Chapter II

Response of Field bean to Heavy metal stress
2.1 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 differences are closely associated with their toxicity (Pinto et al., 2003). Metals having a density higher than 5 g cm\(^3\) are termed as heavy metals (Weast, 1984). Environmental contamination has become a worldwide problem leading to severe agricultural losses and hazardous health effects. The metal pollutants, derived from growing 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. Their mode of action proceeds in two stages: The first, inductive stage, lasting 1-2 days, is confined to the action of heavy metals on the signaling processes, and the direct effect on enzymatic processes or the structure of cell wall and cell membranes. At the second stage, lasting over two days, permanent effects involving biochemical diminution of stress and senescence processes leading to degradation of plant tissues occurs (Maksymiec et al., 2008). Increased metal content of agricultural soil has lead to selection and colonization of resistant plants to a particular area, thus affecting biodiversity (Ye et al., 2003).

The toxic effect generated by heavy metals depend on several factors like solubility of the metal, absorbability, transport, its chemical reactivity, pH of the medium and presence of other ions (Stochs and Bagchi, 1995; Das et al., 1997). Heavy metal toxicity may arise from the binding of the metal with the sulfhydryl groups in proteins leading to inhibition of metabolically important enzymes (Panda and Choudhury, 2005). It could also increase the permeability of the plasma membrane (Janicka-Russak et al., 2008) and expression of heat shock proteins (HSPs) (Heckathorn et al., 2004). Elevated levels of heavy metals causes water imbalance leading to decrease in relative water content and transpiration, the mechanism of which is distinct from that caused by osmotic stress (Krantev et al., 2006). 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 (Yordanova et al., 2009). The activity of
chlorophyll degrading enzyme chlorophyllase is also activated by heavy metals such as cadmium (Cd), lead (Pb), cobalt (Co), nickel (Ni) and manganese (Mn).

Fifty-three of the ninety occurring elements are heavy metals (Weast, 1984). Of these, Fe, Mo and Mn are important as micronutrients, while Zn, Ni, Cu, Co, Cr and Va are trace elements exhibiting toxicity at higher concentrations. Ag, As, Cd, Hg, Pb and Sb have no known functions as nutrients, and are toxic to plants and microorganisms (Niess, 1999). Of the three metals studied, Cd is the most deleterious heavy metal pollutant of the environment due to its high cytotoxicity, solubility in water and longer biological half life (10-30 years). On agricultural land, Cd tends to accumulate due to the excessive use of phosphate fertilizers, the dispersal of sewage sludge and atmospheric sedimentation (Alloway, 1995). Hg is a global pollutant with complex and unusual chemical and physical properties. Hg in agricultural soil seems to originate from the degassing of the Earth’s crust, volcanic emissions, and industrial processes and from various products (e.g. batteries, lamps and thermometers). As Hg can replace metal ions in photosynthetic pigments, it results in decrease of photosynthetic rates (Patra et al., 2004). Major inputs of Zn in soil are from mining and smelting activities, chemical and physical weathering of parent rocks and fossil fuel combustion (Richau and Schat, 2009). Its toxic effects include stunted growth, Fe deficiency induced chlorosis and interference with phosphorus uptake.

The toxic effects of heavy metals are related to its ability to generate reactive oxygen species (ROS) resulting in unbalanced cellular redox homeostasis (Dubey, 2011; Hossain et al., 2012). ROS such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$) (Cakmak, 2000), may lead to unspecific oxidation of proteins and membrane lipids. A product of lipid peroxidation, malondialdehyde (MDA) has been shown to accumulate during heavy metal stress (Naser et al., 2008). The primary constituents of protective mechanisms against ROS-mediated damage include enzymes such as superoxide dismutases (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), and polyphenol oxidase (PPO, EC 1.14.18.1) (Mittler, 2002; Razinger et al., 2007) and free radical scavengers, such as carotenoids, ascorbate, tocopherols, glutathione and total phenols. 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 (Luhova et al., 2003; 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 the 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 (Day and Ludeke, 1981; 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 (Bradshaw and Chadwick, 1980) a study was undertaken with Field bean (Dolichos lablab) which is extensively cultivated in semi-arid regions. Such crop plants tolerant to heavy metals could be of great value in reclaiming valuable lands. The present chapter basically covers information regarding response and tolerance mechanism of Field bean to Cd, Hg and Zn at various concentrations.

2.2 Materials and Methods

2.2.1 Plant material and chemicals

The seeds of Dolichos lablab (cv HA-4) were procured from National Seed Project, University of Agricultural Science, Bengaluru, India. Fine chemicals and reagents used in this study were purchased from Sigma-Aldrich, Bengaluru, India. All other chemicals were of analytical grade.
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2.2.2 Growth conditions

Seeds of *Dolichos lablab* cultivar HA-4 were surface sterilized with 0.1% (w/v) mercuric chloride for 1 min, rinsed immediately with large volume of sterile distilled water and imbibed overnight in distilled water. 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.

2.2.3 Determination of Germination indices under stress

Uniform sized seeds of *Dolichos lablab* cultivar HA-4 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 petriplates lined with a layer of cotton wool wetted with 4.0 ml of the mentioned concentrations of CdCl$_2$, HgCl$_2$ and ZnSO$_4$ respectively in half-strength Hoagland medium. Control seedlings were supplied with same volume of half-strength Hoagland medium. After the stipulated period, the percentage germination was taken and the length of the shoot and root was recorded in centimeter. The percentage of germination (% G) of each set was recorded after 24 h and 5 days as the case might be (ISTA, 1976).

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 hr of treatment (Abdul-Baki and Anderson, 1973).

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

2.2.4 Heavy metal stress and experimental design

Heavy metal stress was induced by transferring ten day old seedlings of uniform size to a hydroponic system of half-strength Hoagland media (Allen, 1968) containing CdCl₂ (5, 10 and 50 µM), HgCl₂ (3, 4 and 5 µM), and ZnSO₄ (100, 300 and 600 µM) for a period of 72 h. Samples of leaves and roots were collected at treatment intervals of 24, 48 and 72 h and frozen until further analysis. Plants grown on half-strength 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 at random factorial scheme, with 10 media regimes (control and CdCl₂ (5, 10 and 50 µM), HgCl₂ (3, 4 and 5 µM) and ZnSO₄ (100, 300 and 600 µM)) and 3 evaluation points (24, 48, 72 h). Each experiment was composed of 60 experimental units (leaf + root samples) and done in triplicate.

2.2.5 Determination of Relative Water Content (RWC)

The relative water content was estimated according to the method of Turner and Kramer (1980) using the equation: RWC = (FW - DW) X 100 / (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.

2.2.6 Determination of H₂O₂ and antioxidants

2.2.6.1 Hydrogen peroxide: Hydrogen peroxide content in control and stressed seedlings were determined according to Velikova et al., (2000). Leaf and root tissues (500 mg) were homogenized in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000 g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm.
2.2.6.2 Ascorbic Acid: Ascorbic acid estimation was carried out according to the procedure of Sadasivam and Manickam (1997). The tissue was homogenized in 4% oxalic acid and centrifuged at 10,000 g for 10 min. The assay mixture consisted of 0.1 ml of brominated sample extract made up to 3.0 ml with distilled water, 1.0 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37 °C for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7.0 ml of 80% sulphuric acid and absorbance was read at 540 nm.

2.2.6.3 Glutathione: GSH was estimated according to Beutler (1963). The tissue was homogenized with 3% metaphosphoric acid. DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] was added to supernatants cleared by centrifugation. The formation of 5-thio-2-nitrobenzoic acid, which is proportional to total glutathione concentration, was monitored at 412 nm at 25 °C against reagent controls.

2.2.6.4 Total Phenolics: Total phenols were estimated by the method of Slinkard and Singleton (1977) with slight modifications. 0.2 ml of sample extract, 0.5 ml FC reagent and 2.0 ml Na$_2$CO$_3$ (20%) were mixed in a vial and boiled for exactly 1 min. Absorption was determined at 650 nm against a suitable blank. Catechol was used for the standard curve.

2.2.7 Determination of stress response factors

2.2.7.1 Proline: The estimation was carried out according to the method of Bates et al., (1973). Free proline was extracted from 0.5 g of fresh tissue in 10 ml sulphosalicylic acid (3%) and the extract was filtered through whatman no. 1 filter paper. A known quantity of the filtrate was mixed with 2 ml of acid Ninhydrin reagent. The contents were boiled for 1 h in a boiling water bath and cooled rapidly on ice. The color was extracted in 4 ml toluene by vigorous shaking and the organic phase recorded at 520 nm against toluene as blank. Standard curve was prepared for different concentrations of proline.

2.2.7.2 Malondialdehyde: The extent of lipid peroxidation was determined according to Heath and Packer (1968) with suitable modification. Briefly, 0.5 g of fresh tissue was ground in 5.0 ml of 0.1% TCA containing 0.5% butylated hydroxytoluene containing 1.0% PVP. The homogenate was centrifuged at 12,000 g for 30 min.
4.0 ml of the supernatant was mixed with 4.0 ml of the substrate (0.5% thiobarbituric acid and 20% TCA). The mixture was boiled for 30 min, chilled on ice, and centrifuged at 12,000g for 10 min. The absorbance of supernatant at 532 nm was measured and the nonspecific absorbance at 600 nm was subtracted. The MDA content was calculated from the extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

2.2.7.3 Total Chlorophyll: The procedure of chlorophyll determination was based on the work of Mackinney (1941) on the absorption of light by aqueous acetone (80%) extracts of chlorophyll with at 663 and 645 nm. The concentrations of total chlorophyll, chlorophyll-$a$, -$b$ and total carotenoids were calculated by the formula of Arnon (1949) as given below:

a. Chl $a$ (mg g$^{-1}$) = \[(12.7 \times A_{663}) - (2.6 \times A_{645})\] \times (V / 1000 \times wt)

b. Chl $b$ (mg g$^{-1}$) = \[(22.9 \times A_{645}) - (4.68 \times A_{663})\] \times (V / 1000 \times wt)

c. Total Chl = \[(20.2 \times A_{645}) + (8.02 \times A_{663})\] \times (V / 1000 \times wt)

d. Carotenoids = $A_{480} + (0.14 \times A_{663} - 0.638 \times A_{645})$

The chlorophyll stability index (CSI) was calculated according to the method of Murty and Majumdar (1962) as the ratio between Chl content in stressed leaves and Chl content in control leaves and expressed in %.

2.2.7.4 Total Soluble Sugars: Frozen samples (0.5 g) were ground and extracted in 2 ml of 2.5 N HCl for 30 min, followed by centrifugation at 10,000×g at 4 °C for 10 min. Total soluble sugar content was estimated colorimetrically using anthrone reagent and glucose as standard (Roe, 1955). Results are expressed as mg soluble sugar/g FW tissue.

2.2.7.5 Starch: Starch was determined using anthrone reagent according to the method of Hodge et al., (1962). Samples (0.5 g) were ground and extracted in hot 80 % ethanol. 6.5 ml of 52 % perchloric acid was added and the mixture was extracted in ice. The extract was centrifuged at 10,000×g for 10 min. The supernatant was made up to 100 ml with distilled water. 1.0 ml aliquots were mixed with 4.0 ml of anthrone reagent, boiled for 8 min, cooled rapidly and read at 630 nm against a suitable blank.
2.2.8 **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 1mM 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 12,000g 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 BSA as the standard.

2.2.9 **Activities of Active Oxygen Quenching Enzymes**

2.2.9.1 **Catalase (CAT, EC 1.11.1.6):** Catalase activity was assayed by following the decline in optical density at 240 nm (ε = 39.4 M⁻¹ 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 H₂O₂ 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 H₂O₂ min⁻¹ under the assay conditions. In-gel assay for CAT isozymes were performed by soaking the gels in 10 mM H₂O₂, and sequentially staining with 2 % potassium ferricyanide followed by 2 % ferric chloride to visualize the bands.

2.2.9.2 **Guaiacol peroxidase (POX, EC 1.11.1.7):** Guaiacol peroxidase 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 H₂O₂ and 100 µl enzyme extract (Chance and Maehly, 1955). The formation of tetraguaiacol was followed by an increase in absorbance at 470 nm (ε = 26.6 mm⁻¹ cm⁻¹). One unit of peroxidase is defined as the amount of enzyme needed to convert 1 µmol of H₂O₂ min⁻¹ at 25 °C. In-gel assay was performed by incubating gels with o-dianisidine followed by 10 mM H₂O₂ until the appearance of bands.

2.2.9.3 **Ascorbate peroxidase (APX, EC 1.11.1.11):** The activity of APX 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 H₂O₂, 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$_2$O$_2$. The oxidation of ascorbate was followed by a decrease in the A290 ($\varepsilon = 2.8$ mM$^{-1}$ 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.

2.2.9.4 Glutathione reductase (GR, EC 1.6.4.2): GR activity was determined by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6220$ M$^{-1}$ 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$_2$, 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. GR isozymes were separated on non-denaturing gels soaked in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg/ml MTT, 1 mg/ml 2, 6-dichlorophenol indophenol, 3.4 nM GSSG, and 0.4 mM NADPH until the appearance of bands.

2.2.9.5 Polyphenoloxidase (PPO, EC 1.14.18.1): PPO 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 ($\varepsilon = 1150$ M$^{-1}$ 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.

2.2.10 Activities of Hydrolytic Enzymes

2.2.10.1 β-Amylase (AMY, EC 3.2.1.1): Activity of β-AMY was measured using the DNS method (Bernfeld, 1955). The reaction mixture consisted 0.5 ml of 2% starch solution dissolved in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract. β-AMY isozymes were visualized by soaking the gels in substrate (2 % soluble starch) followed by incubation in 0.025 % acidified iodine solution.

2.2.10.2 Acid phosphatase (AP, EC 3.1.3.2): AP 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 μmoles of α-naphthol or p-nitro phenol released per minute. In-gel assay was carried out using α-naphthyl phosphate as substrate and fast blue-RR as coupling dye.

2.2.10.3 Invertase (INV, EC 3.2.1.26): Invertase 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).

2.2.11 Electrophoretic analysis

2.2.11.1 Native PAGE

Non-denaturing, discontinuous slab gel electrophoresis was carried out essentially according to the method of Davis (1964). A discontinuous gel system consisting of 10.0% separating gel and 5% spacer gel was used. Samples were mixed with gel loading buffer (100 mM Tris-HCl, pH 6.8; 20% glycerol, 2% bromophenol blue), 50 μg of total soluble protein was applied per lane. The electrophoresis was carried out in electrode buffer (25mM Tris-HCl, 192mM glycine, pH 8.0) at 4 °C. After electrophoresis, the gels were stained for enzyme activity as described earlier.

2.2.11.2 SDS-PAGE

Proteins from extracts were electrophoretically resolved on SDS polyacrylamide gel according to Laemmli (1970). A Gel system consisting of 12% resolving gel and 5% stacking gel were employed. Samples containing 100 μg of total soluble protein were mixed with an equal volume treatment buffer (100 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol, 5% β ME, and 2% bromophenol blue), and denatured in boiling water bath for 4 min. The electrophoresis was carried out in electrode buffer (25 mM Tris-HCl, pH 8.0; 250 mM glycine and 0.1% SDS). The gels were stained with 0.25% Coomassie brilliant blue in 45% methanol, 10% acetic acid, and subsequently destained in 45% methanol, 10% acetic acid.
2.2.12 Polyamine analysis

Total free polyamine levels were determined in leaves and roots of Field 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 15,000g 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 benzyol chloride, samples were vortexed for 20 s, 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 12,000g 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 HPLC (Shimadzu) on a reverse-phase C₁₈ column (Alex-Octadecysilane), 5 particle diameter, 4.6 × 250 mm using methanol:water (64:36) at a flow rate of 1ml/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 with the same method.

2.2.13 Metal Uptake

Total leaf and root accumulation of heavy metal in Field 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 mins, air dried and then oven dried at 80 °C for 2 days. The tissue was ground into a fine powder using a pestle and motar. A known amount of this powder (1 g) was dissolved in binary mixture containing 3 parts of 1M HNO₃ 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.
2.2.14 **Statistical analysis**

The experiment was performed using a randomized design. All data are 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 lowest standard deviations (LSD) test. Comparisons with $P \leq 0.05$ were considered significantly different. The bar in the diagrams represented ± standard error. Each data point represent mean of three replicates analyzed twice, and each value is therefore mean of six estimations ($n=6$).

2.3 **Results and Discussion**

**Growth parameters**

Exposure of Field bean seedlings to heavy metals caused a marked decrease in the growth parameters such as leaf area, shoot and root weight (Table 1 & Fig 2.1). Of the three heavy metals, Hg had greater impact followed by Cd and Zn. A low shoot/root ratio suggested a pronounced effect of metals on shoot growth compared to roots. Other alterations included chlorosis after 48 h of exposure in all the three metals employed; and appearance of dark pigmentation and necrotic lesions on the laminae, which were apparent under Cd stress. These effects are in conformity with those observed in chickpea plants stressed with Cd (Faizan et al., 2011). Observed chlorosis and reduced biomass in heavy metal treated Field bean (Table 1) is attributed to enhanced production and accumulation of ROS, resulting in reduced photosynthetic activity as apparent from reduction in chloroplastic pigments. 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 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 Indian mustard (Shiyab et al.,
Another parameter that gets affected under heavy metal stress is RWC. The observed reduction in RWC of heavy metal treated Field bean (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 could be due to reduction in the size and number of xylem vessels and imbalance in hormone levels imposed by heavy metals (Poschenrieder and Barceló, 1999).

![Fig 2.1](image_url): Effect of heavy metal stress (after 72 h of exposure) in Field bean seedlings treated with different concentrations of Cd (a), Hg (b) and Zn (c). A decrease in the surface area and chlorosis of leaves in stressed seedling can be seen.
Table 2.1: Effect of heavy metal stress on germination indices of Field bean after 72 h stress.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Metal] μM</th>
<th>% G</th>
<th>Length (cm)</th>
<th>Tolerance Index (TI)</th>
<th>Vigor Index (VI)</th>
<th>RWC (%)</th>
<th>Weight of whole plant (g)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Leaf Stem Root</td>
<td>Leaf Stem Root Fresh Dry</td>
<td>Leaf Stem Root</td>
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<td></td>
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<tr>
<td>C</td>
<td>0</td>
<td>90</td>
<td>4.6 ± 0.2   7.2 ± 0.4 2.7 ± 0.9 _</td>
<td>98.53 89.54</td>
<td>97.6 ± 0.5 85.9 ± 2.7</td>
<td>94.9 ± 0.0 94.3 ± 4.2</td>
<td>1.37 ± 0.10 0.80 ± 0.06</td>
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<tr>
<td></td>
<td>5</td>
<td>88</td>
<td>4.3 ± 0.7   6.6 ± 1.3 2.0 ± 0.5 74.07</td>
<td>89.54 72.43</td>
<td>85.9 ± 2.7 83.3 ± 3.6</td>
<td>94.3 ± 4.2 86.9 ± 5.2</td>
<td>1.18 ± 0.09 0.80 ± 0.06</td>
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<td></td>
<td>10</td>
<td>84</td>
<td>3.8 ± 0.3   6.0 ± 0.1 1.9 ± 0.4 70.37</td>
<td>72.43 68.43</td>
<td>83.3 ± 3.6 83.3 ± 3.6</td>
<td>86.9 ± 5.2 84.6 ± 0.9</td>
<td>0.80 ± 0.06 0.61 ± 0.07</td>
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<td></td>
<td>50</td>
<td>76</td>
<td>2.5 ± 0.8   5.1 ± 0.9 1.7 ± 0.2 62.96</td>
<td>68.43 66.7 ± 3.3</td>
<td>63.2 ± 4.1 61.5 ± 2.1</td>
<td>0.61 ± 0.07 0.61 ± 0.07</td>
<td>0.06 ± 0.03 0.11 ± 0.01</td>
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<td>3</td>
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<td>4.2 ± 0.2   6.9 ± 1.1 2.5 ± 0.6 91.43</td>
<td>75.32 86.2 ± 3.7</td>
<td>82.4 ± 1.4 89.3 ± 5.3</td>
<td>1.27 ± 0.10 0.61 ± 0.07</td>
<td>0.06 ± 0.03 0.11 ± 0.01</td>
</tr>
<tr>
<td>Cd</td>
<td>4</td>
<td>65</td>
<td>3.8 ± 0.4   5.5 ± 0.8 2.2 ± 0.8 81.48</td>
<td>61.43 80.9 ± 6.4</td>
<td>84.8 ± 4.6 81.7 ± 2.4</td>
<td>1.08 ± 0.10 0.61 ± 0.07</td>
<td>0.06 ± 0.03 0.11 ± 0.01</td>
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<td>5</td>
<td>51</td>
<td>3.2 ± 0.9   5.1 ± 0.7 1.8 ± 0.1 66.67</td>
<td>56.43 71.5 ± 2.9</td>
<td>78.9 ± 4.0 80.3 ± 5.3</td>
<td>0.93 ± 0.07 0.61 ± 0.07</td>
<td>0.06 ± 0.03 0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90</td>
<td>4.9 ± 0.7   8.1 ± 0.5 3.2 ± 0.7 118.51</td>
<td>92.31 86.6 ± 2.2</td>
<td>87.0 ± 3.0 91.4 ± 4.5</td>
<td>1.29 ± 0.14 0.61 ± 0.07</td>
<td>0.06 ± 0.03 0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>86</td>
<td>4.2 ± 1.1   7.3 ± 0.8 2.6 ± 0.8 96.29</td>
<td>88.42 81.8 ± 3.8</td>
<td>84.3 ± 4.7 77.1 ± 3.2</td>
<td>1.06 ± 0.04 0.61 ± 0.07</td>
<td>0.10 ± 0.02 0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>82</td>
<td>3.8 ± 0.5   6.8 ± 0.5 2.1 ± 0.3 77.78</td>
<td>73.43 75.9 ± 2.5</td>
<td>76.5 ± 2.7 70.2 ± 5.1</td>
<td>1.02 ± 0.08 0.61 ± 0.07</td>
<td>0.09 ± 0.02 0.11 ± 0.01</td>
</tr>
</tbody>
</table>
Metal uptake

Field bean seedlings exposed to heavy metal stress in a hydroponic system showed increasing metal concentrations in the primary leaves as a function of time after the onset of exposure (Fig 2.2). Heavy metal content was relatively higher in roots (~ 28-fold in Cd-50, 3.8-fold in Hg-5 and 18-fold in Zn-600 after 72 h of stress) than in leaves (Fig 2.2). The rate of uptake of metals by Field bean was concentration- and time-dependent and comparable to the response seen in Cd-treated maize (Drazkiewicz et al., 2003), Cd- and Zn-treated Brassica juncea plants (Sridhar et al., 2005), and Hg-treated Indian mustard (Shiyab et al., 2009).

![Fig 2.2: Cd, Hg and Zn levels in leaf and root of control and stressed Field bean seedlings after treatment for 72 h as determined by atomic absorption spectroscopy.](image)

Response of antioxidants and antioxidant enzymes

When the defense system in heavy metal-treated plants is overburdened, the reactive oxygen species (ROS) start to appear in excess and the antioxidative system is strongly activated. The timing of H$_2$O$_2$ occurrence observed almost coincides with the action of other stress factors, in which signal molecules are involved. Redox metals like Cu$^{2+}$ or Fe$^{2+}$ undergo Fenton reactions producing OH$^-$ from H$_2$O$_2$ and O$_2^-$ (Winterbourne, 1982). The OH$^-$ radical is the most reactive and initiates various irreversible modifications in the cells. The hydroperoxyl radical (HO$_2^-$) generated from O$_2^-$ can
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induce lipid peroxidation via protonation reaction (Mithofer et al., 2004). Cd$^{2+}$ and Hg$^{2+}$ are non-redox metals and are unable to replace Cu$^{2+}$ or Fe$^{2+}$ in Fenton reactions. Such metals can affect the pro-oxidant status by depleting the antioxidant glutathione (GSH) pool (Pinto et al., 2003). In the present study, it was found that the H$_2$O$_2$ content increased with increasing time and concentration of the heavy metal (Fig 2.3). The increase in H$_2$O$_2$ content in leaves after 72 h of stress under the heavy metal ranged between 2.5 fold (Zn) and 3.5 fold (Cd and Hg). The increase in H$_2$O$_2$ in roots ranged between 3 fold (Cd and Hg) and 3.5 fold (Zn). This is probably due to the declining phase of GSH levels in a concentration-dependent manner after 48 h of stress in all three metals under study (Fig 2.7). Decrease in GSH as a mechanism to counteract oxidative stress has been reported by a number of researchers (Singla-Pareek et al., 2006; Hossain et al., 2009; Hossain et al., 2011). Cd-induced depletion of GSH has been mainly attributed to phytochelatin synthesis (Sun et al., 2007), decreased GR activity (Aravind and Prasad, 2005), and increased utilization for ascorbate synthesis or for direct interaction with metals like Cd (Pietrini et al., 2003). These results suggested a close relation between GSH and H$_2$O$_2$ contents in leaves of Field 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 malondialdehyde, MDA (Singh, 2006). Formation of lipid peroxides serves as an activation signal for plant defense genes through increased activity of the octadecanoid pathways (Maksymiec, 2007). MDA content of metal-stressed leaves of Field bean exhibited time- and concentration-dependent increase. This relationship has been held good for all the metals studied; Cd treated plants, however showed greater enhancement in MDA than Hg and Zn treated ones (Fig 2.3). Such increase in MDA levels have been reported in pea (Metwally et. al., 2005) and tomato (Cervilla et al., 2007) stressed with Cd. The MDA content in Field bean roots too increased in time- and concentration dependent manner (Fig 2.3).
Fig 2.3: Levels of MDA (mol MDA g\textsuperscript{-1} FW) and H\textsubscript{2}O\textsubscript{2} (mg g\textsuperscript{-1} FW) in leaves and roots of Field bean treated with different concentrations of CdCl\textsubscript{2} (2: 5 μM; 3: 10 μM; 4: 50 μM); HgCl\textsubscript{2} (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl\textsubscript{2} (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
The strength of the oxidative stress relies on the interaction of several factors that determine the antioxidant status of the plant. ASC, the key antioxidant, showed metal specific variations. The increase in ASC levels in leaves exhibited better gradation under Cd stress during the entire period of exposure (Fig 2.4). However, the levels of ASC under Zn and Hg stress did not show significant gradation beyond 24 h of exposure. 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. Although the levels of ASC in roots of Hg and Zn stressed Field bean exhibited a decreasing trend with increasing metal concentration during 24 h and 48 h of exposure, the trend was reversed during 72 h of exposure with remarkable increment in ASC with increasing concentrations of Zn (Fig 2.4). The increases in ASC levels are 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 H₂O₂ radicals non-enzymatically (Lin et al., 2007). The levels of ASC in Field bean suggested that ASC has a major role in the leaf than in the root system, and is mainly synthesized in the leaves. Relative levels of ASC under these three metals also suggested that Cd induced greater oxidative stress, and hence greater elevation in ASC with time and concentration of metals than Zn and Hg. Levels of ASC in leaves under Zn stress also indicated that Zn produced less toxic effect even at 600 mM at 72 h of exposure in Field bean.

GSH levels in leaves treated with Cd showed time- and concentration-dependent decline. Hg had a positive influence on GSH levels in the early exposure period at lower concentrations. In contrast, Zn had a negative influence on GSH, wherein, increasing concentrations of Zn and exposure time lead to greater decline in GSH levels (Fig 2.5). 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). Metal concentration dependent decline of GSH in leaves under Zn and Cd, and also Hg to a lesser extent 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 (Fig 2.5). The observed decrease in the GSH levels in roots could be attributed to its metal-catalyzed oxidation to GSSG (Devi and Prasad, 2005), and also to
**Fig 2.4:** Activity of ascorbate peroxidase (APX) and ascorbate (ASC) in leaves and roots of Field bean treated with different concentrations of CdCl₂ (2: 5 µM; 3: 10 µM; 4: 50 µM); HgCl₂ (5: 3 µM; 6: 4 µM; 7: 5 µM) and ZnCl₂ (8: 100 µM; 9: 300 µM, 10: 600 µM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
**Fig 2.5:** Activity of glutathione reductase (GR) and reduced glutathione (GSH) in leaves and roots of Field bean treated with different concentrations of CdCl₂ (2: 5 μM; 3: 10 μM; 4: 50 μM), HgCl₂ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl₂ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
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the synthesis of metal-chelating proteins, phytochelatins (Yadav, 2010). These observations are in line with those made by Yurekli and Kucukbay (2003) in sunflower 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., 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). Field bean seedlings treated with Cd exhibited considerable increase in APX levels during the early exposure, but the levels dropped with increasing time of exposure. The other two metals, Zn and Hg did not show any significant alteration in APX level except the concentration- and time-dependent decline at extended time of exposure (Fig 2.4). Similar results were reported in Brassica juncea (Singh et al., 2011) and Vigna radiata (Shanker et al., 2004). The GR levels in heavy metal stressed Field bean seedlings showed time- and concentration-dependent increase (Fig 2.5). The coincidence of declining GSH and increasing GR activities under heavy metal stress suggested that ASC/GSH cycle is operative in this plant, thus regenerating the oxidized ASC to DHA. Such coincidence has been reported under heavy metal stress as well as other oxidative stress (Noctor and Foyer, 1998b; Khan et al., 2007; Mobin and Khan, 2007).

Fig 2.6: Zymogram patterns of glutathione reductase (GR) in leaves and roots of Field bean treated with different concentrations of CdCl$_2$ (2: 5 µM; 3: 10 µM; 4: 50 µM; HgCl$_2$ (5: 3 µM; 6: 4 µM; 7: 5 µM) and ZnCl$_2$ (8: 100 µM; 9: 300 µM, 10: 600 µM) compared to control, 1; after different periods of treatment.
CAT is a heme-containing enzyme that catalyzes the dismutation of H\textsubscript{2}O\textsubscript{2} into oxygen and water. The CAT activity decreased significantly in leaves and roots of metal stressed seedlings (Fig 2.7) causing a parallel increase in H\textsubscript{2}O\textsubscript{2}, a stress signaling molecule. These observations are in consonance with those of Hg-treated *Cucumis sativus* (Cargnelutti et al., 2006), Cd-treated *Pisum sativum* (Sandalio et al., 2001) and Zn-treated *Brassica campestris* (Chatterjee et al., 2005). The drop in CAT activity in Field bean could be due to destabilization of peroxisomal membrane as a result of elevation of H\textsubscript{2}O\textsubscript{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 H\textsubscript{2}O\textsubscript{2} destruction is via peroxidases (POX) that are found throughout the cell and have a much higher affinity for H\textsubscript{2}O\textsubscript{2} than CAT (Noctor and Foyer, 1998a). CAT has low substrate affinities when compared to POX and requires simultaneous access of two H\textsubscript{2}O\textsubscript{2} molecules at the active site (Willekens et al., 1997).

Like CAT, POX is involved in detoxifying H\textsubscript{2}O\textsubscript{2}, but at the expense of another substrate being oxidized such as ascorbate. POX is also reported to be enhanced by heavy metal stress and this was positively correlated with heavy metal tolerance (Chen et al., 2007; Eyidogan and Oz, 2007; Arora et al., 2008). The activity of POX in leaves increased in time- and concentration- dependent manner under all the three metals studied, but decreased steadily in roots (Fig 2.7). The enhanced POX activity in leaves of Field bean thus indicated an efficient detoxification of ROS in leaves but not in roots. Further, the counterbalancing of CAT and POX suggested predominant role of POX in antioxidative mechanism of Field 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 (Fig 2.1) could be due to limitation of cell expansion via lignification.
Fig 2.7: Activity of guaiacol peroxidase (POX) and catalase (CAT) in leaves and roots of Hyacinth bean treated with different concentrations of CdCl₂ (2: 5 μM; 3: 10 μM; 4: 50 μM; HgCl₂ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl₂ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
Fig 2.8: Zymogram patterns of guaiacol peroxidase (POX) (a) and catalase (CAT) (b) in leaves and roots of Field bean treated with different concentrations of CdCl₂ (2: 5 μM; 3: 10 μM; 4: 50 μM), HgCl₂ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl₂ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment.
Polyphenolic compounds observed in the aerial part of the plant, seems to play an important role in the bioactivities and growth of any plant and thus manage the plants growth and development during environmental stress (Sirhindi, 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 and synthesis of phenolic compounds (Mayer, 2006). Elevated PPO levels in leaves of Field bean (Fig 2.9) indicated its possible role even during the early exposure to metal stress. Higher PPO activities were seen in leaves stressed with Hg and Zn when compared to leaves of Cd-stressed seedlings. This increase could be possible tolerance mechanism as demonstrated in radish subjected to Cd stress (Mohamed et al., 2009). The increased PPO activity might have caused oxidation of phenolics to quinones, thereby reducing their content in both leaves and roots (Fig 2.9), 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 (Burguieres et al., 2006). PPO activity however, was found to decline in roots with time of exposure in all three heavy metals (Fig 2.9).
Fig 2.9: Activity of polyphenol oxidase (PPO) and total phenol (TP) in leaves and roots of Field bean treated with different concentrations of CdCl₂ (2: 5 μM; 3: 10 μM; 4: 50 μM); HgCl₂ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl₂ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
Response of hydrolytic enzymes and soluble sugars

Apart from antioxidant enzymes, which have been indisputably implicated in stress response, there are also reports of induction of metabolite enzymes such as; β-AMY, AP and INV under abiotic stress (Yang et al., 2007). β-AMY activity was positively influenced with progressive concentrations and time of exposure to Cd (Fig 2.10). 50 µM CdCl₂ caused a 2-fold increase in the activity after 72 h of stress. Plants treated with HgCl₂ showed a concentration-dependent increase in activity up to 48 h. However, the levels did not show any alteration relative to control at 72 h of exposure. Zn stress had no effect on β-AMY (Fig 2.10). Further, isozyme patterns of β-AMY did not show any new isoforms. Nevertheless, the intensity of the bands correlated with in vitro levels (Fig 2.11). There are reports of increased activity of β-AMY leading to accumulation of 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 h 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 heavy metals on carbon metabolism is a result of their possible interaction with the reactive centre of ribulose bisphosphate carboxylase, RuBisCo (Feller et al., 2008).
Fig 2.10: Activity of β-amylase (AMY) and total soluble sugar (TSS) in leaves and roots of Field bean treated with different concentrations of CdCl$_2$ (2: 5 μM; 3: 10 μM; 4: 50 μM); HgCl$_2$ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl$_2$ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
Acid phosphatase widely distributed in plants are 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 (Fig 2.12). Such enhancement in AP could be due to metal induced denovo synthesis (Pereira et al., 2002). The isozyme patterns in root and leaves of Field bean indicated similar expression patterns (Fig 2.13). 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 phosphorylation (Ehsanpour et al., 2003; Fincher, 1989) and may help the plant to survive longer under stress conditions.
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Fig 2.12: Activity of acid phosphatase (AP) in leaves and roots of Field bean treated with different concentrations of CdCl$_2$, HgCl$_2$, and ZnCl$_2$ compared to control, after different periods of treatment, 1, 24 h; 2, 48 h; and 3, 72 h. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P ≤ 0.05).

Fig 2.13: Zymogram patterns of acid phosphatase (AP) in leaves and roots of Field bean treated with different concentrations of CdCl$_2$ (2: 5 μM; 3: 10 μM; 4: 50 μM); HgCl$_2$ (5: 3 μM; 6: 4 μM; 7: 5 μM) and ZnCl$_2$ (8: 100 μM; 9: 300 μM, 10: 600 μM) compared to control, 1; after different periods of treatment.
Invertase activity in both leaves and roots showed a clear decrease in response to increasing concentration of Cd and Hg (Fig 2.14). This inhibition of INV could explain the tendency to accumulate TSS in these stressed tissues (Fig 2.10). However, INV activity was found to increase in leaves of Zn stressed plants indicating that Zn at the concentration chosen may serve as a micronutrient and stimulate carbohydrate synthesis.

![Invertase - Leaf](image1)

![Invertase - Root](image2)

**Fig 2.14:** The levels of invertase (INV) in leaves and roots of Field bean treated with different concentrations of CdCl₂, HgCl₂, and ZnCl₂ compared to control, after different periods of treatment, 1, 24 h; 2, 48 h; and 3, 72 h. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P ≤ 0.05).

*Levels of other stress-response factors: proline, protein, polyamines and pigment content*

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 (Fig 2.15) and TSS (Fig 2.10) in time and concentration dependent manner in all three metals suggests probability of sugar-
mediated proline biosynthesis and acquisition of tolerance (Hare and Cress, 1997). The increased NADP⁺/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 (Kavi Kishor et al., 2005). The simultaneous increase and interdependence of TSS and proline levels have also been reported in pepper (Goicoechea et al., 2000) and poplar plants (He et al., 2012). 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).

Fig 2.15: Levels of free proline (PRO) in leaves and roots of Field bean treated with different concentrations of CdCl₂, HgCl₂, and ZnCl₂ compared to control, after different periods of treatment, 1, 24 h; 2, 48 h; and 3, 72 h. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).
When a plant is subjected to any biotic or abiotic stress factor, the first observed response is a decrease in the normal metabolic activities, with a consequent reduction of growth. In this ‘alarm phase’, protein synthesis is one of the most negatively affected anabolic processes (Bonjoch and Tamayo, 2003). It is opined that abiotic stress may inhibit synthesis of some proteins and promote others (Dubey, 1999) with a general trend of decline in the overall content. The protein content indicated an increase under Cd and a decrease under Hg and Zn stress in Field bean plants (Fig. 2.16). These observations coincide with those of Costa and Spitz (1997) and Choudhury and Panda (2004), who have also reported a decrease in soluble protein content under heavy metal stress in *Lupinus albus* and moss, respectively. The decrease in protein content may be caused by enhanced protein degradation as a result of increased protease activity (Palma et al., 2002) and fragmentation of proteins due to toxic effects of ROS (John et al., 2009). The increase in protein content in Cd treated Field bean coincides with that of Cd treated *Lemna minor* (Mohan and Hosetti, 1997).

![Fig 2.16: Levels of protein in leaves and roots of Field bean treated with different concentrations of CdCl₂, HgCl₂, and ZnCl₂ compared to control, after different periods of treatment, 1, 24 h; 2, 48 h; and 3, 72 h. Data plotted are mean ± SE of duplicates of three separate replicates, mean values were compared by one way ANOVA (P≤ 0.05).](image)
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, Put is considered to be a stress marker in plants. Field bean stressed with three heavy metals exhibited a common pattern in polyamine level with remarkable elevation in Spd levels at highest metal concentrations employed. The other major polyamines, Put and Spm were not affected by metal stress (Fig 2.17). 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; Groppa et al., 2001). This protective role for polyamines was recently 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.

![Fig 2.17: Levels of polyamines in leaves of Field bean treated for 72 h with different concentrations of CdCl₂, HgCl₂, and ZnCl₂ compared to control.](image-url)
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 (Pèrez-Gálvez and Mínguez-Mosquera, 2002). Treatment of Field bean with heavy metals resulted in decreased concentration of chloroplastic pigments (Table 2). Though changes in carotenoid concentration were not significant, the concentration of chlorophylls decreased significantly; the decrease after 72 h of stress was found to be highest in presence of Cd (51 %) followed by 42% and 25% in the presence of Hg and Zn respectively. Both chl a and chl b were decreased 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 ALA (Marschner, 1995).
Table 2.2: Effect of heavy metal stress on pigment content in leaves of Field bean

<table>
<thead>
<tr>
<th>Stress response component</th>
<th>Control</th>
<th>CdCl₂ (µM)</th>
<th>Hg Cl₂ (µM)</th>
<th>ZnSO₄ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Total Chl.</td>
<td>8.2 ± 1.0</td>
<td>7.2 ± 0.5</td>
<td>6.9 ± 0.0</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>Chl (a/b) ratio</td>
<td>1.29 ± 0.2</td>
<td>1.15 ± 0.4</td>
<td>1.21 ± 0.6</td>
<td>1.31 ± 0.1</td>
</tr>
<tr>
<td>CSI</td>
<td>100</td>
<td>87.52</td>
<td>84.87</td>
<td>78.72</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.81 ± 0.05</td>
<td>0.78 ± 0.03</td>
<td>0.78 ± 0.04</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>Total Chl.</td>
<td>8.7 ± 0.9</td>
<td>6.2 ± 0.2</td>
<td>4.9 ± 0.7</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Chl (a/b) ratio</td>
<td>1.15 ± 0.4</td>
<td>1.25 ± 0.2</td>
<td>1.32 ± 0.3</td>
<td>1.38 ± 0.5</td>
</tr>
<tr>
<td>CSI</td>
<td>100</td>
<td>71.26</td>
<td>56.32</td>
<td>49.42</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.83 ± 0.08</td>
<td>0.79 ± 0.04</td>
<td>0.76 ± 0.05</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>Total Chl.</td>
<td>8.4 ± 0.7</td>
<td>4.5 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Chl (a/b) ratio</td>
<td>1.17 ± 0.6</td>
<td>1.27 ± 0.5</td>
<td>1.31 ± 0.6</td>
<td>1.40 ± 0.4</td>
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<tr>
<td>CSI</td>
<td>100</td>
<td>53.57</td>
<td>58.33</td>
<td>50.00</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.82 ± 0.02</td>
<td>0.72 ± 0.06</td>
<td>0.68 ± 0.03</td>
<td>0.63 ± 0.04</td>
</tr>
</tbody>
</table>
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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 Field bean. Plants cells possess a well-organized antioxidative defence 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.

In conclusion, the cellular redox status is affected by heavy metals, and oxidative stress could be an important mechanism of heavy metal toxicity. The early-activated antioxidative defense mechanism controls and establishes the redox balance initially, but after a longer metal exposure, biochemical and morphological alterations do occur, even with relatively low, environmentally realistic heavy metal concentration. As the heavy metals chosen for the study are not redox-active metals, the oxidative stress caused by them is most likely an indirect effect. Therefore, further research on the indirect mechanisms of heavy metal-induced oxidative stress is required to ascertain the underlying molecular and biochemical events.