

## Full Length Research Paper

# Oxidative stress biomarkers and metabolic changes associated with cadmium stress in hyacinth bean (*Lablab Purpureus*)

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Cadmium (Cd)-induced stress in hyacinth bean (*Lablab purpureus*) was investigated by growing seedlings in a nutrient solution containing increasing cadmium concentrations (0 to 50 µM), under strictly controlled growth conditions. Changes consequent to Cd uptake in growth parameters, enzyme activities and other stress response factors directly or indirectly are related to the cellular free radical scavenging systems. Antioxidants and other stress response components were studied in roots and leaves of 10-day old seedlings over 72 h of exposure. A significant decrease in length, relative water content (RWC), weight of shoot and root, and chlorophyll content was observed in seedlings growing on >10 µM CdCl<sub>2</sub>. Oxidative stress markers, H<sub>2</sub>O<sub>2</sub>, malondialdehyde, proline and total soluble sugars were elevated in both leaves and roots. Ascorbate and phenol contents increased in leaves, but, decreased in roots. Reduced glutathione levels declined in both tissues. Cd stress enhanced antioxidant enzymes, guaiacol peroxidase, glutathione reductase, ascorbate peroxidase and polyphenol oxidase in concentration and time dependent manner in leaves. Antioxidant enzymes in roots showed inverse relationship with concentration and time of exposure. Catalase activity was found to decrease in both leaves and roots. Metabolic enzymes β-amylase and acid phosphatase activity increased in both leaves and roots. The results suggest that primary antioxidative response originates from leaves of Hyacinth bean even though roots are involved in direct uptake of heavy metals. The root tends to accumulate Cd and thus excludes its uptake by leaves.

**Key words:** Antioxidants, antioxidant enzymes, cadmium, Hyacinth bean.

## INTRODUCTION

Heavy metals have been considered to be major environmental pollutants due to their toxicity to plants. They are present in the environment with wide range of oxidation states and co-ordination numbers and these

number of diverse anthropogenic sources, have had enormous impact on different ecosystems (Macfarlane and Burchett, 2001). The non-biodegradability of metals has led to their persistence in the environment. Increased

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**Abbreviations:** ROS, Reactive oxygen species; SOD, superoxide dismutases; CAT, catalase; POX, guaiacol peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase; PPO, polyphenol oxidase; ASC, ascorbate; RWC, relative water content; TBARS, thiobarbituric acid reactive substances; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PPO, polyphenoloxidase; DNS, dinitrosalicylic acid; AMY, β-amylase; AP, acid phosphatase; INV, invertase; Put, putrescine; Spd, spermidine; Spm, spermine.

differences are closely associated with their toxicity (Pinto et al., 2003). The metal pollutants, derived from growing metal content of agricultural soil have lead to selection and colonization of resistant plants to a particular area, thus affecting biodiversity (Ye et al., 2003). The toxic effect generated by Cd depends on several factors like solubility of the metal, absorbability, transport, its chemical reactivity, pH of the medium and presence of other ions (Das et al., 1997). Toxicity arises from the binding of the metal with the sulphydryl groups in proteins leading to inhibition of metabolically important enzymes (Panda and Choudhury, 2005), increase in permeability of the plasma membrane (Quartacci et al., 2000) and expression of heat shock proteins (HSPs) (Wollgfehn and Neumann, 1995). Elevated levels of Cd cause water imbalance leading to decrease in relative water content (RWC) and transpiration, the mechanism of which is distinct from that caused by osmotic stress (Barcelo and Poschenrieder, 1988). Higher levels of heavy metals can disrupt the nutritional status of the plant by preventing the uptake of essential metal ions, inhibition of photosynthesis and chlorophyll biosynthesis (Marschner, 1991).

The toxic effects of Cd is related to its ability to generate reactive oxygen species (ROS) resulting in unbalanced cellular redox homeostasis (Panda and Choudhury, 2005). ROS such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $HO^{\cdot}$ ) (Cakmak 2000), may lead to unspecific oxidation of proteins and membrane lipids. The primary constituents of protective mechanisms against ROS-mediated damage include enzymes such as superoxide dismutases (SOD), catalase (CAT), guaiacol peroxidase (POX), glutathione peroxidase, ascorbate peroxidase (APX), glutathione reductase (GR), and polyphenol oxidase (PPO) (Mittler, 2002) and free radical scavengers, such as carotenoids (car), ascorbate (ASC), tocopherols, glutathione (GSH) and total phenols (TP). These are located in various sites throughout the cell. Enzymes of scavenger metabolism such as APX, GR and dehydroascorbate reductase (DHAR) together form the Halliwell-Asada pathway (Halliwell and Gutteridge, 1999). One important feature of these protective mechanisms is that their activity is enhanced when plant cells are exposed to conditions resulting in increased free radical production (Mittler, 2002).

Different plant species show varied degree of accumulation of heavy metals and antioxidative mechanisms. This variability has been observed between the species as well as within species of plants (Richau and Schat, 2009). With increasing anthropogenic enrichment of heavy metals in agricultural soils and rapid reduction in agricultural lands, identification of plants that accumulate heavy metals and tolerate metal stress, has become imperative from the point of view of phytoremediation and agricultural productivity. Legume crops are reported to be tolerant to several heavy metals.

There has been considerable interest in finding legume species that are able to colonize metal-enriched soils for use in land reclamation (Peterson, 1983) or for crop production on marginal soils (Franco and Munns, 1982). Because of the importance of legumes in maintaining soil fertility, and sustaining plant growth in nitrogen deficient soils, a study was undertaken with Hyacinth bean (*Lablab purpureus*) which is extensively cultivated in semi-arid regions. Such crop plants tolerance to heavy metals could be of great value in reclaiming valuable lands.

## MATERIALS AND METHODS

### Determination of germination indices under stress

Uniform sized seeds of *L. purpureus* cultivar HA-4 procured from National Seed Project, University of Agricultural Science, Bangalore, India, were surface sterilized with 0.1% (w/v) mercuric chloride for 1 min followed by three rinses in sterile distilled water. About 25 seeds were imbibed for 24 h in distilled water and then transferred to Petri plates lined with a layer of cotton wool wetted with 4.0 ml  $CdCl_2$  (5, 10 and 50  $\mu M$ ), in 0.5X Hoagland medium. Control seedlings were supplied with same volume of 0.5X Hoagland medium. After the stipulated period, the percentage germination was determined and the length of the shoot and root was recorded in centimeter. Means of shoot and root length were taken by measuring shoot and root length in centimeter of 5-day-old seedlings from 200 seedlings. Tolerance Index (TI) of 5-day-old seedlings was calculated according to the formula of Turner and Marshal (1972) as follows:

$$TI = \frac{\text{Length of longest root in test sample}}{\text{Length of longest root in control}} \times 100$$

Vigour Index (VI) was calculated on the basis of the following formula after 48 h of treatment (Abdul-Baki and Anderson, 1973).

$$VI = \text{Percentage germination at } 48 \text{ h} \times \text{Length of embryonic axis at } 48 \text{ h}$$

The dry mass of shoot and root was recorded from 7-day-old seedlings after keeping them in an oven at 80°C for 48 h.

### Heavy metal stress and experimental design

The overnight soaked seeds were sown in trays containing vermiculite and acid-washed sand (1:1 w/w) and irrigated daily with distilled water. The germination was carried out under natural greenhouse conditions; day/night temperature and relative humidity were 30/25°C, and 75/70%, respectively. The average photoperiod was 12 h light/12 h dark. Cd metal stress was induced by transferring ten day old seedlings of uniform size to a hydroponic system of 0.5X Hoagland media (Allen, 1968) containing  $CdCl_2$  (5, 10 and 50  $\mu M$ ). Samples of leaves and roots were collected at treatment intervals of 24, 48 and 72 h and frozen at -20°C until further analysis. Plants grown on 0.5X Hoagland media without addition of these salts served as control. Samples used for determination of RWC, fresh and dry weight were used immediately after collection. The experimental design used was carried out at random factorial scheme, with four media regimes (control and  $CdCl_2$  - 5, 10 and 50  $\mu M$ ) and three evaluation points (24, 48 and 72 h). Each experiment was composed of 24 experimental units (leaf + root samples) and done in triplicate.

### Relative water content (RWC)

The relative water content was estimated according to the method of Turner and Kramer (1980) using the equation:

$$\text{RWC} = (\text{FW-DW}) \times 100 / (\text{TW-DW}).$$

Leaf discs of 10 mm diameter were weighed to determine the fresh weight (FW), soaked in distilled water at 25°C for 4 h to determine the turgid weight (TW), then oven dried at 80°C for 24 h to determine the dry weight (DW). Similarly, entire shoot and root was taken for analysis and RWC was computed as before.

### Metal uptake

Total leaf and root accumulation of heavy metal in Hyacinth bean was determined after 72 h of treatment in order to estimate the accumulation of metal in roots and translocation into leaves. Leaves and roots were harvested, washed in deionised water for 2 min, air dried and then oven dried at 80°C for two days. The tissue was ground into a fine powder using a pestle and mortar. A known amount of this powder (1 g) was dissolved in binary mixture containing three parts of 1 M HNO<sub>3</sub> and 1 part of 1M HCl (3:1 ratio), and the metal concentration was analyzed by flame photometer (Systronics India Limited, Model No. 128). Different concentrations of metal salts were used as standards.

### Determination of H<sub>2</sub>O<sub>2</sub> and antioxidants

Hydrogen peroxide content in the control and stressed seedlings were determined according to Velikova et al. (2000). Ascorbic acid estimation was carried out according to the procedure of Sadasivam and Manickam (1997). GSH was estimated according to Beutler (1963). Total phenols were estimated by the method of Slinkard and Singleton (1977) using catechol as an authentic standard.

### Determination of stress response factors

Proline content was estimated using ninhydrin reagent according to Bates et al. (1973). The amount of total soluble sugars was estimated colorimetrically at 540 nm using anthrone reagent, according to Roe (1955). Chlorophyll content was determined according to Mackinney (1941). The concentrations of total chlorophyll, chlorophyll-a, and -b were calculated by the formula of Arnon (1949). The extent of lipid peroxidation was determined according to Heath and Packer (1968). The thiobarbituric acid reactive substances (TBARS) content was calculated from the extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>. Total free polyamine levels were determined in leaves and roots of Hyacinth bean according to the method of Flores and Galston (1982) with slight modification. Each 100 mg of tissue was homogenized with 1.0 ml 5% (w/v) cold perchloric acid using a cooled mortar and pestle. The homogenates were kept in an ice bath for 1 h, and then centrifuged at 15,000 g for 30 min at 4°C, the supernatant was transferred to new plastic vials and were stored at -70°C for PA quantification. Plant extracts were benzoylated. 1.0 ml of 2 M NaOH was mixed with 500 µl supernatant. After the addition of 10 µl benzoyl chloride, samples were vortexed for 20 s, and incubated for 20 min at 37°C. Following the high temperature incubation, 2 ml of saturated NaCl was added. Benzoyl-polyamines were extracted in 2 ml diethyl ether and vortexed for 10 s. After centrifugation at 12,000 g for 5 min at 4°C, 1 ml of the ether phase was collected, evaporated to dryness under a stream of warm air, and redissolved in 100 µl methanol. The benzoylated extracts were filtered through a 0.22 µm membrane

filter, and then separated by high performance liquid chromatography (HPLC) (Shimadzu) on a reverse-phase C<sub>18</sub> column (Alex-Octadecylsilane), 5 particle diameter, 4.6 × 250 mm using methanol:water (64:36) at a flow rate of 1 ml/min. Eluted peaks were detected by a spectrophotometer (Shimadzu, UV 254 nm), recorded and integrated by an attached computer. Standards (Putrescine, Spermine, Spermidine and Cadaverine) and plant extracts were determined by the same method.

### Extraction of enzymes

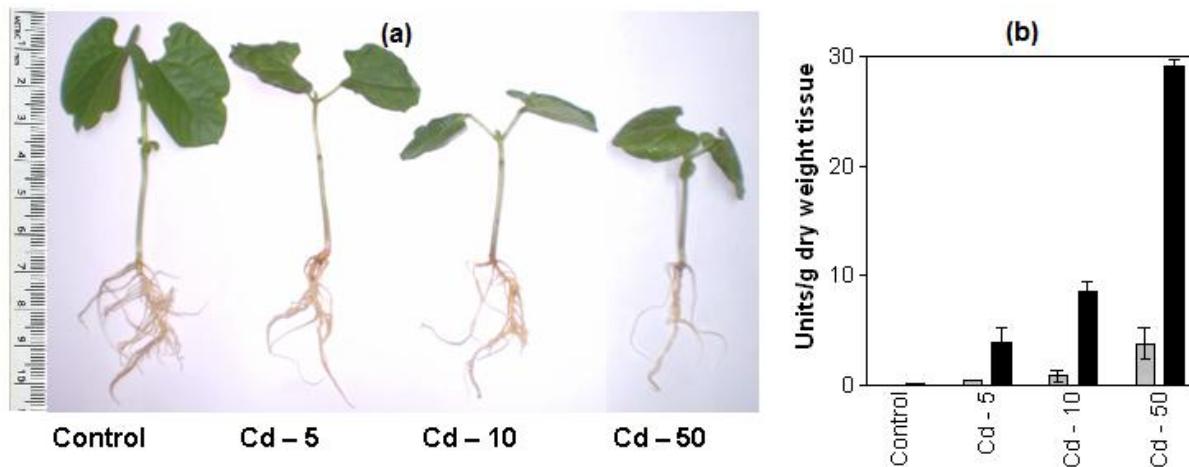
The frozen samples were homogenized with pre-chilled 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA) using pestle and mortar. L-ascorbate was raised to a final concentration of 2 mM for extraction of APX. The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was used as a source of enzymes. Soluble protein content was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

### Activities of active oxygen quenching enzymes

Catalase activity was assayed by following the decline in optical density at 240 nm ( $\epsilon = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$ ) according to the method of Aebi (1984). The reaction mixture consisted of 50 µl of enzyme extract and 50 mM sodium phosphate buffer (pH 7.0). The reaction was started by addition of H<sub>2</sub>O<sub>2</sub> to a final concentration of 10 mM, and its consumption was measured for 2 min. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 µmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> under the assay conditions. Guaiacol peroxidase (POX, EC 1.11.1.7) activity was measured in a reaction mixture of 3.0 ml consisting of 50 mM phosphate buffer (pH 7.0) containing 20 mM guaiacol, 10 mM H<sub>2</sub>O<sub>2</sub> and 100 µl enzyme extract (Chance et al., 1955). The formation of tetraguaiacol was followed by an increase in absorbance at 470 nm ( $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One unit of peroxidase is defined as the amount of enzyme needed to convert 1 µmol of H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> at 25°C. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined spectrophotometrically as described by Webb and Allen (1995). The assay mixture contained 50 mM HEPES buffer (pH 7.0), 1 mM EDTA, 1 mM H<sub>2</sub>O<sub>2</sub>, 0.5 mM sodium ascorbate, and 50 µl of enzyme extract in a total volume of 2.0 ml. The reaction was initiated by addition of H<sub>2</sub>O<sub>2</sub>. The oxidation of ascorbate was followed by a decrease in the A<sub>290</sub> ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One unit of ascorbate peroxidase is defined as the amount of enzyme necessary to oxidize 1 µmol of ascorbate per min at 25°C. Glutathione reductase (GR, EC 1.6.4.2) activity was determined by monitoring the oxidation of NADPH at 340 nm ( $\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ ) according to the method of Carlberg and Mannervik (1985). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 3 mM MgCl<sub>2</sub>, 500 nM GSSG, 200 nM NADPH and 250 µl of enzyme extract in a total volume of 1.5 ml. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH per minute under the assay conditions. Polyphenoloxidase (PPO, EC 1.14.18.1) was assayed spectrophotometrically at 400 nm using tertiary butyl catechol as substrate according to Kanade et al. (2006). The assay mixture consisted of 0.9 ml sodium acetate buffer (pH 5.0), 0.1 ml t-butyl catechol and 0.1 ml enzyme extract. The quinone formed was measured at 400 nm ( $\epsilon = 1150 \text{ M}^{-1} \text{ cm}^{-1}$ ). One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of t-butylquinone per minute under the assay conditions.

### Activities of hydrolytic enzymes

β-Amylase (AMY, EC 3.2.1.1) activity was measured using the



**Figure 1.** Effect of Cd stress (after 72 h of exposure) in Hyacinth bean seedlings treated with different concentrations of  $\text{CdCl}_2$ . A decrease in the surface area and chlorosis of leaves in stressed seedling (a); Cd levels in leaf and root tissue as determined by atomic absorption spectroscopy.

**Table 1.** Effect of Cd stress on germination indices of Hyacinth bean.

Metal ( $\mu\text{M}$ )	Length (cm)		Tolerance index (TI)	Vigor index (VI)	RWC (%)			Weight (g)	
	Shoot	Root			Leaf	Stem	Root	Fresh	Dry
0	7.2 $\pm$ 0.4	2.7 $\pm$ 0.9	—	98.53	97.6 $\pm$ 0.5	87.7 $\pm$ 1.6	94.9 $\pm$ 0.0	1.37 $\pm$ 0.10	0.21 $\pm$ 0.01
5	6.6 $\pm$ 1.3	2.0 $\pm$ 0.5	74.07	89.54	85.9 $\pm$ 2.7	94.3 $\pm$ 4.2	95.6 $\pm$ 4.0	1.18 $\pm$ 0.09	0.12 $\pm$ 0.07
10	6.0 $\pm$ 0.1	1.9 $\pm$ 0.4	70.37	72.43	83.3 $\pm$ 3.6	86.9 $\pm$ 5.2	86.4 $\pm$ 0.9	0.80 $\pm$ 0.06	0.11 $\pm$ 0.01
50	5.1 $\pm$ 0.9	1.7 $\pm$ 0.2	62.96	68.43	66.7 $\pm$ 3.3	63.2 $\pm$ 4.1	61.5 $\pm$ 2.1	0.61 $\pm$ 0.07	0.06 $\pm$ 0.03

dinitrosalicylic acid (DNS) method (Bernfield, 1955). The reaction mixture consisted of 0.5 ml of 2 % starch solution dissolved in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract was used in the assay. Acid phosphatase (AP, EC 3.1.3.2) activity against p-nitrophenyl phosphate was determined by monitoring the release of p-nitrophenol at 410 nm according to the method described by Hoerling and Svensmark (1976). Each unit of activity is defined as the number of  $\mu\text{moles}$  of  $\alpha$ -naphthol or *p*-nitro phenol released per minute. Invertase (INV, EC 3.2.1.26) activity was determined by the method of Sridhar and Ou (1972). 4.0 ml reaction mixture containing 0.025 M sodium acetate buffer (pH 5.0), 0.625% sucrose, and appropriate volume of enzyme extract was incubated at 37°C for 24 h. The reaction was arrested by adding equal volume of DNS reagent and estimated using the method of Miller (1959).

#### Statistical analysis

The experiment was performed using a randomized design. All data were expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using PrismGraph version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by least significant difference (LSD) test. Comparisons with  $P \leq 0.05$  were considered significantly different.

#### RESULTS AND DISCUSSION

Hyacinth bean seedlings exposed to Cd stress in a hydroponic system showed increasing Cd levels in the

primary leaves as a function of time after the onset of exposure (Figure 1b). The metal content was relatively higher in roots (~ 28-fold in Cd-50, after 72 h of stress) than in leaves indicating that the later sequesters the metal ion thereby preventing its accumulation in photosynthetic tissues. The rate of uptake of Cd by Hyacinth bean was concentration- and time- dependent and comparable to the response seen in maize (Drakiewicz et al., 2003), faba bean (Rahoui et al., 2008) and *Brassica juncea* (Sridhar et al., 2005).

Exposure of Hyacinth bean seedlings to Cd caused a marked decrease in the growth parameters such as plant weight, length of shoot and root, tolerance index and vigor index (Table 1). A low shoot/root ratio suggested a profound effect of Cd on shoot growth compared to roots. Other alterations included chlorosis after 48 h of exposure; and appearance of dark pigmentation and necrotic lesions on the laminae (Figure 1a). These effects are in conformity with those observed in chickpea (Faizan et al., 2011). Observed chlorosis and reduced biomass in Cd treated Hyacinth bean (Table 1) is attributed to enhanced production and accumulation of ROS, resulting in reduced photosynthetic activity as apparent from reduction in chloroplastic pigments (Table 2). The decrease in chloroplast pigments is believed to be due to reduced synthesis or increased oxidative degradation because of the oxidative stress imposed during heavy

**Table 2.** Levels of stress markers in leaves and roots of Cd-stressed Hyacinth bean.

Plant part	Time (h)	Stress marker	Control	CdCl <sub>2</sub> (μM)		
				5	10	50
Leaf	24	H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	7.59 ± 2.5	12.1 ± 2.5	12.7 ± 2.6	16.9 ± 3.2
		TSS <sup>a</sup>	37.5 ± 4.6	39.2 ± 5.7	40.6 ± 3.7	53.4 ± 5.8
		TBARS <sup>b</sup>	4.1 ± 1.2	4.1 ± 1.0	4.1 ± 1.0	4.4 ± 1.3
		T.Chl <sup>a</sup>	8.2 ± 1.0	7.2 ± 2.5	6.9 ± 2.0	6.4 ± 1.8
		Chl(a/b) <sup>a</sup>	1.29 ± 0.2	1.15 ± 0.4	1.21 ± 0.6	1.31 ± 0.1
		CSI	100	87.52	84.87	78.72
Leaf	48	Carotenoids	0.81 ± 0.05	0.78 ± 0.03	0.78 ± 0.04	0.71 ± 0.06
		H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	4.4 ± 1.2	10.5 ± 2.7	14.2 ± 4.7	21.1 ± 3.6
		TSS <sup>a</sup>	41.8 ± 6.2	53.4 ± 8.7	55.3 ± 7.6	62.3 ± 8.8
		TBARS <sup>b</sup>	5.6 ± 0.5	10.3 ± 1.5	12.9 ± 2.0	15.9 ± 3.2
		T.Chl <sup>a</sup>	8.71 ± 0.9	6.23 ± 1.2	4.92 ± 1.7	4.37 ± 1.1
		Chl(a/b) <sup>a</sup>	1.15 ± 0.4	1.25 ± 0.2	1.32 ± 0.3	1.38 ± 0.5
Leaf	72	CSI	100	71.26	56.32	49.42
		Carotenoids	0.83 ± 0.08	0.79 ± 0.04	0.76 ± 0.05	0.70 ± 0.03
		H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	5.8 ± 1.2	20.8 ± 5.7	22.7 ± 3.7	24.6 ± 4.6
		TSS <sup>a</sup>	40.9 ± 3.4	54.3 ± 7.2	60.3 ± 6.4	63.4 ± 7.1
		TBARS <sup>b</sup>	5.8 ± 0.8	10.7 ± 1.1	13.5 ± 1.3	18.6 ± 4.0
		T.Chl <sup>a</sup>	8.42 ± 1.7	4.54 ± 2.2	4.93 ± 2.7	4.27 ± 1.3
Root	24	Chl(a/b) <sup>a</sup>	1.17 ± 0.6	1.27 ± 0.5	1.31 ± 0.6	1.40 ± 0.4
		CSI	100	53.57	58.33	50.00
		Carotenoids	0.82 ± 0.02	0.72 ± 0.06	0.68 ± 0.03	0.63 ± 0.04
		H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	1.4 ± 0.2	2.1 ± 0.4	3.1 ± 0.5	4.2 ± 0.6
		TSS <sup>a</sup>	30.9 ± 4.2	45.1 ± 8.2	55.1 ± 4.9	58.2 ± 8.7
		TBARS <sup>b</sup>	2.2 ± 0.1	2.8 ± 0.09	2.9 ± 0.1	3.0 ± 0.2
Root	48	H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	1.8 ± 0.0	5.7 ± 0.8	5.5 ± 1.4	10.2 ± 1.1
		TSS <sup>a</sup>	42.0 ± 3.5	70.9 ± 9.2	77.9 ± 6.5	82.1 ± 7.3
		TBARS <sup>b</sup>	3.0 ± 0.4	6.2 ± 0.3	8.8 ± 1.0	10.3 ± 1.5
		H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	2.2 ± 0.4	5.4 ± 0.8	9.2 ± 1.0	11.3 ± 2.1
		TSS <sup>a</sup>	45.3 ± 5.4	76.7 ± 8.2	78.2 ± 2.6	95.1 ± 9.4
		TBARS <sup>b</sup>	2.6 ± 1.0	8.5 ± 2.3	9.1 ± 0.9	10.1 ± 1.1

<sup>a</sup>mg/g fresh weight tissue; <sup>b</sup>m moles/g fresh weight tissue.

metal stress. Necrosis of chlorotic leaves could be a result of programmed cell death (PCD) which is frequently associated with Cd stress (Garnier et al., 2006). Similar structural alteration in stem, leaf and roots; and reduction in dry weight have been reported in *Brassica juncea* under 10 mM Cd and 100 mM Zn over a period of 15 days (Sridhar et al., 2005) and Hg treated tomato (Cho and Park, 2000). Another parameter that is affected under heavy metal stress is RWC. The observed reduction in RWC (Table 1) could probably be due to inhibition of absorption and translocation of water as has been observed in other plants (Barceló et al., 1988). This inhibition occurs due to reduction in the size and number of xylem vessels and imbalance in hormone levels imposed by the heavy metal (Poschenrieder and Barceló, 1999).

When the defense system in heavy metal-treated plants is overburdened, the ROS start to appear in excess and

the antioxidative system is strongly activated. The timing of H<sub>2</sub>O<sub>2</sub> occurrence observed almost coincides with the action of other stress factors, in which signal molecules are involved. Cd<sup>2+</sup> is a non-redox metal and is unable to replace Cu<sup>+</sup> or Fe<sup>2+</sup> in Fenton reactions (Winterbourne, 1982). Such metals can affect the pro-oxidant status by depleting the antioxidant GSH pool (Pinto et al., 2003). In the present study, it was found that the H<sub>2</sub>O<sub>2</sub> content increased with increasing time and concentration of Cd (Table 2). This is probably due to the declining phase of GSH levels in a concentration-dependent manner (Table 3). Decrease in GSH as a biomarker for oxidative stress has been reported by a number of researchers (Gallego et al., 1996; Groppa et al., and 2001; Balestrasse et al., 2001). Cd-induced depletion of GSH has been mainly attributed to phytochelatin synthesis (Grill et al., 1985), decreased GR activity (Dixit et al., 2001), and increased utilization for ascorbate synthesis or for direct interaction

**Table 3.** Levels of antioxidants in leaves and roots of Cd-stressed Hyacinth bean.

Plant part	Time (h)	Antioxidant	Control	CdCl <sub>2</sub> (μM)		
				5	10	50
Leaf	24	GSH <sup>a</sup>	576.2 ± 5.2	493.9 ± 12.9	466.7 ± 13.4	390.9 ± 11.1
		ASC <sup>b</sup>	0.68 ± 0.0	0.91 ± 0.01	0.93 ± 0.02	1.06 ± 0.01
		Proline <sup>b</sup>	1.09 ± 0.04	1.01 ± 0.02	1.25 ± 0.03	2.22 ± 0.05
		TP <sup>b</sup>	3.92 ± 0.04	4.40 ± 0.04	4.46 ± 0.05	4.45 ± 0.07
	48	GSH <sup>a</sup>	568.1 ± 13.6	570.3 ± 12.2	499.8 ± 11.8	501.2 ± 13.2
		ASC <sup>b</sup>	0.59 ± 0.01	0.99 ± 0.05	1.12 ± 0.02	1.23 ± 0.03
		Proline <sup>b</sup>	0.95 ± 0.04	1.85 ± 0.02	2.28 ± 0.03	3.82 ± 0.07
		TP <sup>b</sup>	2.91 ± 0.07	4.07 ± 0.08	4.46 ± 0.09	6.61 ± 0.1
	72	GSH <sup>a</sup>	529.9 ± 11.6	471.3 ± 10.9	417.4 ± 12.3	380.7 ± 11.2
		ASC <sup>b</sup>	0.81 ± 0.02	1.01 ± 0.05	1.33 ± 0.06	1.51 ± 0.01
		Proline <sup>b</sup>	1.13 ± 0.05	3.29 ± 0.12	3.72 ± 0.22	3.82 ± 0.04
		TP <sup>b</sup>	3.18 ± 0.04	4.89 ± 0.05	5.92 ± 0.06	9.07 ± 0.07
Root	24	GSH <sup>a</sup>	52.6 ± 5.2	64.1 ± 6.3	78.6 ± 4.8	78.4 ± 3.3
		ASC <sup>b</sup>	0.49 ± 0.01	0.59 ± 0.03	0.41 ± 0.01	0.36 ± 0.02
		Proline <sup>b</sup>	1.15 ± 0.02	1.08 ± 0.03	1.23 ± 0.01	1.96 ± 0.00
		TP <sup>b</sup>	0.53 ± 0.05	1.05 ± 0.06	1.01 ± 0.07	0.665 ± 0.04
	48	GSH <sup>a</sup>	57.0 ± 2.8	50.6 ± 2.5	43.1 ± 5.6	41.1 ± 6.9
		ASC <sup>b</sup>	0.32 ± 0.02	0.25 ± 0.01	0.11 ± 0.04	0.10 ± 0.0
		Proline <sup>b</sup>	1.06 ± 0.03	1.41 ± 0.01	1.73 ± 0.02	1.82 ± 0.01
		TP <sup>b</sup>	0.83 ± 0.04	0.97 ± 0.12	8.20 ± 0.06	1.17 ± 0.01
	72	GSH <sup>a</sup>	48.6 ± 3.4	4.11 ± 0.0	1.33 ± 0.0	1.02 ± 0.0
		ASC <sup>b</sup>	0.45 ± 0.03	0.42 ± 0.02	0.16 ± 0.04	0.11 ± 0.03
		Proline <sup>b</sup>	1.22 ± 0.01	1.67 ± 0.04	1.95 ± 0.03	2.34 ± 0.01
		TP <sup>b</sup>	0.71 ± 0.07	0.63 ± 1.3	0.45 ± 0.03	0.32 ± 0.03

<sup>a</sup>μg/g fresh weight tissue; <sup>b</sup>, mg/g fresh weight tissue.

with metals like Cd (Pietrini et al., 2003). These results suggest a close relation between GSH and H<sub>2</sub>O<sub>2</sub> contents in leaves of Hyacinth bean.

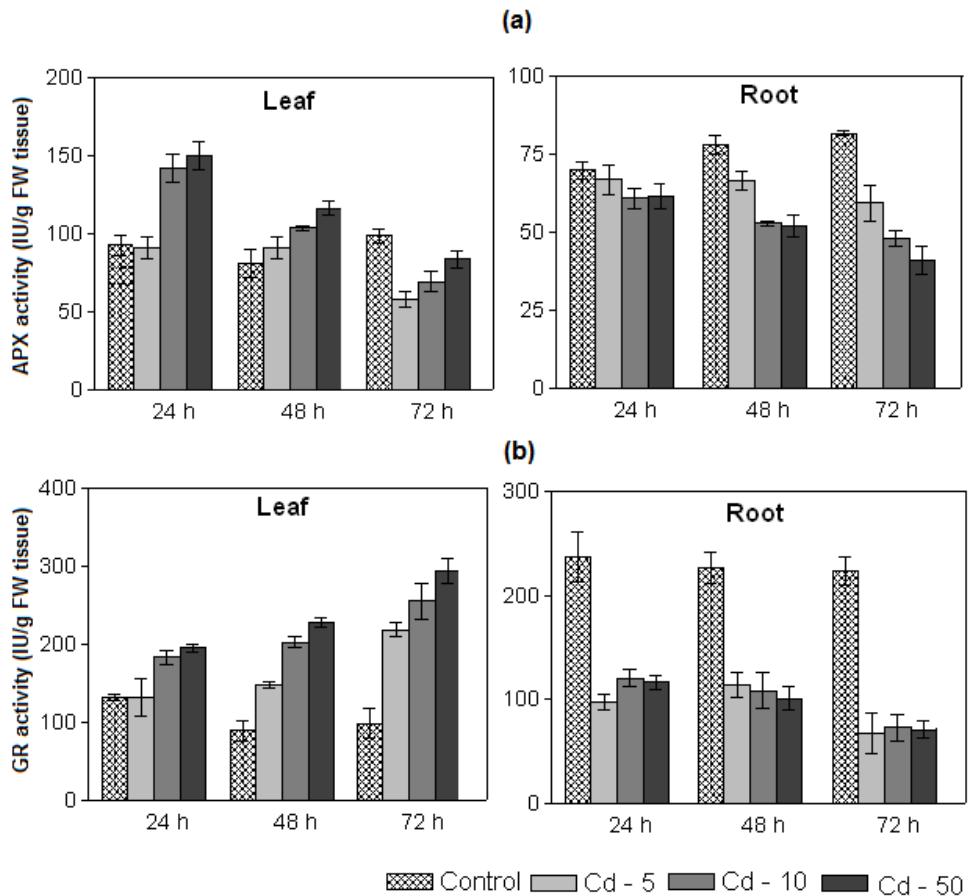
The cell membrane is considered to be the primary site of heavy metal injury. The accumulation of free radicals in stressed plants cause oxidation of polyunsaturated fatty acids in the plasma membrane resulting in the formation of TBARS (Singh, 2006). Formation of lipid peroxides serves as an activation signal for plant defense genes through increased activity of the octadecanoid pathways (Maksymiec, 2007). TBARS content of Cd-stressed leaves and roots of Hyacinth bean exhibited time- and concentration-dependent increase (Table 2). Such increase in TBARS levels have been reported in pea (Metwally et al., 2005) and rice (Shah et al., 2001) stressed with Cd.

The strength of the oxidative stress relies on the interaction of several factors that determine the antioxidant status of the plant. The increase in ASC levels in leaves exhibited better gradation under Cd stress during the entire period of exposure (Table 3). This is indicative of an effective free radical scavenging system in plants. As a powerful reducing agent, ASC maintains chloroplastic α-tocopherol and metalloenzyme activities and acts as reductant in enzymatic reactions and free radical scavenging of superoxide and H<sub>2</sub>O<sub>2</sub> radicals nonenzym-

atically (Lin et al., 2007). The levels of ASC in Hyacinth bean suggested that ASC has a major role in the leaf than in the root system, and is mainly synthesized in the leaves. Unlike in leaves, the ASC levels in roots under Cd stress showed a progressive decrease, with the gradation of decline becoming steeper with increasing time of exposure.

GSH levels in leaves and roots treated with Cd showed time- and concentration-dependent decline (Table 2). Higher GSH concentrations in foliar tissues of plants exposed to environmental stresses are interpreted as an acclimation, supporting the antioxidative defense systems (Polle and Rennenberg, 1992). Cd-dependent decline of GSH could be attributed to sequestration of the heavy metal ions by GSH. The levels of GSH in roots showed slightly reverse trend when compared to leaves (Table 2). The observed decrease in the GSH levels in roots could be attributed to its metal-catalyzed oxidation to oxidized glutathione, GSSG (Devi and Prasad, 2005), and also to the synthesis of metal-chelating proteins, phytochelatins (De Vos, 1992). These observations are in line with those made by Tukendorf and Rauser (1990) in maize seedlings stressed with Cd.

Two major enzymes, APX and GR are involved in the ASC/GSH cycle that operates in chloroplasts, cytoplasm, mitochondria as well as peroxisomes (del Rio et al.,

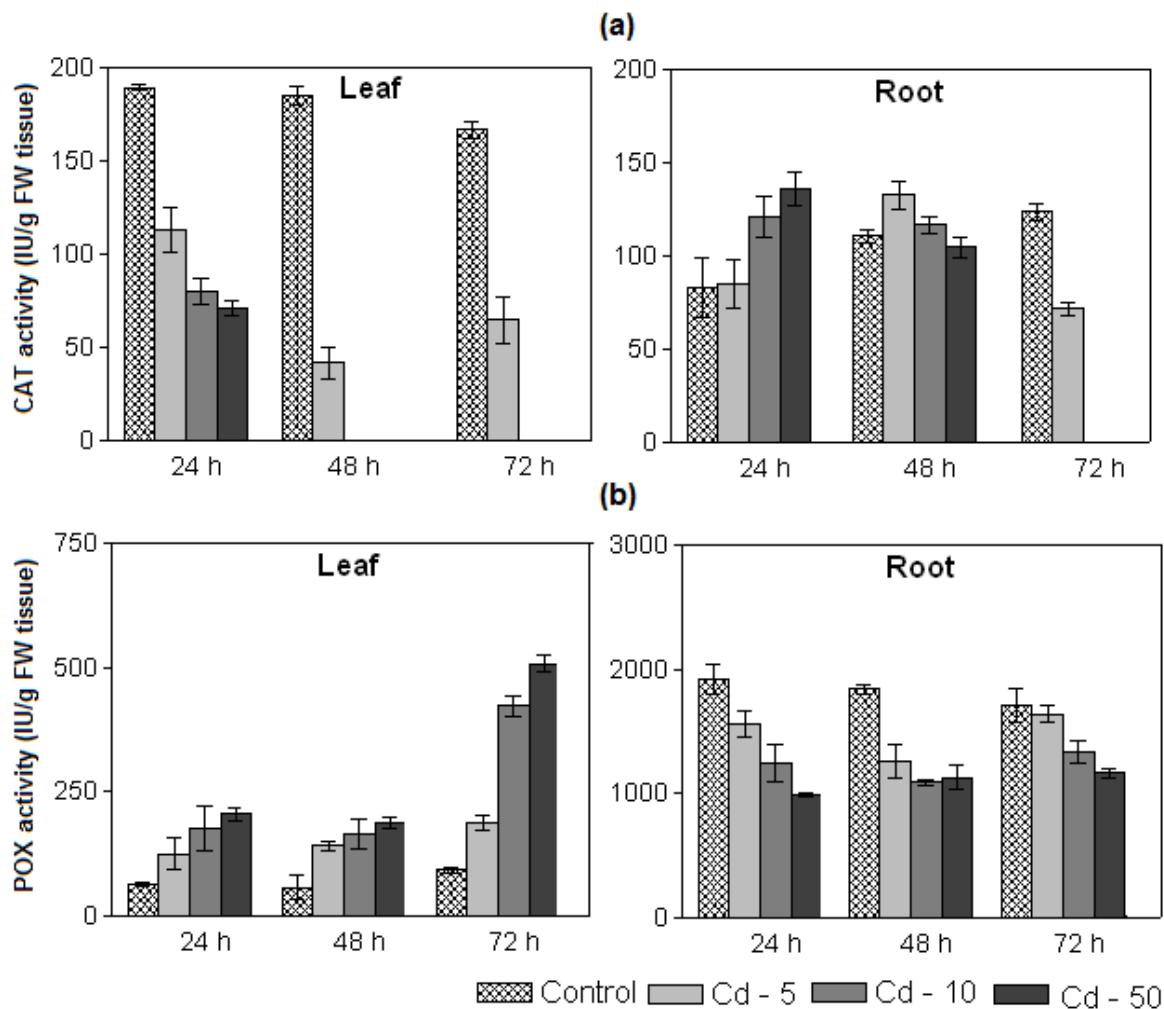


**Figure 2.** The levels of ascorbate peroxidase (APX) (a) and glutathione reductase (GR) (b) in leaves and roots of Hyacinth bean treated with different concentrations of  $\text{CdCl}_2$ . Values are means of triplicates  $\pm$  SE ( $P \leq 0.05$ ).

2006). In chloroplasts, APX reduces  $\text{H}_2\text{O}_2$  using ASC as the electron donor. Efficient working of APX requires rapid regeneration of ASC from dehydroascorbate, which in turn, is dependent upon the availability of GSH. To maintain homeostasis, GSH must be generated from oxidized glutathione by GR at the expense of NADPH. A recent study in Cd-treated plants demonstrated that leaf peroxisomes respond by improving the capacity of antioxidative enzymes involved in the ASC/GSH cycle (Palma et al., 2002). Hyacinth bean seedlings treated with Cd exhibited considerable increase in APX levels during the early exposure, but the levels dropped with increasing time of exposure (Figure 2a). Similar results were reported in *B. juncea* (Singh et al., 2011) and *Vigna radiata* (Shanker et al., 2004). The GR levels showed time-and concentration-dependent increase (Figure 2b). The concurrence of declining GSH and increasing GR activities suggested that ASC/GSH cycle is operative in this plant, thus regenerating the ASC from dehydro-ascorbate, DHA. Such behaviour has been reported under heavy metal stress as well as other oxidative stress (De Vos, 1992; Noctor and Foyer, 1998; Kocsy, 2001; Tausz

et al., 2004).

CAT is a heme-containing enzyme that catalyzes the dismutation of  $\text{H}_2\text{O}_2$  into oxygen and water. The CAT activity decreased significantly in leaves and roots of Cd stressed seedlings (Figure 3a) causing a parallel increase in  $\text{H}_2\text{O}_2$ , a stress signalling molecule. These observations are in consonance with those of Hg-treated *Cucumis sativus* (Cargnelutti et al., 2006) and Cd-treated *Pisum sativum* (Sandhalo et al., 2001). The drop in CAT activity in hyacinth bean could be due to destabilization of peroxisomal membrane as a result of elevation of  $\text{H}_2\text{O}_2$  and resultant lipid peroxidation. The decline in CAT could also be due to photoinactivation as suggested for rye leaves under Cd stress (Streb et al., 1993). An alternative mode of  $\text{H}_2\text{O}_2$  destruction is via peroxidases (POX) that are found throughout the cell and have a much higher affinity for  $\text{H}_2\text{O}_2$  than CAT (Noctor and Foyer, 1998). CAT has low substrate affinities when compared to POX and requires simultaneous access of two  $\text{H}_2\text{O}_2$  molecules at the active site (Willekens et al., 1997). The activity of POX in leaves increased in time- and concentration-dependent manner, but decreased steadily in roots



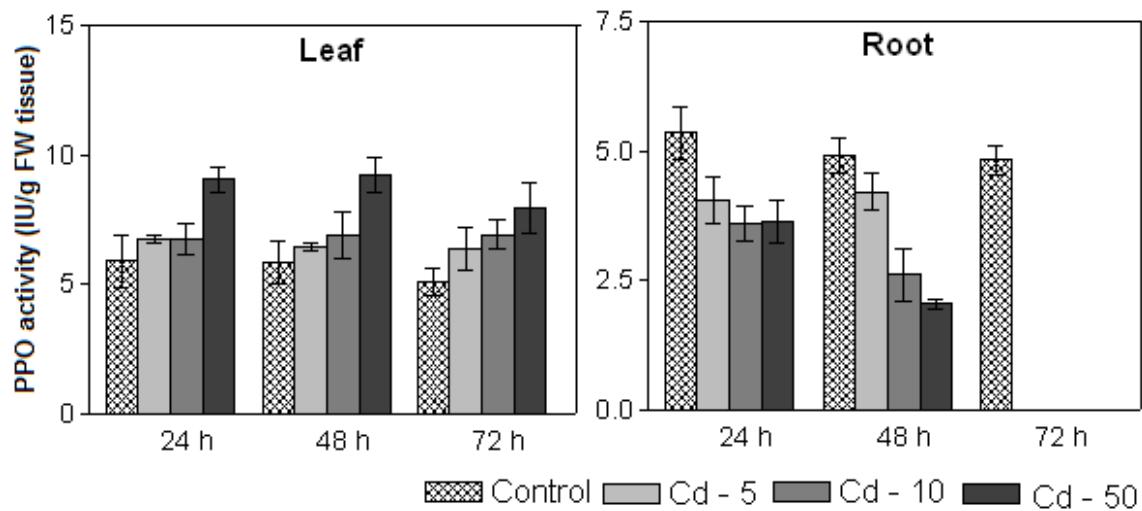
**Figure 3.** The levels of catalase (CAT) (a) and guaiacol peroxidase (POX) (b) in leaves and roots of Hyacinth bean treated with different concentrations of CdCl<sub>2</sub>. Values are means of triplicates  $\pm$  SE ( $P \leq 0.05$ ).

(Figure 3b). The enhanced POX activity in leaves of hyacinth bean thus indicated an efficient detoxification of ROS in leaves but not in roots. Further, the counter-balancing of CAT and POX suggested predominant role of POX in antioxidative mechanism of Hyacinth bean. Elevated levels of POX have been implicated in adaptation to stress by lignification (Smeets et al., 2005). The observed elevation of POX and consequent reduction of growth (Figure 1a) could be due to limitation of cell expansion via lignification.

Polyphenolic compounds observed in the aerial part of the plant, seems to play an important role in the bioactivities and growth of many plant and thus manage the plants growth and development during environmental stress (Sirhindhi, 2003). It has also been noted that PPO activity increased at the onset of abiotic stress. Some studies have related this enzyme with reactions associated to photosynthesis, respiration (Vaughn et al., 1984) and synthesis of phenolic compounds (Robb, 1984). Elevated PPO levels in leaves of Hyacinth bean

(Figure 4) indicated its possible role even during the early exposure to metal stress. This increase could be possible tolerance mechanism as demonstrated in radish subjected to Cd stress (Mohamed et al., 2009). PPO activity however, was found to decline in roots with time of exposure (Figure 4). The increased PPO activity might have caused oxidation of phenolics to quinones, thereby reducing their content in both leaves and roots (Table 3), and conferring resistance to stress by altering peroxidation kinetics leading to a reduction in the fluidity of the membrane and preventing the diffusion of free radicals (Burguières et al., 2006).

Apart from antioxidant enzymes, which have been indisputably implicated in stress response, there are also reports of induction of metabolite enzymes such as;  $\beta$ -AMY, AP and INV under abiotic stress (Yang et al., 2007). AMY activity was positively influenced with progressive concentrations and time of exposure to Cd (Figure 5a). 50  $\mu$ M CdCl<sub>2</sub> caused a 2-fold increase in the activity after 72 h of stress. There are reports of in-



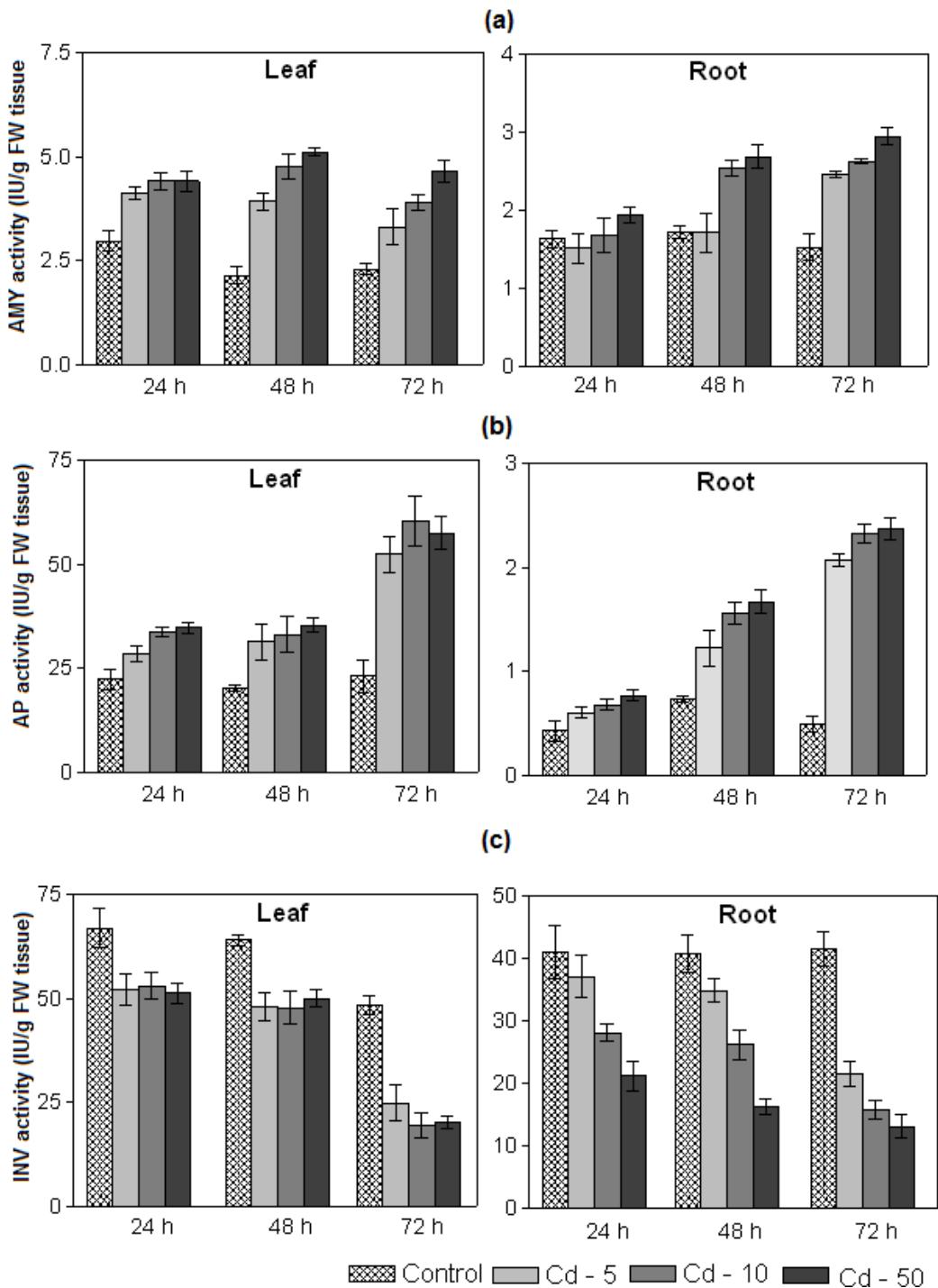
**Figure 4.** The levels of polyphenoloxidase (PPO) in leaves and roots of hyacinth bean treated with different concentrations of  $\text{CdCl}_2$ . Values are means of triplicates  $\pm$  SE ( $P \leq 0.05$ ).

creased activity of AMY leading to accumulation of total soluble sugars (TSS) in the stressed plant (Ahmad et al., 2006). TSS exhibited a concentration dependent increase after 24 h of stress; however, the increment was marginal in leaves after 48 and 72 h of treatment. These results corroborate with the findings of Ahmad et al. (2006) in *Pisum sativum* who attributed photosynthetic inhibition or stimulation of respiration rate as a reason for constancy in TSS values. This negative effect of Cd on carbon metabolism is a result of their possible interaction with the reactive centre of ribulose bisphosphate carboxylase (RuBisCo) (Stiborová et al., 1987). INV activity in both leaves and roots showed a clear decrease in response to increasing concentration of Cd (Figure 5c). This inhibition of INV could explain the tendency to accumulate sugar in these stressed tissues (Table 2).

Acid phosphatase widely distributed in plants is involved in hydrolysis of phosphate esters, an important process in energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways of plant cells (Vincent et al., 1992). AP levels increased in both leaf and root tissue in a concentration- and time-dependent manner (Figure 5b). Such enhancement in AP could be due to metal induced *de novo* synthesis (Pereira et al., 2002). This stimulation is believed to increase the orthophosphate (Pi) availability. Free soluble Pi plays a vital role in many biological processes including photosynthesis, respiration, enzyme regulation, energy transfer, metabolic regulation and nucleotide phospho-rylation (Ehsanpour et al., 2003) and may help the plant to survive longer under stress conditions.

Proline is considered as an important osmoprotectant and its role is valuable under plant abiotic stress response as an antioxidant, regulator of pH, stabilizer of proteins (Pál et al., 2006). The increase in proline (Table

3) and TSS (Table 2) in time and concentration dependent manner suggests probability of sugar-mediated proline biosynthesis and acquisition of tolerance (Hare and Cress, 1997). The increased  $\text{NADP}^+/\text{NADPH}$  ratio mediated by proline biosynthesis enhances the activity of oxidative pentose phosphate pathway that provides precursors for carbohydrate biosynthesis (Hare and Cress, 1997). Sucrose is known to be a positive effector of proline accumulation (Larher et al., 1993). The simultaneous increase and inter-dependence of TSS and proline levels have also been reported in alfalfa (Irigoyen et al., 1992) and pepper plants (Goicoechea et al., 2000). In addition to their role in osmoprotection, TSS play an important role in biosynthetic processes, energy production, stabilization of cellular membranes, maintenance of turgor, and signaling (Nayer and Reza, 2008). Polyamines are implicated in a wide range of regulatory processes such as promotion of growth, cell division, DNA replication and cell differentiation (Bouchereau et al., 1999). Their involvement in biotic and abiotic stresses has been well documented (Bouchereau et al., 1999; Walters, 2003). Among polyamines, putrescine (Put) is considered to be a stress marker in plants. Hyacinth bean stressed with Cd exhibited a common pattern in polyamine level with remarkable elevation in spermidine (Spd) levels at highest metal concentrations employed. The other major polyamines, Put and spermine (Spm) were not affected by metal stress (Figure 6). Accumulation of polyamines in plants exposed to oxidative stress resulting from heavy metals such as Hg, Cd, and Zn has been previously reported (Groppa et al., 2003; Franchin et al., 2007). The protective role of polyamines in plant cells is due to their radical scavenging ability (Sharma and Dietz, 2006) and inhibition of lipid peroxidation (Velikova et al., 2000). This protective role for polyamines

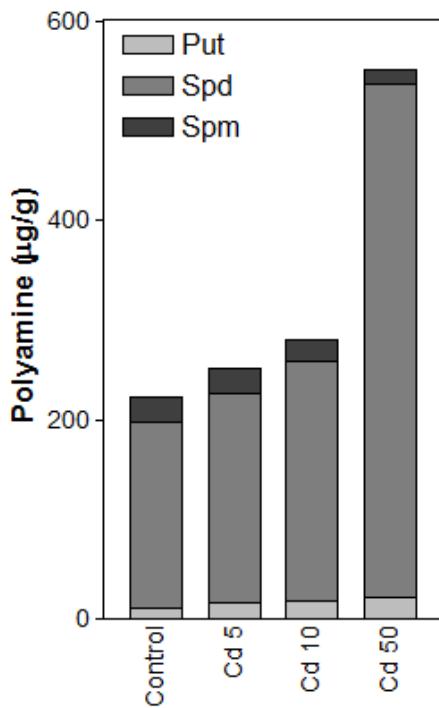


**Figure 5.** The levels of amylase (AMY) (a); acid phosphatase (AP) (b) and invertase (INV) (c) in leaves and roots of Hyacinth bean treated with different concentrations of  $\text{CdCl}_2$  compared to control. Values are means of triplicates  $\pm$  SE ( $P \leq 0.05$ ).

was confirmed by Groppa et al. (2001), Rhee et al. (2007) and Wang et al. (2007) who showed increase of tolerance to copper and cadmium by exogenously supplying polyamines.

ROS cause chlorophyll degradation and membrane lipid peroxidation. Decrease in chlorophyll and carotenoid

contents of leaves in response to salt stress is a general phenomenon (Parida and Das, 2005). Carotenoids are potent quenchers of singlet oxygen and hence protect the cell from oxidative damage (Pérez-Gálvez and Mínguez-Mosquera, 2002). Treatment of Hyacinth bean with Cd resulted in decreased concentration of chloroplastic pig-



**Figure 6.** The levels of polyamines in leaves of hyacinth bean treated for 72 h with different concentrations of CdCl<sub>2</sub>.

ments (Table 2). Though changes in carotenoid concentration were not significant, the concentration of chlorophyll decreased significantly; the decrease after 72 h of stress was found to be 51%. Both chl a and chl b were lowered but chl a/b ratio was not affected significantly. Decreased concentrations of chloroplastic pigments that lead to chlorosis of leaves may be an outcome of reduced synthesis and/or enhanced oxidative degradation of these pigments by imposed oxidative stress. Inhibition of ALA dehydratase in Cd-stressed plants as reported by Noriega et al. (2007) could have led to decreased synthesis of chlorophyll. This enzyme catalyzes the rate-limiting step of sequential pathway of heme and chlorophyll synthesis from its precursor aminolevulinate, ALA (Marschner, 1995).

From the results presented in this study, exposure to environmentally realistic heavy metal concentration seems to disturb the cellular redox status in the leaves and roots of Hyacinth bean. The plant possesses a well-organized antioxidative defense system which operates with the sequential and contemporaneous action of antioxidative enzymes and metabolites. Even before visible effects of toxicity could be observed, the capacities of several enzymes increased. Antioxidative as well as metabolic enzymes were activated. Furthermore, the ascorbate-glutathione cycle appeared to play an important role against heavy metal-induced oxidative stress. As Cd is not a redox-active metal, the oxidative stress caused is most likely an indirect effect. Therefore, further

research on the indirect mechanism of heavy metal-induced oxidative stress is required to ascertain the underlying molecular and biochemical events.

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