Research Article

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Molecular modulations and influence of acclimation of Ni on acute Ni toxicity in *Plectonema boryanum*

Ni moleküler modülasyonunun ve etkisinin Ni ile şartlandırılmış *Plectonema boryanum* üzerindeki akut Ni toksisitesi

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Abstract

Objective: In this study, metal induced accumulation of antioxidants, proline and phenol were proposed during acclimation, to provide defense, protection and resistance against oxidative stress on Ni acclimated cyanobacterium.

Methods: *Plectonema boryanum* was used as a test organism in the present study and the experiments were performed in two sets (1) cells differentially exposed to Ni (2) cells acclimated with Ni and then further exposed to Ni in the plant tissue culture laboratory.

Results: Dose-dependent formation of peroxide in Ni-treated cells increased the activity of superoxide dismutase (SOD) and also enhanced accumulations of non-enzymatic antioxidants such as proline and total phenols at low concentrations of Ni. Down regulation of most of the polypeptides at 20 μM of Ni demonstrated the severe toxicity of the metal while a slight up-regulation of peptide depicted its role in metal tolerance. Ni acclimated cells also showed reduction in peroxide and an increase in proline and total phenol after exposure to 4 μM of Ni.

Conclusion: Our results for the first time demonstrated that accumulation of phenol and proline during the acclimation process could provide tolerance to the cyanobacterium from deleterious effects of the metal stress if it is further exposed to the same metal.

Keywords: Antioxidants; Nickel; Cyanobacteria; Phenolic Compounds; Proline.

Özet

Amaç: Bu çalışmada, antioksidanlar, prolin ve fenolün metal bağlı birikimini Ni ile şartlandırılmış siyanobakterinin oksidatif strese karşı savunma, koruma ve direnç sağlama gibi özellikler gösterdiğini belirlemiştir.

Metot: Bu çalışmada *Plectonema boryanum* test organizması olarak kullanılmıştır ve deneyler iki set olarak; diferansiyel olarak hücrelerin nikel endikasyonu ile ve nikel ile şartlandırılması şeklinde gerçekleştirilmiştir, sonrasında daha fazla nikel maruz kalma işlemi bitki doku kültürü laboratuvarında gerçekleştirilmiştir.

Bulgular: Doz bağımlı peroksit oluşumu nikel ile muamele edilmiş hücrelerde süperoksidisit dismutaz (SOD) aktivitesini artırmış, aynı zamanda non-enzimatik antioksidanlar proline ve total fenollerin birikimini de düşüktü konsantrasyondaki Ni miktarlarında artırmıştır. Peptidin metal toleransı ile regülyasyonu artıcı etkisi düşünülmüştür, çoğu polipeptid üretimi 20 μM Ni konsantrasyonunda ciddi toksite göstergesiyle baskılmıştır. 4 μM Nikel'e maruz bırakılmış, Ni ile şartlandırılmış hücrelerde peroksit
Introduction

In recent years, unrestricted developmental activities have brought an economical evolution to the world, but at the same time, it is the major cause of environmental pollution [1]. The environmental pollutants are spread through different anthropogenic activities and many of them enter into the food chain of livestock to man [2]. Pollutants, mainly heavy metals are gaining importance due to their widespread uses in industries like textile, leather, metal processing, paints, and steel fabricating industries. These industries may discharge a large quantity of heavy metals, toxic waste, and untreated effluents, which become a major source of soil and water pollution [3]. Ni has gained considerable attention due to its increasing concentration in water, soil, and air. Although, Ni is an essential metal that play an important role in cellular physiology for eukaryotes and prokaryotes. Also, it is a necessary cofactor for enzymatic function in prokaryotes [4], but high concentrations could be potentially harmful.

Cyanobacteria is well known to accumulate heavy metals from industrial effluents and quickly respond and adapt to stress conditions. It has been shown that cyanobacteria are highly sensitive to heavy metal pollution [5] and therefore, they can serve as an indicator to measure the level of metal pollution. There are currently no generally accepted mechanisms that could explain effects of heavy metals on the cyanobacterial cells, although hypothetical mechanisms have been suggested.

The formation of reactive oxygen species (ROS) in various sites of cells after heavy metal treatment could be the possible hypothesis, because it is potentially oxidative in nature and easily reactive with cellular components, such as lipids, proteins, and DNA [6]. Proteins are important target of ROS and an alteration in the expression and activities of proteins act as the most important molecular biomarker or indicator of environmental stress. The functioning of some proteins is inhibited or lost and that of others are enhanced or induced while facing such stress level. However, the metal-induced peptides act as metal-chelating agents for the excess of toxic metals in the cells. This could play an important role in metal metabolism in aquatic organisms and particularly in the detoxification mechanisms [7].

In general, organisms are able to adapt after small fluctuations in their environment, or even resistant to and can survive dramatic changes [8]. With an increasing anthropogenic metal pollutants discharged into aquatic ecosystems and then aquatic biota may develop acclimation or adaptation strategies to cope with elevated metal concentrations [9]. Cyanobacterial acclimation due to metals are the result of several physiological and biochemical mechanisms and which may result to other changes at the molecular level [10, 11]. Thus, the present study deals with the biochemical and molecular responses of cyanobacterium *P. boryanum* towards Ni stress and the involvement of some non-enzymatic antioxidants like proline and phenol in the stress acclimation process.

Materials and methods

Chemicals and reagents

All the chemicals were purchased from Sigma, Himedia, Merck (Worli, Mumbai).

Organism and growth conditions

*P. boryanum*, a non-heterocystous cyanobacterium, was obtained from the laboratory of Dr. Sheo Mohan Prasad, University of Allahabad, Department of Botany, Allahabad, India. The strain was originally isolated from rice field, Allahabad, India. Axenic culture of *P. boryanum* was maintained in the culture room at 27 ± 2°C. For regular experiments, cultures were grown in BG-11 medium (pH 7.5) without nitrogen source under photosynthetic photon flux density (PPFD) of 75 μmol m⁻² s⁻¹ and 14 h photoperiod. The cultures were shaken gently twice or thrice daily.

Experimental design

The experiments were performed in two sets (1) cells differentially exposed to Ni, (2) cells acclimated with Ni and then further exposed to Ni. For the acclimation of the *P. boryanum*, cells were initially subjected to very low dose
(0.1 μM) of Ni, which was the lowest nontoxic concentration and tolerated by the test cyanobacterium (result of the screening experiment to determine the dose not shown) and subsequently transferred every 15 days to the higher concentrations (2, 4 and 8 μM) with regular growth study at each step of cells transfer to the higher concentration (data not shown). Physiological acclimation of the control strain was obtained by successive sub-cultivation at increasing doses of Ni up to 8 μM (hereafter referred to as the acclimated strain) as described by Rai et al. [12]. All the parameters used in the investigation were analyzed from the same culture.

**Effect of Ni on the Ni-acclimated P. boryanum**

The Ni-acclimated P. boryanum was further treated with Ni (4 μM) for 4 days to see the modulation of Ni toxicity in these acclimated strain. Selected parameters such as total peroxides, phenol and proline were measured in control, Ni-acclimated, Ni alone and Ni-acclimated Ni treated P. boryanum as per the methods described above. The Ni concentration and incubation time were selected on the basis of its moderate toxicity in the dose-and time-dependent experiments.

**Metal treatment**

The stock solution, 1000 μM of (NiCl₂.6H₂O) was prepared in glass-distilled water and sterilized by passing through the Millipore membrane filter (0.22 μm). Required concentrations of Ni was prepared in BG-11 medium from stock solution. The Ni-acclimated P. boryanum was treated with the Ni (4 μM) for 4 days to see the modulation of Ni toxicity in these acclimated strain. The dose of Ni and an incubation time was selected on the basis of its moderate toxicity. Selected parameters such as peroxide, total phenol and proline were measured in control, Ni-acclimated, Ni alone and Ni-acclimated Ni treated P. boryanum as per the methods described below. All the experiments were conducted in triplicate and repeated thrice to confirm the reproducibility of the results.

**Growth measurement**

Growth was measured in the terms of chlorophyll estimation from Myers and Kratz [13]. Measurements were on alternate days by estimating protein content at regular intervals for 10 days.

**Photosynthetic pigment determination**

For extraction of Chla and carotenoids, equal volume of each organism was centrifuged and pellet was suspended in a desired volume of 80% (acetone: water, v/v). After overnight incubation at 4°C, suspension was centrifuged and supernatant was used for measuring Chla and carotenoids. The absorbance of pigment extracts were measured by spectrophotometer where Chla at 665 nm and carotenoids at 480 nm. The specific coefficient as given by Myers and Kratz [13] were used for the calculation of Chla and carotenoids concentrations in cultures. Quantitative estimation of these pigments in terms of g/L was measured by using formulae 1.

\[
C = \frac{D}{d \alpha}
\]

Where \( \alpha = \) absorption coefficient (value of \( \alpha \) for chla is 82.04 and carotenoids is 200), \( D = \) Optical density, \( d = \) inside path length of spectrophotometer in (cm), \( C = \) Concentration of pigment in g/L⁻¹.

**Phycocyanin measurement**

A known volume of cyanobacterial cultures were centrifuged and the pellet was re-suspended in 80% of acetone. After 1 h of incubation in dark, cultures were centrifuged again and their residue was used for measuring phycocyanin. Absorbance was recorded by spectrophotometer at 620 nm from the method of Blumwald and Tel-Or [14]. The phycocyanin monomer at pH 7 measured maximum absorption at ~615 nm and a molar extinction coefficient of \( 2.3 \times 10^5 \) M⁻¹ cm⁻¹.

**Estimation of total protein**

Protein concentration was measured by the method of Lowry et al. [15] and modified by the method of Herbert et al. [16]. The amount of protein was estimated from a standard curve prepared by using BSA as a source of protein.

**Peroxide estimation**

The level of peroxide in the cell was measured by the method of Sagisaka [17]. For total peroxide estimation, 10 mL culture was extracted in 3.5 mL of 5% tri-chloro acetic acid. Thereafter, centrifuged at 8000 rpm for 20 min. Total peroxide level in the supernatant was measured by spectrophotometer at 480 nm. The peroxide content
was quantitatively estimated using the standard curve of hydrogen peroxide (H$_2$O$_2$) and results were expressed as μmol (g dry weight)$^{-1}$.

**Superoxide dismutase (SOD)**

SOD level was measured by the method of Giannopolitis and Ries [18]. The initial rate of reaction was measured by the differences in an increase in absorbance at 560 nm in the presence or absence of extract was proportional to the amount of enzyme. The unit of superoxide dismutase (SOD) activity was defined as the amount of enzyme which may cause a 50% inhibition of the reaction observed in the absence of enzyme. The activity was calculated by using the formula:

$$U/mL = [(V_o/V) - 1] \times \text{(dilution factor)}$$

where, $V_o$ = $A_{560}$ of Control; $V$ = $A_{560}$ of sample.

The results were expressed as Unit (mg protein)$^{-1}$.

**Proline and total phenols measurement**

Proline accumulation and total phenols was measured by the procedure of Bates et al. [19] and Singleton and Rossi [20], respectively.

**Proline measurement**

Twenty milliliter of exponential phase culture was taken and centrifuged at 8000 rpm. The cells were homogenized in 10 mL of 3% sulfosalicylic acid, thereafter centrifuged at 8000 rpm for 10 min to remove cell debris. 2 mL of supernatant (2 mL each of acid ninhydrin and glacial acetic acid) was added and incubated at boiling temperature for 1 h. The mixture was extracted with toluene, and proline was quantified spectrophotometrically at 520 nm from the organic phase.

Concentration of proline was estimated by referring to a standard curve of proline. The amount of proline in the sample was calculated in μg (g dry weight)$^{-1}$ of samples.

**Total phenols measurement**

Ten milliliter culture of cyanobacterium was centrifuged and cells were extracted in 2 mL of methanol (80%) at 4°C. The extracted sample was centrifuged at 8000 rpm. Thereafter, 0.5 mL of supernatant with 2 mL of sodium bicarbonate, 0.3 mL DDW, 0.2 mL folin reagents were added and incubated in water bath until blue color developed. The amount of phenol was calculated by measuring the optical density at 750 nm. The concentration of total phenols was calculated by standard curve of gallic acid and expressed as mg (g dry weight)$^{-1}$.

**Protein analysis by SDS-PAGE**

The total crude protein was extracted from cyanobacterial biomass by the method of Ivleva et al. [21] after some modification. 100 mL of logarithmic phase culture of *P. boryanum* optical density 0.5 at 750 nm was taken and the cells were spun down for 10 min at 1500 g. The pellets were washed by re-suspending in 2 mL of PBS buffer and repeating the spin. The pellets were again re-suspended in 200 μL of PBS. The cells were frozen at −80°C and then quickly thawed at 37°C to allow partial cell breakage. Thereafter, the samples were kept on ice in the presence of protease inhibitors. 100 μL of PBS buffer was added and suspension was spun at 1000 g. The supernatant fraction was collected and again spun at 1000 g for 10 s to pellet residual glass beads. The resulting green supernatant fraction was a crude cell extract.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cyanobacterial protein was performed on 12% polyacrylamide resolving gel after 5 days of metal treatment and carried out in a vertical system (Bangalore Geni) according to the method of Laemmli [22]. After loading approximately 25 μg protein from treated and untreated extracts in each wells, electrophoresis was started by turning on power (DC) on a gel of about 1 mm thickness and about 14 cm length, with an applied voltage of about 80 V when samples are in stacking gel then on 120 V through running gel. At the end of electrophoresis (when the dye front reached the bottom of the gel), protein bands in the gel are visualized by staining with coomassie brilliant blue dye. After about 24 h, with gentle agitation and several changes of destaining solution, the gel background became colorless and protein bands of blue, purple color is visible. Finally, the whole cell protein profiles of the samples were visualized under trans white light and captured by Gel Doc (Bio-Rad).

Protein concentration of the treated and untreated samples were estimated by the method of Lowry et al. [15]. Bio-Rad gel documentation system was used to scan the developed one dimensional gel. Densitometric analysis of the bands was performed by using Quantity one imaging and analysis software.
Statistical analysis

All experiments were performed by using exponentially growing cultures and repeated three times to ascertain the reproducibility of the result. Values are represented as mean ± SEM. Statistical analysis was carried out by using the SPSS program (SPSS Inc., version 10.) and student’s t-test (non-parametric analysis). p < 0.05 was considered statistically significant.

Results and discussion

The result represents the possible mechanism between metal toxicity and oxidative stress (OS). Oxidative stress can be defined by the free radical formation within cells which may actively participate in an enhancing the reactive oxygen species (ROS). Excess ROS formed within cells due to metal stress can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and DNA. These changes lead to oxidative injuries and result in the reduction of organism growth and development. Figure 1, illustrates the overall molecular alterations under the metal stress and the same has been supported by many previous studies which have focused on metal-induced toxicity, emphasizing the role of metallic pollutants in the formation of ROS in biological systems and the significance of this therein [5].

Growth measurement

In the growth measurement, when the cells were treated with a dose of 2 μM of Ni, lag phase occurred for 2 days then log phase continued upto 10 days (Figure 2). At 4 μM concentration, the decline in growth commenced only after 2 days. Upto 6 days, the growth was almost constant after which the growth declined continuously till 10 days. At the concentration of 8 and 16 μM, there was a sharp decline phase after 2 days. No significant decline was noticed till 6 days. The decline phase became prominent after 6th day and continued upto 10 days.

The decrease in growth of cyanobacterium following Ni exposure can be regarded as a general response associated with the metal toxicity [23]. The decreasing trends in growth following metal exposure might be due to the arrest of processes like photosynthesis, carbon fixation and protein synthesis in cyanobacteria [24].

Photosynthetic pigments

Chla, carotenoids and phycocyanin in P. boryanum were estimated in the cells treated at different concentrations of Ni after 4 days to investigate the damaging effect as presented in Table 1. The results indicated that photosynthetic pigments were affected in a dose-dependent manner in response to metal exposure, except in case of carotenoids which was increased by 5% at low concentration (2 μM).

Figure 1: Ni induced oxidative stress and its consequences.
Scavenging of free radicals is an important function of carotenoids present in cyanobacterial thylakoid membranes [25] and an increased content at low concentration proves its role as an antioxidant. The deleterious effect was more pronounced on Phycocyanin followed by Chl a and Carotenoids. After 4 days of treatment, the lowest concentration (2 μM) of Ni reduced Chl a and Phycocyanin contents by 16 and 18%, respectively. The declining trend in the pigment contents continued with the rising concentration of Ni as 16 μM of Ni sharply lowered Chl a, carotenoids and phycocyanin contents by 54, 47 and 50%, respectively. Similar to this, damaging effect of heavy metals on photosynthetic pigments has been reported by Shukla et al. [26] and Bajguz [27].

### Free radicals formation

The primary response of ROS formation on organisms after exposure to high levels of heavy metals were observed. Various metals either generate ROS directly through Haber-Weiss reactions or indirectly by interaction with the antioxidant system [28], disrupting the electron transport chain [29] or disturbing the metabolism of essential elements.

Results presented in Figure 3 showed an increase in peroxide radical in Ni treated *P. boryanum*. An increase was directly proportional to the concentration of Ni. Very low concentration of Ni was difficult to promote total peroxide content after 4 days of exposure. There was significant increase (55%–180%) in peroxide level at the selected concentrations of Ni (from 2 to 16 μM) as compared to control. The high dose of Ni treatment stimulated ROS significantly. In response to the deleterious effect of ROS, an organism develops defense mechanism for protection.

### Enzymatic and non-enzymatic antioxidants

Data related to the SOD activity in *P. boryanum* are presented in Figure 4. In case of Ni treated cells, the dose dependent increase in SOD activity was noticed up to a concentration of 4 μM. Further increasing the
concentration of the Ni from 8 to 16 μM, the SOD activity decreased by 17%–24%, respectively as compared to control. The increase in the activity of SOD of the *P. boryanum* observed in our study following metal treatment could be ascribed to the increased production of superoxide anion (O$_2^-$) in the cells. A similar increase in SOD activity has been demonstrated in the leaves of *Arabidopsis thaliana* and in marine microalgae *Tetraselmis gracilis* following Cd treatment [30]. A study conducted by Polit et al. [31] also showed that metal-induced O$_2$ and H$_2$O$_2$ triggered the activity of antioxidant enzymes, such as SOD in *Chroococcus* sp at 5 mg Cr (VI).

The content of non-enzymatic antioxidants such as total phenol and proline, which were scavenged the superoxide and peroxide radicals accumulated in Nickel treated *P. boryanum* after 4 days of treatment (Figures 5 and 6). Accumulation of total phenol in *P. boryanum* is presented in Figure 5. The exposure of *P. boryanum* to Ni (2–4 μM) showed dose dependent increase in total phenol. At the high concentration of Ni (8 μM and 16 μM) percentage showed a decline depicting cell death. At the highest dose of Ni (16 μM) the total phenol content showed a decline of about 12% as compared to control. Although, ROS scavenging activity of phenolic metabolites by cooperation with antioxidative enzymes e.g. peroxidases in H$_2$O$_2$ [32].

Similar to the phenol, proline also increased upto 8 μM of Ni treatment as observed in the present study (Figure 6). At high concentration of Ni (16 μM) the proline content showed a decline of about 22% as compared to control.

An increase in proline content as a result of metal accumulation in *Brassica* and *Cajanus* was also observed by Sgherri et al. [33]. Proline besides acting as a metal chelator and osmolyte, also scavenge hydroxyl radicals and singlet oxygen and thus providing protection against ROS-induced cell damage [34].
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Alterations in protein profile of *P. boryanum* under Ni Stress

The electrophoretic pattern of crude extract of protein isolated from the control and Ni-treated cyanobacterial cells after 5 days of treatment is shown in Figure 7. The resolved bands were classified according to their molecular weight markers and density of bands was estimated by the densitometric analysis as described in the material and methods section. The Lane II and Lane III showed protein profiles in control and Ni-treated *P. boryanum*, respectively. The result indicates that polypeptide of ~29.41, ~30.26 and ~39.25 KDa were completely disappeared when treated with the selected dose of Ni (20 μM). Down regulation of most of the polypeptides at 20 μM of Ni demonstrated the severe toxicity of the metal, while there was slight up regulation in intensity of peptide having molecular weight 27.49 KDa was observed, which is expected to be that of SOD as demonstrated by Matysik et al. [35]. The disappearance of protein bands could be due to the interference of metal ions at the level of transcription, post-translation degradation and translational level [36]. However, slight up regulation of polypeptides may be playing a possible role in metal tolerance, but their precise role is yet to be explored. Further studies are required to investigate in detail about the metal induced peptide through 2D gel electrophoresis and their role in stress tolerance.

Acclimation of *P. boryanum* to Ni

The gradual increase in protein content after each acclimation starting from 0.1 to 8 μM concentration of Ni showed that the acclimation with an increasing concentrations were successful and the *P. boryanum* tolerated and adapted well (Figure 8). Further in order to prove our hypothesis that Ni adaptation would confer cross protection to Ni. The Ni acclimated cells and control cells not acclimated to Ni were exposed to 4 μM Ni and parameters relating to ROS generation and antioxidant were measured. Exposure of cyanobacteria successively increasing the concentration of metals confers physiological
tolerance to that metal, which can be termed as acclimation. Affenzeller et al. [37] showed that the toxic effects of Ni on the Ni-acclimated strain was much less as compared to its effect on the non-acclimated Anabeana doliolum.

The levels of total peroxide presented in Figure 9 shows that the cellular level of peroxides was increased about 40% following the exposure of 4 μM Ni. In contrast to this, the Ni acclimated Ni-exposed cells showed a reduction of 18% in the level of total peroxides when compared to that of Ni alone. Acclimation of P. boryanum to 8 μM of Ni caused an enhancement of 30% proline as compared to non-acclimated P. boryanum. The Ni alone treatment induced the intracellular proline by approximately 10% when compared to control. However, when Ni acclimated P. boryanum was exposed to 4 μM of Ni, there was an additional increase of 10% in the proline content as compared to Ni alone (Figure 10). Acclimation of P. boryanum to 8 μM of Ni caused an enhancement of 35% phenol as compared to non-acclimated P. boryanum. The Ni alone treatment induced the intracellular phenol by 15% when compared to control, however when the Ni acclimated P. boryanum was exposed to 4 μM Ni there was an additional increase of 10% in the phenol content as compared to Ni alone (Figure 11). Most compatible solutes like proline and phenols also seem to play an important role in hydroxyl radical scavenging (Figures 10 and 11), thus defending plants against oxidative damage, which is a common consequence of many abiotic stresses [38].

Several researchers have reported metal-tolerance or Metallo-adaptive response [39]. Adaptive responses are regulated by coordinated cascades of events, involving different cellular compartments and structures, several kinds of biomolecules and various signaling systems.

**Conclusions**

In conclusion, Ni-induced adaptive response demonstrated in the present study could therefore possibly be attributed to the involvement of antioxidant defense especially proline and proline-mediated expression of some stress proteins. The precise mechanism underlying the Ni adaptive response, however remains obscure at this moment warranting further research at the molecular level.

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**Conflict of interest:** The authors declare that they have no conflict of interest regarding this study.

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