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Impact of cadmium and lead on *Catharanthus roseus* - A phytoremediation study

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Abstract: *The Madagascar Periwinkle, Catharanthus roseus (L.) G. Don (a valued medicinal plant) was exposed to different concentrations of heavy metals like, $CdCl_2$ and $PbCl_2$ with a view to observe their bioaccumulation efficiency. Germination was inhibited by both the heavy metals in the seeds previously imbibed in GA_3 and KNO_3 for 24 hr. EC_{50} (the effective concentration which inhibits root length by 50%) was recorded as 180 µM for $CdCl_2$ and 50 µM for $PbCl_2$. Both α-amylase and protease activity were reduced substantially on treatment of seeds with increasing concentrations of $CdCl_2$ and $PbCl_2$. Malondialdehyde (MDA) a product of lipoxigenase (LOX) activity also increased due to the treatment of both $CdCl_2$ and $PbCl_2$. When two-months-old plants grown in normal soil were transferred to soils containing increasing amounts of these two heavy metals, senescence of lower leaves and extensive chlorosis were noticed after four days of transfer. However, plants gradually acclimatized and after 20 days the chlorophyll content was almost comparable to normal. Plants receiving $CdCl_2$ treatment (250 µg g^{-1} and less) became acclimatized after two weeks and started normal growth. But $PbCl_2$ of 432 µg g^{-1} and less could not affect the plant growth throughout, after a preliminary shock was erased. In case of $CdCl_2$ treatment, a stunted growth with reduced leaf area, reduced biomass and sterility were recorded after six months, while plants show normal growth and flowering in case of $PbCl_2$ treatment. Total alkaloid was also found to be decreased in the roots of $CdCl_2$ treated plants. No change was observed in case of $PbCl_2$. GA_3 treatments to the $CdCl_2$ treated plants show internode elongation and increase in leaf area with relatively elongated leaves and thinning of stem diameter. AAS analyses of leaves of treated plants exhibited 5-10% accumulation of cadmium, but there was no accumulation of lead at all.*

Key words: *Toxicity Cd and Pb stress, Phytoremediation, Catharanthus*
PDF file of full length paper is available with author

Introduction

Among the abiological stresses, the heavy metal stress is of serious concern in industrial and mining areas. Plants encounter a variety of metals in natural aquatic and terrestrial habitats. Heavy metals are those metallic elements, which have their atomic weight more than 20 or in other words, with a density higher than 5 g cm^{-3}. There are 90 naturally occurring elements that are heavy metals (Weast, 1984), but not all of them are biologically significant. Based on their solubility under physiological conditions, 17 heavy metals may be available for living cells and of importance for organisms and ecosystems (Weast, 1984). Among these metals, Fe, Mo and Mn are important as micronutrients. Zn, Ni, Cu, V, Co, W and Cr are toxic elements with high or low importance as trace elements. As, Hg, Ag, Sb, Cd, Pb and U have no known function as nutrients in the living system and seem to be more or less toxic (Goldbold and Huttermann, 1985; Breckle, 1991; Nies, 1999; Sogut *et al*., 2005; Baek *et al*., 2006). However, elevated concentrations of both essential and nonessential metals can result in growth inhibition and toxicity symptoms (Akinola and Ekiyoyo, 2006).

Of the highly toxic heavy metals cadmium and lead are very important as far as their persistence in the environment is concerned. Metal contaminated soils are notoriously hard to remediate. Current technology resort to soil excavation and either land filling or soil washing followed by physical or chemical separation of the contaminants. Because of the high cost associated with these processes, there is need for the less- expensive cleanup technologies. Phytoremediation is emerging as a cost-effective alternative. It is a process in which plants are used to clean up contaminated soil. More importantly, the process *in situ* avoids dramatic landscape disruption and preserves the ecosystem. Depending on plant species, metal tolerance may result from two basic strategies; metal exclusion and metal accumulation (Baker, 1981). The exclusion strategy comprise the avoidance of metal uptake and restriction of metal transport to the shoot (De Vos *et al*., 1991), and the accumulation strategy comprises of import of heavy metals through root epidermal cells and their accumulation in the vacuoles and further transport to different parts of the plant body.

In the present study we have tried to analyse the phytoremediating efficiency of *Catharanthus roseus* with respect to heavy metals such as cadmium and lead. Periwinkle *Catharanthus roseus* (L.) G. DON. [= Syn. *Vinca rosea* (L.) is a diploid (2n = 16)] is a perennial herb belonging to the family Apocynaceae. It is pantropical in distribution and is found throughout India on wastelands and sandy tracts, especially in the coastal areas and is often grown in gardens for its pink and white flowers that bloom throughout the year. It has erect stem with flexible branches bearing leaves that are simple axillary and terminal clusters. Fruit is a cylindrical follicle with many black seeds. The commercial crop is a mixture of populations having wide morphological and chemical variations with various range of

heritability (Kulkerni *et al.*, 1984) due to fair amount of outcrossing existing in the species (Krishnan *et al.*, 1979). The genus *Catharanthus* has now gained considerable reputation in the therapeutic world for its wide assemblage of over 100 alkaloids including vincristin, vinblastin, ajmaline, ajmalicine and serpentine which are extremely important. Since stress condition provide suitable environment for synthesis and accumulation of secondary metabolites, *Catharanthus* was chosen to study its phytoremediation efficiency with respect to alkaloid production.

Materials and Methods

Seeds of *Catharanthus roseus* var. *rosea* were collected from the experimental gardens of the Botany Department, Burdwan University and were dried under the sun. Then they were stored in screw cap vials at 10°C. With the seeds following experiments were performed.

Germination studies: Few seeds were soaked in water for overnight in dark and then treated with 0.2% (w/v) bavistin solution and allowed to germinate on two sets of petridishes. Petridishes contained double layer whatman no.1 filter discs moistened with distilled water and different concentration of $CdCl_2$ and $PbCl_2$ solutions (0, 10, 50, 100, 200, 500 and 1000 μM) 5 ml each. There were three replicates for each of these concentrations. All the petriplates were incubated at 25±1°C in dark in an incubator.

Estimation of $CdCl_2$ and $PbCl_2$ on α-amylase and protease activity: After 48 hr of incubation for germination, seeds were taken and extracted with 5 ml of chilled 0.1 M sodium phosphate buffer (pH-6.8) at 4°C according to Snell and Snell (1971). Amylase and protease activities of the extracts were measured in term of subsequent analysis of residual substrate according to the method of Khan and Faust (1967) and Lowry *et al.* (1951) respectively. For the assay of these two enzymes, the blank was taken as zero-time control and the activity of the enzyme was expressed as :-

$$\frac{(\Delta A \times T_V)}{t \times v}$$

Where ΔA is the absorbance of sample after incubation minus the absorbance of zero time control. T_V is the total volume of filtrate taken for incubation. 't' is the incubation time in minutes and 'v' is the volume of filtrate taken for incubation (Fick and Qualset, 1975).

Determination of membrane integrity with respect to MDA: The membrane lipid peroxidation of treated seedlings was estimated in terms of malondialdehyde (MDA, a product of lipid peroxidation) content by thiobarbituric acid (TBA) reaction according to Heath and Packer (1968). The leaf tissue was homogenized in 0.1% trichloroacetic acid (TCA) following centrifugation at 10,000 rpm for 5 minutes. The supernatant was treated with 0.5% (w/v) TBA at 95°C and cooled quickly. The absorbance was measured at 530 nm wavelength. The non-specific turbidity was corrected by subtracting A_{600} from A_{530} value. The concentration of MDA was calculated from its extinction coefficient of 155 μM cm^{-1}. The MDA content is finally expressed in mole g^{-1} dry mass of tissue.

Studies on morphological changes: Seeds were soaked over night in distilled water. Then the seeds were treated with 0.2% bavistin (w/v) and sown in the heavy metal negative garden soil. When plants were two months old they were transferred to finely grinded soil containing variable concentrations of $CdCl_2$ and $PbCl_2$. There were three replicates for each treatment. Pots were kept in open natural temperature and sunlight. Pots were watered twice daily. Morphological changes were observed thereafter.

Gibberellin treatment: The $CdCl_2$ treated 6 months old plants, which showed stunted growth and sterility, were then sprayed with 100 ppm of GA_3 (Sigma) at every alternate day. This treatment was performed with one of the replica experimental set up.

Estimation of chlorophyll content: After 4 days of transferring plants in different concentration of the heavy metals on soil 2-3 lower leaves were plucked and chlorophyll content was estimated according to the method of Sadasivam and Manikam (1996). For this 1 g of freshly collected leaves were taken and homogenized properly in a mortar with 10 ml of 80% acetone. This was then centrifuged at 5000 rpm for 5 minutes at room temperature. The supernatant was taken and the pellet was reextracted with 80% acetone until pellet becomes colorless. The final volume was made up to 100 ml with 80% acetone. Finally the absorbance was measured at 645, 663, and 470 nm wavelength in a double beam UV-VIS spectrophotometer (Systronics spectrophotometer 2101).

Chlorophyll content was calculated according to the following formula:-

$$\text{Mg of Chl-a/g tissue} = 12.7(A_{663}) - 2.69(A_{645}) \times \frac{V}{1000 \times w}$$

$$\text{Mg of Chl-b/g tissue} = 22.9(A_{645}) - 4.68(A_{663}) \times \frac{V}{1000 \times w}$$

Where, A = absorbance at specific wavelength, V = final volume of the extract in 80% acetone and W = fresh weight of tissue extract.

Soil sample preparation: Soil sample was prepared according to the instruction manual. 5 g soil sample was taken and air-dried properly. The dried soil was then ground and sieved. 5 g soil sample (sieved) was collected in an Erlen Meyer flask and 20 ml of extracting solution (0.5N HCl + 0.025N H_2SO_4) was added. This was shaken for 15 minutes on a mechanical shaker. Then the extract was filtered through whatman no. 42 filter paper into 50 ml volumetric flask. The final volume was made up to 50 ml with extracting solution. This solution was used for estimation of heavy metals present in the soil.

Plant sample preparation: This was also prepared according to the instructional manual. One gram of tissue sample (leaf) was dried completely and then gro und in a mortar. This sample was taken in a beaker and 10 ml of concentrated HNO_3 was added into it and allowed to stand for overnight. This was then heated carefully on a hot plate until the production of red NO_2 fumes have ceased to come out. The beaker was then cooled and a small amount of (2-4 ml) of 70% $HClO_4$ was added. This was then heated again and allowed to evaporate to a small volume. The sample was transferred to a 50 ml volumetric flask and diluted to volume with distilled water. It was then subjected to AAS analysis.

Estimation of total alkaloid: For this, 200 mg dried root samples were powdered in a mortar and then extracted with 1% H_2SO_4 for overnight. The next day samples were shaken for 30 min and then centrifuged. The supernatant was saved and the pellet was reextracted twice with H_2SO_4. The starch and other impurities were removed by precipitating with small amount of $Ca(OH)_2$ solution by continuous shaking. Precipitates were removed by centrifugation. The supernatant was then evaporated on hot water bath and the slurry was solubilized finally in n-butanol:HCl (98:2) (Farooqi and Sreeramu, 2001). Standard curve was prepared with a mixture of ajmalicine and ajmaline.

Results and Discussion

Effect on germination and growth: It is evident that the germination is reduced tremendously as the concentration increased and it is 50% of the control at the concentration of 500 μM $CdCl_2$ and 200 μM $PbCl_2$. Germination is inhibited completely at 1000 μM concentration of $CdCl_2$ and 500 μM $PbCl_2$. The growth analysis was done by measuring the relative root elongation (Fig. 1). Relative root elongations have been calculated according to the following formula: -

$$\text{Relative root elongation (RRE)} = \frac{\text{Root elongation with } Cd^{+2} \text{ or } Pb^{+2}}{\text{Root elongation without } Cd^{+2} \text{ or } Pb^{+2}} \times 100$$

The EC_{50} (the effective concentration which inhibits root length by 50%) of treated seeds were found to be 180 μM for $CdCl_2$ and 50 μM for $PbCl_2$.

Effect of $CdCl_2$ and $PbCl_2$ on amylase and protease activity: An important parameter to analyze the germinability of a seed is to determine the concentrations of amylase, protease, phytase etc because these enzymes are secreted in more amount during the time of germination for breaking down and mobilizing the stored food in the endosperm of the seed. Seeds pretreated with different concentrations of cadmium and lead were incubated at 25°C in dark and after that the concentrations of amylase and protease (in terms of mg of substrates hydrolyzed /g dry weight / minute) were determined as stated in the materials and methods. The results shows that both amylase and protease activity was reduced considerably on increasing the concentrations of $CdCl_2$ and $PbCl_2$ (Fig. 2).

Effect on malondialdehyde (MDA) content: MDA level, which is a measure of membrane lipid peroxidation and lipoxigenase activity was found to increase in the seedlings of $CdCl_2$ and $PbCl_2$

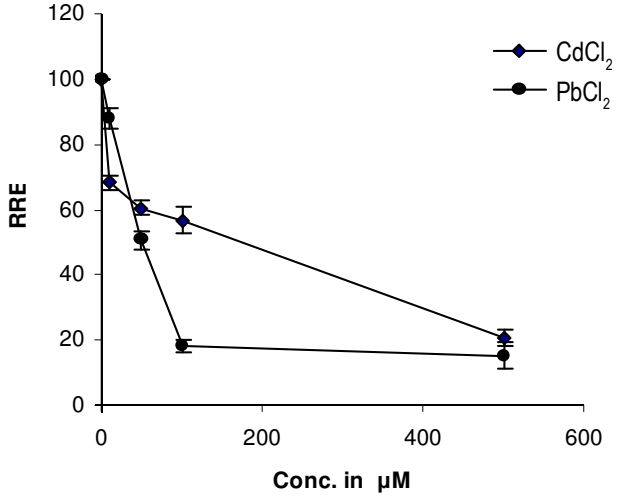

Fig. 1: Relative root elongation of $CdCl_2$ and $PbCl_2$ treated seeds. The EC_{50} was found to be 180 μM for $CdCl_2$ and 50 μM for $PbCl_2$. Relative root elongation (RRE) was calculated based on the root elongation after 10 days of germination with exposure to different concentrations of cadmium and lead relative to elongation without the heavy metals

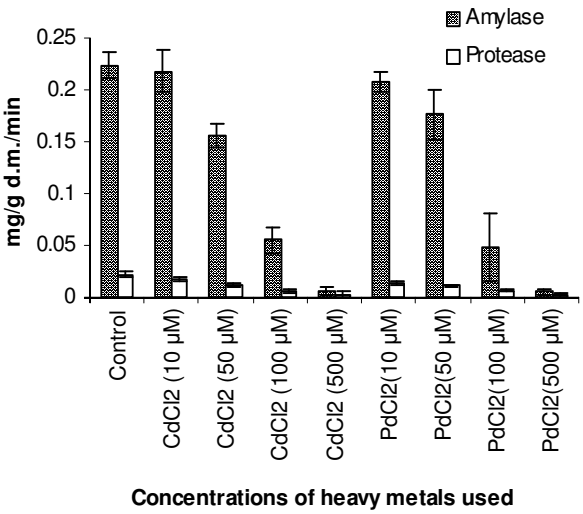

Fig. 2: Effect of $CdCl_2$ and $PbCl_2$ onthe amylase and protease activities of germinating seeds (24 hr after imbibition)

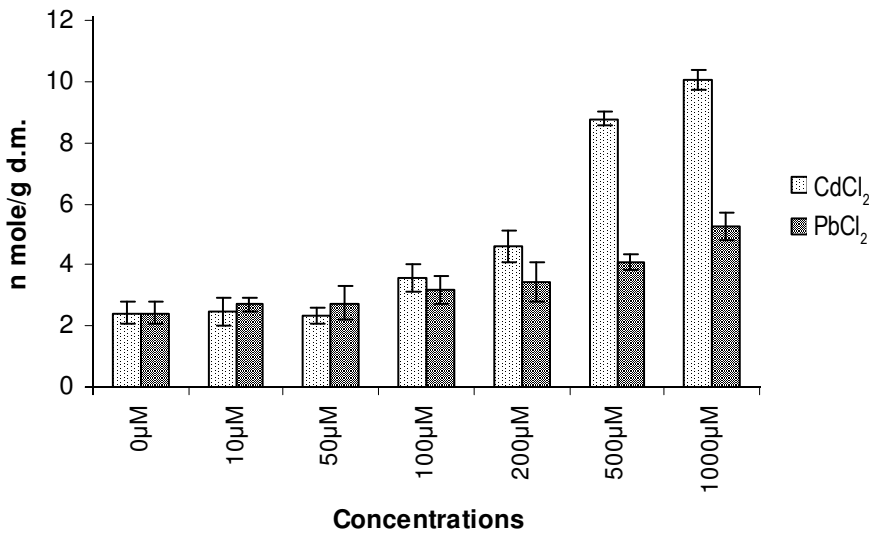

Fig. 3: Effect of cadmium and lead on the membrane lipid peroxidation (in terms of malondiaaldehyde, MDA) in *C. roseus* seedling, 10 days old

Fig. 4: The figure shows 8 months old roots. A = Control, B = $PbCl_2$ treated, C = $CdCl_2$ (1X), D = $CdCl_2$ (2X) and E = $CdCl_2$ (3X)

treated plants and the increase in MDA concentration was proportional to concentrations of heavy metals used (Fig. 3).

Effect of heavy metals ($CdCl_2$ and $PbCl_2$) on plant morphology and biomass: When two months old plants grown first in heavy metal free soil were transplanted to soil containing variable concentrations of cadmium and lead showed at the beginning the symptoms of chlorosis and senescence. But after a period of two weeks of transfer, the plants gradually adapted and grew well at least in case of lead treated plants. It seemed in case of lead treated plants that after an initial shock plants grew at the same way as the control plants. However, after 6 months of growth cadmium treated plants showed stunted growth, with reduced numbers of leaves, deformities in the bark and most importantly sterility. Plants treated with lead on the other hand grew normally even after 6 months (data not shown). Biomass of 8 months old plants were determined weighing the different plant parts separately. Table 1 shows the reduction of biomass in all the plant parts. Reduction in biomass of root has also been shown in the Fig. 4.

Morphological changes due to Gibberellin treatment: Dramatic change was noticed on GA_3 treatment. The plants showing stunted growth pattern, started growing very fast showing increase in branching, internode elongation, change in leaf area and morphology (leaves become elongated and narrower) (Table 2).

Effect on chlorophyll content: Chlorosis was further confirmed by estimating chlorophyll content after 4 days and after 20 days of transplantation. After 4 days both chlorophyll-a and chlorophyll-b

Table - 1: Effect on dry weight of different plant parts (with and without GA_3)

Treatment	Leaf (in g)	Stem (in g)	Root (in g)
Control	0.876	2.678	1.657
$PbCl_2$ 3X	0.645	2.414	1.668
$CdCl_2$ 1X*	0.255	0.865	1.374
$CdCl_2$ 2X	0.205	0.767	0.620
$CdCl_2$ 3X	0.084	0.409	0.406
$CdCl_2$ 2X (GA_3)	0.565	1.205	0.607
$CdCl_2$ 3X (GA_3)	0.165	0.439	0.462

*1X = 80, 2X = 160, 3X = 240 and 4X = 320 µg g^{-1}

Table - 2: *Effect on leaf area and internode elongation (with and without GA_3)

Treatment	Leaf area (in mm^2)	Size of internode (avg. of first 5) (in mm)
Control	725	4.8
$CdCl_2$ (1X)	368	4.8
$CdCl_2$ (2X) + GA_3	720	21.2

* As measured in the month of March

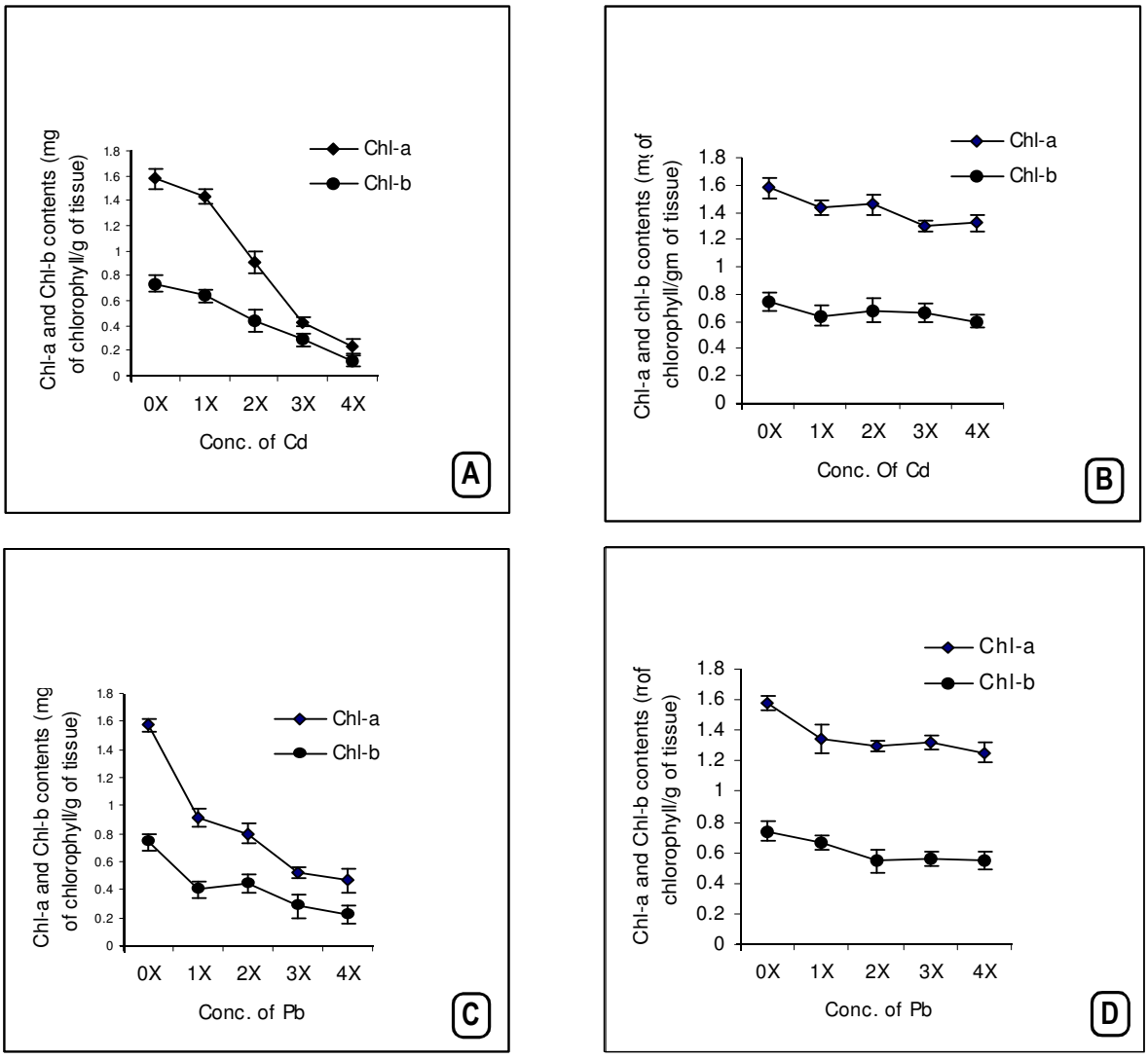

Fig. 5: Effect on chlorophyll content. The panels A and B are chlorophyll content of $CdCl_2$ treated plants at 4 days and 20 days interval respectively and panels C and D are $PbCl_2$ treated plants at 4 days and 20 days interval respectively

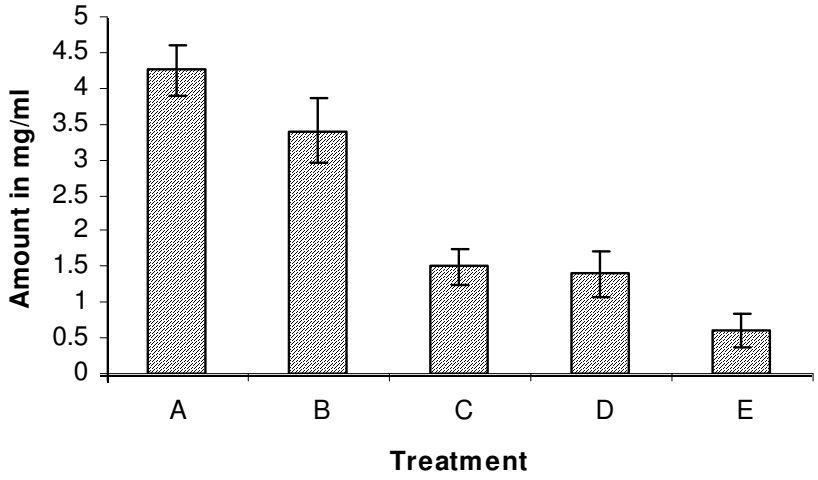

Fig. 6: Effect of $CdCl_2$ and $PbCl_2$ on total alkaloid of roots. A = Control, B = $PbCl_2$ treated, C = $CdCl_2$ (1X), D = $CdCl_2$ (2X) and E = $CdCl_2$ (3X)

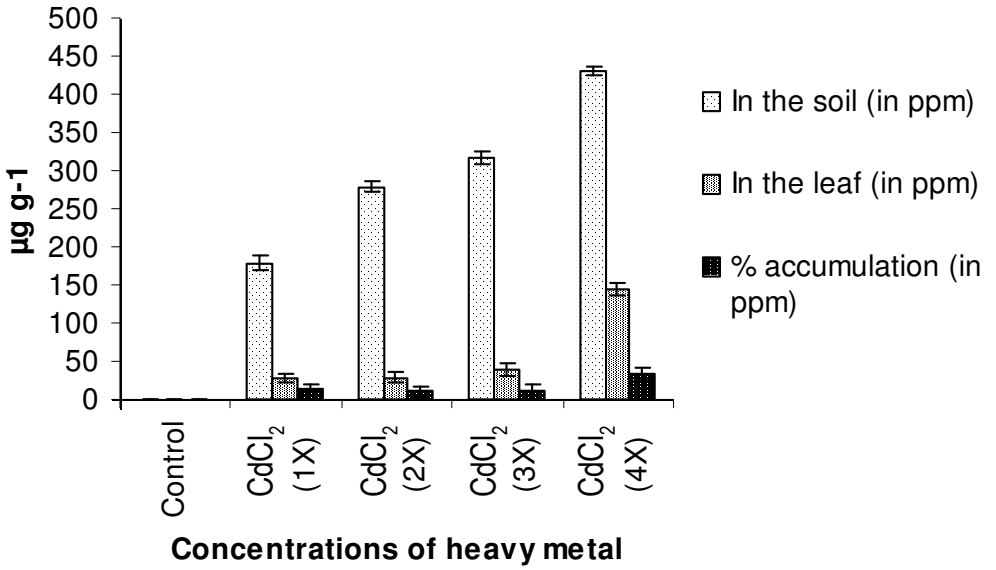

Fig. 7: Concentrations of Cd^{2+} in the soil and the amount of Cd^{2+} accumulated in the leaves. The black bar with white spots shows the percentage of heavy metal ultimately accumulated. 1X, 2X, 3X, and 4X are concentrations 100 µg g^{-1}, 150 µg g^{-1}, 250 µg g^{-1} and 350 µg $g^{-1}$of Cd^{2+} added in the soil. Plants were grown in earthen pots

contents were found to be reduced tremendously in both the cases. However, chlorophyll contents were near normal when estimated after 20 days showing that the plants have acclimatized by that time (Fig. 5).

Effect on alkaloid content: Amount of total alkaloid was found to decrease tremendously in $CdCl_2$ treated plants. Decrease in total alkaloid was also observed in case of $PbCl_2$ although less (Fig. 6).

Estimation of Cd and Pb through atomic absorption spectroscopy (AAS): Although plants did not show any absorption of Pb^{2+}, but they showed a good amount of Cd^{2+}accumulation in the leaves as estimated by AAS. The AAS data showed that the periwinkle could absorb up to about 12% of the amount of Cd^{2+} present in the soil. Soil, which contained a concentration of 320 µg $g^{-1}$of Cd^{2+} (4X in figure), did not support plant growth and the plants died in that concentration (Fig. 7).

The plant *C. roseus* is found to be resistant to heavy metal contamination in soil particularly to cadmium. They show tolerance to Cd^{2+} concentration up to 500 µM with respect to germination and up to 250 µg g^{-1} soil when the plants are two months old. However, it must be noted that the heavy metal contamination is highly dangerous as it reduces germination percentage of these plants, decreases biomass in all respect, induces sterility and finally decreases total alkaloid content also. The AAS data shows that periwinkle absorbs cadmium up to 12% whereas it does not accumulate lead. This indicates that periwinkle avoids lead present in the soil and shows normal growth. It may be because the import proteins in the root cells for lead are not expressed in *Catharanthus* or it may also be that, it is absorbed by the root but is not transported to the leaf. Therefore, it seems that *Catharanthus roseus* can be used as phytoremediator at least in these two cases where cadmium and lead contamination has occurred. The strategy the plant uses for both these metals are different. For lead it is an excluder, whereas for cadmium it is an accumulator. As far as inhibition at the level of germination is concerned, it will be a better idea to grow the plants in nursery in soil devoid of any heavy metal for two months and then transplanting it to contaminated site, because the inhibitory effect during the time of germination is high due to high level of surface absorption. It has been known that under stress condition plants generally shift a major portion of their metabolic activities towards secondary metabolite synthesis, so an increase in alkaloid content was expected. But in this case decrease in total alkaloid was recorded in both the treatments. This indicates that the concentrations that have been used are toxic for the vital metabolic ATP generating processes of the cell. However, further research in this area is required. The increase in the concentrations of MDA is in accordance with the observation of (Somashekaraiah *et al.*, 1992 and Piqueras *et al.*, 1999) and is due to the increased activity of lipid peroxidation caused by the enzyme lipoxigenase. The increased activity of lipid peroxidation is due to Cd^{2+} induced generation of reactive oxygen species (ROS), which leads to the increased synthesis of catalase, peroxidase *etc.* (Bhattacharjee *et al.,* 1996). The increased activity of lipoxigenase may also indirectly contribute to the depletion of chlorophyll content (Singh and Tiwari, 2003). Decrease in the concentrations of chlorophyll a and chlorophyll b when plants are subjected to high concentrations of lead and cadmium is in accordance with the observation of Stobart *et al.,* 1985; Phetsombat *et al*., 2006. Cadmium is involved in the inhibition of heme biosynthesis and chlorophyll synthesis by interacting with sulphydryl requiring enzymes involved in the pathway. The use of GA_3 is found to have an ameliorating effect as evidenced by an increase in the overall biomass of the aerial parts, morphological changes like elongation of the internode and leaves and increase in the number of leaves etc. However, the biomass of the root remains unaffected. This must be mentioned that the data were recorded in the month of March, which is dry season for the plants.

It can be concluded that the toxic effects of cadmium and lead with respect to *C. roseus* is maximum during germination and the plant gradually becomes more resistant to these heavy metals as it attains maturity. This plant seems to be a cadmium accumulator and lead avoider.

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