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Effects of Cr, Cd, and Pb on Ultrastructure, GSH and Free Cysteine in *Typha angustifolia*

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Abstract

Pot experiments were conducted to study the potential of *T. angustifolia* plants for Cr, Cd and Pb toxicity on the ultrastructure, Reduced Glutathione (GSH) and Free Cysteine on *T. angustifolia* (Narrow Cattail) after 30 d exposure of 1 mM Cr, Cd or Pb. Cr, Cd and Pb toxicity stress distort the ultrastructure and the treated seedlings showed that the vacuoles were expanded and endoplasmic membrane appeared to be rough. The levels of chloroplast in the plant cell were visible and prominent, yet the heavy metal stress caused an imbalance. The levels of free cysteine and GSH in shoots were significantly decreased in content especially in Cr-treatment and Cd-treatment, except for Pb which showed marked increase of free cysteine compared to the control. Meanwhile, no PCs were detected in the shoots of *T. angustifolia* exposed to Cr, Cd and Pb-treatments during analysis.

Keywords: Ultrastructure; Heavy metal; Reduced glutathione; Free Cysteine; *T. angustifolia*

Introduction

Heavy metals have been described by many studies as important limiting factors for agricultural production. Several heavy metals including, Cd, Pb, Cu and Cr, are considered hazardous waste metals that can be accumulated in the human body with a relatively large half-life. Salt et al. [1] have stated for example, that Cd has a half-life of 10 years once in the human body. Furthermore, some species of Cd, Cr, and Cu have been associated with health effects ranging from dermatitis to various types of cancer. Heavy metals like Cr, Cd and Pb can be incorporated into food chain and their levels can increase through biological magnification Cardwell et al. [2].

Several studies have demonstrated that toxic effect of Cd include chlorosis, growth inhibition, damage to root tips, reduction in water and nutrient uptake and change to proteins. The most general symptoms are stunting growth, chlorosis and alteration of anatomical, morphological, physiological and biochemical properties of leaf, stem and roots Godbold et al. [3], Liu et al. [4].

One other important heavy metal Cr is a nonessential element to the plants. It is stated that its compounds are toxic and detrimental to growth and development of plants. Chromium and its compounds have multifarious uses. Their accumulation by plants can reduce growth, induce chlorosis in young leaves, reduce pigment content, alter enzymatic function, damage root cells and cause ultrastructural modifications of the chloroplast cell membrane Panda and Choudhury [5]. Cr toxicity can reduce seed germination and ridicule growth in plants Nayari et al. [6], Shanker et al. [7]. Cr exists in hexavalent (Cr^{6+}) and trivalent (Cr^{3+}) forms, the previous form being more toxic than later one. Cr is toxic to most of the higher plants at100 g/kg dry weight Davies et al. [8]. The maximum quantity of element contaminant was always contained in organs. The reason of the high accumulation in roots could be its immobilization in the vacuoles of the root cells, which renders it less toxic, hence may be a natural toxicity response of the plant Shankers et al. [7]. Since both Cr(VI) and Cr(III) must cross the endodermis via symplast, the Cr(VI) in cells is probably readily reduced to Cr(III) which is retained in the root cortex cells under low concentration of Cr(VI) which in part explains the lower toxicity of Cr(III). Chromium is also one of the most difficult elements for plants to be translocated from root to shoot Jana [9].

Exposure to environmental sources of lead (Pb) is a serious public health concern. In humans chronic Pb exposure produces neurotoxicity, anemia, and kidney damage, and acute lead toxicity can be fatal. Pb presents potential high risks to human health but the cleanup of Pb contaminated soil is one of the most difficult tasks for environmental engineering. Pb has limited solubility in soil and is generally not available for plant uptake due to complexation with organic matter, sorption on oxides and clays or precipitation as carbonates, hydroxides and phosphates Lim et al. [10]. The major limitation to Pb phytoremediation have been attributed to low bioavailability in soil and poor translocation from roots to shoots. Pb hyperaccumulator plant species are those able to concentrate 0.1 percent or more of this metal in their dry stems and leaves without suffering stress or toxic consequences. In addition, these plants have to grow quickly and be able to produce a large amount of biomass Piechalak et al. [11], Sahi et al. [12]. In order for a plant to suffer the toxic effects of Pb, the Pb must be taken into the cell membrane. Many of the mechanisms (such as the formation of insoluble Pb phosphate) allow the Pb compound to be stored outside the cell membrane, somewhere along the cell wall. This type of storage would not harm the plant, but would result in elevated Pb concentrations. Pb is a widespread metal pollutant in soils Mushak [13].

In this research we use Narrow-cattail species (*Typha angustifolia*) as our focus for the study. The narrow leaf cattail inhabits unstable wetlands with slightly salty or calcareous soil, or in water that is slightly deeper than the common water habitat for the broad leaf species T. latifolia [14]. Narrow-leaved cattail is eaten by waterfowl and muskrats. Muskrats also construct their lodges with cattail, and black birds use cattail for perches. Extensive monotypic stands of Cattail are poor habitat for wildlife. In Vietnams it is eaten by people known as bon bon.

Materials and Methods

Plant materials and treatments

The pot experiment was carried out May to September, 2008.

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Agricultural soil was collected from the experimental farm (depth 0-15 cm) on Huajiachi Campus of Zhejiang University, Hangzhou, China. The soil was air-dried and mixed daily until 8% water content was reached. Air-dried soil of 4 kg was weighed and loaded into a plastic pot (5 L, 20 cm height). Pots were kept in a greenhouse under natural light condition during 60 d after sowing.

Healthy seeds of *T. angustifolia* were sown in the above mentioned pots, and irrigated with equal volume of tap water to keep humid. At the 60 day seedlings were thinned to leave 10-15 uniform, healthy seedlings per pot, and then pots were transferred to a growth incubator with light intensity of 300 μm m-2 s-1 and day/night temperature of 20±0.5°C /25±0.5°C with 14 h of day time. There were 2 application dates for heavy metal treatments: D90 and D130, in which seedlings were allowed to grow for another 30, and 70 d (i.e. 90 and 130 d after sowing), respectively, before Cd, Cr and Pb application. During this period, soils in the pots were kept humid (90-100% water holding capacity) for the first 20 and 60 d, respectively, and then stop irrigation to reach the water holding capacity at about 50%. Before irrigation was stopped the plants were supplied with 500 ml of nutrition solution. When the soil was having limited amount of water (90, and 130 d after sowing), 500 ml of distilled water (control, no addition of heavy metal), 1 mM $K_2Cr_2O_7$, 1 mM $CdCl_2$, and 1 mM Pb $(NO_3)_2$ solution were added to each pot, and 10 d later the corresponding solution was added again to form 4 treatments of control, Cr, Cd and Pb for the two different growth period plants of 90 and 130 days after sowing (denoted as Control-D90, Cr-D90, Cd-D90, Pb-D90, and Control-D130, Cr-D130, Cd-D130, Pb-D130, respectively). The soil was kept humid thereafter. All reagents were analytical grade and all stock solutions were made with deionized water. The experiment was laid in a randomized block design, and 2 plants from each pot were marked for final harvest. To avoid the effect of positioning, the pots were shifted periodically.

Sampling and measurements

After 30 d of heavy metal application (the two different growth period plants of 120 and 160 d after sowing) plants were investigated of leaf and root ultrastructure using Transmission electroscopy.

Investigation of leaf and root ultrastructure: Fresh roots (3 mm in length, 2-3 mm behind the apex) and leaves (1 mm^2, top middle section of the fully expanded leaf) from 160 d after sowing were hand sectioned and fixed for 6-8 h in 100 mM (pH 7.0) PBS containing 2.5% glutaraldehyde (v/v), and washed three times with the same PBS. The samples were post-fixed in 1% osmium tetroxide (OsO4) for 1 h and washed in PBS for 1 h. Subsequently, samples were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) with 15-20 min interval and followed by acetone (100%) for 20 min, and then infiltrated and embedded in Spurr's resin overnight. Finally, the specimen sections were stained by uranyl acetate and alkaline lead citrate for 15 min, respectively, and ultra-thin sections (80 nm) were prepared and mounted on copper grids and viewed under a transmission electron microscope (JEOL JEM-1230 EX, Japan).

Determination of PCs and other low molecular weight: Shoots were cut 0.5 cm above surface of soil, washed thoroughly with deionised water, and immediately frozen in liquid nitrogen and then stored frozen at -80°C for the determination of Cys, GSH and PCs

Extraction and analysis of PCs and other low molecular weight SH were performed according to the method described by Sneller et al. [15] with a slight modification. All reagents were of reagent grade. The monobromobimane (mBBr) was prepared daily, and others were prepared weekly and stored at 4℃. Milli-Q water (18.3 MΩ) was used. Frozen plant shoots were homogenized with mortar and pestle with liquid HNO_3 in 2 mL 6.3 mM DTPA(diethylenetriamine-pentacetic acid) with 0.1% TFA (CF_3CO_2H) at 4°C. The homogenate was centrifuged at 14,000 g at 4°C for 12 min. The clear supernatants were collected and immediately used for the assay of PCs and other low molecular weight SH by high-performance liquid chromatography (HPLC), using pre-column derivatization with a fluorescent probe mBrB. 250 mL of supernatant was mixed with 250 mL of 200 mM HEPPS (N-[2-hydroxyethyl] piperazine-N'-[3-propane sulfonic acid]) buffer, at pH 8.2, with 6.3 mM DTPA, and 10 mL of 25 mM mBrB. Derivatization was carried out in the dark at 45°C for 30 min. The reaction was terminated by adding 300 mL of 1 M MSA. The samples were stored in the dark at 4°C until HPLC analysis. Reagent blanks were used to identify the reagent peaks. The bimane derivatives were separated using a binary gradient of mobile phase A (0.1% TFA) and B (100% ACN) at room temperature (22.7°C). Fluorescence was detected at 380 nm excitation and 470 nm emission wavelengths. The flow rate was 0.5 mLmin-1. The derivatized sample (25 mL) was run in a linear gradient from 12% to 25% B for 15 min, then 25-35% B for 14 min, and subsequently 35-50% B for 21 min. Before injecting a new sample, the column was cleaned (5 min, 100% B) and equilibrated (10 min, 12% B). The post time was 5 min resulting in a total analysis time of 70 min. All solvents were degassed before use. The analytical data were integrated by using HP Chemstation.

The retention times of PCs and other low molecular weight SH (GSH, Cys) in biological samples were checked using (g-GluCys)2-Gly (PC2), (g-GluCys)3-Gly (PC3), (g-GluCys)3-Gly (PC4), GSH, gEC, and Cys standards. PC2, PC3 and PC4 standard were obtained from Shanghai science peptide biological technology co., Ltd. The standards were run after every six samples to monitor the slight shift of PCs peaks in retention time.

Individual PC subtype was quantified by using the relationship peak vs. concentrations of GSH standard solutions. Corrections for differential derivatization efficiencies were made according to the methods stated by Sneller et al. [15].

Statistical Analysis

All data presented are the mean values. The measurement was done with 3 replicates on plant growth traits and metal concentrations and 4 replicates on all enzyme activities and PCs and other low molecular weight. Statistical analyses were performed with Data Processing System (DPS) statistical software package using ANOVA followed by the least significant difference test (LSD) according to Fisher to evaluate significant treatment effects at significance level of $P \leq 0.05$.

Results and Analyses

Transmission electron microscopy

The Transmission electron microscopy images of an ultrathin section of *T. angustifola* roots tips dosed with or without Cr, Cd and Pb are shown in Figure 1A-1D. The Transmission electron microscopy observation on root tips of the control seedlings showed that the cytoplasm and cell wall are stick together and it seems that the materials inside the mitochondrion are many and intact. Meanwhile, from the observation it was obvious that damages occurred due to exposure of the seedlings to Cr, Cd and Pb as it was vividly shown as changes were recorded when compared with the control. The cell wall and cytoplasm appeared to be separated, materials inside the mitochondrion seems to be a bit little and appears to be swollen because of the stress conditions the seedlings were subjected to by exposure to Cr, Cd and Pb treatment. Furthermore, in the treated seedlings it was realized that the vacuoles were expanded, endoplasmic membrane appears to be rough and root

epidermis cracked which might be as a result of toxicity symptom in the root cells. The Transmission electron microscopy images of an ultrathin section of *T. angustifola* roots tips dosed with or without Cr, Cd and Pb are shown in Figure 1.

The ultrathin sections of the leaves treated with or without Cr, Cd and Pb are shown in Figures 1E-1H. The ultrastructural studies of the leaves of the control showed more or less an intact plant cell as almost all the true features of the cell were very prominent and alive including the nucleus, nucleolus, chloroplast, cell wall, cell membrane, vacuole, amyloplast and starch grain. However, for Cr-treated seedlings in comparison to the control revealed advanced vacoulation and many more starch grains were prominent. As for Cd-treated seedlings starch grains were not prominent and vacuole slightly expanded in comparison to the control. Furthermore, in Pb-treated plant the vacuole was also expanded and starch grains not prominent. These symptoms presented by Cr, Cd and Pb-treated seedlings can be as a result of stress caused by toxicity of the heavy metals. The damage caused by the Cd-treatment to the seedlings seems to be lesser in comparison to Cr and Pb-treatment. Meanwhile, in all treatments chloroplast was very visible and prominent only that the degree varies. The Transmission electron microscopy images of an ultrathin section of *T. angustifola* Shoot dosed with or without Cr, Cd and Pb are shown in Figure 2.

Effect of Cr, Cd and Pb stress on free Cysteine and GSH

GSH and other non-protein thiols have been shown to be affected by the presence of several metals. Rauser [16] describe GSH as the assembling block of phytochelatins (PCs), a family of cysteine-rich peptides that accumulate under metal exposure. GSH and other non-protein thiols are known to be essential metabolite in the cellular redox homeostasis.

The levels of free cysteine and GSH in *T. angustifolia* shoots are shown in Table 1. Exposure to Cr, Cd and Pb resulted in a significant decrease in content of free cysteine and GSH especially in Cr-treatment and Cd-treatment, except for Pb which showed marked increase of free cysteine compared to the control. Further analysis revealed the level of free cysteine and GSH content decreased by3%, 40% and 25%, 5% after Cr-treatment, by 62%, 16% and 29%, 10% after Cd-treatment and GSH by 65% and 8% and Cysteine increased by 92% and 9% after Pb-treatment respectively compared to that of control. Meanwhile, no PCs were detected in the shoots of *T. angustifolia* exposed to Cr, Cd and Pb-treatments during analysis.

Discussion

Plant species differ significantly in capacity for uptake and tolerance to Cd and other heavy metals Roosens et al. [17], Bert et al. [18]. The effect of heavy metals on cellular organization is an important factor in understanding the physiological alterations induced by heavy metals due to complementarity of structure and function Jin et al. [19].

The transmission electron microscopy observation on root tips revealed that the cell wall and cytoplasm were both disturbed; the vacuoles were expanded with rough endoplasmic membrane and root epidermis cracked. The mitochondrion which is the power house of the plant was also disturbed. All of these put together might be as a result of toxicity symptom in the root cells and that plant under stress need to expend extra energy for maintaining normal function, hence

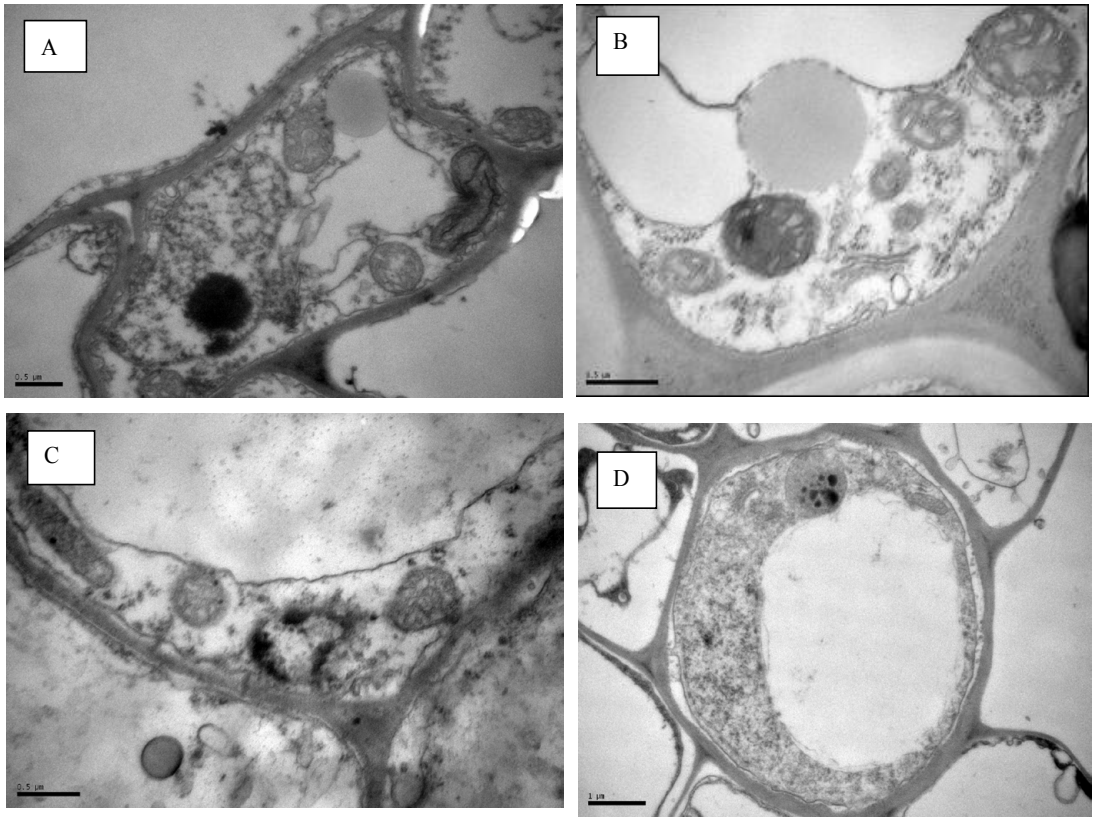

Figure 1: The Transmission electron microscopy images of an ultrathin section of T.angustifola roots tips dosed with or without Cr, Cd and Pb are shown in TEM images of an ultrathin section of Typha angustifolia roots treated with or without Cr, Cd and Pb treatment after 30 d. A-D represents roots with A-Control, B- Cr (Chromium), C- Cd (Cadmium) and D- Pb (Lead)

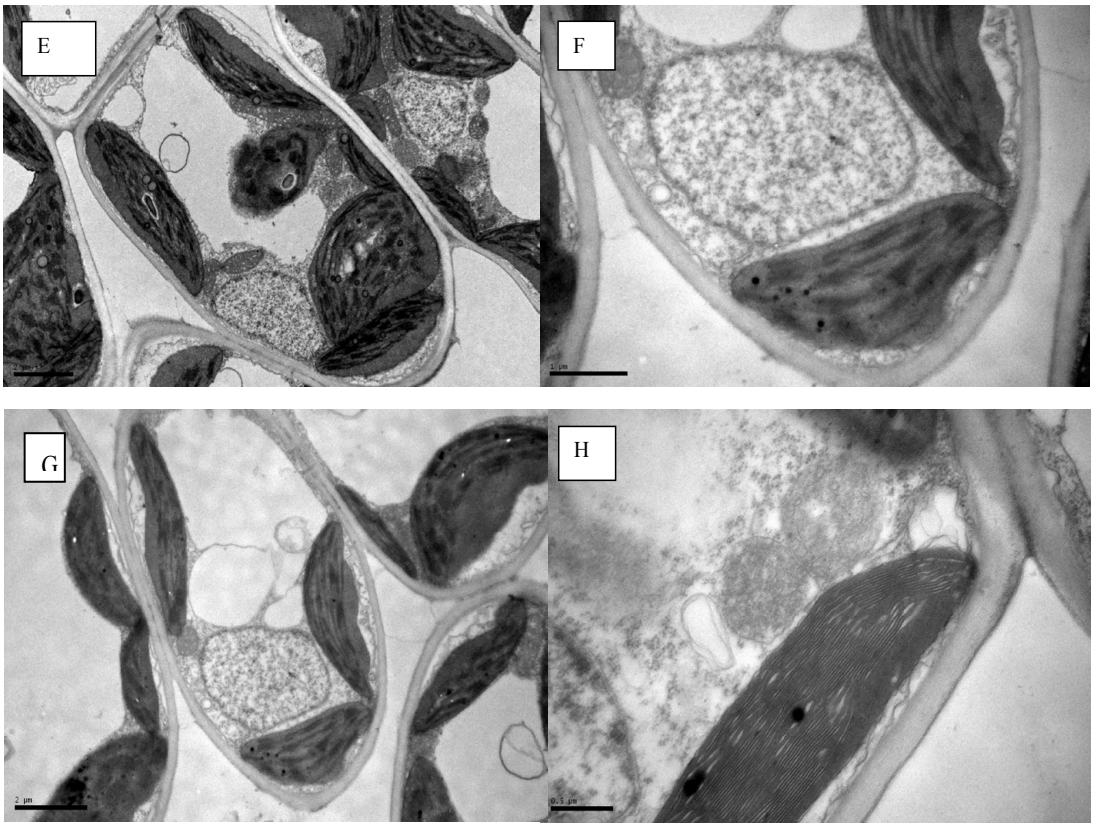

Figure 2: *T. angustifola* Shoot dosed with or without Cr, Cd and Pb are shown in TEM images of an ultrathin section of Typha angustifolia leaves treated with or without Cr, Cd and Pb treatment after 30 d. E-H represent leaves with E-Control, F- Cr (Chromium), G- Cd (Cadmium) and H- Pb (Lead).

Treatment	Free cysteine	GSH
Control-D90	25.5 ± 1.3	372 ± 11.6
Cr-D90	24.5 ± 1.3	276 ± 6.3
Cd-D90	10 ± 1.8	266 ± 11.6
Pb-D90	50 ± 2.7	130 ± 6.3
Control-D130	43 ± 2.7	183.5 ± 5.8
Cr-D130	25.5 ± 3.1	173.5 ± 4.9
Cd-D130	35.5 ± 1.3	164.5 ± 3.1
Pb-D130	46.5 ± 3.1	168.5 ± 4.0

Control., Cr, Cd and Pb correspond to distilled water (no addition of heavy metal), 1 mM $K_2Cr_2O_7$, 1 mM $CdCl_2$, and 1 mM Pb $(NO_3)_2$, respectively; D90 and D130 referred to the first application date of heavy metals: 90 and 130 days after sowing. Data were means of three independent replications (Mean ± SD).

Table 1: Effect of Cr, Cd, and Pb on Free Cysteine and GSH activities in shoots of the *T. angustifolia* plants for the two application times.

the distortion in the structure of the mitochondrion. According to Dietz et al. [20] tolerant plants showed enhanced avoidance and homeostatic mechanisms to prevent the onset of stress. The response pattern evoked after stress is the characteristic stress syndrome and such processes are homeostatic, in that they aim at normalizing the plant vital functions and at raising its powers of resistance. Therefore, in agreement with Ernst et al. [21]. The expanded vacuolation observed in our results was a mechanism by the plant to deal with high levels of Cr, Cd and Pb. The ultrastructural studies of the shoots also showed advanced vacuolation and that though chloroplast was visible and prominent, yet the heavy metal stress caused an imbalance which was reflected on the plant heights, root lengths and biomass. These effects were more prominent with Cr-treated plants. Chromium-induced morphological and ultrastructural changes have been reported in several aquatic vascular plants: In Lemna minor and Ceratophyllum demersum, chromium-induced changes in chloroplast fine structure disorganized thylakoids with loss of grain and caused formation of many vesicles in the chloroplast. Chromium (VI) has caused stunting and browning of roots produced from the chromium-treated excised leaves of *Limnanthemum cristatum*. At 226_g/g Cr dry wt leaf tissue concentration, development of brown coloration in the hydathodes of juvenile leaves of *Limnanthemum cristatum* is a characteristic chromium induced alteration. Studies on barley leaves showed that rapid compartmentalization of Cd in to the vacuole was an important mechanism for dealing with high levels of Cd. Our results suggested a reduction in photosynthesis rates especially under Cr stress. Since non-specific effects of stress are alterations in membrane properties (membrane potential, transport of substances), increased respiration, inhibition of photosynthesis, reduced dry matter production and growth disturbances we can safely say that *T. angustifolia* when subjected to Cr, Cd and Pb treatment exhibit stress conditions in multifaceted manner. We also observed that the nature and intensity of response of individual plants to heavy metal stress factor varied considerably depending on age and type of metal treatment. From our results, we can conclude that the damaged caused by Cd-treatment to the seedlings seem to be lesser in comparison to Cr and Pb-treatment.

Glutathione (GSH) is a low-molecular weight thiol tripeptide, involved in cellular defense against the toxic action of xenobiotics, oxyradicals as well as of metal cations Meister and Anderson [22]. It is able to modify metal toxicity by chelating metal ions in cells; it plays a key role in protecting macromolecules from damage by free radicals by trapping them in an aqueous phase Freedman et al. [23].

GSH acts as a first line of defense against metal toxicity by complexing metals before the induced synthesis of PCs arrives at effective levels Freedman et al. [23], Singhal et al. [24].

GSH exists predominantly in the reduced form with estimates varying from 70% in barley chloroplasts to 90% in pea chloroplasts Bielawski and Joy [25]. In our research it was shown that exposure to Cr, Cd and Pb also in agreement Bielawski and Joy [25] resulted in a significant decrease in content of free cysteine and GSH especially in Cr-treatment and Cd-treatment, except for Pb which showed marked increase of free cysteine compared to the control. Further analysis revealed the level of free cysteine and GSH content decreased by3%, 40% and 25%, 5% after Cr-treatment, by 62%, 16% and 29%, 10% after Cd-treatment and GSH by 65% and 8% and Cysteine increased by 92% and 9% after Pb-treatment respectively compared to that of control at both growth stages There was marked decline in GSH and Free Cysteine under Cr and Cd stress which may have occurred as a result of interconversion of reduced and oxidized forms of glutathione to maintain redox status of the cell as to scavenge free radicals, hence would have probably caused a decrease in GSH. Since GSH can function as an antioxidant in many ways and can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger, the marked decline in GSH and Free Cysteine in our results may have stabilize the membrane structure by removing acyl peroxides formed by lipid peroxidation reactions in agreement with Price et al. [26]

Conclusion

The ultrastructural studies of the shoots showed expanded vacuolation and that the chloroplast was visible and prominent, yet the heavy metal stress caused an imbalance in the plant in several ways. The mitochondrion which is the power house of the plant was also disturbed.

We also observed that the nature and intensity of response of individual plants to heavy metal stress factor varied considerably depending on age and type of metal treatment. We can conclude that the damaged caused by Cd-treatment to the seedlings seem to be minimal in comparison to Cr and Pb-treatment

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