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# Antioxidative response of *Lemna polyrrhiza* L. to cadmium stress

**Riffat John[1], Parvaiz Ahmad[2], Kasturi Gadgil[1] and Satyawati Sharma*[2]**
***satyawatis@hotmail.com**

[1]Centre for Energy Studies, Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110 016, India
[2]Centre for Rural Development and Technology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110 016, India

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***Abstract:*** *Growth, lipid peroxidation, different antioxidative enzymes and metal accumulation were studied in Lemna polyrrhiza treated with different concentrations (1-40 ppm) of $CdSO_4$. The growth of the plant was slightly enhanced with 1 ppm, while higher concentrations retarted growth and multiplication of fronds, the effect being concentration and dose dependant. Increase in malondialdehyde content was insignificant after the first week but a prolonged exposure led to significant ($p<0.05$) increase of about 38% and 45% over the control in 20 and 30 ppm, respectively, after four weeks. Catalase (EC 1.11.1.6; CAT) activity increased at low concentration, but it declined to 42% and 54% at 40 ppm after 6 and 30 days, respectively. Superoxide dismutase (EC 1.15.1.1; SOD), ascorbate peroxidase (EC 1.11.1.11; APx) and glutathione reductase (EC 1.6.4.2) increased at both low as well at high concentrations, but a prolonged exposure to high concentration of Cd (40 ppm) led to significant ($p<0.05$) decline in the mean activities of these antioxidant enzymes. Accumulation of Cd in biomass was concentration and time dependant. However at high concentration of 40 ppm, Cd accumulation did not increase significantly ($p<0.05$) with time. Increased activities of antioxidant enzymes in Cd treated plants suggest that metal tolerance in L. polyrrhiza might be associated to the changes of antioxidant enzymatic activities.*

***Key words:*** *Antioxidant enzymes, CAT, SOD, APx, Cd, Lemna polyrrhiza*
*PDF file of full length paper is available with author*

## Introduction

Cadmium is a toxic trace pollutant for human, animals and plants, which enters the environment mainly from industrial processes and phosphate fertilizers and then is transferred to food chain with potential consequences for human health (Jarup, 2003). Biotechnological efforts are underway to improve plant metal tolerance and ability to extract heavy metals from the soil (Salt *et al.,* 1995). In order to devise new strategies for phytoremediation and improved tolerance, it is important to understand as to how heavy metals are taken up and act at cellular and tissue level (Schutzendubel and Polle, 2002; Beak *et al.*, 2006).

The plant antioxidative defense system involves several enzymes and low molecular weight quenchers that are present in plant cells. Superoxide radicals generated in plant cells are converted to hydrogen peroxide by the action of superoxide dismutase (SOD). Accumulation of hydrogen peroxide, a strong oxidant, is prevented in the cell by either catalase or ascorbate glutathione cycle, where ascorbate peroxidase reduces it to water (Iannelli *et al.,* 2002). Cadmium indirectly promotes generation of reactive oxygen species (Romero-Puertas *et al.,* 2002; Sandalio *et al.,* 2001). The enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase are involved in the detoxification of $O^{2-}$ and water respectively, thereby preventing the formation of $OH^-$ radicals. Ascorbate peroxidase (APx) is an important component of ascorbate glutathione cycle involved in $H_2O_2$ detoxification (Jimenez *et al.,* 1997). The toxicity of Cd has been related with oxidative damages and alterations in antioxidant systems in *Phaseolus aureus* (Shaw, 1995), *Helianthus annus* (Gallego, 1996), *Pisum sativum* (Sandalio, 2001) and *Salvina natans* (Mohan and Hosetti, 2006).

In this work, the effect of different concentrations of $CdSO_4.7H_2O$ on lipid peroxidation, antioxidant enzymes and Cd accumulation by *Lemna polyrrhiza* L. was studied in order to understand the mechanisms involved in the plant tolerance to cadmium.

## Materials and Methods

**Plant material:** Young plants of *Lemna polyrrhiza* L. were collected from domestic sewage pond Wazirabad, New Delhi and maintained as stock in a tank at the Micromodel Indian Institute of Technology, Delhi. To the pond was added small amount of solid compost and tap water was regularly added at the intervals. The experiments were done in plastic trays (8 liters capacity). Different concentrations of cadmium used were 1 ppm, 10 ppm, 20 ppm, 30 ppm and 40 ppm each in triplicate. Control experiments without metal treatment were set parallely. Plants were taken out from plastic trays after interval of one week and dried by slightly wrapping in blotting sheet and fresh weight was taken and expressed in grams. The plant material from each tray was analyzed for different parameters at intervals of 7 days.

**Measurement of lipid peroxidation:** The level of lipid peroxidation in fronds was determined as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fronds (1.0 g) were homogenized in

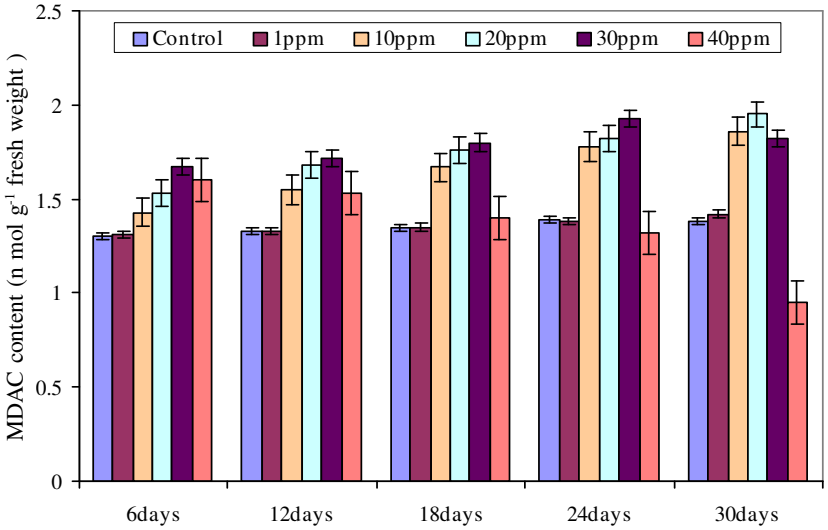


**Fig. 1:** Effect of different Cd concentrations on MDA content with time CD at p>0.05 between different Cd concentrations is 0.34 CD at p<0.05 between different time intervals (weeks) is 0.665

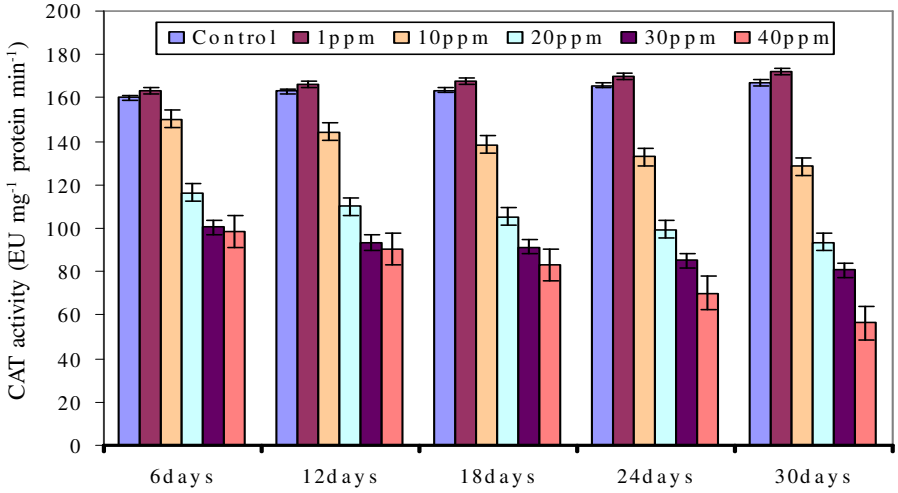


**Fig. 2 (A):** Effect of different Cd concentrations on CAT activity with time CD at p>0.05 between different Cd concentrations is 4.84 CD at p<0.05 between different time intervals (weeks) is 3.22

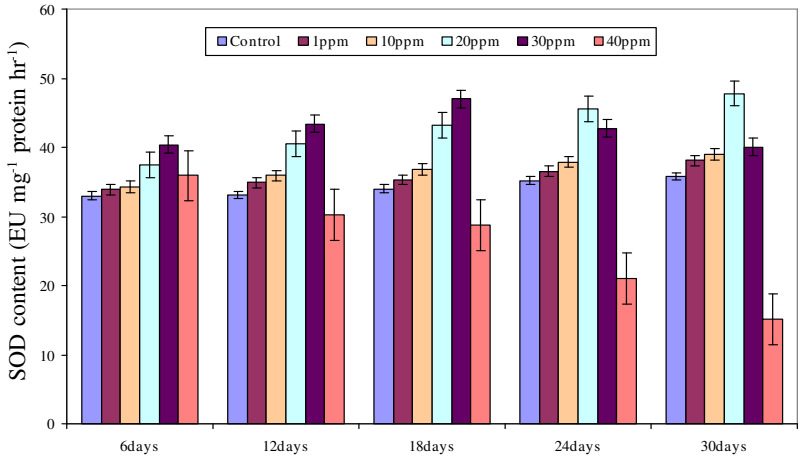


**Fig. 2(B):** Effect of different Cd concentrations on SOD activity with time CD at p>0.05 between different Cd concentrations is 0.63 CD at p<0.05 between different time intervals (weeks) is 0.58

0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 min. To 1 ml supernatant, 4 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 90°C for 30 min and then quickly cooled on ice. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant at 532 nm was read, and the value for the non specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient (∈) of 155 $mM^{-1}$ $cm^{-1}$.

**Assay of catalase (CAT) activity:** Catalase activity in leaves was estimated by the method given by Aebi (1984) with slight modification. 0.2 gm of fresh tissue was homogenized in 2 ml of extraction buffer under cold conditions. The homogenate was centrifuged at 10,000 rpm for 20 min at 40°C. The supernatant was used for quick assay. Catalase activity was determined by observing the disappearance of $H_2O_2$ by measuring the spectrophotometer. Reaction, carried out in total volume of $H_2O_2$, was allowed to run for 3 min. Activity was calculated by using extinction coefficient (∈) 0.036 $mM^{-1}$ $Cm^{-1}$.

**Assay of superoxide dismutase (SOD) activity:** SOD activity was measured by the method described by Dhindsa *et al.* (1981). 0.2 g of fresh leaf material was homogenized in 2.0 ml of extraction buffer with the help of precooled mortar and pestle. The homogenate was centrifuged at 15,000 rpm at 4°C and supernatant was stored at 4°C. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium. The test tubes containing assay mixture (1.5 ml reaction buffer, 0.2 ml of methionine, 0.1 ml enzyme extract with equal amount of $NaCO_3$, NBT solution, riboflavin, EDTA and 1.0 ml DDW) were incubated in light under 15 W inflorescent lamps for 15 min, illuminated and non-illuminated reactions without supernatant served as calibration standard. Absorbance of samples along with the blank was read at 560 nm wavelength. One unit of enzyme activity was defined as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes (supernatant).

**Assay of ascorbate peroxidase (APx) activity:** Ascorbate peroxidase (APx) activity was determined as described by (Nakano and Asada, 1981) by the decrease in the absorbance of ascorbate at 290 nm of 1 ml reaction mixture containing 0.1mM EDTA and enzyme extract. The reaction was allowed to run for 3 min at 25°C. Activity was calculated by using extinction coefficient (∈) 2.8 $mM^{-1}$ $cm^{-1}$.

**Assay of glutathione reductase activity:** Glutathione reductase activity was determined by the method of Foyer and Halliwell (1976). 0.5 g of the fresh *L. polyrrhiza* fronds were ground in 2 ml of extraction buffer and centrifuged at 10,000 rpm for 10 mins. The supernatant was collected and used for assay. GR activity was determined by monitoring the glutathione-dependant oxidation of NADPH at 340 nm wavelength on spectrophotometer. 1ml reaction mixture containing NADPH, GSSG and enzyme extract was allowed to run for 3 min at 25°C. Corrections were made for any GSSG oxidation in the absence of NADPH. Activity was calculated by using extinction coefficient (∈) 6.2 $mM^{-1}$ $cm^{-1}$, and expressed in enzyme units (mg protein)$^{-1}$. One unit of enzyme is the amount necessary to decompose 1 µmol of NADPH per min at 25°C.

**Accumulation of Cd:** Plants were dried and slowly digested for about 3 hr in 5 ml of a 1:1 mixture of perchloric acid and nitric acid. The volume of digested samples was adjusted with distilled water. Cd analysis carried out with an atomic absorption spectrophotometer (Perkinelmer, AAnalyst 200) using flame emission technique.

**Statistical analysis:** In all the experiments three replicates were performed for each concentration. Mean and standard deviations were calculated from triplicate measurement of three preparations. The two way analysis of variance in completely randomized block design involving six treatments (One control and five treatments) and five durations was performed to confirm the validity of the data. Critical difference was calculated to compare between various treatments. Significant differences of the means were accepted for $p<0.05$.

## Results and Discussion

**Effect of Cd on biomass yield:** As compared to control biomass yield, in plants given 1 ppm Cd treatment was higher. 10 ppm Cd have little effect on growth while 40 ppm Cd retarded growth significantly ($p<0.05$) by 89% above control levels after 4 weeks (Table 1).

**Malondialdehyde content:** Increase in the MDA content was concentration and time based. At 40 ppm MDA content declined significantly ($p<0.05$) by 25% over the control (Fig. 1 A).

**Table - 1:** Effect of Cd on the growth of *Lemna polyrrhiza* L

| Treatment | Biomass fresh weight (g) | | | | | |
|---|---|---|---|---|---|---|
| | 0 day | 6 days | 12 days | 18 days | 24 days | 30 days |
| Control | 5.00 ± 0 | 7.52 ± 0.04 | 9.52 ± 0.05 | 12.54 ± 0.19 | 20.93 ± 1.10 | 35.30 ± 2.8 |
| 1 ppm | 5.00 ± 0 | 7.61 ± 0.03 | 10.89 ± 0.32 | 15.32 ± 0.50 | 25.9 ± 1.7 | 39.98 ± 3.02 |
| 10 ppm | 5.00 ± 0 | 6.87 ± 0.09 | 9.35 ± 0.12 | 11.61 ± 0.37 | 18.66 ± 1.04 | 30.01 ± 2.04 |
| 20 ppm | 5.00 ± 0 | 5.10 ± 0.08 | 6.01 ± 0.3 | 8.32 ± 0.07 | 10.54 ± 0.65 | 14.56 ± 0.22 |
| 30 ppm | 5.00 ± 0 | 4.21 ± 0.06 | 3.95 ± 0.04 | 2.5 ± 0.07 | 2.1 ± 0.03 | 1.24 ± 0.09 |
| 40 ppm | 5.00 ± 0 | 3.5 ± 0.03 | 2.21 ± 0.09 | 1.51 ± 0.10 | 0.98 ± 0.05 | 0.55 ± 0.03 |
| CD at 5% | 0.01 | 0.25 | 0.32 | 0.46 | 0.53 | 0.77 |

The values represent mean ± SD (n = 3)

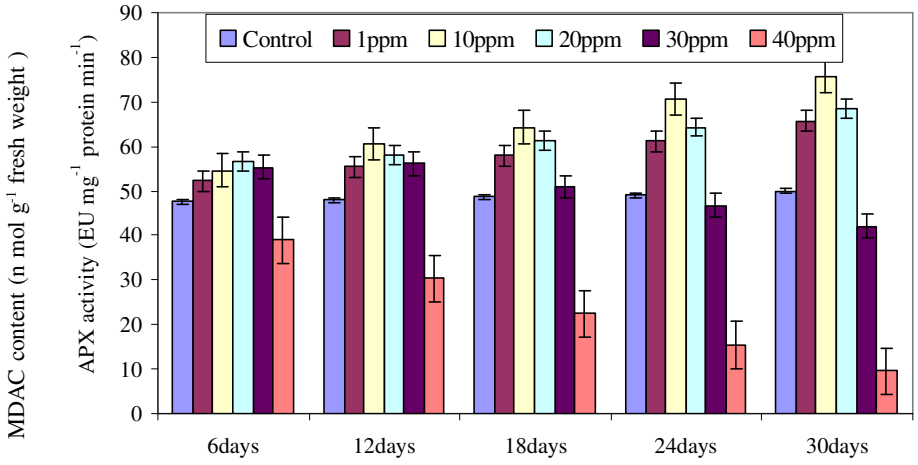


**Fig. 2 (C):** Effect of different Cd concentrations on APX activity with time CD at p>0.05 between different Cd concentrations is 0.81 CD at p<0.05 between different time intervals (weeks) is 0.74

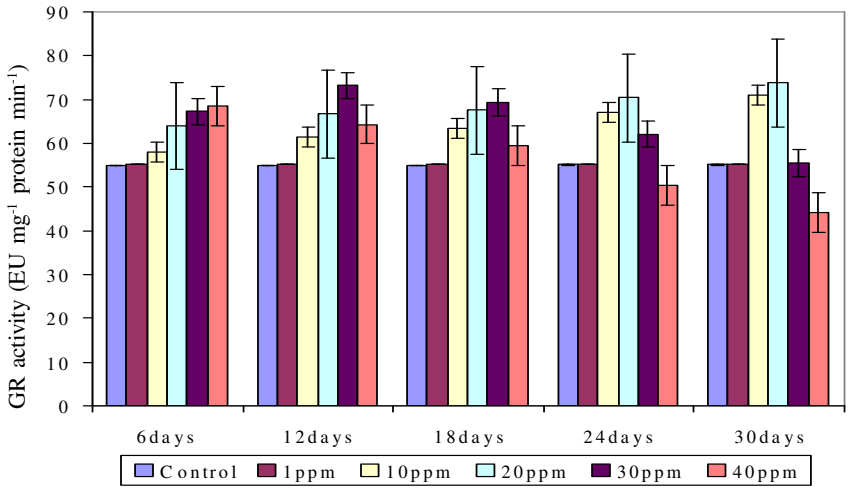


**Fig. 2 (D):** Effect of different Cd concentrations on GR activity with time CD at p>0.05 between different Cd concentrations is 0.77 CD at p<0.05 between different time intervals (weeks) is 0.70

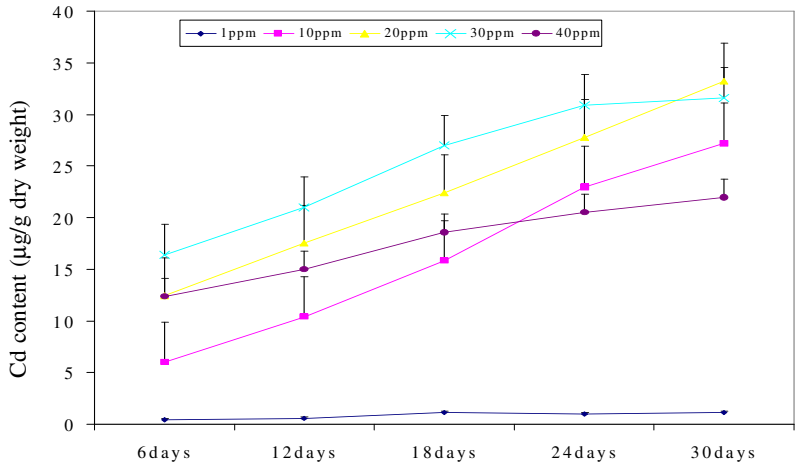


**Fig. 3:** Effect of time and concentration on bioaccumulation of Cd by *L. polyrrhiza* CD at p>0.05 between different Cd concentrations is 0.34 CD at p<0.05 between different time intervals (weeks) is 0.665

**Catalase activity:** In plants treated with 1ppm Cd, mean catalase activity slightly increased, while it decreased at higher concentrations (Fig. 2 A). Maximum mean activity of enzyme was found after 4 weeks in 1ppm treatment, which was insignificant. 40 ppm Cd was most effective in retarding the activity significantly ($p<0.05$) by 54% over the control.

**Superoxide dismutase:** SOD activity increased in all treatments except at the highest concentration of Cd (40 ppm), the activity declined even after 6 days. Mean enzymatic activity of SOD rose significantly ($p<0.05$) by 23 % in 20ppm treatment after four weeks. 30 ppm Cd treatment led to the increased enzymatic activity but after 3 weeks there was a decline of about 12% of mean activity, as compared to the control (Fig. 2 B).

**Ascorbate peroxidase (APx) activity:** Exposure of *L. polyrrhiza* to 1 ppm and 10 ppm Cd enhanced the mean activity of APx with significant ($p<0.05$) increase of 50% after 30 days as compared to control. Higher concentrations reduced the activity of 40ppm being most toxic ($p<0.05$) causing 50% decline in the mean enzymatic activity of APx (Fig. 2 C).

**Glutathione reductase (GR) activity:** After 6 days, there was insignificant increase in the mean activity of GR except at 40 ppm which caused a significant ($p<0.05$) decline of 24%. Prolonged exposure to Cd led to increase in activity. Exposure to 30 ppm showed a significant ($p<0.05$) 71 % increase in the mean enzymatic activity of GR after 30 days whereas the activity declined significantly ($p<0.05$) by 72% at 40 ppm (Fig. 2 D).

**Bioaccumulation of Cd:** Absorption of Cd was concentration and time dependant. Cd accumulation increased steadily as Cd concentration in the media increased from 1ppm to 40 ppm. Maximum absorption (55.21 mg/g dry wt.) occurred at the treatment of 20 ppm. After 30 days, a high concentration of 40 ppm, *L. polyrrhiza* caused significant ($p<0.05$) 40% decline in Cd content as compared to one with 20 ppm (Fig. 3).

Heavy metals in small amount may stimulate growth of some plants (Dou, 1988). *L. polyrrhiza* grown with 1 ppm of cadmium and lead showed good budding and regeneration. Our results showing a good growth of *L. polyrrhiza* at low Cd concentration (1 ppm) are corroborated by the findings of Mohan and Hosetti (1997) with *L. minor* plants grown in domestic sewage having 0.25 mM lead solution and 0.25 mM lead + 0.5 μM cadmium concentration. *L. polyrrhiza* regenerated by budding after eight days of treatment possibly due to the development of resistance of the plant in growing media. The good growth of plants at low concentrations of metals, as observed in the present study may partially be due to sulphur added as a consequence in the form of a sulphate salt (Jain *et al.,* 1989) besides having growth stimulating effect (Dou, 1988).

Oxidative stress leads to programmed cell death, which is initiated and propagated through the generation of ROS (Jacobson, 1996). Low doses induce antioxidant enzymes; however, having reached a certain threshold, concentration of ROS activites a signal transduction pathway, which results in PCD, (Solomon *et al.,* 1999; Desikan *et al.,* 1998). Malondialdehyde is considered to be the general indicator of lipid peroxidation, which can be started by the redox-active metal ions themselves. This phenomenon can be imitated by the iron containing enzyme lipoxygenase (Thompson *et al.,* 1987). This membrane bound enzyme, which is known to produce free radicals, may be responsible for increase in MDA level on Cd treatment (Somashekaraiah *et al.,* 1992).

Our results are in conformity with those of Shaw (1995), describing a decrease of catalase activity and an increase of ascorbate peroxidase activity in *Phaseolus aureus* treated with Cd. Varying responses of Cd induced oxidative stress are probably related both to levels of Cd supplied and to concentration of thiolic groups already present or induced by Cd treatment. Thiols possess strong antioxidative properties, and consequently counteract oxidative stress (Pichorner *et al.,* 1993). We observed increase in CAT activity upto 1 ppm Cd but there was a decrease at higher concentrations. CAT activity has been shown to decrease in *Phaseolus aureus* (Shaw, 1995), *Lemna minor* (Mohan and Hossetti, 1997) and *Amaranthus lividus* (Bhattacharjee, 1998) following the application of Cd. Decline in CAT activity, a general response to many stresses, is supposedly due to inhibition of enzyme synthesis or change in assembly of enzyme subunits (Mac Rae and Ferguson, 1985).

SOD activity enhances under a variety of stressful conditions including Cu, Al, Mn, Fe and Zn toxicities (Prasad, 1997) for maintenance of overall defense system of plants subjected to oxidative damage (Slooten *et al.,* 1995). We observed a decrease in SOD enzyme activity after prolonged exposure to 40 ppm Cd, which may be due to the enhanced level of $H_2O_2$ and its derivative AOS (active oxygen species), as reported by Luna *et al.* (1994) in oat leaves under Cu stress. Several lines of evidence indicate that phytotoxic amounts of certain metals, *i.e.* Cd (10-100 μM) (Somashekariha *et al.,* 1992), Cu (0.5-5 mM) (Gora and Clijisters, 1989), Zn (2.5-7.5 μM) (Gora and Clijisters, 1989) and Al (40 μM) in soils can cause inhibition of several peroxidases (Assche and Clijisters, 1990). But induction of peroxidases is a general response of higher plants, which is considered to play a significant role in stress metabolism (Severi, 1997).

Enhanced level of GR in *L. polyrrhiza* under toxicity suggests its active participation in detoxification of oxygen species and free radicals directly (Asada and Takahashi, 1987). The increase in GR activity level in our study may be because synthesis of GSH is a

demand-driven process, it is used for phytochelatin formation that triggered increased sulphur uptake and its own synthesis (May *et al.,* 1998). We observed a declining mean enzymatic activity of GR after prolonged exposure to high Cd concentrations of 30 ppm and 40 ppm. Heavy metal (Fe, Cu and Cd) induced loss in glutathione reductase has been observed in sunflower (Gallego *et al.,* 1996) in *L. minor* by Cu (Teisseire and Guy, 2000). Glutathione reductase contains a highly conserved disulphide bridge between $Cys^{76}$ and $Cys^{81}$ (Creissen *et al.,* 2000), which may undergo cleavage by heavy metals at toxic concentrations.

The present study suggests that higher Cd concentrations (10 ppm, 20 ppm, 30 ppm) caused oxidative stress in *L. polyrrhiza,* whereas 40 ppm concentration was unable to invoke antioxidant defense system, which resulted in accelerated necrosis and death of the plant. The oxidative stress was an indirect effect of Cd toxicity leading to ROS production which increased tissue level of SOD, APx and GR. Understanding the biochemical detoxification strategies of *L. polyrrhiza* against stress induced by accumulated metal ions is a key to manipulating heavy metal tolerance in plants.

## Acknowledgments

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