Phytohormone up-regulates the biochemical constituent, exopolysaccharide and nitrogen metabolism in paddy-field cyanobacteria exposed to chromium stress

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Sanjesh Tiwari
Ranjan Plant Physiology and Biochemistry Laboratory
tiwarsanjesh@gmail.com

Anuradha Patel
University of Allahabad

Sheo Mohan Prasad
University of Allahabad

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Abstract
Current study deals with the assuaging effects of two phytohormones; indole acetic acid (IAA; 290 nM) and kinetin (KN; 10 nM) on growth, phycobiliproteins, status of nitrogen metabolism and biochemical constituents; protein, carbohydrate and exopolysaccharide contents in two diazotrophic cyanobacteria *Nostoc muscorum* and *Anabaena* exposed to chromium (Cr\text{VI}) stress (100 μM and 150 μM). Chromium individually at both the tested doses expressively declined the growth, chlorophyll *a* to carotenoid ratio and contents of phycobiliproteins; phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE). With distinctive impact on status of nitrogen metabolism chromium significantly reduced the nitrate (NO\textsubscript{3}–) and nitrite (NO\textsubscript{2}–) uptake rate and foremost decrease in nitrate and ammonia assimilating enzyme; nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT) except glutamate dehydrogenase (GDH). However, beneath alike condition, exogenous application of IAA and KN exhibited noteworthy assuaging effects on growth-regulating parameters in both the paddy field cyanobacteria, which consummately occurred as a result of substantial decrease in Cr uptake and inducing signaling responses and also enhances the growth parameter i.e. nitrogen metabolism as a result of considerable lowering in Cr induced damaging effect on nitrogen metabolism and uptake rate, and the alleviating effect was more pronounced with the lower dose of Cr, efficient in *N.muscorum* than *Anabaena*.

Introduction
Industrial commotions have largely contributed to the accretion of toxic substances including toxic heavy metals that contaminate terrestrial as well as aquatic ecosystems. Among countless heavy metals, Cr is most commonly occurring in earth’s crust (Prado et al., 2016) which subsists in various oxidation states ranging from (−2 to +6) but the trivalent chromite (Cr\text{III}) and hexavalent chromate (Cr\text{VI}) are the most toxic and stable forms (Ashraf et al., 2017). Further (Cr\text{VI}), contrary to (Cr\text{III}) has high solubility and mobility across biological membranes and thus well-thought-out a potent carcinogen, mutagen and strong oxidizing agent that persists in soil for a longer time (Coren˜o-Alonso et al., 2014; Viti et al., 2014; Desai et al., 2008). Increased industrial and modern agricultural practices (Butera et al., 2015; Chen et al., 2016) on an average 137,000 metric tons of Cr is produced
every year and increasing continuously (USGS, 2016) which is exceeding the threshold limit (Vogel et al., 2015). The Cr concentration is reported to be 50 µg L\(^{-1}\) in drinking water (Lilli et al., 2015) and in the aquatic ecosystem its concentration is in the range of 0.1—117 mg L\(^{-1}\) in freshwater and 0.2—50 mg L\(^{-1}\) in seawater (Nriagu, 1988). This contaminated water is used to irrigate the agricultural field and thus enters the field.

In India, crop fields are mainly irrigated with canal systems through river water which is contaminated by industrial waste encompassing heavy metals that enters the agricultural fields. Paddy (Oryza sativa) is a staple food crop that fulfills the food demand of about two-third of the world's human population, particularly in Asia after maize and sugarcane (Kuenzer and Knauer, 2013). Rice fields are provided with the flooded condition before transplantation that gives the natural environment for the growth of cyanobacteria. Cyanobacteria are endowed with the exclusive assets of fixing atmospheric nitrogen in form of nitrate that is easily available to rice plants thereby boosts the fertility of paddy fields and act as a bio-fertilizer (Zehr, 2011; Singh et al., 2016). Besides, its role as bio-fertilizer, they are potent source of carbohydrates, lipids, phenolics, vitamins, amino acids, sugars as well as regulatory substances that directly or indirectly enhance the crop yield (Prasanna et al., 2008).

Chromium induced negative effects on photoautotrophs are extensively studied by many workers (Gupta and Ballal, 2015; Tiwari et al., 2018; Singh and Prasad, 2019). It has been well studied that elevated level of Cr significantly affects the physiology and biochemistry of cyanobacteria such as Haplosiphon (Bano et al., 2012), Scenedesmus (Kovacik et al., 2015a), Oscillatoria (Munagamage et al., 2016) and Haematococcus (Peng et al., 2019). Elevated concentrations of Cr affects many physiological processes such as photosynthetic pigments (Banerjee et al., 2004), photosynthesis (Prasad et al., 1991) including nitrogen metabolism. Among various factors, nitrogen metabolism is prominent because nitrogen (N\(_2\)) is considered as key element in regulating the growth and development (Parween et al., 2011) as it is involved in the synthesis of nucleotides, amino acids, pigments, vitamins, enzymes and other compounds (Popovic et al., 2005). Chromium negatively affects the nitrogen metabolism by reducing the inorganic nitrogen uptake associated with decreased
nitrogen assimilating enzymes viz; nitrate reductase and nitrite reductase and ammonia assimilating enzymes that is associated with increased alternative pathway of ammonia assimilating enzyme glutamate dehydrogenase and altered the nitrogen requirement of cyanobacteria and reduced the growth. Hence, nitrogen metabolism is of central importance under stressful conditions. Earlier reports also indicate that biochemical constituents such as protein, carbohydrate and exopolysaccharides are also negatively affected by Cr stress (Bano et al., 2012; Gupta and Ballal, 2015).

Plants including micro-organisms survive in metal contamination sites by secreting growth stimulating substances such as phytohormones that act as a signaling molecule and mediate the overall development (Hunt et al., 2011) even at low concentrations. Maintaining the micro-flora of aquatic ecosystem or management of aquatic vegetation by the application of natural or synthetic growth regulators is a new step towards understanding the role of plant hormones (auxins, gibberellins, cytokinins, abscisic acid, ethylene, and brassinosteroids) in cyanobacteria by mediating an array of morphological, physiological, and developmental processes. In plants, leaf abscission is mediated by auxin (Jin et al., 2015) while cytokinin regulates the process of cell division, and chloroplast development (Tarakhovskaya et al., 2007). These phytohormones indeed present in plants as well as in micro-organism including cyanobacteria and several reports has been published concerning the presence of noble phytohormones in microalgae (Sergeeva et al., 2002; Lu and Xu, 2015; GirdhariBabu et al., 2017). Similar to plants, the role of phytohormones in micro-algae is controversial because there is less literature available. Thus, the present study is an endeavor to provide a comprehensive account of the ameliorating effects of phytohormones i.e IAA and KN on physiological and biochemical attributes with special reference to nitrogen metabolism of Nostoc muscorum and Anabaena PCC 7120 under chromium (Cr\textsuperscript{VI}) exposure.

Materials And Methods

2.1 Growth and treatment conditions

The cultures of Nostoc muscorum and Anabaena were maintained in BG-11 medium at 25 ± 2°C under 75 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} in well maintained culture room. Potassium dichromate (K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}) was
used as the source of Cr and in range from 5-350 µM concentrations and on the basis of this the two doses i.e. 100 and 150 µM of Cr\textsuperscript{VI} were selected that caused reduction by 10% (LC\textsubscript{10}) and 30% (LC\textsubscript{30}) for \textit{N. muscorum} and by 15% (LC\textsubscript{15}) and 35% (LC\textsubscript{35}) for \textit{Anabaena}. Further, doses for IAA and KN were also screened out and stimulatory concentrations of IAA i.e. 290 nM and KN i.e. 10 nM enhanced the growth by 9 and 14% for \textit{N. muscorum} and 7 and 10% for \textit{Anabaena} were selected for detailed study. There were three replicates (n=6) for every treatment and all the parameters were analyzed after 96 h of experiment.

2.2 Measurement of growth attributes

Growth was measured in terms of culture absorbance by taking absorbance at 750 nm by using UV-Visible Double beam-1700 Spectrophotometer.

2.3 Estimation of photosynthetic pigment contents

The amount of chlorophyll \textit{a} and carotenoids was estimated by subsequent methodology of Porra et al. (1989) and Goodwin (1954), respectively by recording absorbance of pigment at 665 nm for chlorophyll \textit{a} and at 450 nm for carotenoids with the help of UV-Visible Double beam-1700 Spectrophotometer, Shimadzu, Japan.

For the estimation of phycobiliproteins (PBPs), cells were treated with toluene and extracted with 2.5 mM potassium phosphate buffer (pH 7.0) and the absorbance was read out at 615, 652 and 562. The amount of PBPs was determined by using equation given by Bennett and Bogorad (1973).

2.4 Estimation of cellular accumulation of chromium

The pellets were washed with 1 mM EDTA and re-suspended in chilled buffer for 15 min to remove apo-plastic Cr\textsuperscript{VI}, respectively, and then pellets were oven-dried at 70-80 °C for 3 days until it completely dries. Dried samples were digested by adding 5 ml of tri-acid mixture (HNO\textsubscript{3}, H\textsubscript{2}SO\textsubscript{4} and HClO\textsubscript{4} in ratio of 5:1:1) at 80 °C until a transparent solution obtained. The Cr\textsuperscript{VI} was estimated by using atomic absorption spectrophotometer (iCE 3000 series, Model 3500 AAS, Thermo Scientific, UK). The instrument was calibrated by using standard solutions of Cr\textsuperscript{VI}.

SEM technique
Surface morphology of the dry adsorbent before and after Cr\textsuperscript{VI} treatment was also visualized by scanning electron microscope (Double beam FEI Nova Nano SEM-450).

2.5 Estimation of NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} uptake

For estimation of NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} uptake the cyanobacterial, cells were pre-incubated with 100 µM KNO\textsubscript{3}/ KNO\textsubscript{2} under their respective growth conditions for 24 h and thereafter cells were harvested for uptake study.

2.5.1 Nitrate (NO\textsubscript{3}\textsuperscript{−}) uptake

The NO\textsubscript{3}\textsuperscript{−} uptake rate in control and treated cyanobacterial cells was estimated by measuring the depletion of NO\textsubscript{3}\textsuperscript{−} from the external medium at 210 nm using the method of Cawse (1967). Samples were withdrawn after 4 h of incubation, subjected to centrifugation at 4,000 g for and the cell-free supernatants were examined for residual NO\textsubscript{3}\textsuperscript{−}.

2.5.2 Nitrite (NO\textsubscript{2}\textsuperscript{−}) uptake

The NO\textsubscript{2}\textsuperscript{−} uptake rate was restrained by the depletion of NO\textsubscript{2}\textsuperscript{−} from the external medium through spectrophotometer at 540 nm using the method of Snell and Snell (1949). the experiments were started by the addition of 100 µM of KNO\textsubscript{2} to the cell suspension at zero time for NO\textsubscript{2}\textsuperscript{−} uptake. Samples were withdrawn after 4 h of incubation, subjected to centrifugation at 4000 g for 10 min and the cell-free supernatants considered for residual NO\textsubscript{2}\textsuperscript{−}.

2.6 Nitrate assimilating enzymes

2.6.1 Estimation of nitrate reductase (NR) and nitrite reductase (NiR) activity

The NR/NiR activity was carried out with dithionite-reduced methyl viologen as a reductant in cells by adding mixed alkyltrimethyl ammonium bromide (MTA) to the reaction mixture according to the method of Herrero et al. (1981; 1984; 1986). The reaction mixture was incubated for 5 min at 25 °C, and NO\textsubscript{2}\textsuperscript{−} was estimated in corresponding cell-free media. For the measurement of NR and NiR
activity in heterocystous cyanobacteria *N. muscorum* and *Anabaena* were acclimatize in BG-11 medium containing KNO$_3$ and NaNO$_2$ at beginning of the experiment to induce NR and NiR enzymes, respectively. One unit of NR activity is defined as 1 nmol NO$_2^-$ formed min$^{-1}$ and one unit of NiR activity is defined as 1 nmol NO$_2^-$ consumed min$^{-1}$.

2.7 Ammonium assimilating enzymes activity

2.7.1 Estimation of glutamine synthetase (GS) activity

Glutamine synthetase activity was determined by the formation of $\gamma$-glutamylhydroxamate (transferase assay) following the method of Mérida et al. (1991). After centrifugation, cells were disrupted by sonication (Sonic Vibra Cell, Model VCX-130PB, USA) and homogenate was centrifuged at 15000 g for 20 min at 4 °C (Model CPR-30, Remi, India) and the resulting supernatant constituted the cell extract. One unit of GS activity is defined as 1 nmol-$\gamma$-glutamylhydroxamate formed min$^{-1}$.

2.7.2 Estimation of glutamate synthase (NADH-GOGAT and FD-GOGAT) activity

The glutamine 2-oxoglutarate aminotransferase (GOGAT) activity was restrained by following the method of Meers et al. (1970) and Navarro et al. (1995). After centrifugation, cells were re suspended in Tris-HCl buffer (pH 7.6) and then disrupted thoroughly by sonication (Sonics Vibra Cell Model VCX-130PB, USA). The reaction was determined by measuring oxidation of NADH and FD at 340 nm. One unit of GOGAT activity is defined as 1 nmol NADH and FAD oxidized min$^{-1}$.

2.7.3 Estimation of glutamate dehydrogenase (NADH-GDH) activity

Glutamate dehydrogenase activity (aminating) was quantified as per the method given by Chávez and Candau (1991). The cells were crushed in HEPES-NaOH buffer (pH 7.0) and the supernatant obtained was used as the enzyme extract. The reaction was started by the addition of NH$_4$Cl and oxidation of NADH was recorded at 340 nm. One unit of GDH activity is defined as 1 nmol NADH oxidized min$^{-1}$.

2.8 Estimation of protein

The protein content of each sample was measured by the method of Bradford (1976). After centrifugation at 4,000g the pellets homogenized with potassium phosphate buffer (pH 7.8) and
centrifuged at 10,000 g for 10 min at 4 °C, and supernatants were used for the estimation of protein content by taking absorbance at 595 nm.

2.9 Estimation of exopolysaccharides content

Exopolysaccharides (EPS) content in both the cyanobacteria was determined by following the method of Sharma et al. (2008). The treated and untreated cultures were centrifuged at 3,000 g for 15-20 min, and the cell-free suspension was taken and concentrated to 10 folds by evaporation at 40 °C. Followed by washing with isopropanol thrice and further left for drying at 37 °C. Then the hydrolysates was analyzed for glucose and content calculated as per the standard curve obtained for glucose (Seifer et al., 1959).

2.10 Estimation of carbohydrate content

Carbohydrate content in each sample was estimated by adopting the method of Dubois et al. (1956). The samples were centrifuged at 10,000 g for 10 min. the sample was prepared of which 1.0 ml of supernatant and 1.0 ml of 5 % phenol and 5 ml H₂SO₄ were added. The absorbance was recorded at 490 nm, using a spectrophotometer and compared with the standard curve prepared with pure glucose.

2.11 Scanning electron micrography

Before and after Cr exposition the surface morphology of the dry were studied by Scanning electron microscope (Double beam FEI Nova Nano SEM-450). After repeated washing in organic solvents, dry samples were mounted on stubs and coated with gold-palladium of thickness 100–1500A and then transferred to the sample chamber of the instrument. This was operated at 20 kV and 5.5 WD in high vacuum mode.

2.12 Statistical Analysis

Results were statistically analyzed, one-way ANOVA was performed to test significance level (Duncan multiple range tests, DMRT) at p < 0.05. Further to confirm Two-way ANOVA test was also performed to show the differential action of Cr and KN alone as well as in combination. The results presented are meant ± standard error of three replicates (n = 3) and SPSS-16 software was used for DMRT.
Experimental Findings

4.1 Biomass accumulation

Result pertaining to growth (measured in terms of culture absorbance at 750 nm) of tested cyanobacteria i.e. Nostoc muscorum ATCC 27893 and Anabaena PCC 7120 with and without exogenous supplemented phytohormones i.e. IAA or KN has been presented in Figures 1A-B. Chromium (Cr\textsuperscript{VI}) at both the tested doses i.e. 100 µM (lower dose) and 150 µM (higher dose) significantly declined the growth that corresponds to LC\textsubscript{10} (10%) and LC\textsubscript{30} (30%) in N. muscorum and LC\textsubscript{15} (15%) and LC\textsubscript{35} (35%) in Anabaena as shown in growth response curve (Figures 1A-B). Results clearly showed that Cr\textsuperscript{VI} at 150 µM caused maximum growth inhibition and the damage was more pronounced in Anabaena. Exogenous application of IAA in Cr (100 and 150 µM) resulted in inhibition in growth of only 4 and 19% in N. muscorum and 7 and 26% in Anabaena respectively. Under similar Cr\textsuperscript{VI} treatment, exogenous KN addition in cultures caused only the inhibition of 1 and 14 % in N. muscorum and 4 and 21% in Anabaena respectively. The ameliorating effect of IAA and KN against Cr toxicity was greater in N. muscorum as compared to Anabaena. Furthermore, exogenous supplementation of phytohormones i.e. IAA and KN in Cr\textsuperscript{VI} untreated samples enhanced the growth by 9 and 14% in N.muscorum, while in Anabaena it was 6 and 10% respectively as compared to respective phytohormones and Cr\textsuperscript{VI} untreated samples (control) (Figure1A).

4.2 Chromium accumulation

The result related to the intracellular accumulation of Cr has been depicted in Figures 2 and was found to increase in cells of both the cyanobacteria in Cr exogenous concentration-dependent manner. The cellular accumulation of Cr was increased from 173±3.0 µg Cr g\textsuperscript{-1} dry weight to 216±3.6 µg Cr g\textsuperscript{-1} dry weight in N. muscorum and from 198±2.6 µg Cr g\textsuperscript{-1} dry weight to 267±6.9 µg Cr g\textsuperscript{-1} dry weight in Anabaena when concentration of Cr was raised from 100µM to 150 µM, respectively. Upon IAA and KN (alone) supplementation to the cultures, the intracellular accumulation of Cr was significantly declined than the values recorded under-tested doses of Cr\textsuperscript{VI} without
phytohormone and lowering in the cellular accumulation of Cr was more pronounced under KN supplementation than IAA in both the cyanobacteria and the lowering in intracellular Cr accumulation following either of phytohormone exposure was greater in case of *N. muscorum*.

SEM images proved that contrary to control, both cyanobacterial strains grown under the Cr\(^{VI}\) stress produced a significantly high amount of EPS (Figure 6A). Figures clearly show presence of depression and groves as exopolysaccharide exudate provide high surface area of the bio-sorbent that gives binding sites visible as white encrustations. SEM images also show alteration in cell morphology as decrease in cell size and shrinkage was observed under Cr\(^{VI}\) stress (Figure 6A).

### 4.3 Photosynthetic pigments

Results pertaining to the ratio of Chl a by Car and contents of phycobiliproteins of Cr\(^{VI}\) stressed cultures of *N. muscorum* and *Anabaena* have been depicted in Tables 1. The result clearly reveals that Cr\(^{VI}\) at both the tested doses a significant reduction in ratio of Chl a to Car by 4 and 10 % in *N. muscorum* and by 6 and 13% in *Anabaena*, respectively. Similarly, phycobiliproteins (PBPs; PC, APC and PE) were also found to be majorly affected under Cr\(^{VI}\) stress (Tables 1). Among the three components of phycobiliproteins the PC content was severely affected as it showed inhibition of 14 and 37 in *N. muscorum* and 22 and 47% in *Anabaena*. Under similar conditions, there is no significant reduction in the PE content while APC content follows similar pattern. Further exogenous supplementation of phytohormone; IAA and KN (alone) caused significant improvement in ratios by 2 and 4% in *N. muscorum* and 1 and 3% in *Anabaena* respectively, however with tested doses of Cr\(^{VI}\) reduction in values were recorded but still lower than the values recorded with control and also with samples lacking phytohormone treatment. Moreover the PBPs showed significant enhancement under stress condition followed with IAA and KN treatment and similar pattern was recorded for APC and PE but PC is majorly affected in both the cyanobacteria, prominent in *Anabaena*.

### 4.4 Inorganic nitrogen uptake

For the estimation of inorganic nitrogen uptake both the cyanobacteria were grown in NO\(_3^-\) and NO\(_2^-\)
containing BG-11 medium for 24 h previous to record NO$_3^-$ and NO$_2^-$ uptake rate and activity of nitrate reductase (NR) and nitrite reductase (NiR) activity.

### 4.4.1 Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) uptake

Data related to the NO$_3^-$ and NO$_2^-$ uptakes in both the tested cyanobacteria have been depicted in Figure 3A. The result reveals that Cr$^{VI}$ at 100 and 150 µM declined the uptake of NO$_3^-$ by 12 and 28% and NO$_2^-$ by 10 and 25% in *N. muscorum* and the corresponding decrease in NO$_3^-$ by 17 and 33% and NO$_2^-$ by 15 and 30% in *Anabaena*, respectively, over the control values. Under similar condition (100 and 150 µM Cr$^{VI}$) exogenous supplementation of IAA exhibited alleviating effect as the reduction was only 6 and 20% for NO$_3^-$ and 4 and 17% for NO$_2^-$ in *N. muscorum* while in *Anabaena* it was 10 and 22% for NO$_3^-$ and 8 and 20% for NO$_2^-$ respectively. Similar results were also obtained under KN treatments; however, KN showed greater alleviating effect as compared to IAA in both the cyanobacteria.

### 4.5 Nitrate assimilating enzymes

#### 4.5.1 Nitrate and nitrite reductase activity

The NR activity in both the tested cyanobacteria has been depicted in Figure 3B. Result reveals that NR activity was inhibited by 13 and 31% in *N. muscorum* and by 18 and 38% in *Anabaena* after 100 and 150 µM Cr$^{VI}$ treatments respectively. A similar pattern of inhibitory effect was noticed for NiR activity under Cr$^{VI}$ stress (100 and 150 µM) showing 15 and 33% in *N. muscorum* and by 17 and 35% in *Anabaena*, respectively. Upon IAA supplementation, a significant recovery in the activity of both the enzymes was noticed, as it was decreased by only 6 and 18% in NR and by 7 and 22% in NiR in *N. muscorum*, and by 10 and 23% in NR and 11 and 28% in NiR in *Anabaena* at 100 and 150 µM Cr$^{VI}$ treatments, respectively. Likewise, KN supplementation significantly alleviated the activity of NR and NiR in both the cyanobacteria however, the ameliorating effect was more pronounced in *N. muscorum* under Cr$^{VI}$ stress.
4.6 Ammonia assimilating enzymes

4.6.1 Glutamine synthetase and glutamate synthase (GOGAT) activity

Results pertaining to the GS and GOGAT activity in Cr\textsuperscript{VI} stressed \textit{N. muscorum} and \textit{Anabaena} supplemented with IAA and KN have been depicted in Figure 4. Chromium at both the tested doses (100 and 150 µM) suppressed the activity of GS by 14 and 29% in \textit{N. muscorum} and by 15 and 32% in \textit{Anabaena}, respectively, above control values.

Likewise, similar doses of Cr\textsuperscript{VI} lowered down the GOGAT activity by 11 and 30% in \textit{N. muscorum} and by 14 and 35% in \textit{Anabaena}, respectively, over the values of the respective control. Exogenous supplementation of both the phytohormones IAA and KN caused significant improvement in the activity of both the enzymes and the alleviating effect against Cr\textsuperscript{VI} toxicity to these enzymes was greater in \textit{N. muscorum}.

4.6.3 Glutamate dehydrogenase (GDH) activity

Data related to alternative GDH activity have been depicted in Figure 4. As compared to the activity of other enzymes studied, a reverse trend was observed for GDH in Cr\textsuperscript{VI} stressed both cyanobacteria. Results exhibited that GDH activity was enhanced by 17 and 36% in \textit{N. muscorum} and by 28 and 42% in \textit{Anabaena}, respectively after 100 and 150 µM of Cr\textsuperscript{VI} treatments. Further, on IAA and KN supplementation to Cr\textsuperscript{VI} stressed cultures a declining trend in GDH activity was noticed in both the cyanobacteria, however, the activity was still considerably greater than that of control.

4.7 Protein content

The results pertaining to the effect of IAA or KN supplementation on the protein content of Cr\textsuperscript{VI} stressed \textit{N. muscorum} and \textit{Anabaena} have been depicted in Figure 5. The Cr\textsuperscript{VI} at 100 and 150 µM doses significantly decreased the protein content by 12 and 30% in \textit{N. muscorum}, and by 18 and 35% in \textit{Anabaena}, respectively, over the control values. However, IAA or KN supplementation to Cr\textsuperscript{VI} stressed cyanobacteria considerably lowered the inhibitory effect of Cr\textsuperscript{VI} on protein content but values were still less than control. Furthermore, the alleviating effect on protein content by the application of
phytohormone was more pronounced in *N. muscorum*.

**4.8 Exopolysaccharides (EPS) content**

The results pertaining to EPS content has been depicted in **Figure 5**. Results reveal that at the lower dose of Cr\textsuperscript{VI} i.e. 100 µM, EPS content was enhanced by 11% in *N. muscorum*, and 8% in *Anabaena*, respectively. Contrary to this, 150 µM of Cr\textsuperscript{VI} caused a significant reduction in EPS content by 11% in *N. muscorum*, and 16% in *Anabaena*, respectively, over the control values. Upon IAA or KN supplementation to Cr\textsuperscript{VI} stressed cyanobacterial cultures, EPS content was further enhanced at lower dose (100 µM Cr\textsuperscript{VI}) and partial alleviation in EPS content was recorded at higher dose (150 µM Cr\textsuperscript{VI}) in tested cyanobacteria.

**4.9 Carbohydrate content**

The carbohydrate content in both the cyanobacteria was lowered at both the doses of Cr\textsuperscript{VI} (100 and 150 µM), as it was decreased by 13 and 19% in *N. muscorum*, and by 18 and 25% in *Anabaena*, respectively (**Figure 5**). The supplementation with the IAA or KN to Cr\textsuperscript{VI} treated cells, exhibited appreciable improvement in carbohydrate content in both the cyanobacteria but the values were still less than that of control.

**Discussion**

In the present study the cyanobacteria *viz.*, *Nostoc muscorum* and *Anabaena* PCC 7120, were treated with the doses (Cr\textsuperscript{VI}; 100 and 150 µM) of hexavalent Cr with/without the supplementation of phytohormones; auxin (indole-3-acetic acid; IAA) and cytokinin (6-furfuryl amino acid/kinetin; KN). Chromium at both the tested doses showed a significant decline in growth (**Figure 1A-B**). The reduction in cyanobacterial growth under Cr\textsuperscript{VI} stress might be due to (i) significant increase in intracellular accumulation of Cr (Figure 2) due to its uptake *via* sulfate transporters (Liu et al., 2008), (ii) reduction in the light-harvesting pigments such as Chl a (published elsewhere) and phycobiliproteins (PBPs; PC, APC and PE) (**Tables 1**), (iii) reduction in inorganic nitrogen contents and reduction in nitrate (NR and NiR) and ammonia assimilatory enzymes (GS and GOGAT) except the enzyme (GDH) involved in alternative ammonia assimilating pathway (**Figures 3, 4**), followed by the
damaging effects on macromolecules such as protein, carbohydrate and exopolysaccharides (EPS) (Figure 5). The similar reason behind growth reduction was also explained by Rochetta et al. (2012) due to enhanced Cr uptake inside the cells as well as variations in defense system that actively inhibited the cell division (Zou et al., 2006). Our results are in agreement with the other findings where Cr significantly caused growth reduction in Oscillatoria (Jayashree et al., 2012) and Spirulina platensis (Gupta et al., 2014) and in Haematococcus (Peng et al., 2019). Furthermore, Cr induced toxicity on growth in Anabaena was greater than that of N. muscorum which could be correlated with the more accumulation of Cr in the Anabaena cells (Figure 2). Higher accumulation of Cr in Anabaena than that of N. muscorum could be explained on the basis of absence of thick gelatinous sheath in case of Anabaena as compared to N. muscorum (Desikachary, 1959). Adverse environmental factors like metal toxicity decrease the endogenous level of auxin, cytokinin and gibberellic acid as reported by Atanasova et al (2004), which could also be correlated with growth retardation (Figure 1).

Indole acetic acid (IAA) or kinetin (KN) supplementation had an ameliorative impact on all the studied growth parameters. The amelioration of CrVI induced toxicity in the presence of IAA or KN can be correlated with (i) significant decline in cellular accumulation of Cr (Figure 2), (ii) significant improvement in major photosynthetic pigment and accessory pigment contents (Table 1), (iii) improvement in nitrogen and ammonium assimilating enzymes (Figures 3, 4), protein, carbohydrate and first protective barrier EPS contents against Cr (Figure 5) in both the tested cyanobacteria, however the degree of alleviation was greater in N. muscorum as compared to Anabaena. Under IAA supplementation, improvement in the growth of both the tested cyanobacteria under CrVI stress is not yet clearly known but it has been suggested that the major function of IAA is to mediate the cell division as well as cause cell expansion that is associated with the enhancement in growth associated with KN (Del Pozo et al., 2005; Ha et al., 2012). IAA supplementation also enhances the fatty acid content in Scenedesmus which might be another reason for improved growth (Salama et al., 2014). Similarly, KN induced enhancement in the lipid and carbohydrate content was also reported by
Renuka et al. (2017) in microalga *Acutodesmus obliquus*. In cyanobacteria *Nostoc entophytum*, *Hapalosiphon stuhlamanii* and *Nostoc muscorum* the KN induced growth and pigment contents were also reported by other workers (Giriyappanavar, 2013; Tiwari et al., 2018). Upon IAA or KN supplementation the substantial decrease in intracellular Cr in tested cyanobacteria might have occurred due to the up-regulation of sulfate transporter protein which is primarily responsible for sulfate uptake inside the cell. Hence, under this condition there might have been greater competition between the uptake of sulfate and chromate resulting in an appreciable decline in intracellular Cr accumulation (*Figure 2*).

Chromium at tested doses (100 and 150 µM) caused a substantial decrease in major pigments content Chl a to accessory pigment Car ratio (*Table 1*) and main light-harvesting pigment (phycobiliproteins) in cyanobacteria that play important role in photosynthesis (Kumar et al., 2015). Chromium declined the pigments contents either destroying its precursor or inhibiting the enzyme involved in Chlorophyll biosynthesis (Drazkiewicz, 1994; Vajpayee et al., 2000) or due to overproduction of ROS (Pinto et al., 2003; Gupta and Ballal, 2015). Carotenoids are the accessory pigments and act as light-harvesting components. The decrease in ratio of chla/Car show spartan toxicity of Cr VI on photosynthetic pigments leading dysfunctioning of the light-harvesting antenna complex.

Furthermore, in cyanobacteria light-harvesting photosynthetic antenna complexes comprise phycobiliproteins (PBPs) have three distinct components i.e. phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) and they are proteinaceous in nature and located to the outer surface of the thylakoid lamellae. Under both the tested doses of Cr VI a significant reduction in all the three components of PBPs was observed (*Table 1*). This reduction might be due to alteration in the PBPs biosynthesis at precursor level, in enzyme inactivation level and/or damage caused to PBPs by Cr due to its easy accessibility for heavy metals. Similarly, decrease in pigment content associated with growth retardation was also reported in *Spirulina* (Jetley et al., 2004) and *Synechocystis* (Page et al., 2012) and also in *Oscillatoria* under nitrogen starved condition (Parmar et al., 2011). Among all the
three PBPs, PE was least affected under Cr\textsuperscript{VI} stress and the increasing order of damage followed the trend as- PE<APC<PC.

Further, IAA or KN supplementation to Cr\textsuperscript{VI} stressed cultures improved the content of the pigment and this stimulatory effect of both the phytohormones could be attributed to (i) inhibition in degradation of δ-aminolevulinic acid, (ii) stimulation in chlorophyll biosynthesis (Cortleven and Schmülling, 2015) and (iii) stabilization in the thylakoid membrane (Chattopadhayay et al., 2002). The significant increase in ratio of chla/Car and PBPs contents under IAA/KN supplementation similar with the results of Bajguz and Piotrowska-Niczyporuk (2013) and Mansouri and Talebizadeh (2015).

Being a macronutrient, nitrogen is involved in the biosynthetic processes of proteins and nucleic acids, and nitrogen metabolism is an important physiological process that has a direct impact on growth of cyanobacteria. Cyanobacteria use nitrate (NO\textsubscript{3}⁻) and nitrite (NO\textsubscript{2}⁻) as source of nitrogen easily available to them. The activity of the nitrogen assimilation enzymes as well as inorganic nitrogen contents was found to be affected by Cr\textsuperscript{VI} stress. The activity of NO\textsubscript{3}⁻ assimilating enzyme in both the tested cyanobacteria under Cr\textsuperscript{VI} stress was found to be decreased, mainly due to reduced uptake of N in the form of NO\textsubscript{3}⁻ (Figure 3A). The NO\textsubscript{3}⁻ and NO\textsubscript{2}⁻ uptake in cells of cyanobacteria is directly mediated by ABC-type transporter which is ATP dependent (Flores and Herrero 1994). The reason behind reduced NO\textsubscript{3}⁻ and NO\textsubscript{2}⁻ uptake might be due to declined ATP pool as a significance of damaged photosynthetic electron transport chain (Rai et al., 1995). Our results are in agreement with Sheeba et al. (2011) where reduced NO\textsubscript{3}⁻ uptake in Nostoc and Phormidium was reported due to altered photosynthetic electron transport chain. In another study done by Devriese et al. (2001) it was found that the NO\textsubscript{3}⁻ uptake rate decreased significantly by heavy metals such as Cd, Cu, Zn, Fe and Co in green algae Chlamydomonas. After entering in cell, reduction of NO\textsubscript{3}⁻ involves two-step (i) NO\textsubscript{3}⁻ is reduced into NO\textsubscript{2}⁻ catalyzed by nitrate reductase (NR) and (ii) NO\textsubscript{2}⁻ is now reduced into NH\textsubscript{4}⁺ catalyzed by nitrite reductase (NiR) (Herrero and Guerrero, 1986). Chromium stress significantly
decline the NO$_3^-$-assimilating enzymes *viz.*, NR and NiR in dose dependent manner but the effect was more pronounced in *Anabaena* (Figure 3B). A significant decrease in NR and NiR activity under Cr$^{VI}$ stress might be due to increased cellular accumulation of Cr (Figure 2). Our results are in agreement with Sangwan et al. (2014) where Cr$^{VI}$ significantly declined the nitrate assimilating enzymes. Furthermore, in present study reduction in NR activity is also noticed (Figure 3B) which might be due to (i) reduced carbon fixation, (ii) decrease in NO$_3^-$ uptake by cell, and (iii) altered electron transport that provides Fd, an electron donor to reduce NO$_2^-$ (Kumar and Joshi, 2008).

As a result of NO$_3^-$ assimilation NH$_4^+$ ions are rapidly assimilated into organic compounds *via* ammonia assimilating enzymes. Likewise in plants, cyanobacteria also assimilate NH$_4^+$ into amino acids through GS-GOGAT pathway (Sanz-Luque et al., 2015). In current study the GS/GOGAT activities were found to decrease significantly under Cr$^{VI}$ stress (Figure 4), which might be due to over-accumulation of free radicles that directly inhibited the enzyme activities (Tiwari et al., 2018). Since Cr$^{VI}$ causes impairment in electron transport chain, hence ETS would not be able to produce ATP sufficiently (Prasad et al., 1991), and thus decrease in ATP supply might be a reason for decline in GS-GOGAT activity, as, ATP acts as a cofactor in ammonia assimilation process. Moreover, under Cr$^{VI}$ stress decreased activity of GS-GOGAT may also be responsible for accumulation of NH$_4^+$ in cells that had resulted in altered intracellular pH leading to the disturbance in osmotic balance and inhibited photosynthesis hence caused appreciable decrease in growth (Figure 1) as reported in earlier finding (Dai et al., 2008) Under this condition, excessive accumulation of NH$_4^+$ might have triggered the activity of GDH enzyme (alternative route for NH$_4^+$ assimilation) (Figure 4). Activation of GDH under stressful environment confirms its role in decreasing the toxicity induced by NH$_4^+$ as well as in maintaining the glutamate level that further involves in the synthesis of non-enzymatic antioxidants *viz.*, proline and phytochelatins. Supplementation of IAA/KN significantly reduced the cellular
accumulation of Cr as well as enhance the uptake of NO$_3^-$ and NO$_2^-$(Figure 3A), associated with enhance activity of NO$_3^-$ assimilating enzymes in both the tested cyanobacteria (Figure 3B), that eventually improved N status hence, improved the growth of both the cyanobacteria under Cr$^{VI}$ stress (Figure 1A-B). A significant increase in uptake of NO$_3^-$ in cell following IAA /KN supplementation stimulated the NR activity because it is a substrate-induced enzyme. Further, under IAA or KN supplementation expression of specific NR and NiR proteins might have also increased that manifests the increase in NR and NiR activity. Improvement in GS-GOGAT activity under IAA/KN supplementation together with Cr$^{VI}$, stress lowered the GDH activity (high Km value) in comparison to Cr$^{VI}$ treatment individually in both the tested cyanobacteria (Figure 4) that could be explained on the basis of lesser availability of substrate i.e. NH$_4^+$, as it is mainly assimilated by GS/GOGAT pathway.

Protein and carbohydrates are an important component of photosynthetic organisms that affects the growth of tested cyanobacteria under adverse environmental conditions. In the current study decline in protein content under Cr$^{VI}$ stress (Figure 5) might be due to direct impact of Cr on protein synthesis, as observed by Shashirekha et al. (2015) and also supports the earlier findings where reduction in protein content was observed under Cr stress in Nostoc (Shashirekha et al., 2015) and Oscillatoria (Jayashree et al., 2012). Furthermore, reduced level of carbohydrate is observed in present study under-tested doses of Cr$^{VI}$ in Nostoc and Anabaena (Figure 5). Significant decrease in carbohydrate content might have occurred due to reduction in rate of photosynthesis and degradation of photosynthetic pigments (Tiwari et al., 2018) that resulted in decline in sugar accumulation, and our results are in parallel with the findings of Bajguz (2011) in Chlorella vulgaris and in Spirulina platensis Gupta et al. (2014).

Further, to overcome the negative effects induced by heavy metal cyanobacteria secretes a high molecular weight polymer, termed as exopolysaccharides (EPS) that acts as physical barrier against heavy metals (Planchon et al., 2013). In present study, a significant decrease in carbohydrate content is associated with increase in EPS content at 100 µM Cr$^{VI}$ (Figure 5). Having positive charge on the
CrVI, the EPS could chelate the CrVI ion due to presence of negative charge on the ion on the outer cell layers (De Philippis and Micheletti, 2009). A similar increase in EPS content was also noticed in Lyngbya and Nostoc linckia and under Cr/Co stress (Kiran and Kaushik, 2008; Mona and Kaushik, 2015). An increase in EPS content gives tolerance against heavy metal stress but under higher dose reduction in EPS content was noticed. Moreover, upon IAA/KN supplementation significant increase in EPS content was noticed (Figure 5) associated with increase in carbohydrate content. An increase in EPS contents suggests its role in removing CrVI from contaminated water and considered as “green “materials and behaves as bio-remediate and our results of EPS secretion are confirmed by SEM images (Figure 6A).

Table 1S displays two-way ANOVA results to validate the interactive effect of CrVI and IAA/KN on growth in N. muscorum and Anabaena. The two-way ANOVA intricate that CrVI and IAA/KN alone significantly affected all the treatment, while in combination all the parameters showed insignificant relation (except NiR and GDH) hence, pointing towards relieving role of IAA/KN against CrVI prompted toxicity in N. muscorum and Anabaena.

Conclusion
Findings signpost the noteworthy role of phytohormones; indole acetic acid (IAA) and kinetin (KN) in curtailing the negative effects induced CrVI in paddy field cyanobacteria N. muscorum and Anabaena. Negative effects on growth, the ratio of chlorophyll a to carotenoids and contents of phycobiliproteins (PBPs) and nitrogen metabolism are considered as gauge of heavy metal toxicity and in the current study tested doses of CrVI caused damaging effects on these parameters. Further, CrVI also declined the biochemical constituents such as protein, carbohydrate and EPS. Exogenous supplementation of IAA/KN alleviated CrVI induced toxicity on growth and its related parameters such as inorganic N contents and improvement in nitrate and ammonia assimilating enzymes as well as protein content. Overall findings propose that phytohormone plays major role in alleviating and enhancing the adaptation capability of tested paddy field cyanobacteria under CrVI stress and enhancing the N content in paddy fields that increased fertility and productivity of soil (Figure 6B,C).
Declarations

Declaration of competing for interest

The No conflict of interest declared by authors.

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Author contribution

SMP planned the experiment and ST and AP designed and performed the experiment, AP and ST analyzed the data and SMP, ST and AP wrote the manuscript.

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Table 1. Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and kinetin; KN) on photosynthetic pigment contents of *Nostoc muscorum* and *Anabaena* PCC 7120 exposed to CrVI stress.

| Photosynthetic pigment contents (µg mg\(^{-1}\) dry weight) | Treatments | Ratio | Contents |
|-------------------------------------------------------------|-------------|-------|----------|
| IAA / KN Car / (Chl a) / (PC) / (APC) (PE) | N. muscorum Anabaena | N. muscorum Anabaena | N. muscorum Anabaena |
| N. muscorum Anabaena | Anabaena | Anabaena | Anabaena |
Data are means±standard error of three replicates (n=3). Values followed by different superscript letters show significant difference according to Duncan multiple range test (DMRT) at P<0.05 level.
Figures

Figure 1

(A,B). Dose response curve of Nostoc muscorum and Anabaena exposed to different concentrations of chromium (CrVI) with and without exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN). Data are means±standard error of three replicates (n=3). Lines followed by different letters how significant difference at P<0.05 according to Duncan multiple range test (DMRT).
Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN) on intracellular chromium accumulation of Nostoc muscorum and Anabaena exposed to CrVI stress for 96 h. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference at P<0.05 according to Duncan multiple range test (DMRT). nd = not detected.
Figure 3A

Figure 3B

Figure 3
Figure 3A. Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN) on nitrate and nitrite uptake rate of Nostoc muscorum and Anabaena exposed to CrVI stress for 96 h. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference at P<0.05 according to Duncan multiple range test (DMRT).

Figure 3B. Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN) on nitrate and nitrite reductase activities of Nostoc muscorum and Anabaena exposed to CrVI stress for 96 h. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference at P<0.05 according to Duncan multiple range test (DMRT).
Figure 4

Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN) on glutamine synthetase (a, b), glutamate synthase (c, d) and glutamate dehydrogenase (e, f) activities of Nostoc muscorum and Anabaena exposed to CrVI stress for 96 h. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference at P<0.05 according to Duncan multiple range test (DMRT).
Figure 5

Impact of exogenous supplementation of phytohormones (Indole acetic acid; IAA and Kinetin; KN) on protein (a, b), carbohydrate (c, d) and exopolysaccharides (e, f) contents of Nostoc muscorum and Anabaena exposed to CrVI stress for 96 h. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference at P<0.05 according to Duncan multiple range test (DMRT).
Figure 6

Figure 6A. Scanning electron micrograph (SEM) of dry algal absorbent of (A) Nostoc muscorum ATCC 27893 and (B) Anabaena PCC7120 treated with chromium with and without exogenous supplementation of (Indole acetic acid; IAA and Kinetin; KN); Cellular structure–I, without treatment (control) – II, chromium – III, IAA – IV, KN – V. 6B. Diagrammatic representation of toxicity mediated by chromium and up regulation of antioxidant nitrogen metabolism enzyme in alleviating Cr toxicity.

Supplementary Files
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