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Hexavalent Chromium Induced Histological Alterations in *Bacopa monnieri* (L.) and Assessment of Genetic Variance

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Abstract

Chromium, a transition metal exists in nature in several oxidation states, the most important being Cr (III) and Cr (VI) species. The two forms display quite different chemical properties as Cr (VI) is highly toxic to most organisms whereas Cr (III) is relatively innocuous. Cr (VI) exposures are responsible for carcinogenic and mutagenic effects in humans also. The present study investigates the changes in cortical and vascular tissues of *Bacopa monneiri* after short-term exposure to Cr (VI). $K_2Cr_2O_7$ (0.05M) was added to Murashige and Skoog's (MS) media to which *Bacopa* explants were introduced and the detrimental effects were studied after 21 days of incubation under different stress conditions. Histological results revealed a time-dependent noxious effect of Cr (VI) on cortical cells. The amount of damage in vascular region was observable but not much defined. Changes included enlargement of the cortical cells and accumulation of chromium within the live tissues. The molecular interpretation of damage due to Cr (VI) was done by PCR amplification of different antioxidant genes and analysing the products on 1% agarose gel. The genes were unable to show expression with cDNA template from explants exposed to Cr (VI) while with cDNA from explants exposed to bacterial isolates and chromium, expression of antioxidant genes was observed. The study will provide glimpses of Cr (VI) induced damage in plant tissue at cellular and genetic levels.

Keywords: Cr (VI); Histological changes; *Bacopa monneiri;* Cr (VI) reduction

Introduction

Industrial effluents contain a large number of toxicants such as salts of heavy metals, acids, organic matter, and pesticides etc. which deteriorate the physico-chemical characteristics of water. These pollutants build up in the food chain affecting the inhabitant and nearby flora and fauna. Among heavy metals, chromium (Cr) is one of most toxic metals. Chromium occurs in oxidation states of Cr (II) to Cr (VI) but only Cr (III) and Cr (VI) are of biological significance as stable forms. An essential micronutrient for human diet, Cr (III) is relatively less toxic than Cr (VI), which is toxic, mutagenic and carcinogenic [1]. Cr (VI) is derived from the oxidation of ores and also from the combustion of the fossil fuels, wood and paper and is relatively stable in water. It causes irritation to sensitive epithelial lining and results in ulceration [2]. Cr (VI) is equally toxic to plants and affects various aspects of plant metabolism [3]. Accumulation of Cr (VI) by plants can reduce growth, induce chlorosis, reduce pigment content, alter enzymatic functions and can cause ultra structural modifications in cell membrane [4]. Cr (VI) toxicity can reduce seed germination and radical growth in plants due to inhibition of cell division by inducing chromosomal aberrations [5]. Hence, it is almost impossible to grow plants at or near Cr (VI) contaminated soils. Therefore, suitable methods need to be developed for remediation of Cr (VI) contaminated soils. Also it becomes relevant to study the impact of chromium on antioxidant defence system and other biochemical changes in plant metabolism.

Chromium (Cr) is discharged into the environment through improper disposal of wastes from industries like leather tanning, metallurgical and metal finishing, textiles and ceramics, pigment and wood preservatives, photographic sensitizer manufacturing etc. Effluents from these processes are strongly acidic and may contain the toxic Cr (VI) or the less toxic trivalent form [6]. Cr (VI) remains stable for several months in the soil without changing its oxidation state. Cr (VI) is accumulated by plants and its accumulation is biomagnified at different trophic levels through food chain [7]. The medicinal plants constitute, a large group of plants (both lower and higher) providing raw materials for use in drug formulation and related industries. If such plants are either naturally grown or cultivated in metal contaminated regions, there is a danger that the heavy metal accumulation by plants of medicinal value may cause serious health hazards to patients using metal adulterated herbal drugs. There are a number of reports on heavy metal accumulation by some essential oil yielding and other medicinal plants [8]. The contamination of heavy metals in market samples of some plant-based drugs has also been reported [9]. Hence, it becomes necessary that medicinal plants are first tested for metal contamination before exploiting them for medicinal uses.

Bacopa monnieri (L.), commonly known as "Brahmi" is a creeping herb from the family Scrophulariaceae. It is commonly found on the banks of rivers and lakes widely used to evaluate the physiological changes in the contaminated ecosystems. *B. Monnieri*, the medicinal plant has been used for centuries in folklore and traditional system of medicine as a memory enhancer, anti-inflammatory, analgesic, antipyretic, sedative and anti-epileptic agent, posing its importance as a medicinal plant [10]. In addition to its unique medicinal use, *B. monnieri* has also been linked to phytoremediation programmes for the removal of heavy metals such as cadmium and chromium [11].

Histological studies are a noteworthy and promising field to understand the changes in structural organization that occur due to pollutants in the environment. These structural changes vary with the

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complexity of tissue, nature of the pollutant, medium and duration of exposure [12]. Mostly such studies are focussed on the aquatic fishes assessing haematological, biochemical and immunological parameters. However, scientific evidence of toxicological impact of chromium associated with nearby flora is still rare. Amongst various remedial strategies, bioremediation is a better choice for rehabitation and reclamation of chromium contaminated sites. Genes from microbes, plants, and animals are being used successfully to enhance the ability of plants to tolerate, remove, and degrade pollutants. The most important criteria in successful bioremediation employing microbes are the ability of these microbes to possess desired metabolic activity i.e. Cr (VI) tolerance for surviving in polluted environment. Endophytic microbes are thus evaluated for bioremediation studies, as they share a common environment with their host plants, offering a better range of adaptation.

The present study focuses on understanding the histological and genetic changes associated with Cr (VI) in a common medicinal plant *B. monneiri*. The impact of chromium tolerant endophytic bacteria, *Bacillus megaterium* (NCBI Acc. No. JQ585719) and CIM 1 in neutralization of chromium disaster and their influence on plant growth and survival are evaluated. The exploration will also brighten the histological alterations occurring in flora, inhabiting the contaminated sites.

Materials and Methods

Medium and culture conditions

Murashige and Skoog's, MS medium [13] of half nutrient strength was used for this study. This was prepared by adding 3% (w/v) sucrose (Hi-Media, Mumbai, India) to MS basal salts; pH of the medium was adjusted to 5.8 using 0.1 M NaOH before adding 0.6% (w/v) agar (Bacteriological grade) (Hi- Media, Mumbai, India) and the medium was autoclaved at 121°C for 15 mins. Based on the experiment, two different endophytic bacteria (Table 1) having 50 µg/ml tolerances for Cr (VI) were added to the MS basal medium with pH5.6, before adding the agar. The endophytic bacteria selected were *Bacillus megaterium* (NCBI Acc. No. JQ585719) and CIM 1, possessing plant growth promoting activities. A preliminary study for shoot induction was carried out for 21 days after which the observations were recorded.

Plant material

B. monnieri plants were collected from the medicinal plant garden at CIMAP, CSIR-India. Leaf and internode explants were prepared according to surface decontamination procedure [14]. Briefly, shoots were washed in sterile water for 15 mins, soaked in Triton-100 for 5 mins and finally washed three times with sterile distilled water. Surface decontamination was performed by immersing shoot tips in 70% (v/v) alcohol for 30 seconds, treated for 3 mins with 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) and washed six times with sterile distilled water. Shoots with a single node (2 cm) were placed in the MS medium. Plants produced from this culture (after 3 weeks) were used for the subsequent experiments.

Chromium (VI) concentration

The explants were subjected to various treatments after exposing to 50 µg/ml of chromium (VI) ($K_2Cr_2O_7$). The effect of dark photoperiod, UV-light and temperature on the growth of explants were also studied with reference to control plants. The efficiency of growth promotion ability of both the endophytic bacteria (*B. megaterium* and CIM 1) under stress of toxic Cr (VI) concentration and also temperature (30°C) was evaluated. The various treatments observed are illustrated (Table 1).

Histological analysis

Thin transverse section of treatment no. 1, 2, 3 and 4 (Table 1) were cut with the help of a sharp scalpel and double stained for better resolution of the cells. The sections were observed at 100 x magnification by OLYMPUS, Japan microscope and recorded with the help of Catcam microscope eyepiece digital camera.

Evaluation of heavy metals tolerance and Cr (VI) reduction ability among endophytes

The Minimum Inhibitory Concentration (MIC) was determined on Luria agar (LA) plates amended with Cr (VI) concentration ranging from 25-100 µg/ml. The minimum concentration of the metal inhibiting complete growth after 24 hrs of incubation at 25°C was taken as the MIC. For estimation of Cr (VI) reduction, the endophytes were grown in peptone broth supplemented with 50 µg/ml concentrations of Cr (VI). All the inoculated flasks and controls (in triplicate) were incubated for 72 hrs at 10°C with shaking at 100 rpm. To measure the Cr (VI) reduction, 1 ml culture was centrifuged (6000 rpm, 10 mins at 10°C) and Cr (VI) in the supernatant was estimated according to the 1, 5-diphenyl carbazide method [15].

Statistical analysis

A record for shoot length, fresh weight and dry weight for all the treatments was maintained and was analysed by one- way ANOVA using Assistat software version 7.6 beta (2011). The significance of the data was analysed at $p > 0.005$.

RNA isolation and amplification of antioxidant genes

Total RNA was isolated from the explants of treatment 1, 2, 3 and 4 by Trizol Mini-Prep method. First strand cDNA synthesis was done following the manufacturer's protocol (In-Vitrogen, India). The integrity of cDNA was checked by the amplification of actin house-keeping genes and further quantification of different antioxidant genes (SOD and Catalase) was done using equivalent amount of cDNA template.

Results

Histological results

Histological studies provide an insight into cellular processes and clues for the proposal of hypothesis for further experimentation. In the control explants (Treatment 1), no damage to the tissues was observed. The transverse section of control plant showed continuous mass of

Experiment	Parameter details
Treatment 1	Control with no Cr VI.
Treatment 2	Explant with 50 µg/ml Cr (VI).
Treatment 3	Explant with 50 µg/ml Cr (VI) and *Bacillus megaterium* .
Treatment 4	Explant with 50 µg/ml Cr (VI) and endophyte CIM 1.
Treatment 5	Explant without Cr VI incubated in dark conditions.
Treatment 6	Explant with no Cr VI exposed toUV- light.
Treatment 7	Explant with *Bacillus megaterium* incubated at 20°C.
Treatment 8	Explant with endophyte CIM 1 incubated at 20°C.
Treatment 9	Explant with *Bacillus megaterium* incubated at 30°C.
Treatment 10	Explant with endophyte CIM 1 incubated at 30°C.

Table 1: Treatment details and Cr (VI) concentrations.

parenchymatous cells in the cortical region arranged in a compact manner without intercellular spaces. The cells maintained their polygonal shape, intact cell wall and homogeneous cytoplasm (Figure 1). In contrast, the damage in explants with Cr (VI) alone (Treatment 2) might be due to the systemic toxicity caused by high dose of Cr (VI) (50 µg/ ml) (Figure 2). The cells showed intracellular accumulation of Cr (VI) in the cortical tissues in a particulate fashion within the cortical tissues. Cortical cells became hypertrophied with formation of dark ringed structures while the vascular region was not much affected. In the case of excessive damage, ringed structures were observed at the intercellular locations. The histological changes can be concluded as strong reduction in meristematic activity and hypertrophy of cortical parenchyma. Explants having Cr (VI) and *B. megaterium* (Treatments 3) and explants having Cr (VI) and endophyte CIM 1 (Treatment 4) showed less cellular degradation as compared to explants with 50 µg/ ml Cr(VI) alone (Treatment 2) as is depicted in Figure 3 and 4 respectively. The cortical cells were less enlarged and ringed structures were not observed in the transverse sections of explants having Cr (VI) and *B. megaterium* in the growth media. Figure 4 depicts the transverse sections of explants having Cr (VI) and CIM 1 treatment where microbial inhabitation as hazy growth was observed in the hypodermal regions.

Minimum inhibitory concentration and Cr (VI) reducing capability

During the initial screening process, 81 bacterial strains were isolated, out of which two isolates were specifically chosen based on their relatively higher growth efficiency and enhanced tolerance, up to the concentration 50 µg/ml of Cr (VI). Figure 5 depicts the ability of endophytes to tolerate different concentrations of various heavy metals. The results depicted enhanced tolerance capability for the trivalent form of chromium in the microbes as compared to Cr (VI). Both the endophytes (*B. megaterium* & CIM 1) were able to tolerate cent percent concentrations of Mg and Mn. *B. megaterium* was more tolerant to metals like Pb, Ni and Cu as compared to endophyte CIM 1. These isolates were able to reduce Cr (VI), up to 34% (*B. megaterium*) and 20.5% (CIM 1) after 24 hours incubation while more than 50% Cr (VI) reduction was observed after 72 hours incubation (Figure 6).

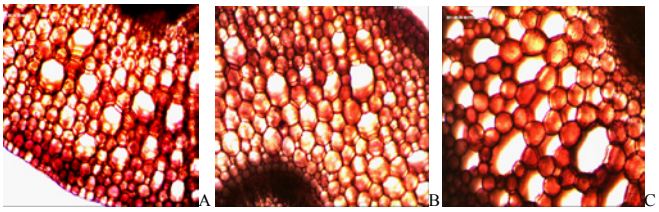

Figure 1: Transverse section of control explants (without Cr) showing normal cells with intact cytoplasm.

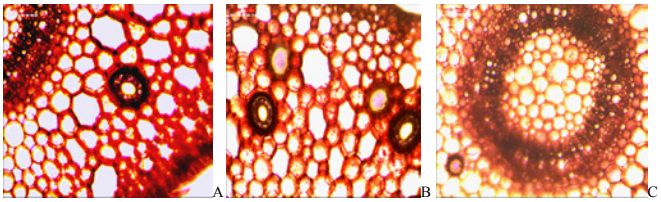

Figure 2: Light micrographs of Cr (VI) (50 µg/ml) exposed explants (incubation time 21 days).

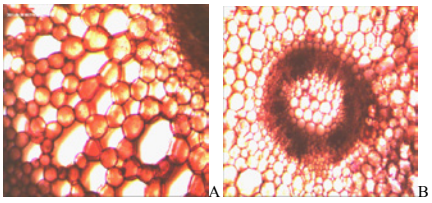

Figure 3: T. S. of explants having Cr (VI) and *B. megaterium* in the growth media.

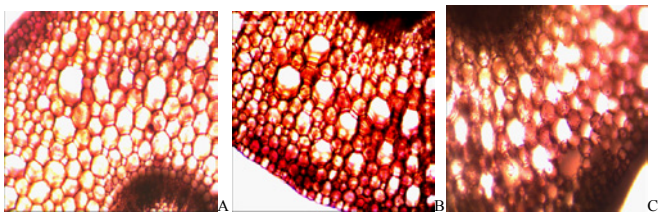

Figure 4: Transverse sections of explants with Cr (VI) and CIM 1.

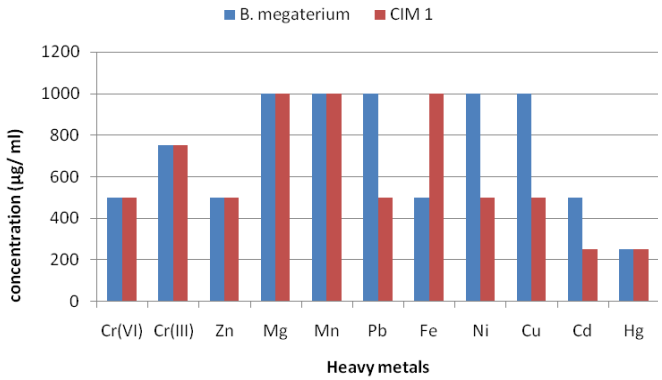

Figure 5: Tolerance levels for different heavy metals by endophytic bacteria (*B. megaterium* & CIM 1).

Statistical analysis

The Duncan test at a level of 5% of probability was applied. One way analysis of variance ANOVA results for growth promotion revealed significant mean differences against control for plant height, fresh weight and dry weight. Means were compared using the Least Significant Difference (LSD) at probability level (0.05) (Table 2). For plant height, explants incubated at 20°C (Treatments 7&8) showed almost same effects while explants exposed to Cr (VI) were severely affected. Also, temperature variation was not much affecting the plant growth. The explants with different treatments also varied significantly in their fresh and dry weight. CV% values for plant height provides a quick sight for enhanced plant growth which is not so depicted in the case of fresh and dry weight of the explants. Table 3 depicts a quick analysis for different traits with the F- value showing the significance of the analysis.

RNA isolation and PCR amplification results

Total RNA when analysed on 1% agarose gel revealed integrity in the treatments 1, 3 and 4 while damage was detected at RNA level in explants exposed to Cr (VI) concentrations (Treatment 2) alone which

was confirmed by cDNA expression with house- keeping actin genes. The cDNA amplification results for SOD and catalase genes (Figure 7) depict amplification in all treatments except for explants exposed to Cr (VI). The loss of expression in treatment 2 may be attributed to a probable DNA damaging action of Cr (VI).

Discussion

Histological methods contribute significantly to our understanding of *in vitro* culture systems. *In vitro* selection of plants tolerant to toxic ions contained in the soil may lead to production of plants that are better adapted to environmental pollution and can enable better management of degraded soil for e.g. industrial areas and highways. Plant sensitivity to toxic metal ions depends on the kinds of compounds present in the substrate and on their concentrations. Numerous *in vitro* experiments have focused on the effects of high concentrations of heavy metals on the regeneration of plants tolerant or sensitive to industrial pollution [16]. Selection of plants under natural conditions of environmental pollution or *in vitro* may result in the selection of clones tolerant to toxic metal ions. The present study will enable one to select suitable condition and media *in vitro*. Cr (VI) induced oxidative stress has been reported as one of the factors resulting in cellular toxicity [17]. Cr (VI) on reduction generates intermediate species viz. Cr (V) and Cr (IV) which further react with H_2O_2 to generate more amounts of Reactive Oxygen Species (ROS). It is likely that these ROS may interact with various tissues resulting in their damage. The histological results revealed a significant damage in cortical cells with accumulation of chromium which proves that once inside living tissues; chromium converts its oxidation state and generates ROS [18]. The explants having interface with bacteria were able to withstand Cr damage indicating that microbial interaction is of assistance to plants. In juxtaposition with many molecular biology methods, histological techniques can provide the necessary information for selection of explants in transformation experiments.

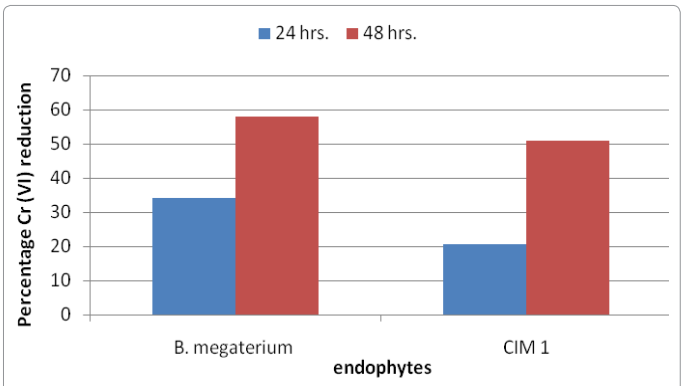

Figure 6: Cr (VI) Reduction ability of endophytic bacteria (*B. megaterium* & CIM 1).

Treatment	Plant height (cm)	Fresh shoot weight (mg)	Dry shoot weight (mg)
Treatment 1	7.37^{c}	50.67^{a}	10.27^{a}
Treatment 2	5.73^{f}	46.00^{d}	8.37^{cd}
Treatment 3	6.67^{de}	47.13^{cd}	8.77^{bc}
Treatment 4	6.47^{e}	46.73^{d}	9.03^{b}
Treatment 5	8.77^{ab}	42.50^{e}	7.87^{d}
Treatment 6	7.07^{cd}	50.03^{ab}	10.53^{a}
Treatment 7	8.23^{b}	46.47^{d}	8.83^{bc}
Treatment 8	8.80^{a}	48.60^{bc}	9.07^{b}
Treatment 9	7.40^{c}	46.87^{d}	8.83^{bc}
Treatment 10	8.73^{ab}	47.63^{cd}	9.07^{b}
LSD	**0.52**	**1.53**	**0.53**
CV%	**4.07**	**1.90**	**3.43**

CV% = Variation of coefficient; LSD = least significant difference.

Values in vertical columns followed by different superscripted letters are significantly different at $p < 0.05$. Means showing the same letters are not significantly different (P=0.05) according to Duncan's multiple range test.

Table 2: Growth promotion effect depicted by difference in mean values, their least significant differences (LSD) and coefficient of variation (CV%).

Source of variation	Degrees of Freedom (df)	Plant height (cm)	Fresh weight (mg)	Dry weight (mg)
Treatment	9	31.34	139.66	17.16
Error	20	1.87	16.05	1.93
Total	**29**	**33.21**	**155.71**	**19.09**
F- value		**30.17 ****	**19.34 ****	**19.79 ****

** Significative at a level of 5% of probability ($p < 0.05$)

Table 3: ANOVA table for different growth promoting traits.

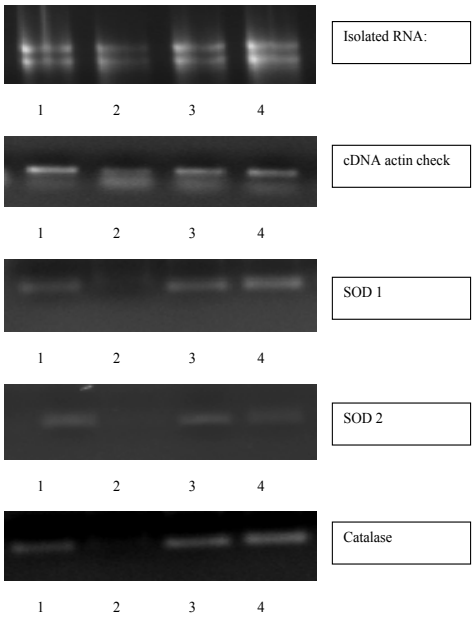

Figure 7: Agarose gel results (Lane1: control explants, Lane 2: with Cr (VI) (50 µg/ml), Lane 3: Cr (VI) with *B. megaterium* and Lane 4: Cr (VI) with CIM 1): depicting expression of RNA and different antioxidant genes.

Endophytic bacteria are the bacteria that colonize the internal tissues of the plant showing no external sign of infection or negative effect on their plant host. Bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of the plants in contaminated sites, which means that endophytes have a role in metabolizing these substances. The importance of endophytic bacteria in chromium reduction and their ability to promote the plant growth in a metal-contaminated environment has been observed earlier also for bioremediation studies by *Rhizobium, Pseudomonas*, and *Proteous* species [19]. The experimentation evaluates the ability of these microbes to show resistance to different heavy metals. The reduced plant growth at chromium concentrations is likely related

to the alteration of genetic material. These bacteria significantly alter the metabolic and physiological reactions of plant in a precise manner which is revealed through histological, statistical and molecular results. The results are in confirmation with the earlier publications [20]. There are several possible mechanisms by which the bacteria could influence the growth of host plants. The mechanisms include: reduction of Cr (VI) to Cr (III) by which it reduces the toxic effects of Cr (VI) to the plants; synthesis of siderophore, which can solubilise and sequester iron from the soil; production of phytohormones, which can enhance the growth of plants and solubilisation of phosphorus. Hence, the plant growth–promoting characteristics by the Cr tolerant Cr (VI) reducing endophytes should be screened for their multifarious roles in remediation strategies.

Chromium stress can induce three possible types of metabolic modification in plants (i) alteration in the production of pigments which are involved in the life sustenance of plants (e.g., chlorophyll) ; (ii) increased production of metabolites (e.g., glutathione, ascorbic acid) as a direct response to Cr stress which may cause damage to the plants and (iii) alterations in the metabolic pool to channelize the production of new biochemically related metabolites which may confer resistance or tolerance to Cr (VI) stress (e.g., phytochelatins, histidine). The effect of Cr (VI) on the plant processes during early growth and development culminates in reduction of yield and total dry matter as a consequence of poor production, translocation and partitioning of assimilates to the economic parts of the plant. The negative effect on yield and dry matter is essentially an indirect effect of Cr (VI) on plants. The results for one way analysis of variance are in confirmation with these findings, showing enhanced growth in treatments having microbial support. Adverse effects of Cr on plant height and shoot growth were detected in our investigation which is in coordination of earlier reports [21]. In a study conducted on *Vallisneria spiralis* to evaluate the Cr (VI) accumulation and toxicity in relation to biomass production, it was found that dry matter production was severely affected by Cr (VI) concentrations [22]. Synergistic effect of microbes on efficient shoot induction has been well documented and proved in the following plant species: *Dianthus chinesis; Rhodiola rosea; Salvia nemorosa*. [23,24].

Induction and activation of Superoxide dismutase (SOD) and catalase are some of the major metal detoxification mechanisms in plants [25]. Alteration of antioxidant enzymes due to metal inhibition has been reported [26] which are in accordance to the present results. A high concentration of ROS generated due to Cr (VI) at cellular level is because of oxidative stress which explains most of the visual Cr (VI) toxicity symptoms observed at whole plant level. High ROS production by Cr (VI) could set in motion a chain of signalling response at gene expression level which in turn could increase active scavenging. Higher energy allocation for active scavenging could deprive the plant of its quota of energy required for normal growth.

Conclusions

Having revised the overall picture of Cr (VI) toxicity in plants, it is clear that the species of Cr (VI) are toxic at different degrees at different stages of plant growth and development and also that the toxicity is concentration and medium dependent. The toxic properties of Cr (VI) originate from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr (VI) to Cr (III) occurring inside the cell. Thus, one of the future challenges to understand Cr (VI) toxicity would be to unravel the complete picture of interconversion of the Cr (VI) species within the plant system, after its uptake, on a time course at environmentally relevant concentrations with emphasis at different stages of plant development. In conclusion, the present study elucidates the adverse effects of Cr (VI) on the cellular integrity in *B. monneirri*; results also suggest that the overall toxic impacts occur at multiple sites and the long-term exposures to this heavy metal might pose a potential risk to native flora in the vicinity of contaminated sites.

Significance of the Work

Chromium is an extensively used anthropogenic pollutant causing severe damage to living cells. In aqueous systems, Cr (VI) exists as oxyanions (CrO_4^{2}) which are structurally analogous to sulphate and phosphate ions thus can be easily incorporated with the anionic transport system. The entry of Cr (VI) in living cells results in ROS (reactive oxygen species generation) causing damage to DNA and exerting mutagenic and teratogenic effects. The present study accounts for investigating the cellular level changes in *Bacopa monneiri* after short-term exposure to Cr (VI) so as to assess the toxicity induced due to chromate. *Bacopa monneiri* is a medicinally important plant inhabiting the moist places nearby rivers and lakes. If such plants are either naturally grown or cultivated in metal contaminated regions, there is a danger that the heavy metal accumulation by plants of medicinal value may cause serious health hazards to patients using metal adulterated herbal drugs. The histological studies are noteworthy to understand the changes in structural organization that occur due to pollutants in the environment. The damage due to Cr (VI) was assessed on DNA so that the molecular relationship could be established.

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