and DHAR

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by following the method of Nakano and Asada⁵³. Decrease in the absorbance was determined spectrophotometrically at 290 nm and enzyme activity was calculated by using an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit (U) of enzyme activity is defined as 1 nmol ascorbate oxidized per minute.

Glutathione reductase (GR; EC 1.6.4.2) activity was assayed according to the method of Schaedle and Bassham⁵⁴. Decrease in the absorbance was measured spectrophotometrically at 340 nm, and GR activity was quantified by using an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit (U) of enzyme activity is defined as 1 nmol NADPH oxidized per minute.

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity in tested cyanobacterial cells was estimated by following the method of Hossain et al.⁵⁵. Decrease in the absorbance due to oxidation of NADH was measured spectrophotometrically at 340 nm, and MDHAR activity was quantified by using an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit (U) of enzyme activity is defined as 1 nmol NADH oxidized per minute.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was determined by following the method of Nakano and Asada⁵⁶. An increase in the absorbance was measured spectrophotometrically at 265 nm, and DHAR activity was calculated by using an extinction coefficient of $7.0 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit (U) of enzyme activity is defined as 1 nmol DHA reduced per minute.

Measurement of ascorbate and glutathione

The measurement of total ascorbate (AsA + DHA), reduced ascorbate (AsA) and dehydroascorbate (DHA) were carried out by following the method of Gossett et al.⁵⁷. In this assay, reduction of Fe^{3+} into Fe^{2+} with ascorbic acid in acidic medium took place and formation of red chelate between Fe^{2+} and 2,2'-bipyridyl occurs. Dehydroascorbate was determined by subtracting AsA from AsA + DHA. Ascorbate content was calculated by using standard curve prepared with l-ascorbic acid. Total (GSH + GSSG), reduced (GSH) and oxidized glutathione (GSSG) were quantified by the enzyme-recycling method of Brehe and Burch⁵⁸. This method followed the chronological oxidation of GSH by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and reduction of GSSG in the presence of NADPH and glutathione reductase. Oxidized glutathione was quantified by pre-incubating 1 ml of 1:20 diluted extract with 40 ml of 2-vinylpyridine for 1 h at $25 \pm 2^\circ \text{C}$. The pre-incubated samples were used for determination of GSSG content. The GSH content was measured by subtracting GSSG from GSH + GSSG using as standard curve prepared with GSH.

Statistical analysis

The results presented are the means of three replicates ($n = 3$) to confirm the reproducibility of the data. Since the results showed normal distribution, comparison between control and treatment's means was carried out by using one-way ANOVA to test significance level (Duncan's multiple range tests, DMRT) at $P < 0.05$.

Conclusions

The result showed that both the doses of cypermethrin declined the growth of both the organisms under cypermethrin stress by depleting the growth of organisms and the components of AsA-GSH cycle quickly even with the accelerated activity of APX, GR, DHAR and MDHAR. This persists because of residing the ROS generation inside the cell which was clearly visual in the cells of both the cyanobacteria. Exogenous

application of KN and IAA significantly enhanced the growth of both the organisms stressed under cypermethrin by improving the rate of AsA-GSH cycle.

Declarations

Acknowledgement

Santwana Tiwari is very much thankful to the University Grant Commission for providing the financial support to complete this work. Authors are also thankful to Prof. D K Chauhan for providing the lab facility for capturing the microscopic photograph and Bishwajit Kumar Kushwaha for assisting to handle the microscope for photography.

Author contributions

S.M.P. hypothesizes and designed the experiments. S.T. performed the experiments. S.M.P. and S.T. wrote and concluded the final manuscripts.

Competing interests

The authors declare no competing interests.

References

1. Prasad, S. M., Zeeshan M. & Kumar, D. Toxicity of endosulfan on growth, photosynthesis, and nitrogenase activity in two species of Nostoc (*Nostoc muscorum* and *Nostoc calcicola*). *Toxicol Environ Chem.* **93**, 513-525 (2011).
2. Prasad, S. M. & Kumar, S. Differential responses of growth, antioxidant enzymes and oxidative stress in two species of Azolla (*Azolla microphylla* and *Azolla pinnata*) exposed to pretilachlor and enhanced UV-B radiation. *J Chem Pharm Res.* **3**: 974-985 (2011).
3. Singh, S., Verma, E., Tiwari, B. & Mishra, A. K. Exopolysaccharide production in *Anabaena* PCC 7120 under different CaCl_2 regimes. *Physiol Mol Biol Plants.* **22**, 557-566 (2016).
4. Latifi, A., Ruiz, M. & Zhang, C. Oxidative stress in cyanobacteria. *FEMS Microbiol Rev.* **33**, 258-278 (2009).
5. Foyer, C. H. & Noctor, G. Ascorbate and glutathione: the heart of redox hub. *Plant Physiol.* **155**, 2-18 (2011).
6. Gill, S. S. & Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **48**, 909-930 (2010).
7. Jiang, H., Grenley, M. O., Bravo, M. J., Blumhagen, R. Z. & Edgar, B. A. EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in *Drosophila*. *Cell Stem Cell* **8**, 84-95

(2011).

8. Das, K. & Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants *Front Environ Sci.* **2**, 1-13 (2014).
9. Bashri, G. & Prasad, S. M. Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed *Trigonella foenum-graecum* seedlings: Toxicity alleviation by up-regulation of ascorbate glutathione Cycle. *Ecotoxicol Environ Saf.* **132**, 329-338 (2016).
10. Sarker, U. & Oba, S. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Rep.* **8**, 1-12 (2018).
11. Singh, S., Singh, A., Srivastava, P. K. & Prasad, S. M. Cadmium toxicity and its amelioration by kinetin in tomato seedlings vis-à-vis ascorbate-glutathione cycle. *J Photochem Photobiol B: Biol.* **178**, 76-84 (2018).
12. Noctor, G. & Foyer, C. H. Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol.* **49**, 249-279 (1998).
13. Foyer, C. H. & Noctor, G. Ascorbate and glutathione: the heart of redox hub. *Plant Physiol.* **155**, 2-18 (2011).
14. Anjum, S. A., Tanveer, M., Hussain, S., Ashraf, U. & Khan, I. Alteration in growth, leaf gas exchange, and photosynthetic pigments of maize plants under combined cadmium and arsenic stress. *Water Air Soil Pollut.* **228**, 13 (2017).
15. Pandey, P., Ramegowda, V. & Senthil-Kumar, M. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. *Front Plant Sci.* **6**, 723 (2015).
16. Ozyigit, I. I., Dogan, I., Igdelioglu, S., Filiz, E., Karadeniz, S. & Uzunova, Z. Screening of damage induced by lead (Pb) in rye (*Secale cereale*) – a genetic and physiological approach. *Biotechnol Biotechnol Equip.* **30**, 1-8 (2016).
17. Kumar, J., Singh, V. P. & Prasad, S. M. An investigation on involvement of the ascorbate-glutathione cycle in modulating NaCl toxicity in two cyanobacteria photoacclimatized to different photosynthetic active radiation. *Algal res.* **32**, 70-78 (2018).
18. Lou, L., Li, X., Chen, J., Li, Y., Tang, Y. & Lv, J. Photosynthetic and ascorbate-glutathione metabolism in the flag leaves as compared to spikes under drought stress of winter wheat (*Triticum aestivum*). *PLoS One.* **13**:e0194625 (2018).
19. Padhy, R. N., Nayak, N. & Rath, S. Antagonism at combined effects of chemical fertilizer and carbamate insecticides on the rice field N₂-fixing cyanobacterium *Cylindrospermum* sp. In vitro. *Interdisciplinary Toxicol.* **7**, 101-107 (2014).
20. Pathak, J., Rajneesh, Maurya, P. K., Singh, S. P., Häder, D. P. & Sinha, R. P. Cyanobacterial farming for environment friendly sustainable agriculture practices: innovations and perspectives. *Environ. Sci.* **6**, 7 (2018).
21. Wang, G., Hao, ZJ, Anken, R. H., Lu, J. Y. & Liu, Y. D. Effects of UV-B radiation on photosynthesis activity of *Wolffia arrhiza* as probed by chlorophyll fluorescence transients. *Adv Space Res.* **45**, 839-

845 (2010).

22. Chahid, K., Laglaoui, A., Zantar, S. & Ennabili, A. Antioxidant-enzyme reaction to the oxidative stress due to alpha-cypermethrin, chlorpyrifos, and pirimicarb in tomato (*Lycopersicon esculentum* Mill.). *Environ. Sci. Pollut. Res.* **22**, 18115-18126 (2015).
23. Singh, V. P., Srivastava, P. K. & Prasad, S. M. UV-B induced differential effect on growth and nitrogen metabolism in two cyanobacteria under copper toxicity. *Cell Mol Biol.* **58**, 85-95 (2012).
24. Srivastava, P. K., Singh, V. P. & Prasad, S. M. Compatibility of ascorbate-glutathione cycle enzymes in cyanobacteria against low and high UV-B exposures, simultaneously exposed to low and high doses of chlorpyrifos. *Ecotoxicol Environ Saf.* **83**, 79-88 (2012).
25. Singh, V. P., Kumar, J., Singh, S. & Prasad, S. M. Dimethoate modifies enhanced UV-B effects on growth, photosynthesis and oxidative stress in mung bean (*Vigna radiata* L.) seedlings: Implication of salicylic acid. *Pesticide Biochem Physiol.* **116**, 13-23 (2014).
26. Saygideger, S. & Deniz, F. Effect of 24-epibrassinolide on biomass, growth and free proline concentration in *Spirulina platensis* (Cyanophyta) under NaCl stress. *Plant Growth Regul.* **56**, 219-223 (2008).
27. Bashri, G. & Prasad, S. M. Indole acetic acid modulates changes in growth, chlorophyll *a* fluorescence and antioxidant potential of *Trigonella foenum-graecum* grown under cadmium stress. *Acta Physiol Plant.* **37**, 1-14 (2015).
28. Bashri, G., Parihar, P., Singh, R., Singh, V. P. & Prasad, S. M. Physiological and biochemical characterization of two *Amaranthus* species under Cr (VI) stress differing in Cr(VI) tolerance. *Plant Physiol Biochem.* **108**, 12-23.(2016).
29. Sharma, A., Kumar, V., Thukral, A. K. & Bhardwaj, R. Epibrassinolide imidacloprid interaction enhances non-enzymatic antioxidants in *Brassica juncea* *Indian J Plant Physiol.* **21**, 70-75 (2016).
30. Singh, S. & Prasad, S. M. Growth, photosynthesis and oxidative responses of *Solanum melongena* seedlings to cadmium stress: Mechanism of toxicity amelioration by kinetin. *Sci Horticult.* **176**, 1-10 (2014).
31. Stirk, W. A. *et al.* Changes in endogenous cytokinins concentrations in *Chlorella* (Chlorophyceae) in relation to light and the cell cycle. *J Phycol.* **47**, 291-301 (2011).
32. Stirk, W. A. *et al.* Auxin and cytokinin relationships in 24 microalgal strains. *J Phycol.* **49**, 459-467 (2013).
33. Abdel-Raouf, N., Al-Homaidan, A. A. & Ibraheem, I. B. M. Agricultural importance of algae. *Afri J Biotechnol.* **11**, 11648–11658 (2012).
34. Lu, Y. & Xu, J. Phytohormones in microalgae: a new opportunity for microalgal biotechnology. *Trends Plant Sci.* **20**, 273-282 (2015).
35. Zizkova, E. *et al.* Control of cytokinin and auxin homeostasis in cyanobacteria and algae. *Annal Bot.* **119**, 151-166 (2017).

36. Wu, X., Zhu, Z., Li, X. & Zha D. Effects of cytokinin on photosynthetic gas exchange, chlorophyll fluorescence parameters and antioxidative system in seedlings of eggplant (*Solanum melongena*) under salinity stress. *Acta Physiol Planta*. **34**, 2105-2114 (2012).
37. Singh, S. & Prasad, S. M. Growth, photosynthesis and oxidative responses of *Solanum melongena* seedlings to cadmium stress: Mechanism of toxicity amelioration by kinetin. *Sci Horticul*. **176**, 1-10 (2014)..
38. Singh, S., Singh, A., Srivastava, P. K. & Prasad, S. M. Cadmium toxicity and its amelioration by kinetin in tomato seedlings vis-à-vis ascorbate-glutathione cycle. *J Photochem Photobiol B: Biol*. **178**, 76-84 (2018).
39. Singh, M., Bashri, G., Prasad, S. M. & Singh, V. P. Kinetin alleviates UV-B-induced damage in *Solanum lycopersicum*: implications of phenolics and antioxidants. *J Plant Growth Regul*. 1-11 (2019).
40. Tiwari, S. & Prasad, S. M. Regulation of insecticide toxicity by kinetin in two paddy field cyanobacteria: Physiological and biochemical assessment. *Environ Pollut*. **259**, 113806 (2020).
41. Tiwari, S., Verma, N., Prasad, S. M. & Singh, V. P. Cytokinin alleviates cypermethrin toxicity in *Nostoc muscorum* by involving nitric oxide: Regulation of exopolysaccharides secretion, PS II photochemistry and reactive oxygen species homeostasis. *Chemosphere*, **259**, 127356 (2020).
42. Sharma, M., Kaushik, A., Bala, S. K. & Kamra, A. Sequestration of chromium by exopolysaccharides of Nostoc and Gleocapsa from dilute aqueous solutions. *J Hazard Mater*. **157**, 315-318 (2008).
43. Tiwari, S., Verma, N., Singh, V. P. & Prasad, S. M. Nitric oxide ameliorates aluminium toxicity in *Anabaena* PCC 7120: Regulation of aluminium accumulation, exopolysaccharides secretion, photosynthesis and oxidative biomarkers. *Environ Exp Bot*. **161**, 218-227 (2019)..
44. Rossi, F. & De Philippis, R., Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. *Life*, **5**, 1218-1238 (2015).
45. Prasad, S. M., Kumar, S., Parihar, P., Singh, A. & Singh, R. Evaluating the combined effects of pretilachlor and UV-B on two Azolla species. *Pestic Biochem Physiol*. **128**, 45-56 (2016).
46. Ahanger, M. A. *et al*. Potential of exogenously sourced kinetin in protecting *Solanum lycopersicum* from NaCl-induced oxidative stress through up-regulation of the antioxidant system, ascorbate-glutathione cycle and glyoxalase system. *PLoS ONE* **13**: e0202175 (2018).
47. Bashri, G. & Prasad, S. M. Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed Trigonella foenum-graecum L. seedlings: Toxicity alleviation by up-regulation of ascorbate glutathione Cycle. *Ecotoxicol Environ Saf*. **132**, 329-338(2016).
48. Dolatabadian, A. & Jouneghani, R. S. Impact of exogenous ascorbic acid on antioxidant activity and some physiological traits of common bean subjected to salinity stress. *Not Bot Horti Agrobot Cluj Napoca*. **37**, 165-172 (2009).
49. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the Folin phenol reagent. *J Biol Chem*. **193**, 265-275 (1951).
50. Herbert, D., Phipps, P. J. & Strange, R. E. Chemical analysis of microbial cells. In: Norris, J.R., Ribbons, D.W. (Eds.), *Methods in Microbiology*. Academic Press, London, pp. 209-344 (1971).

51. Förster, B., Osmond, C. B. & Pogson, B. J. Improved survival of very high light and oxidative stress is conferred by spontaneous gain-of-function mutations in *Chlamydomonas*. *Biochim Biophys Acta*. **1709**, 45-57 (2005).
52. Pompella, A., Maellaro, E., Casini, A.F., Ferrali, M., Ciccoli, L. & Comporti, M. Measurement of lipid peroxidation in vivo: a comparison of different procedures. *Lipids* **22**, 206-211 (1987).
53. Nakano, Y. & Asada, K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **22**, 867-880 (1981).
54. Schadle, M. & Bassham, J. A. Chloroplast glutathione reductase. *Plant Physiol.* **59**, 1011-1012 (1977).
55. Hossain, M. A., Nakano, Y. & Asada, K. Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol.* **25**, 385-395 (1984).
56. Nakano, Y. & Asada, K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **22**, 867-880 (1981).
57. Gossett, D. R., Millhollon, E. P. & Cran, L. M. Antioxidant response to NaCl stress in salt-sensitive cultivars of cotton. *Crop Sci.* **34**, 706-714 (1994).
58. Brehe, J. E. & Burch, H. B. Enzymatic assay for glutathione. *Anal.* **74**, 189-197 (1976).

Tables

Table 1

Impact of KN (20 nM) on total ascorbate (T-ASA) contents and reduced/oxidized (ASA/DHA) ratio of *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. Control: - Cyp, Cyp₁: 2 µg ml⁻¹ and Cyp₂: 4 µg ml⁻¹. Data are means ± standard error of three replicates (n = 3). Values followed by different letters within same column show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Treatments		<i>Anabaena</i> PCC 7120		<i>Nostoc muscorum</i>	
Cypermethrin		T-ASA	ASA/DHA ratio	T-ASA	ASA/DHA ratio
Without KN	- Cyp	101.08 ± 2.33 ^b	5.76 ± 0.13 ^b	111.51 ± 2.57 ^b	7.52 ± 0.17 ^b
	Cyp ₁	92.41 ± 2.13 ^c	4.55 ± 0.10 ^c	103.38 ± 2.38 ^c	6.52 ± 0.15 ^c
	Cyp ₂	77.10 ± 2.45 ^d	3.24 ± 0.07 ^e	88.84 ± 2.05 ^d	3.91 ± 0.09 ^e
With KN	- Cyp	106.44 ± 2.45 ^{ab}	6.20 ± 0.14 ^a	119.42 ± 2.75 ^a	8.47 ± 0.19 ^a
	Cyp ₁	109.11 ± 2.51 ^a	5.73 ± 0.13 ^b	125.20 ± 2.89 ^a	8.62 ± 0.19 ^a
	Cyp ₂	85.59 ± 1.97 ^c	3.82 ± 0.08 ^d	102.19 ± 2.35 ^c	4.84 ± 0.11 ^d

Table 2

Impact of IAA (240 nM) on total ascorbate (T-ASA) contents and reduced/oxidized (ASA/DHA) ratio of *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. Control: - Cyp, Cyp₁: 2 µg ml⁻¹ and Cyp₂: 4 µg ml⁻¹. Data are means ± standard error of three replicates (n = 3). Values followed by different letters within same column show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Treatments		<i>Anabaena</i> PCC 7120		<i>Nostoc muscorum</i>	
Cypermethrin		T-ASA	ASA/DHA ratio	T-ASA	ASA/DHA ratio
Without KN					
- Cyp		102.42 ± 2.36 ^a	5.01 ± 0.11 ^b	111.07 ± 2.56 ^b	7.57 ± 0.17 ^b
	Cyp ₁	93.36 ± 2.15 ^b	4.05 ± 0.09 ^c	103.12 ± 2.38 ^c	6.53 ± 0.15 ^c
	Cyp ₂	78.23 ± 1.08 ^c	2.80 ± 0.06 ^e	88.97 ± 2.05 ^d	3.87 ± 0.08 ^e
With KN					
- Cyp		105.98 ± 2.44 ^a	5.33 ± 0.12 ^a	116.88 ± 2.69 ^{ab}	7.98 ± 0.18 ^{ab}
	Cyp ₁	108.40 ± 2.50 ^a	4.99 ± 0.11 ^b	122.51 ± 2.82 ^a	8.21 ± 0.18 ^a
	Cyp ₂	84.20 ± 1.94 ^c	3.65 ± 0.08 ^d	97.21 ± 2.24 ^c	4.47 ± 0.10 ^d

Table 3

Impact of KN (20 nM) on total glutathione (T-GSH) contents and reduced/oxidized (GSH/GSSG) ratio of *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. Control: - Cyp, Cyp₁: 2 µg ml⁻¹ and Cyp₂: 4 µg ml⁻¹. The 100% T-GSH correspond to 43.40 ± 1.00 µM (g dry weight)⁻¹ and 50.10 ± 1.15 µM (g dry weight)⁻¹ for *Anabaena* PCC 7120 and *Nostoc muscorum* respectively. Data are means ± standard error of three replicates (n = 3). Values followed by different letters within same column show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Treatments			<i>Anabaena</i> PCC 7120		<i>Nostoc muscorum</i>	
Cypermethrin			T-GSH	GSH/GSSG ratio	T-GSH	GSH/GSSG ratio
Without	KN	- Cyp	43.40 ± 1.00 ^{bc}	5.91 ± 0.13 ^b	50.16 ± 1.15 ^c	6.25 ± 0.14 ^b
		Cyp ₁	38.96 ± 0.89 ^d	4.58 ± 0.10 ^c	46.20 ± 1.06 ^d	5.13 ± 0.11 ^c
		Cyp ₂	35.52 ± 0.82 ^e	3.57 ± 0.08 ^d	42.45 ± 0.98 ^e	4.30 ± 0.09 ^d
With KN		- Cyp	45.97 ± 1.06 ^b	6.06 ± 0.14 ^b	54.12 ± 1.25 ^b	6.60 ± 0.15 ^b
		Cyp ₁	51.23 ± 1.18 ^a	6.65 ± 0.15 ^a	60.37 ± 1.39 ^a	7.79 ± 0.18 ^a
		Cyp ₂	40.72 ± 0.94 ^{cd}	4.37 ± 0.10 ^c	49.80 ± 1.15 ^{cd}	5.30 ± 0.12 ^c

Table 4

Impact of IAA (240 nM) on total glutathione (T-GSH) contents and reduced/oxidized (GSH/GSSG) ratio of *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. Control: - Cyp, Cyp₁: 2 µg ml⁻¹ and Cyp₂: 4 µg ml⁻¹. The 100% T-GSH correspond to 43.40 ± 1.00 µM (g dry weight)⁻¹ and 50.10 ± 1.15 µM (g dry weight)⁻¹ for *Anabaena* PCC 7120 and *Nostoc muscorum* respectively. Data are means ± standard error of three replicates (n = 3). Values followed by different letters within same column show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Treatments			<i>Anabaena</i> PCC 7120		<i>Nostoc muscorum</i>	
Cypermethrin			T-GSH	GSH/GSSG ratio	T-GSH	GSH/GSSG ratio
Without IAA	-	Cyp	43.95 ± 1.01 ^b	5.91 ± 0.13 ^b	100.00 ± 2.3 ^c	6.19 ± 0.14 ^b
		Cyp ₁	39.36 ± 0.90 ^c	4.55 ± 0.10 ^c	92.20 ± 2.1 ^d	5.06 ± 0.11 ^c
		Cyp ₂	36.07 ± 0.83 ^d	3.60 ± 0.08 ^e	84.73 ± 1.9 ^e	4.26 ± 0.09 ^d
With IAA	-	Cyp	45.61 ± 1.05 ^b	5.88 ± 0.13 ^b	108.03 ± 2.4 ^b	6.37 ± .014 ^b
		Cyp ₁	51.20 ± 1.18 ^a	6.42 ± 0.14 ^a	120.49 ± 2.7 ^a	7.31 ± 0.16 ^a
		Cyp ₂	40.33 ± 0.93 ^c	4.13 ± 0.09 ^d	99.39 ± 2.2 ^{cd}	5.04 ± 0.11 ^c

Figures

Figure 1

Impact of KN and IAA on growth and content of protein in *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. – Cyp (Control), Cyp₁ (2 μg ml⁻¹) and Cyp₂ (4 μg ml⁻¹). Data are means ± standard error of three replicates (n = 3). The bars followed by different letters show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Figure 2

Impact of KN and IAA on exopolysaccharides (EPS) content in *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. – Cyp (Control), Cyp₁ (2 µg ml⁻¹) and Cyp₂ (4 µg ml⁻¹). Data are means ± standard error of three replicates (n = 3). The bars followed by different letters show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Figure 3

In-vivo visualization (cellular) of superoxide anion (nitroblue tetrazolium: NBT), H₂O₂ (3, 3' diaminobenzidine: DAB) and MDA equivalent contents (Schiff's reagent) accumulated in KN and IAA treated *Anabaena* sp. PCC 7120 and *Nostoc muscorum* ATCC 27893 exposed to cypermethrin (Cyp) stress. – Cyp (Control), Cyp₁ (2 µg ml⁻¹) and Cyp₂ (4 µg ml⁻¹).

Figure 4

Impact of KN on activity of ascorbate peroxidase (APX) and glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) in *Anabaena* sp. PCC 7120 and *Nostoc muscorum* ATCC 27893 exposed to cypermethrin (Cyp) stress. – Cyp (Control), Cyp₁ (2 µg ml⁻¹) and Cyp₂ (4 µg ml⁻¹). Data are means ± standard error of three replicates (n = 3). The bars followed by different letters show significant difference at P<0.05 significance level according to the Duncan's multiple range test.

Figure 5

Impact of IAA on activity of ascorbate peroxidase (APX) and glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) in *Anabaena* PCC 7120 and *Nostoc muscorum* exposed to cypermethrin (Cyp) stress. – Cyp (Control), Cyp₁ (2 µg ml⁻¹) and Cyp₂ (4 µg ml⁻¹). Data are means ± standard error of three replicates (n = 3). The bars followed by different letters show significant difference at P<0.05 significance level according to the Duncan's multiple range test.