CHAPTER-XI

RESPONSE OF HEAVY METAL INDUCED PEPTIDES OF *VIGNA RADIATA* ON SEEDLING TOLERANCE AND OXIDATIVE STRESS MITIGATION

11.1 INTRODUCTION

Heavy metals are the fundamental elements with a relatively high density (5 g/m^3) and are toxic or poisonous at relatively low concentrations. These are mercury (Hg), Cadmium (Cd), Arsenic (As), Chromium (Cr), Lead (Pb), Copper (Cu), Zinc (Zn) etc. These are among the important pollutants causing world wide environmental contaminations and human health problem. Heavy metal pollutants have been discharged into the environment through various means including mining, metal work industries, urban traffic power station and agricultural fertilizers along with pesticides.

Though some heavy metals (Cu, Zn) are also essential for normal growth and development of plant as they are the constituents of many enzymes and other proteins but elevated concentrations of both essential and non-essential metals can result in growth inhibition and toxicity symptoms. It is already established that heavy metals exert adverse effect on physiological and biological activities of plants (Fornazier *et al.*, 2002). Similar to various other stresses, heavy metal tends to increase the permeability of tissues due to membrane destabilization in plants (Pandey and Sharma *et al.*, 2002). The toxicity symptoms observed in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular/molecular level. Toxicity may result from the binding of metals to sulphydroxyl groups in proteins, leading to inhibition of activity or disruption of structure, or displacing of an essential element resulting in deficiency symptoms. Heavy metals excess may stimulate the formation of free radicals and reactive oxygen species perhaps resulting in oxidative stress. Free radical induced lipid peroxidation is considered to be an important mechanism of leaf senescence, membrane leakage and apoptosis in plant system (Hall 2002; Hall and Williams, 2003).

Based on chemical and physical properties three different molecular mechanisms of heavy metal toxicity can be distinguished: (a) Production of reactive oxygen species (ROS) by autoxidation and Fenton reaction, this reaction is typical for transitional metals such as Fe, Cu. (b) Blocking of essential functional groups in biomolecules. This reaction has mainly been reported for non-redox reactive heavy metals such as cadmium and mercury. (c) Displacement of essential metal ions from biomolecules, the later reaction occurs with different kinds of heavy metals. Exposure of plants to non-redox reactive

metals also resulted in oxidative stress as indicated by lipid peroxidation, H_2O_2 accumulation and an oxidative burst.

Cadmium and some other metals are the cause of a transient depletion of Glutathione and an inhibition of anti-oxidative enzymes which indirectly promote the generation of reactive oxygen species. Generally high Cd level inhibit the system involved in H_2O_2 removal by inhibiting the expression of Glutathione and Ascorbate, which are the main non-enzymatic antioxidants and also antioxidant enzymes like Catalase, Glutathione reductase, Peroxidase and causes H_2O_2 accumulation. Metabolic modelling suggests that this loss of antioxidant defences is sufficient to explain the observed H_2O_2 accumulation. Cadmium actually inhibits enzymes which contain sulphur containing Ligand -SCH_3 and -SH in methionine and cysteine amino acids binding with them and replacing Zn from enzymes. It is generally observed that higher level of Cadmium accumulation in plant causes reduction in photosynthesis, diminish water and nutrient uptake and results in visible symptoms of injury in plants such as chlorosis, growth inhibition, browning of root tips, secondary metabolism stimulation, lignifications and finally cell death (Schutzendubel and Polle, 2002). Cadmium also decreases leaf area, stomatal frequency, stomatal index and pigment content in 7 days old mung bean seedlings grown under laboratory conditions (Vassilev *et al.,* 1998). The metal promotes reduction of chlorophyll content, uncoupling of electron transport in chloroplast and perturbation of carbon reduction cycle (Woolhouse, 1983).

Copper can be toxic by their participation in redox reaction producing hydroxyl ($^{\cdot}OH$) radicals which are extremely toxic to living cells. Catalase and Glutathione Reductase activity are also reduced by excess copper (Streb *et al.,* 1993). Excess copper affect the equilibrium between photo-inhibition and repair, resulting in a decrease in steady state concentration of active photosystem.

It has been observed that mercury also produce equivalent grades of toxicity in plant system. Mercury affects water channel proteins or aquaporins and seriously hampers leaf growth and hydraulic conductance (Yukutake *et al.*, 2008). Bivalent mercurial ions probably react with free sulphydryl groups of aquaporins protein associated with root membrane water channels and result in their closure.

A promising new technology referred to as phyto-remediation, offers promise for cleanup of polluted areas in a cost-effective and environment friendly manner. This technology involves removal of toxic heavy metals from contaminated soils and water or rendering them harmless by accumulating, chelating or transforming these contaminants into biologically inactive forms through green plants. One possible approach for phyto-remediation is to use 'hyper accumulator' plant species that have evolved to accumulate high concentration of heavy metals in their biomass. But due to natural limitations, genetic and molecular investigations of plant defence mechanisms involved in heavy metal stress have been under way to improve the efficiency of phyto-remediation (Lee *et al.*, 2003).

Though some of the genetic approaches are experimentally successful in laboratory for efficient heavy metal trapping system and switching to its metabolically non-toxicated forms, lots of other investigations including bioactive antioxidants and peptides are essentially required for proper phytoremediation. My investigation is particularly concentrated on those bioactive peptides which can able to combat these heavy metal stresses. Biologists very well know that the peptides like Glutathione (GSH), Phytochelatin (PCs) and Metallothionins (MTs) have extraordinary efficiency regarding the intoxication of heavy metal stress. Therefore this study aimed at exploring the interaction of peptides and heavy metal (Cd, Hg, and Cu) stress. The low molecular weight peptides that are isolated from 7 days old *Vigna* seedlings under heavy metal stress are characterized according to their solvent partitioning profile in paper chromatography and their efficacy values are monitored under stressful conditions, derived from the presence of excess heavy metals. These peptides can efficiently manage the stressful environment and the plants grown in these peptides containing medium with heavy metals or priming seeds with these peptides before sowing perform better response and execute lesser toxic symptoms than their counterparts, grown in same environment but without peptides. Though the exact mechanism of these responses are not studied due to shortage of time, still I think that the findings which are obtained from my works will essentially carry some values related to heavy metal tolerance in plant system.

11.2 MATERIALS AND METHODS

11.2.1 Plant Material

Seeds of mung bean [*Vigna radiata* (L) Wilczek. cv sonali B1(sensitive variety to heavy metal stress)] were collected from same agriculture research station mentioned in earlier chapters and stored at 10° C cooling temperature inside freeze in the desiccator until further use.

11.2.2 Culturing of Plants

Seeds were surface sterilized with 4% sodium hypochlorite solution for 3 minutes and then washed thoroughly with sterile distilled water three times under laminar air flow. Seeds were allowed to culture in sterile petriplates with absorbent cotton supplied with modified Hoagland solution with one-half strength major nutrients and full-strength micronutrients. The nutrient solution was renewed weekly. Experiments were conducted inside a controlled environmental growth chamber with 14-h light period (350 μmol m^{-2} s^{-1}), 25°/20°C day/night temperature, and 80% relative humidity.

11.2.3 Induction of heavy metal stress

Modified Hoagland was intentionally contaminated with 5 mM Cu^{2+} as copper sulphate [$CuSO_4$, 5H2O] and 2mM each of Cd^{+2} & Hg^{+2} in the form of cadmium chloride [$CdCl_2$, H_2O] and mercuric chloride solutions in separate flasks. Just after germination *Vigna* seedlings were cultured for one week in this heavy metal inoculated medium for expression of heavy metal stress induced peptides.

11.2.4 Isolation and purification of low molecular weight peptides

Isolation and purification of low molecular weight peptides (3k Da to 0.5 k Da) were performed as described in Chapter III Section 3.2.2. Mung been seedlings, which were grown on heavy metal stressed condition, were used for peptide extraction. The extracted peptides were mercury stress induced peptides isolated and purified from plants cultured in acute mercury stressed conditions. Similarly cadmium and copper stress induced peptides were isolated and purified in same manner from seedlings that acutely faced the respective heavy metal stresses.

11.2.5 Aseptic culturing of *Vigna* seedlings with peptides

After isolation of heavy metal stress induced peptides, *Vigna* seedlings were co-cultured in the peptide & heavy metal containing medium in fully aseptic condition. For that, seeds were surface sterilized and aseptically transferred in a suitable medium for observing the morphological and biochemical changes associated with heavy metal stress.

11.2.5a *Seed germination:*

Before germination, seeds were surface sterilized successively with 2% sodium hypochlorite and 70% alcohol followed by 4 times washing with sterile distilled water. The seeds were then aseptically transferred to sterile Petri plates containing cotton. The Petri dishes were placed inside the germinator under 25°/20°C day/night temperature, 90% relative humidity and 14 hrs / 10 hrs. light / dark cycle.

11.2.5b *Aseptic seedling transfer to modified MS medium:*

After emergence of radical, 10 seeds were transferred aseptically with sterile forceps to each sterile flask containing modified MS medium with different concentrations of heavy metals and peptides inside laminar air flow. The basic composition of modified MS medium was half strength MS Major, full strength MS minor, MS Iron and MS Vitamin with 0.5% sucrose and 0.9% agar (bacteriological grade). No hormones were added inside that MS medium. After proper sterilization and autoclaving, heavy metals were inoculated inside this MS medium through proper melting and mixing, the final concentrations of which were enlisted Table 11.1

Table 11.1 Concentrations of heavy metals introduced in MS-medium

Heavy Metals	Conc. of Mother Stock	Mother soln. added to modified Hoagland	Final conc. of Heavy Metals	Vol. of soln. added in each Petri dishes for culturing
$CuSO_4.5H_2O$	1 (M)	1 µL	50 µM	20 ml
		4 µL	200 µM	
$CdCl_2.H_2O$	0.5 (M)	1 µL	25 µM	20 ml
		4 µL	100 µM	
$HgCl_2$	0.5 (M)	1 µL	25 µM	20 ml
		4 µL	100 µM	

Similarly peptides were aseptically introduced inside the MS medium with their respective concentrations mentioned Table 11.2

Table 11.2 Amount of *Vigna radiata* peptides introduced in MS-medium

Peptide Sample	Conc. of peptide mother stock (Fresh Wt. Eq/ml)	Mother stock added to MS medium in each flask	Volume of MS medium in each flask	Final conc. of peptide in MS medium (Fresh Wt. Eq/ml.)
Copper stress induced peptides	100 gm/ml	200 µl	20 ml	1 gm/ml
	1 gm/ml	200 µl	20 ml	10 mg/ml
	10 mg/ml	200 µl	20 ml	100 µg/ml
Cadmium stress induced peptides	100 gm/ml	200 µl	20 ml	1 gm/ml
	1 gm/ml	200 µl	20 ml	10 mg/ml
	10 mg/ml	200 µl	20 ml	100 µg/ml
Mercury stress induced peptides	100 gm/ml	200 µl	20 ml	1 gm/ml
	1 gm/ml	200 µl	20 ml	10 mg/ml
	10 mg/ml	200 µl	20 ml	100 µg/ml

In case of control, plants were cultured without any heavy metal or peptide contaminations.

11.2.5c *Maintenance of seedlings:*

After inoculation of seeds, the flasks were kept under fluorescent tube for 12 hours and placed under dark for another 12 hours. 24 °-26 ° C temperatures were always maintained.

11.2.6 Pre-soaking or priming of seeds of *Vigna radiata*

After proper surface sterilization, fifty seeds of *Vigna radiata* were primed with 10 ml each of water, 500µM of salicylic acid and 1gm/ml of two different peptide solutions (isolated from cadmium and mercury stress induced plants) separately for 7 hours. During this period agitation was constantly maintained for preventing anaerobiosis.

Priming was stopped before radical emergence. Temperature of 25°C and light of 350 μmol m^{-2} s^{-1} was also maintained within this time. After priming, seeds were completely air-dried and kept in refrigerator till further use.

11.2.7 Culturing of primed seeds in heavy metal containing medium

For observing the performance of primed seeds, they were aseptically transferred to different flasks containing 50, 100, 150, 200, 250 ppm of cadmium, copper and mercury separately. For nutritional supply to seedlings modified Hoagland solution containing one-half strength major and full-strength micronutrients was used as basal medium. The flasks were aseptically placed inside the controlled environmental growth chamber with 14-h light period, 25°C/20°C day/night temperature and 80% relative humidity. Morphological, biochemical and enzymological performance of seedlings were measured after 6 days of culturing in these physiological conditions.

11.2.8 Study of Morphological parameters

The records of morphological parameters as length of the shoot, hypocotyls and shoot, area of leaf, lateral root number, weight of a pair of cotyledons and embryo proper were measured after one week of culture with the help of suitable instruments (millimetre scale, digital balance etc.). Photographs were also taken for those seedlings that truly reflect the observed morphological changes among lots of plants.

11.2.9 Induction/repression of antioxidative enzymes

Following enzymes will be assayed after extraction from plant tissues with or without challenging peptides and priming with salicylic acid, water or peptides:

11.2.9a *Catalase (EC 1.11.1.6):*

Catalase (CAT) activity was estimated as described by Patterson et al. (1984). Here the decomposition of hydrogen peroxide was monitored considering Δε 43.6 mM cm^{-1} at 240 nm. 3 ml of Reaction mixture contained 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 ml enzyme extract at 25 °C. The decrease in absorbance at 240 nm was used to calculate the activity. One

unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of H_2O_2 min^{-1} at 25 °C.

11.2.9b *Ascorbate Peroxidase (EC 1.11.1.11):*

Ascorbate Peroxidase (APX) activity was determined according to the method of Nakano and Asada (1981). The reaction mixture (2.0 ml) consisted of 0.05 M potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H_2O_2. The reaction was initiated by the addition of 0.1 ml enzyme extract at 25 °C. The decrease in absorbance at 290 nm for one minute was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.8mM cm^{-1}. The unit of activity is expressed as micromole of ascorbic acid oxidized min^{-1} at 25 °C.

11.2.9c *Guaiacol Peroxidase (EC 1.11.17):*

Guaiacol Peroxidase (GPX) activity was determined spectrophotometrically at 25 °C according to the method mentioned by Tatina *et al.* (1999). 2.0 ml of reaction mixture contained 0.05 (M) potassium phosphate buffer (pH 7.0), 2 mM H_2O_2 and 2.7 mM guaiacol. The reaction was started by the addition of 0.1 ml enzyme extract. The initial rate of oxidation of guaiacol was determined by the rate of formation of tetraguaiacol and was measured at 470 nm ($\Delta\varepsilon$ = 26.6 mM cm^{-1}). One unit is defined as the amount of enzyme required to catalyze the conversion of one micromole of H_2O_2 with guaiacol as hydrogen donor, per minute under specified conditions.

11.2.9d *Glutathione Reductase (EC 1.6.4.2):*

Glutathione reductase (GR) activity was measured spectrophotometrically at 25°C by determining the rate of NADPH oxidation as a decrease in absorbance at 340 nm ($\Delta\varepsilon$ = 6.2 mM cm^{-1}) according to the method of Halliwell and Foyer (1978). The reaction mixture (1.0 ml) consisted of 100 mM Tris-HCl buffer (pH 7.8), 21 mM EDTA, 0.005 mM NADPH, 0.5 mM oxidized glutathione, and the enzyme extract. NADPH was added to start the reaction. Unit activity is defined by the expression reduced 1.0 mmol of oxidized glutathione per minute under standard assay conditions.

11.2.9e *Superoxide Dismutase (EC 1.15.1.1):*

Superoxide Dismutage (SOD) activity was determined spectrophotometrically. The reaction mixture containing Tris-HCl Buffer (pH 8.9) EDTA, tetra-ethylene diamine, BSA and nitro-blue tetrazolium (NBT) with riboflavin in KOH were illuminated under

fluorescent light and increase in absorbance was monitored at 560 nm (Giannopolitis & Reis, 1997).

11.2.10 Estimation of total protein content

Proteins were extracted by homogenizing 500 mg of fresh seedlings in 10 ml of 50 mM pre-chilled Tris-HCl (pH 8.0) and centrifuged at 10,000 rpm for 15 min at 4°C and an aliquot from the supernatant was mixed with an equal volume of ice cold 10% trichloroacetic acid (TCA; w/v) and incubated at 0°C for 1 h to precipitate the proteins. The protein pellet was collected by centrifugation at 5,000 rpm for 15 min at 4°C and dissolved in 1 (M) NaOH. Protein content was estimated by the procedure of Lowry *et al.* (1951) with BSA used as a standard. Protein content was quantified immediately after harvest.

11.2.11 Paper chromatography

100 µl (1 g fresh weight equivalent peptide) of each isolated heavy metal stress induced peptide solution was loaded onto Whatman No-1 chromatography paper (size-46 cm x 57 cm, thickness-0.16 mm), and separated by descending chromatography with mobile solvent phase [Isopropanol: Ammonia: Water (10:1:1 v/v or 8:1:1 v/v)] in one-dimension; and for two-dimensions, two solvents were used separately [solvent1-Isopropanol: Ammonia: Water (10:1:1 v/v or 8:1:1 v/v) and solvent 2-n-Butanol:Acetic acid :water (4:1:1 v/v or 6:1:2 v/v)]. The chromatograms were stained with freshly prepared ninhydrin location reagent (Hirao *et. al.*, 2000). The retardation factor (R_f) values were determined as before.

11.2.12 Determination of biochemical parameters

Following biochemical events were estimated with seven days old seedlings of *V. radiata* after seed priming with water, salicylic acid or peptides.

11.2.12a *MDA determination:*

MDA content was measured by the thiobarbituric acid (TBA) as described by Heath and Packer (1968). After homogenizing the leaves with 5% TCA, the homogenate is directly used for MDA estimation. 1 ml of 5% TCA and 4 ml of TBA reagent (0.5% in 20%

TCA) was mixed and used as a blank. For correction of blank, 1 ml of homogenate and 4 ml of 20% TCA and for sample 1 ml of homogenate and 4 ml of TBA reagent were mixed. After heating at 95°C for 30 min in a water bath the mixture was cooled and centrifuged 4,000 rpm for 10 min. The absorbance was determined at 532 nm and corrected for non-specific absorbance at 600 nm and for the absorbance at 532 nm of the correction blank. MDA content was calculated by using an extinction coefficient at 155 $mM^{-1}.cm^{-1}$. MDA level is routinely used as an index of lipid peroxidation and was expressed as nmol g FW^{-1}.

11.2.12b *Peroxide estimation:*

100 mg of fresh seedlings were homogenized with 5% TCA and centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was used for peroxide estimation by ferri-thiocyanate methods mentioned earlier by Sagisaka (1976). Reaction mixture contained 2 ml tissue extract, 0.4 ml of 50% TCA, 0.4 ml ferrous ammonium sulphate and 0.2 ml potassium thiocyanate. The absorbance of ferrithiocyanate complex was determined spectrophotometrically at 480 nm and compared to H_2O_2 standard. Peroxide content was expressed as μmol g FW^{-1}.

11.2.12c *Estimation of proline:*

Spectrophotometric determination of proline content was estimated according to the method of Bates *et al.* (1973) based on reaction of proline with ninhydrin. For proline estimation, a 1:1:1 solution of proline, ninhydrin and glacial acetic acid was incubated at 100°C for 1 hour. The reaction was arrested in an ice bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined spectrophotometrically. 0.5 g of tissues was mixed with 2 ml of 3% sulfosalicylic acid. The supernatant after centrifugation was mixed in a 1:1:1 ratio with ninhydrin and glacial acetic acid. The method was calibrated for each determination with standard proline solutions within the detection range of the method (0-100 μg. ml^{-1}).

11.2.12d *Determination of ascorbic acid:*

Ascorbic acid of mung bean seedlings was measured using 2,6-dichloroindophenol (DIP) titrimetric method, as reported by Dewanto *et al.*, (2002) and modified slightly. Briefly, 2 g of seedlings was blended in a Waring blender using 30 ml of chilled 2% (w/v) metaphosphoric acid for 5 min. Samples were then homogenized for 3 min. The

homogenates were centrifuged at 2000g for 5 min. The extract solutions were filtered (Whatman no. 1) for titration. A total of 1 ml of ascorbic acid standard solution (1 mg/ml) was added to 50 ml conical flask, and then mixed with 10 ml of 2% (w/v) metaphosphoric acid. Ascorbic acid was titrated with 2, 6-dichloroindophenol solution until the colour turned pink and persisted for at least 15 sec. The measurement was expressed as milligram of ascorbic acid per 100 grams of sample. Data were reported as mean ± SD for at least three replicates.

12.2.12e *Determination of total soluble phenolics:*

The total phenol contents of seedlings of *V. radiata* were determined by the Folin-Ciocalteu colorimetric method (Singleton, 1999). All extracts were diluted appropriately in order to obtain readings that ranged within the standard curve concentration range of 0.0 to 600.0 µg gallic acid ml^{-1}. For each analysis, 100 µl of the standard gallic acid solution or extracts was added to 0.4 ml of deionized water in a test tube. Folin-Ciocalteu reagent (0.1 m) was added to the solution and allowed to react for 10 min to ensure that the Folin-Ciocalteu reagent reacted completely with the oxidizable phenolates in the sample. Then, 1 ml of 7% sodium carbonate solution was added to raise the pH, and 0.8 ml of deionized water was added into the test tubes to adjust the final volume to 2.4 ml. The samples were mixed and allowed to stand for 90 min at room temperature. After the colour was developed, absorbance was read at 760 nm using a UV-VIS spectrophotometer (Systronics, 2201). Results were calculated based on the standard curve of gallic acid concentrations and expressed as milligram per 100 grams of dry weight for triplicate.

11.2.13 Statistical analysis

All analysis was carried out in triplicate. Results were presented as mean ± standard deviation. Bivariate Pearson's correlation co-efficient was performed through SPSS (Version 21, IBM SPSS Inc., Chicago, USA). Significant differences were declared at $p < 0.05$. The results were submitted to PCA analysis using XLSTAT 2009 compatible with Excel versions of Windows 2007.

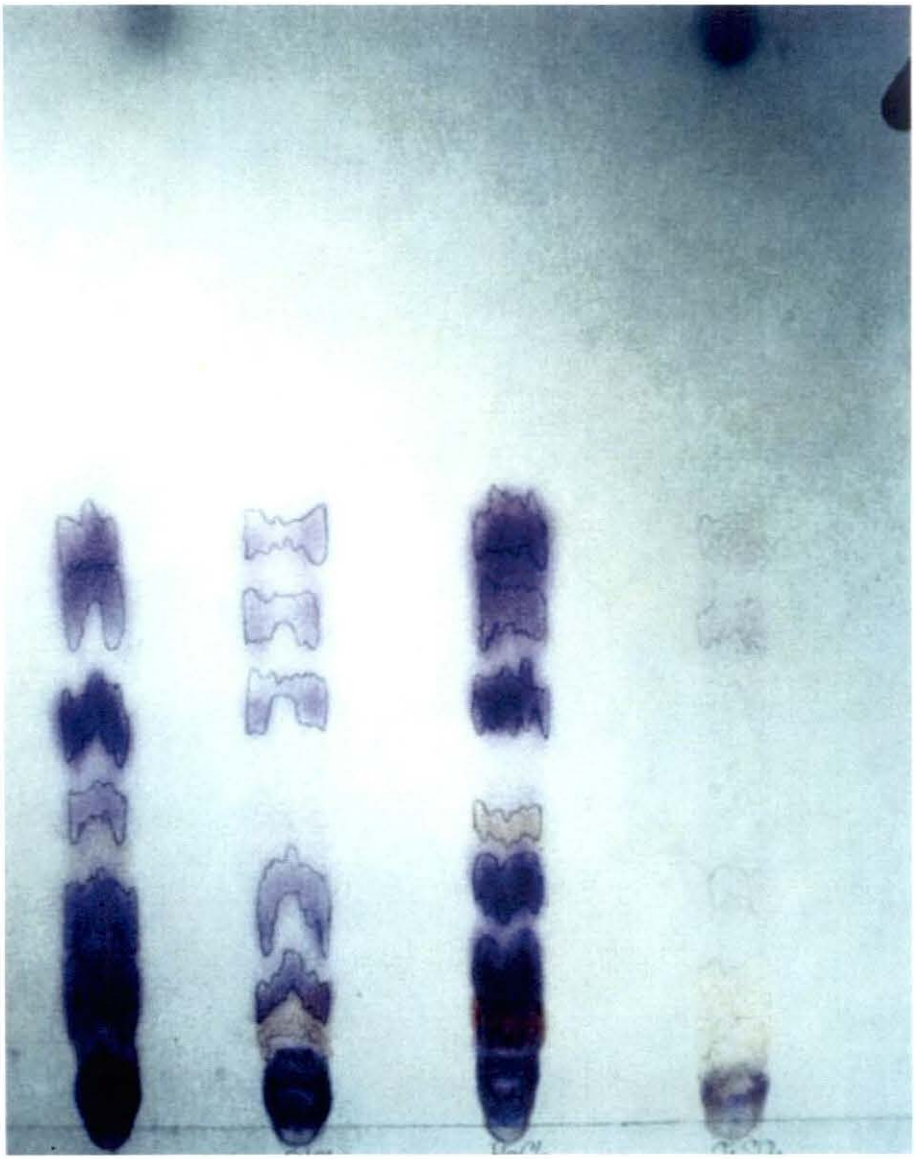

Figure 11.1 One-dimensional paper chromatography of peptides elicited with cadmium, mercury and copper salts

11.3 RESULTS & DISCUSSION

11.3.1 Paper chromatography of heavy metal stress induced peptides

One dimensional paper chromatography was done with isolated peptides in solvent mixture of Isopropanol : Ammonia : Water :: 10 : 1 : 1. After some permutation and combinations of solvent ratio, it was observed that best resolution and greater number of imaginary separation planes were achieved in above mentioned solvent ratio. The peptides that were expressed in normal condition were mostly suppressed in copper stress. Though the apparent pattern of expression of peptides during cadmium stress is more or less same, the intensity of spots were reduced when R_f values were greater than 0.10 (Figure 11.1). One additional spots were appeared in each of $HgCl_2$ and $CuSO_4$ lane, which indicate that new peptides may be expressed during mercury and copper stress (Table 11.3). It was also observed that apparent migration of expressed peptides in this solvent mixture was slow and the R_f values were restricted within 0.6 probably due to lower affinity of peptides in alkaline solvent (Table 11.3).

Table 11.3 Distribution of spots of heavy metal expressed peptides depending on R_f values [Solvent- Isopropanol: Ammonia: Water (10:1:1 v/v)]

Spot no.	R_f values	Control	$CdCl_2$	$HgCl_2$	$CuSO_4$
1	0.03	++++	++++	+++	++
2	0.05	++++	++++	++++	++
3	0.09	+++	++	+++	+
4	0.13	++++	++	++++	+
5	0.22	+++	++	+++	+
6	0.28	*Absent*	*Absent*	++	+
7	0.31	++	*Absent*	*Absent*	*Absent*
8	0.39	+++	++	+++	+
9	0.48	+++	++	+++	+
10	0.56	+++	++	+++	+

+ denotes apparent colour intensity of spots

Table 11.4 2D Paper Chromatographic Fingerprint of Cadmium Chloride induced peptides expressed in one week old *Vigna radiata* (L) Wilczek. cv sonali B1

Vertical No. of spots	SPOTS IN GROUP							
	A		B		C		D	
	Apparent Intensity	R_f	Apparent Intensity	R_f	Apparent Intensity	R_f	Apparent Intensity	R_f
1	+	(0.06,0.13)	-	-	-	-	-	-
2	++++	(0.14, 0.15)	+++	(0.19,0.15)	-	-	-	-
3	+	(0.01,0.19)	+	(0.04,0.19)	+	(0.07, 0.19)	+	(0.15, 0.20)
4	+	(0.10, 0.22)	-	-	-	-	-	-
5	+	(0.13,0.25)	+++	(0.16,0.25)	++	(0.42, 0.26)	-	-
6	++++	(0.26,0.34)	-	-	-	-	-	-
7	+++	(0.48,0.49)	-	-	-	-	-	-
8	++	(0.59,0.57)	-	-	-	-	-	-
9	++	(0.62,0.61)	-	-	-	-	-	-

Table 11.5 2D Paper Chromatographic Fingerprint of Copper Sulfate induced peptides expressed in one week old *Vigna radiata* (L) Wilczek. cv sonali B1

Vertical No. of spots	Apparent Intensity represented by no. of '+' symbols.				SPOTS IN GROUP					
	A		B		C		D		E	
	AI	R_f	AI	R_f	AI	R_f	AI	R_f	AI	R_f
1	++	(0.14, 0.09)	++	(0.19, 0.09)	-	-	-	-	-	-
2	+	(0.17, 0.17)	-	-	-	-	-	-	-	-
3	+	(0.26, 0.26)	-	-	-	-	-	-	-	-
4	+	(0.44, 0.43)	-	-	-	-	-	-	-	-
5	+	(0.55, 0.51)	-	-	-	-	-	-	-	-
6	+	(0.57, 0.55)	-	-	-	-	-	-	-	-
7	+	(0.35, 0.88)	++	(0.38, 0.88)	+	(0.42, 0.88)	+	(0.54, 0.94)	+	(0.64, 0.94)
8	+	(0.59, 0.97)	-	-	-	-	-	-	-	-

Figure 11.2 Two-dimensional paper chromatogram of copper sulphate induced peptides

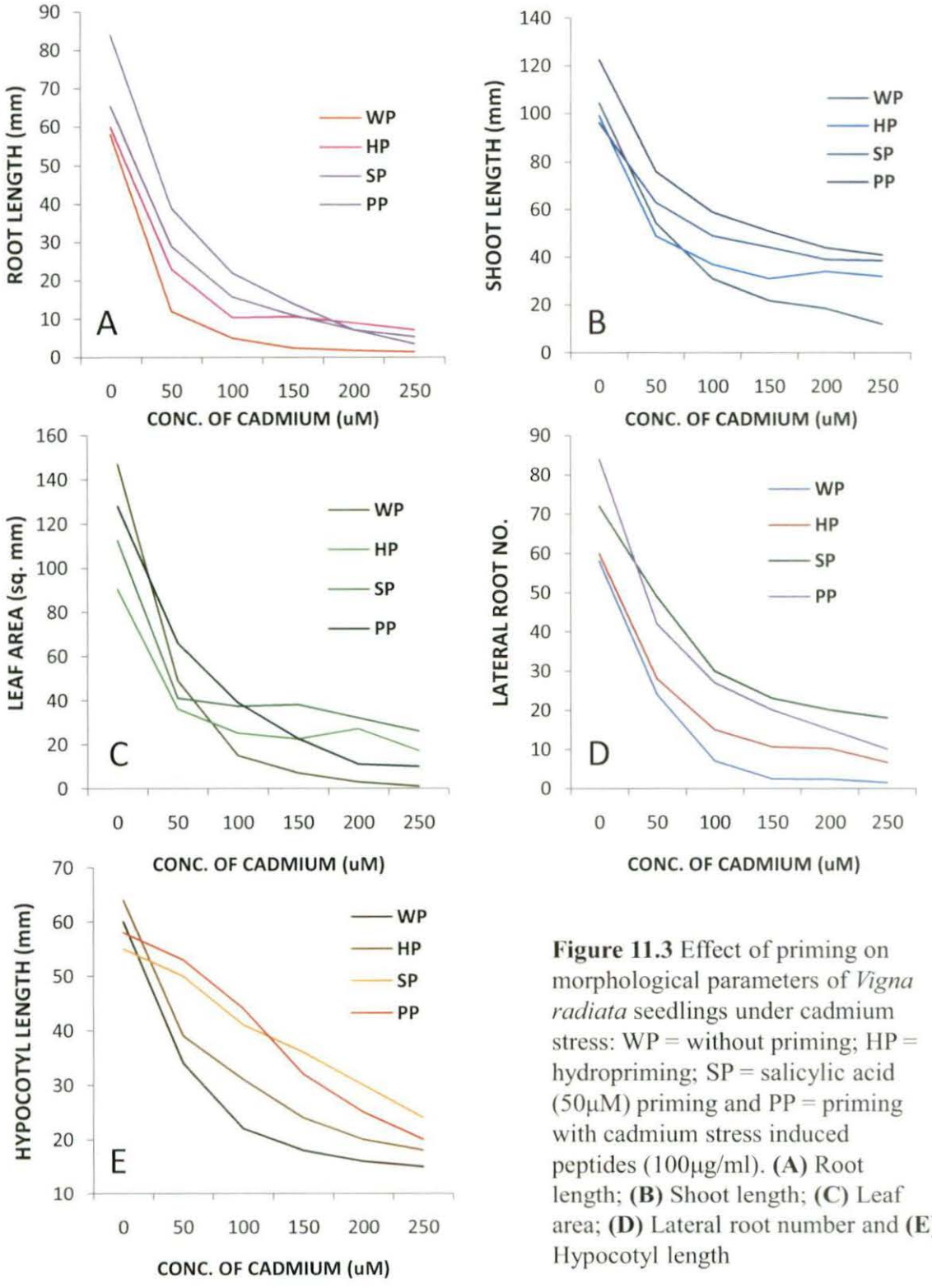

Figure 11.3 Effect of priming on morphological parameters of *Vigna radiata* seedlings under cadmium stress: WP = without priming; HP = hydropriming; SP = salicylic acid (50µM) priming and PP = priming with cadmium stress induced peptides (100µg/ml). **(A)** Root length; **(B)** Shoot length; **(C)** Leaf area; **(D)** Lateral root number and **(E)** Hypocotyl length

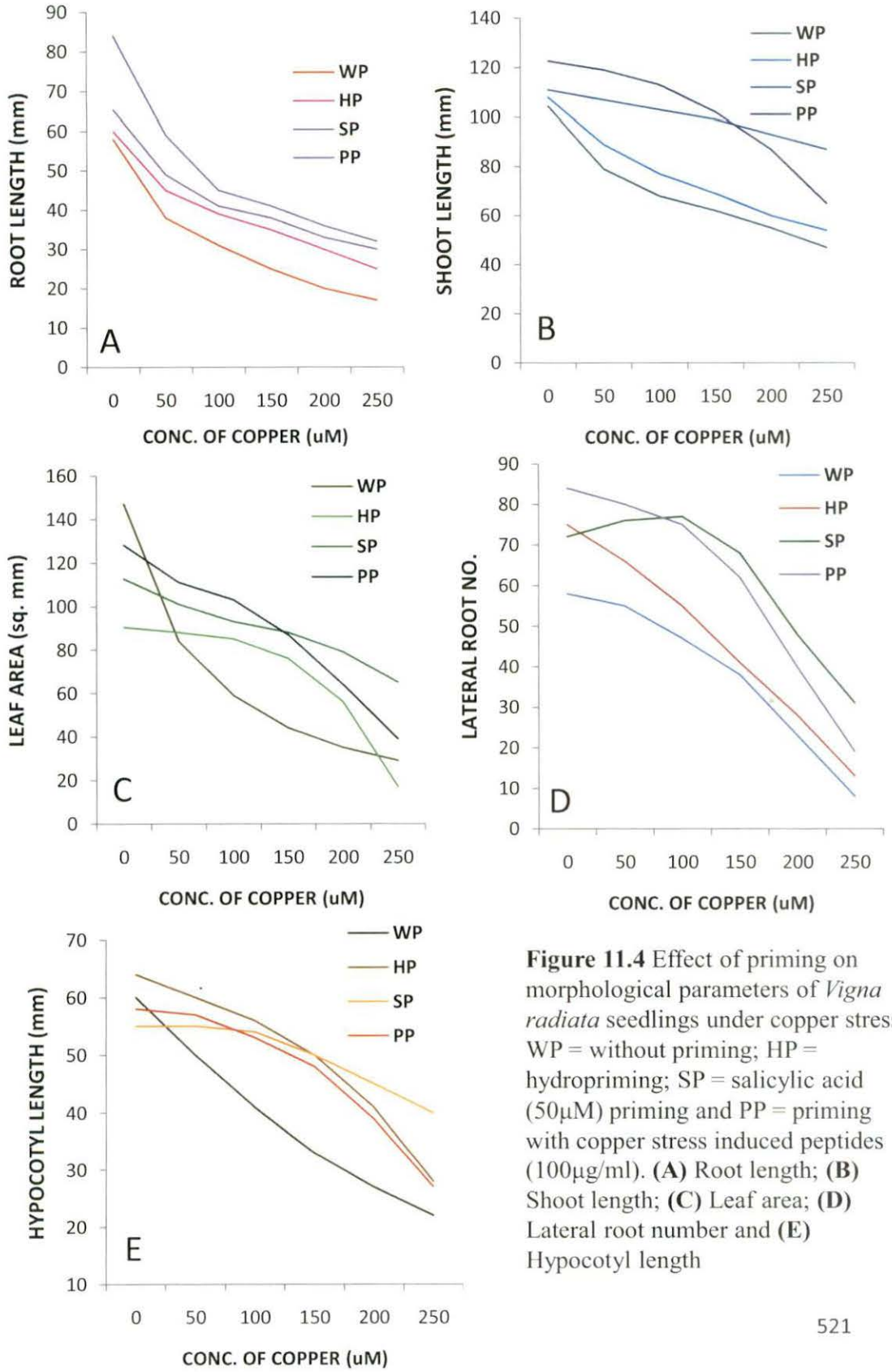

Figure 11.4 Effect of priming on morphological parameters of *Vigna radiata* seedlings under copper stress: WP = without priming; HP = hydropriming; SP = salicylic acid (50µM) priming and PP = priming with copper stress induced peptides (100µg/ml). **(A)** Root length; **(B)** Shoot length; **(C)** Leaf area; **(D)** Lateral root number and **(E)** Hypocotyl length

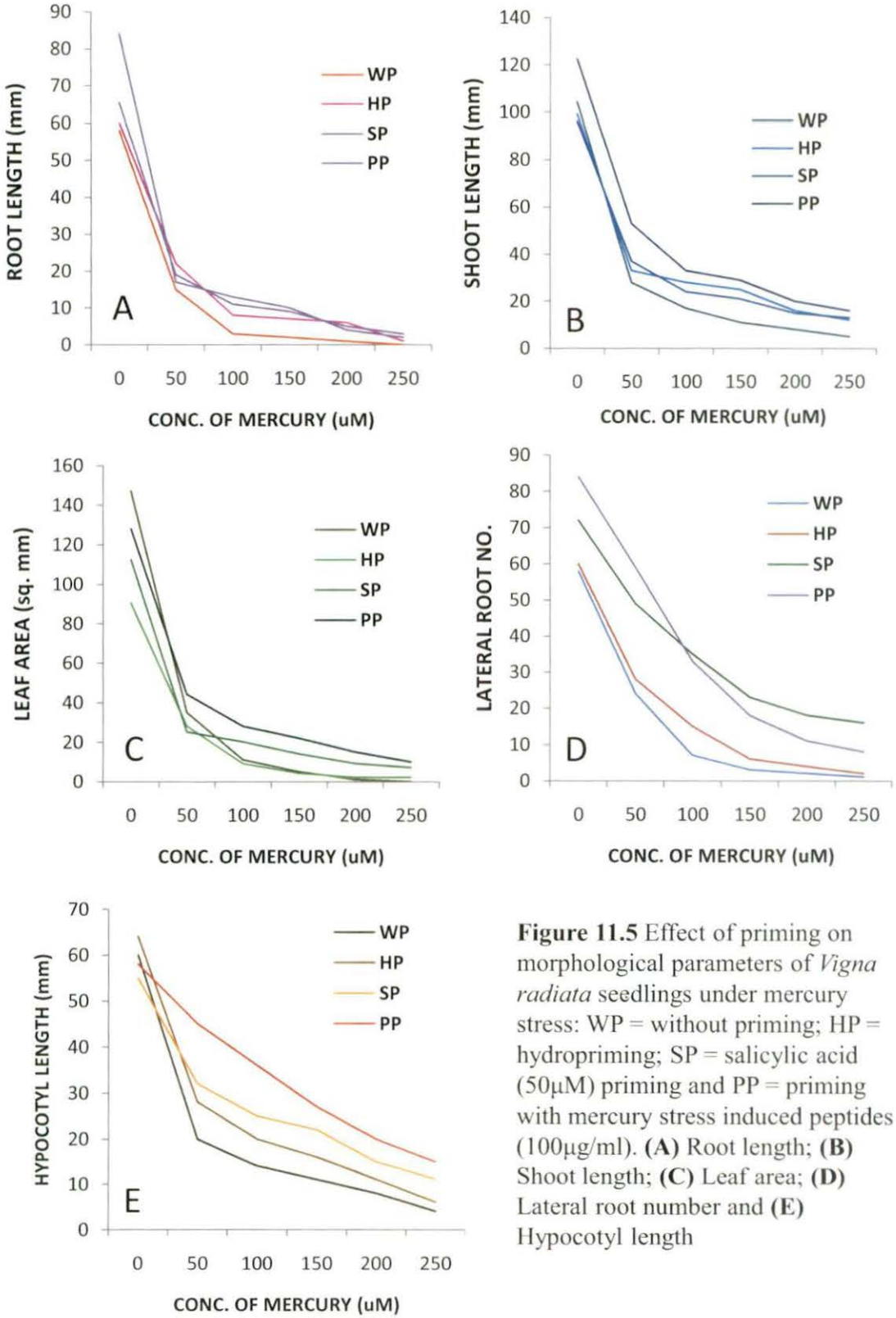

Figure 11.5 Effect of priming on morphological parameters of *Vigna radiata* seedlings under mercury stress: WP = without priming; HP = hydropriming; SP = salicylic acid (50µM) priming and PP = priming with mercury stress induced peptides (100µg/ml). **(A)** Root length; **(B)** Shoot length; **(C)** Leaf area; **(D)** Lateral root number and **(E)** Hypocotyl length

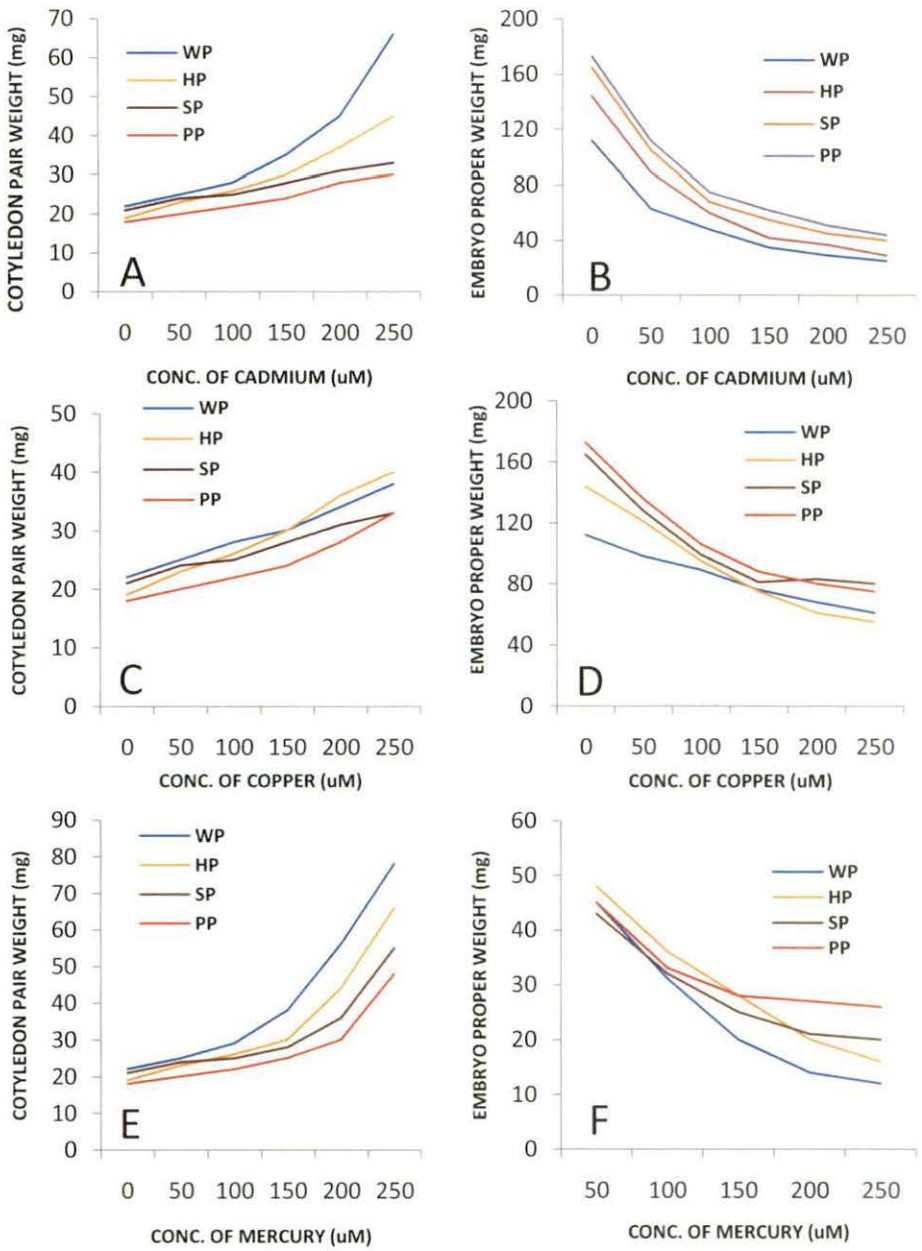

Figure 11.6 Effect of priming on morphological parameters of *Vigna radiata* seedlings under heavy metal stress: WP = without priming; HP = hydropriming; SP = salicylic acid (50μM) priming and PP = priming with heavy metal stress induced peptides (100μg/ml). **(A)** Cotyledon pair weight under cadmium stress; **(B)** Embryo proper weight under cadmium stress; **(C)** Cotyledon pair weight under copper stress; **(D)** Embryo proper weight under copper stress; **(E)** Cotyledon pair weight under mercury stress; **(F)** Embryo proper weight under mercury stress.

In two dimensional paper chromatography, Isopropanol : Ammonia : Water in 8:1:1 and Butanol : Acetic Acid : Water in 6 : 1 : 2 ratios resolved the spots best; 15 spots were developed from cadmium (Table 11.4), 14 from mercury and 13 from copper induced peptides (Table 11.5). If all the conditions were same and stable, these solvent mixtures produce dependable fingerprint of respective heavy metal stress induced peptides. Like 1D, 2D chromatogram also shows low apparent intensity of spots in $CuSO_4$ induced peptides (Figure 11.2). Among $CuSO_4$ induced peptides, the migration distance of some were high in alkaline front ($R_f > 0.80$) which was not observed in other cases. In normal as well as in cadmium and mercury induced peptides, intense spots were distributed near its origin which indicate that a significant quantity of expressed peptides have low R_f value (<0.5) in both alkaline and acidic solvents.

11.3.2 Induction of phytotoxicity under heavy metal stress and possible remediation through application of peptides

All the three heavy metals viz. cadmium, copper and mercury executed potential phytotoxicity on mung bean from the very beginning of germination phase. In all cases of heavy metal contaminations root length, shoot length, leaf area and embryo proper weight was drastically reduced (Figure 11.3-11.6). Even after one week, cotyledons of seedlings held considerable weight in heavy metal contaminated samples (Figure 11.6). This indicates that cotyledonary reserve foods were not appropriately utilized by embryo proper and these heavy metals perturbed the mobilization of storage reserves from cotyledon to embryo proper. Clearly the heavy metals have some visible phytotoxicity which might be operated through malfunctioning of basic physiological systems and ultimately hindered the normal atmosphere of seedling growth and development.

Although copper is an essential micronutrient for plants, it was already documented that uptake of excess copper beyond threshold could be harmful for most plants (Fernandes and Henriques, 1991). Copper mediated free radical formation has been reported in intact roots (De Vos *et al.*, 1993), in detached leaves (Luna *et al.*, 1994) and in intact leaves (Weckx and Clijsters, 1996). Excess copper has been shown to induce leaf senescence (Dhindsa *et al.*, 1981) and lipid peroxidation. The activities of enzymes involved in active oxygen metabolism like catalase, glutathione reductase & superoxide

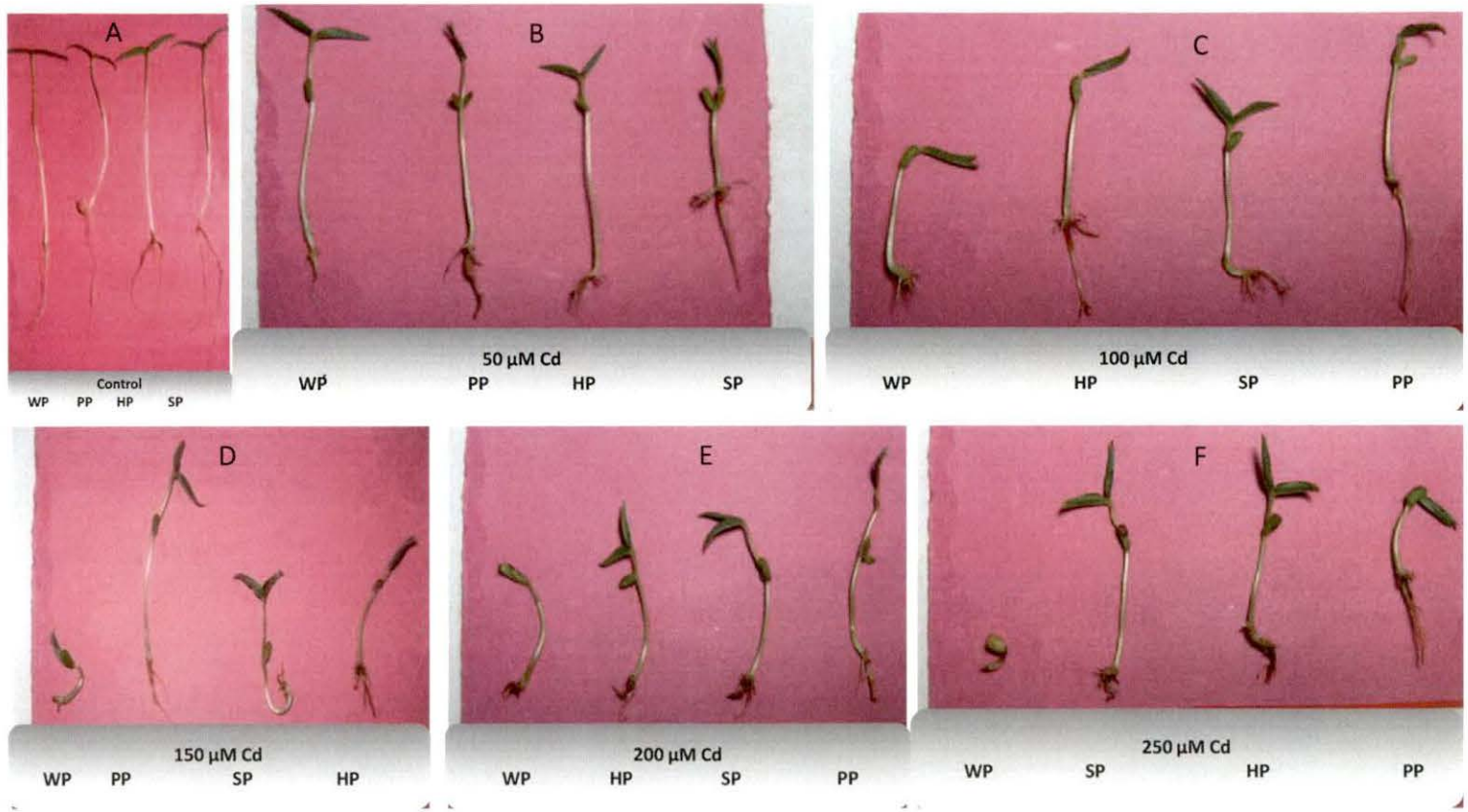

Figure 11.7 Changes of seedling vigour of five-days old *Vigna radiata* plant after treatment with different concentrations of cadmium salt on seeds primed with water, salicylic acid and peptides; WP= Without priming, PP=Peptide priming, HP=Hydropriming and SP=Salicylic acid priming: **(A)** Untreated control; **(B)** to **(F)** Treated with cadmium chloride: **(B)** 50 µM; **(C)** 100 µM; **(D)** 150 µM; **(E)** 200 µM; **(F)** 250 µM

Figure 11.8 Changes of seedling vigour of five-days old *Vigna radiata* plant after treatment with different concentrations of mercury salt on seeds primed with water, salicylic acid and peptides; WP= Without priming, PP=Peptide priming, HP=Hydropriming and SP=Salicylic acid priming: **(A)** Untreated control; **(B)** to **(E)** Treated with mercuric chloride: **(B)** 50µM; **(C)** 100 µM; **(D)** 150 µM; **(E)** 250 µM

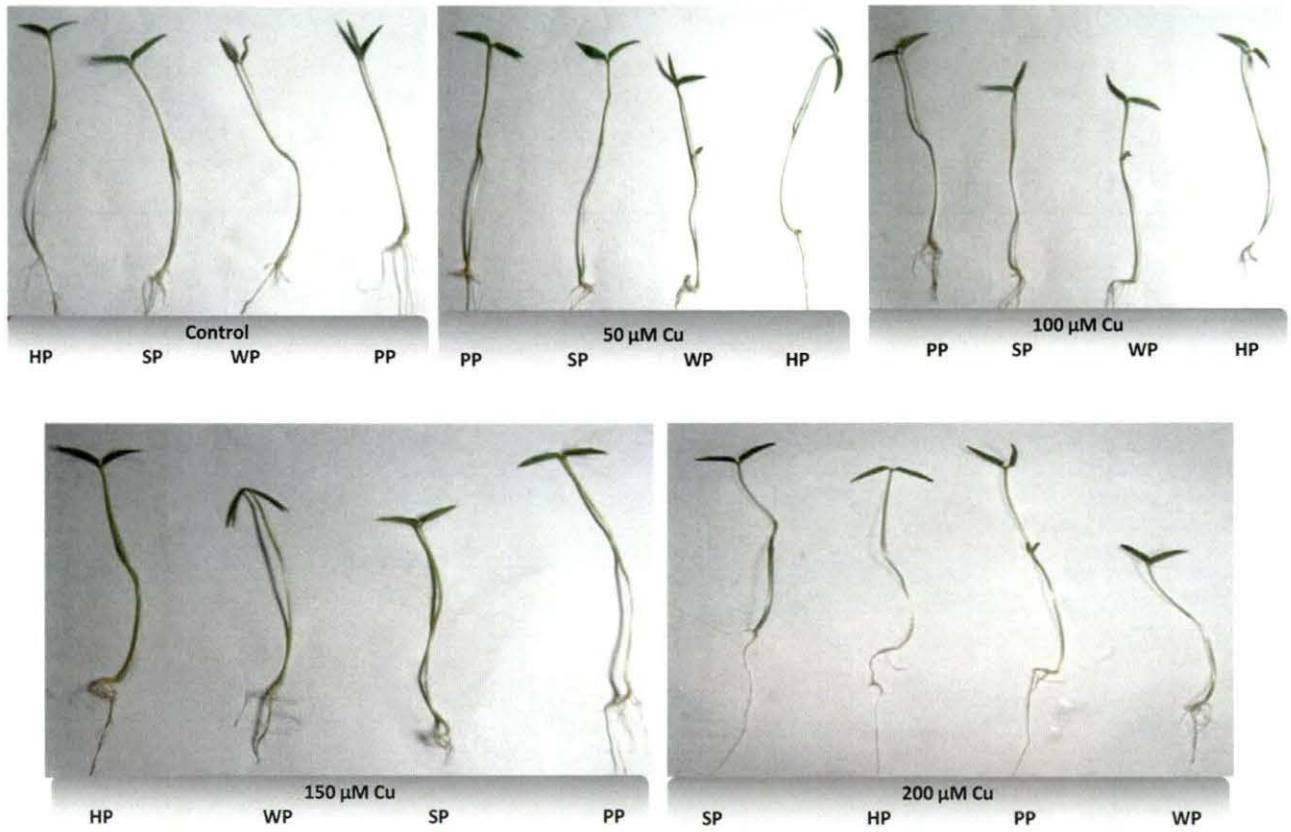

Figure 11.9 Changes of seedling vigour of five-days old *Vigna radiata* plant after treatment with different concentrations of copper salt on seeds primed with water, salicylic acid and peptides; WP= Without priming, PP=Peptide priming, HP=Hydropriming and SP=Salicylic acid priming: **(A)** Untreated control; **(B)** to **(E)** Treated with copper chloride: **(B)** 50 µM; **(C)** 100 µM; **(D)** 150 µM; **(E)** 200 µM

dismutase were reduced or not affected by excess copper (Chen *et al.*, 2000). It was also established that copper induced phytotoxicity and lipid peroxidation is strongly associated with free radicals. As because copper is a transitional metal, copper induced generation of hydroxyl radical and other free radicals are mainly operated through Fenton reaction. Application of non-enzymatic antioxidants like ascorbate, thiourea and sodium benzoate strongly reduced copper mediated phytotoxicity, senescence and lipid peroxidation in rice leaves (Chen *et al.*, 2000).

The story of cadmium and mercury is somewhat different. Both the heavy metals are well known environmental pollutants and induce high level of phytotoxicity even in case of trace of contaminations. Cadmium accumulation causes reduction in photosynthesis, diminishes water and nutrient uptake (Sanita and Gabbrielli, 1999) and results in visible symptoms of injury in plants such as chlorosis, growth inhibition, browning of root tip and finally death (Kahle, 1993). Unlike copper, contrasting results have been reported in case of cadmium induced antioxidative enzyme expression. Cadmium also caused lipid peroxidation suggesting that the tissues suffered from oxidative stress (Chaoui *et al.*, 1997). Cadmium doesn't belong to the group of transitional metals like copper, iron, and zinc which may induce oxidative stress via Fenton type reactions. Accumulation of hydrogen peroxide was observed during cadmium toxicity and that was probably due to inhibition of enzymes involved in H_2O_2 removal like catalase and ascorbate peroxidase (Polle, 2001). Cadmium binds to thiol groups and thereby inactivates thiol-containing enzymes such as glutathione reductase (Creissen and Mullineaux, 1995; Mullineaux and Creissen, 1997). The same inhibition mechanism may be possible for ascorbate peroxidase being sensitive to thiol reagents (Chen and Asada, 1989).

Vigna radiata seedlings grown in presence of 50 to 250 μM Cd^{2+}, Hg^{2+} or Cu^{2+} showed significant growth reduction in both root and shoot lengths along with embryo proper weights after one week, which were also visually distinct (Figure 11.7-11.9). Among three heavy metals, copper induced stress was minimized (Figure 11.9), whereas the stresses imposed by mercury were mostly prominent at concentrations beyond 100 μM (Figure 11.8). In the control, fresh weight of both root and shoot increased linearly over a 96 hours period. Thereafter it gradually reached the plateau phase for next three

days. These three heavy metals under 100 μM concentrations did not affect the growth of either root or shoot significantly over the first 24 hours; next the fresh weight increased linearly with the time, though with lower rates than control. After 5 days of cadmium and mercury treated plants, the visible symptoms of chlorosis and necrotic areas began to be evident at their tips. Roots did not show any apparent damage at 100 μM heavy metal concentrations. Though chlorosis was common but marginal necrosis was not observed in case of copper stress. At higher concentrations of heavy metal (above 100 μM) exposure, growth of the root was more hampered than shoot (Figure 11.3-11.5 A and B). Root fresh weight improved with the increment of 8.4 mg d^{-1} under control growth conditions and at 7 days, root growth has reached up to 58 mm. The treatment with 100 μM of cadmium, mercury or copper decreased root growth from 58 mm to 5, 31 and 3 mm respectively (Figure 11.3-11.5 A). So growth of roots was drastically reduced in respect to control whereas 68%, 30% and 82% retardation of shoot growth was observed after seven days under these heavy metal stresses (cadmium, copper and mercury respectively) (Figure 11.3-11.5 B). If root growth is hampered and number of lateral roots is minimized, ion absorption and uptake of essential ions from soil can be reduced. Decrease in leaf area as revealed from our experiments (Figure 11.3-11.5 C) means hampering of rate of photosynthesis which ultimately affect in production of triose sugars which means that the seedlings are largely dependent on stored carbohydrate present in cotyledons. Weight reduction of cotyledons was also to some extent paralysed under heavy metal stress (Figure 11.6 A, C and E) which means the blockage of energy source from both photosynthesis and stored foods. Priming particularly helps embryo in recovering from this breathless situation.

In all cases of culturing plants with peptides or pre-soaking the seeds with peptides decreased the heavy metal induced phytotoxicity. The beneficial effect of peptides was seen with all growth parameters and was shown to be statistically significant. In case of pre-soaking, seeds were hydro primed, and for the same period, seeds were also primed with peptides and salicylic acids. Both salicylic acid and peptides alleviated the cadmium, copper and mercury induced phytotoxicity in hydroponics culture (Figure 11.3-11.9). As already stated, phytotoxicity of mercury among seedlings was highest; whereas the copper induced toxicity was less significant. Primed seeds

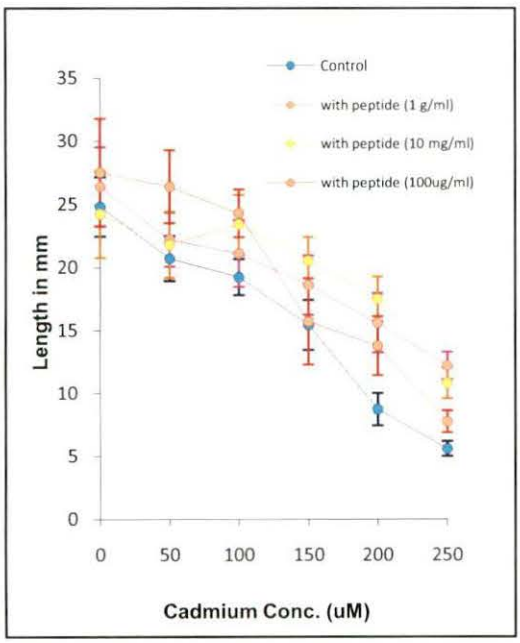

Figure 11.10 Effect of cadmium on root length of mung bean

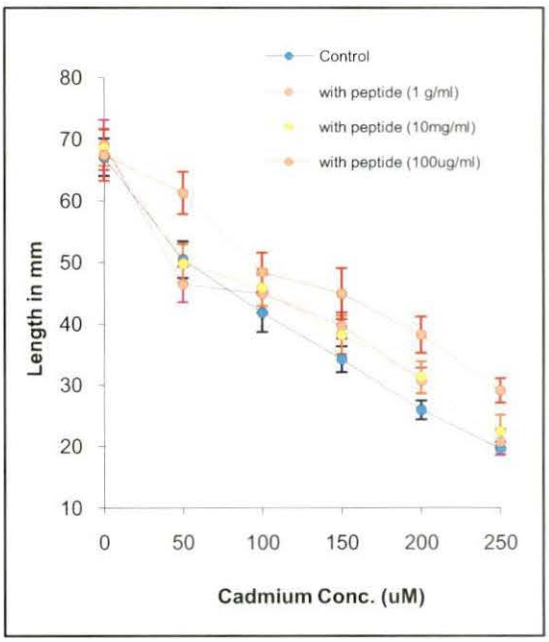

Figure 11.11 Effect of cadmium on shoot length of mung bean

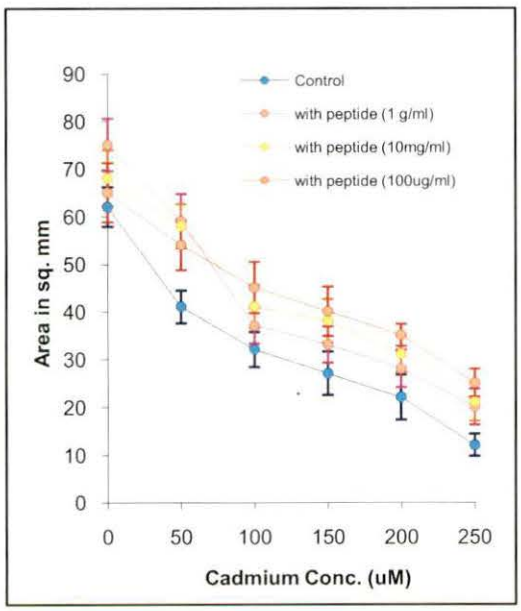

Figure 11.12 Effect of cadmium on leaf area of mung bean

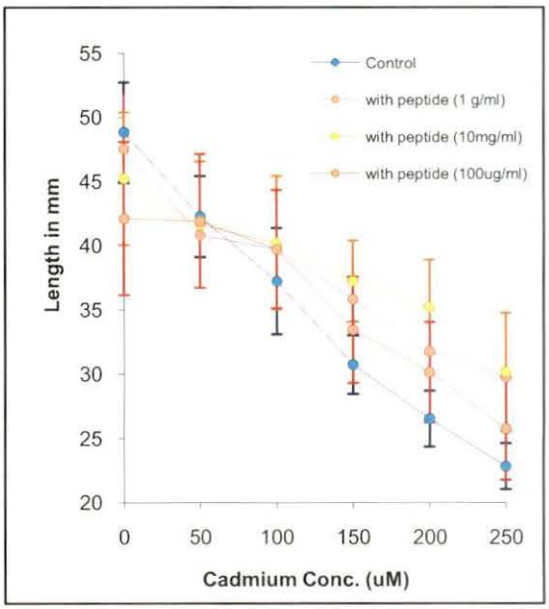

Figure 11.13 Effect of cadmium on hypocotyl length of mung bean

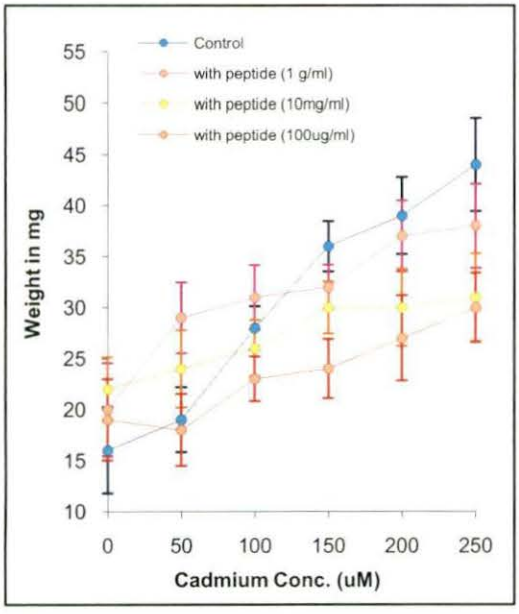

Figure 11.14 Effect of cadmium on cotyledon weight of mung bean

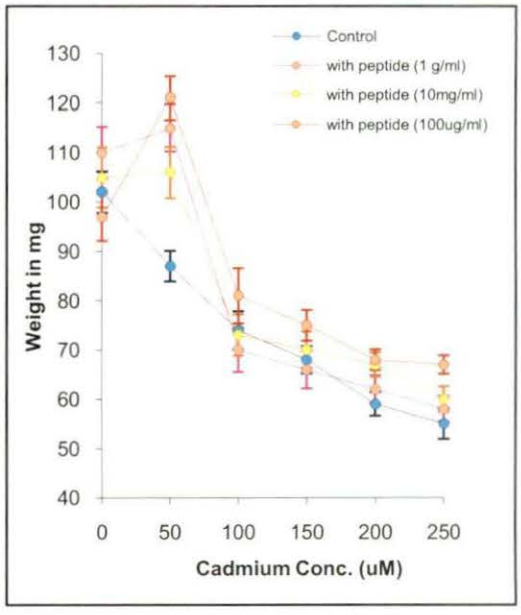

Figure 11.15 Effect of cadmium on embryo proper weight of mung bean

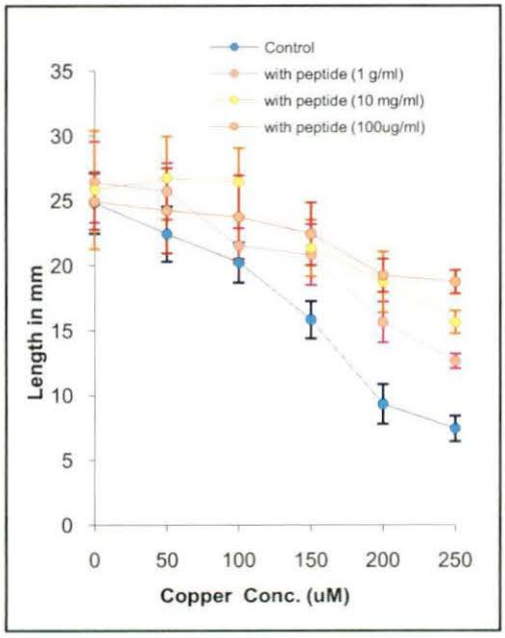

Figure 11.16 Effect of copper on root length of mung bean

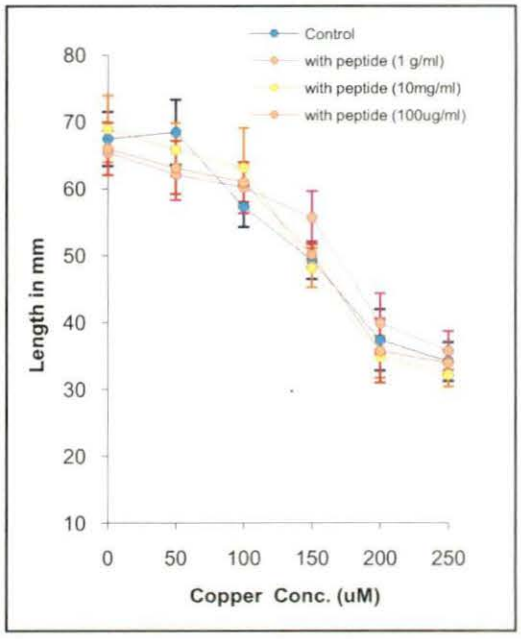

Figure 11.17 Effect of copper on shoot length of mung bean

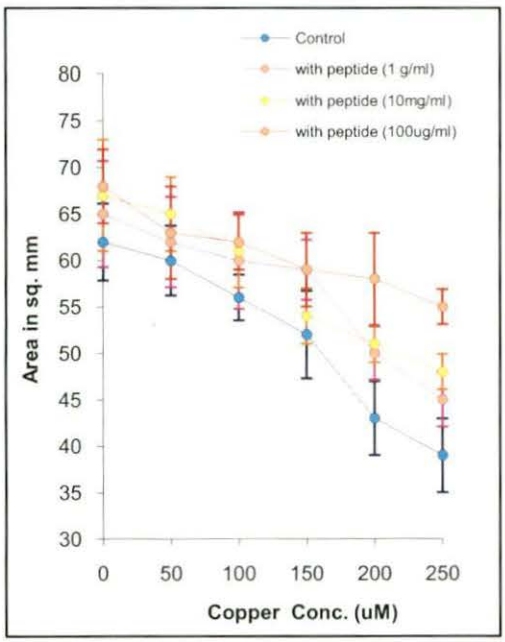

Figure 11.18 Effect of copper on leaf area of mung bean

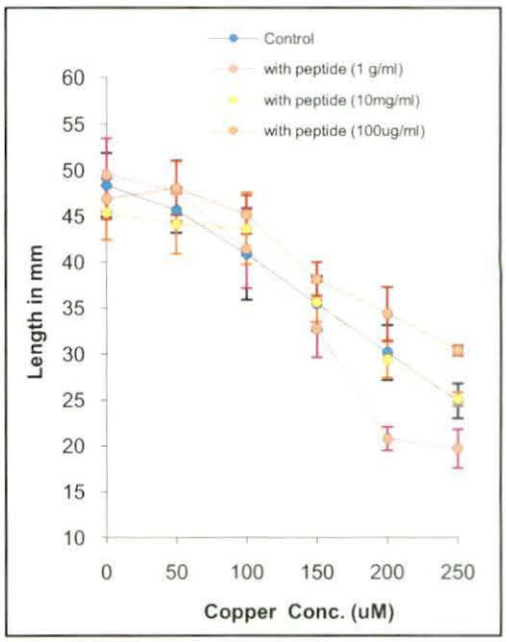

Figure 11.19 Effect of copper on hypocotyl length of mung bean

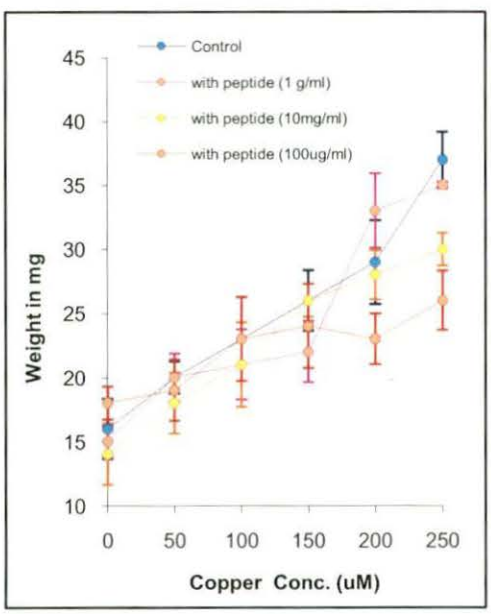

Figure 11.20 Effect of copper on cotyledon weight of mung bean

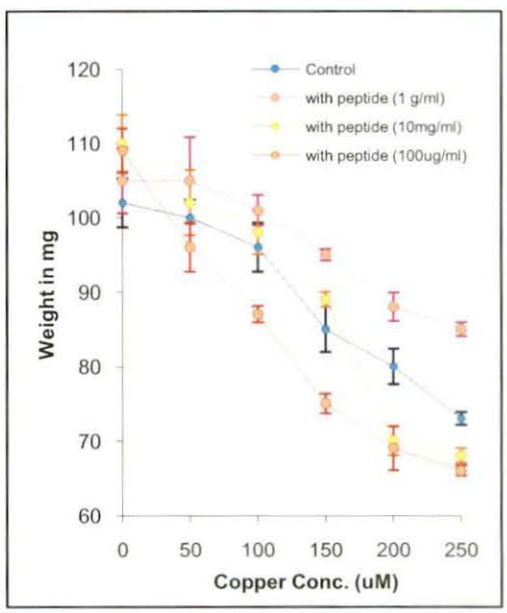

Figure 11.21 Effect of copper on embryo proper weight of mung bean

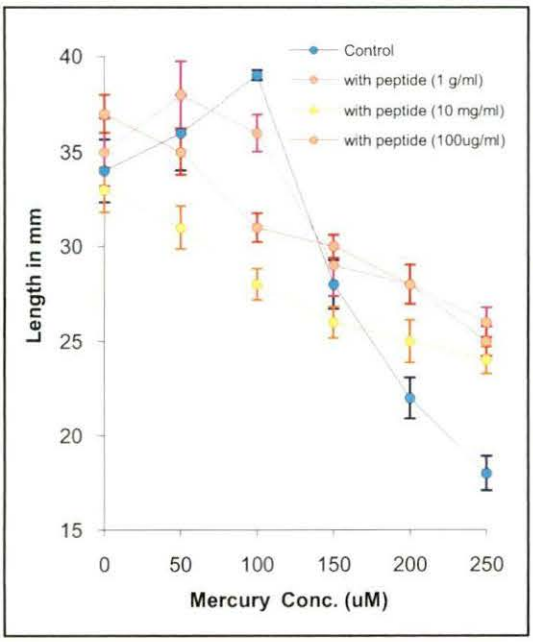

Figure 11.22 Effect of mercury on root length of mung bean

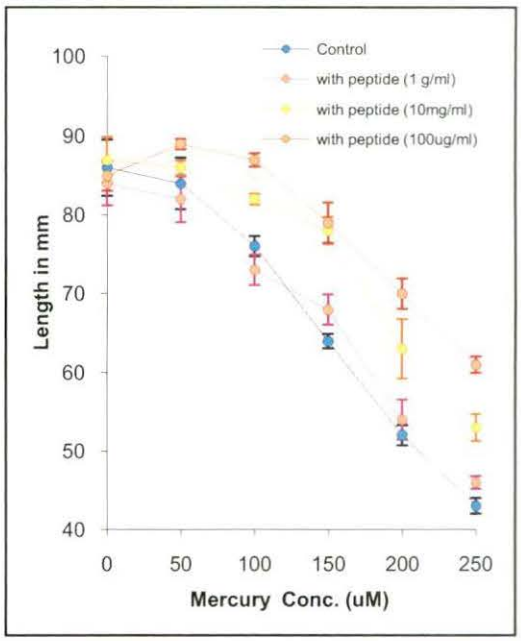

Figure 11.23 Effect of mercury on shoot length of mung bean

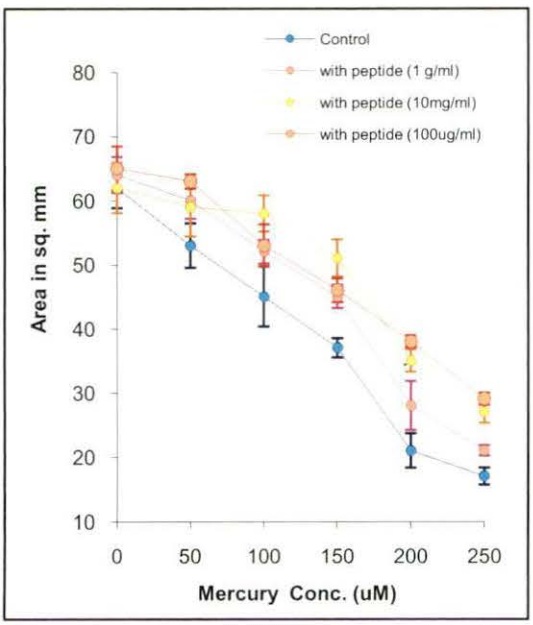

Figure 11.24 Effect of mercury on leaf area of mung bean

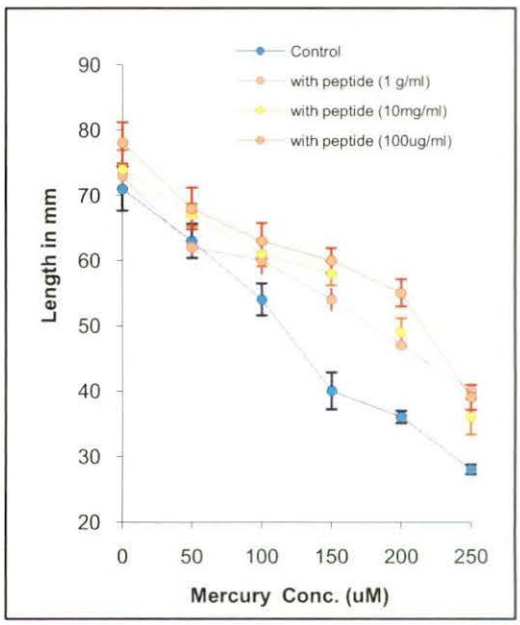

Figure 11.25 Effect of mercury on hypocotyl length of mung bean

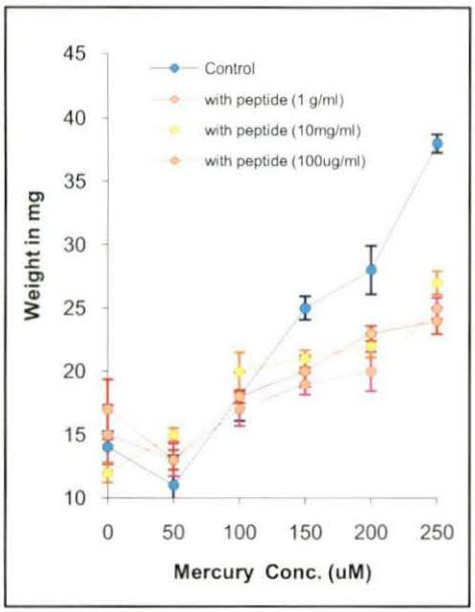

Figure 11.26 Effect of mercury on cotyledon weight of mung bean

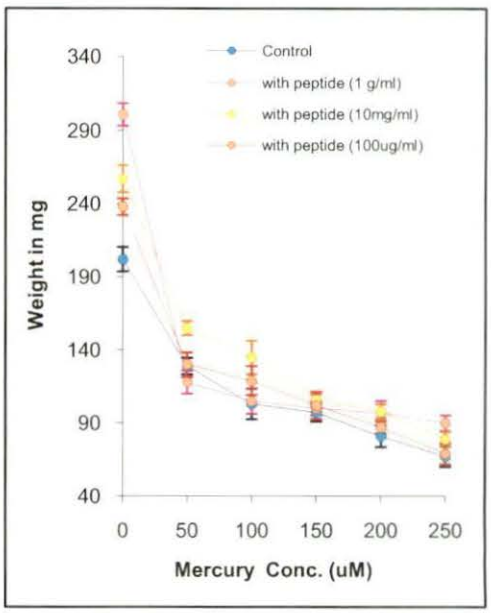

Figure 11.27 Effect of mercury on embryo proper weight of mung bean

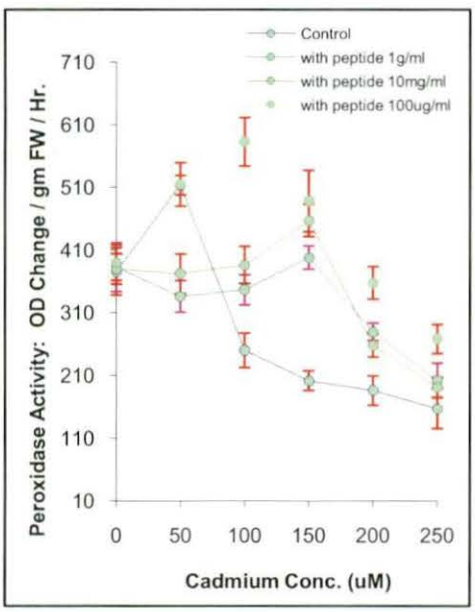

Figure 11.38 Effect of cadmium on peroxidase activity of cotyledon of mung bean

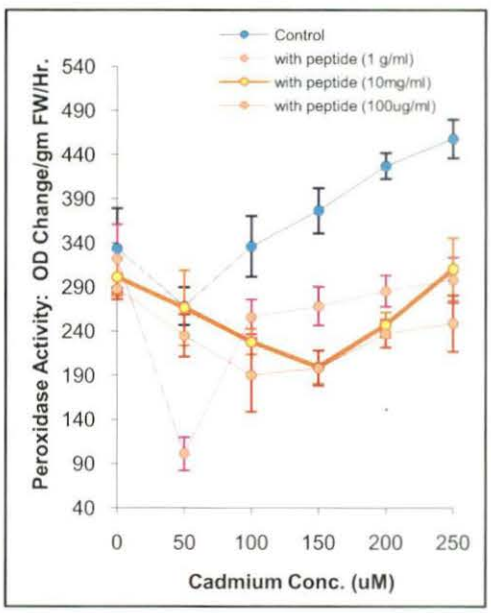

Figure 11.39 Effect of cadmium on peroxidase activity of embryo proper of mung bean

executed significant deviation from unprimed seeds during growth and development of seedlings up to 200 ppm of cadmium, copper and mercury. Seeds under peptide priming at different doses performed best tolerance and their root, shoot and hypocotyls length, lateral root number, leaf area and embryo proper weight was enhanced (Figure 11.3-11.9). Peptide priming with lower doses (10 to 0.1 ppm) significantly improved root and shoot length under cadmium and copper stress (Figure 11.10-11.11 and 11.16-11.17), whereas priming with higher concentrations of peptides (1000 ppm) was required for alleviation of mercury phytotoxicity might be due to higher sensitivity of *Vigna radiata* seedlings towards mercury toxicity (Figure 11.22-11.23). It was also observed that leaf area was remarkably improved under heavy metal toxicity at lower doses of peptide priming (Figure 11.12, 11.18 and 11.24), but for improvement of hypocotyl length under cadmium toxicity, priming with higher dose of peptides were essentially required (Figure 11.13). In all cases of heavy metal toxic situation, cotyledon weight was enhanced with higher dose of metal concentration whereas the reversal of this situation was observed in case of embryo proper, i.e. the embryo proper weight was deteriorated seriously along with higher concentrations of heavy metal treatment (Figure 11.14-11.15, 11.20-11.21 and 11.26-11.27). It may happens that heavy metal toxicity drastically perturb the activity of hydrolytic enzymes present in cotyledons. Therefore the nutrient polymers present in cotyledons might be less utilized by embryo proper which could ultimately hamper the growth of embryo proper. Peptide priming at lower doses improved this situation in case of cadmium (Figure 11.14-11.15) and copper stresses (Figure 11.20-11.21), but here also priming with higher dose of peptides were required for mitigation of mercury toxicity (Figure 11.26-11.27). Embryo proper weight was improved significantly with lower dose of peptide pre-soaking up to 50 ppm toxic concentration of cadmium (Figure 11.15) and up to 250 ppm in case of copper (Figure 11.21), but higher dose of peptides were necessary as pre-soaking agent. Unfortunately, no significant improvement of embryo proper was observed with peptide per-treatment in mercury phytotoxicity (Figure 11.27).

Therefore, it can be assumed that, after peptide priming, seeds were capable for better absorption and survival than unprimed seedlings, which means better physiological acclimation under heavy metal stress. Seed priming before the emergence of radicle trained embryo the strategies of survival in desiccated conditions. When the embryo and

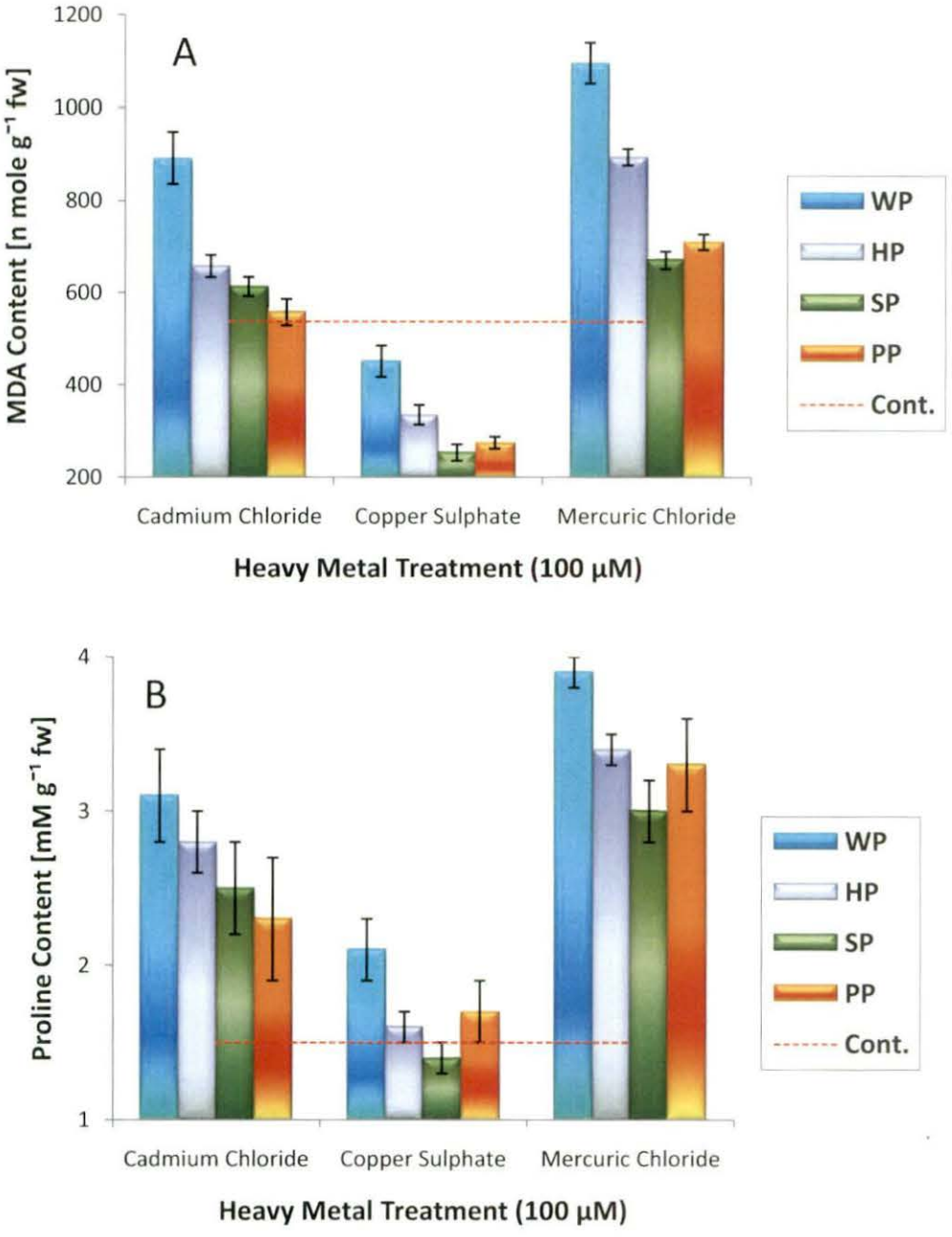

Figure 11.28 Biochemical changes associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment. (**A**) Malondialdehyde content and (**B**) Proline content

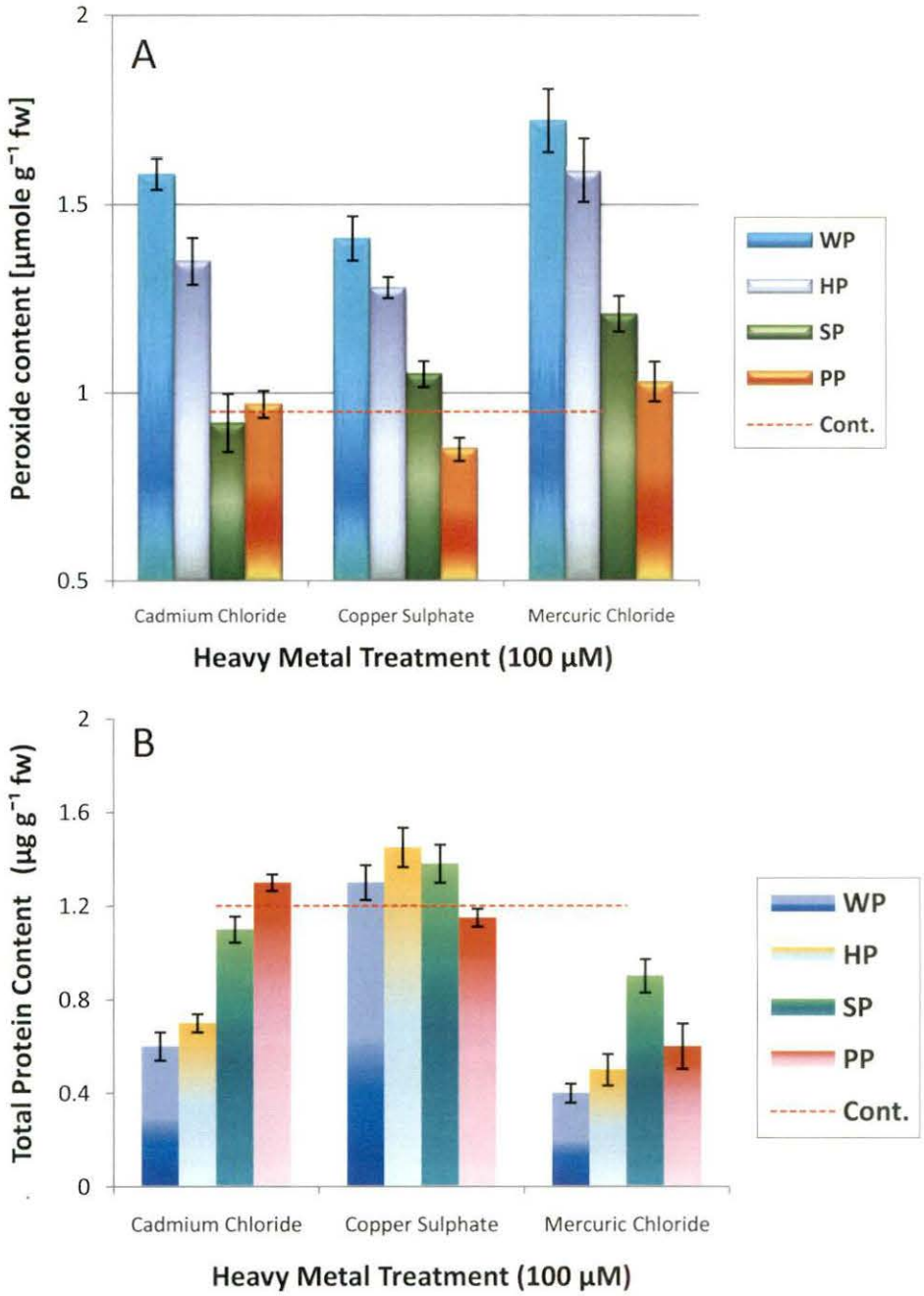

Figure 11.29 Biochemical changes associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment. (**A**) Peroxide content and (**B**) Total soluble protein content

seed is hydrated, the basic programme for seed germination has started and several structural protein and hydrolytic enzymes are released in a planned genetic fashion which is essential for growth and development of embryo. Now when these seeds are going to be dehydrated slowly after hydration for 5-6 hours, the growing embryo feel extreme desiccation stress and the embryo then generate stress tolerant protein like LEA protein, hormone like abscisic acid and antioxidative enzymes, which help the embryo to tolerate against these desiccation. The survived embryo can able to manage the extreme environment in heavy metal toxicity in a better reformed fashion.

Now the basic understanding of heavy metal induced toxicity and tolerance in plants is related to the experimental design performed in this section and the available reports from existing literature performed by different authors in different plants. Any types of stress fundamentally induce oxidative stress in plant system. Even cadmium and mercury, which are not transitional metals, may cause oxidative stress. Enhancement of lipid peroxidation and accumulation of malonaldehyde (MDA) particularly proves this. In this investigation, the MDA accumulation which was observed during stress conditions was partially minimized after priming indicating lesser lipid peroxidation (Figure 11.28 A). Cadmium induced malonaldehyde accumulation was also proved in Scots pine roots (Schutzendubel and Polle, 2002), supporting our opinion. Proline was generally accumulated in higher amount during stress conditions and may be designated as oxidative stress markers. In case of heavy metal induced phytotoxicity, proline accumulation was observed in unprimed seedlings, whereas after priming proline content in seedlings was reduced (Figure 11.28 B). The detoxification of oxygen free radicals may be achieved through several paths. One of the metabolic fates of this active oxygen is the generation of hydrogen peroxide. In our experiments, peroxide content was significantly accumulated during heavy metal stress conditions, but priming particularly with salicylic acid and peptides significantly reduced this peroxide accumulation in seedlings (Figure 11.29 A). In association with improved oxidative homeostasis after priming, total protein content also enhanced predominantly after salicylic acid and peptide pre-treatment (Figure 11.29 B). This hydrogen peroxide may induce phenyl propanoid path and accumulate polyphenols and flavonoids which may take part in redox reactions. Lignification is another manifestation of this process. During heavy metal

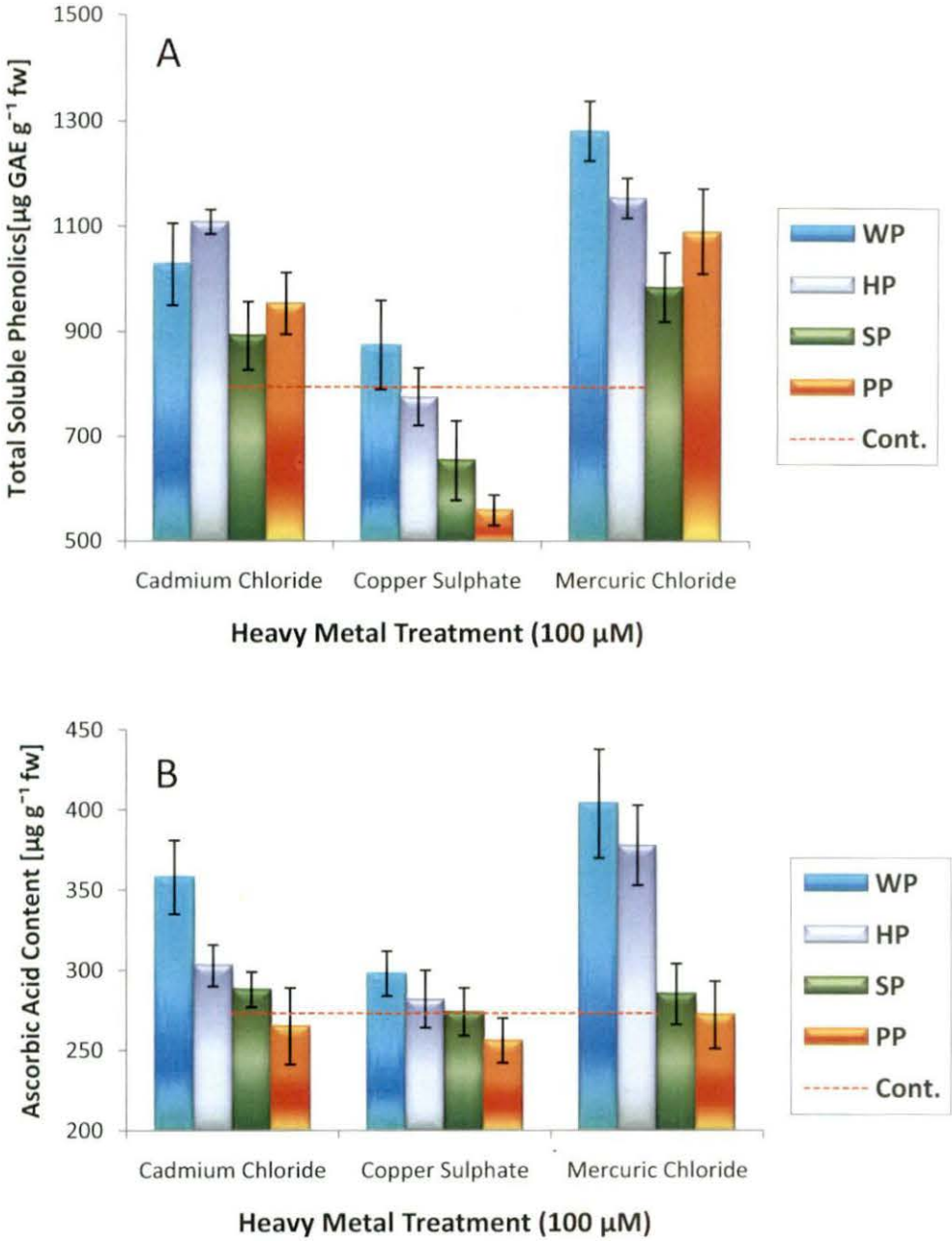

Figure 11.30 Biochemical changes associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment. (**A**) Total soluble phenolics content and (**B**) Ascorbic acid content

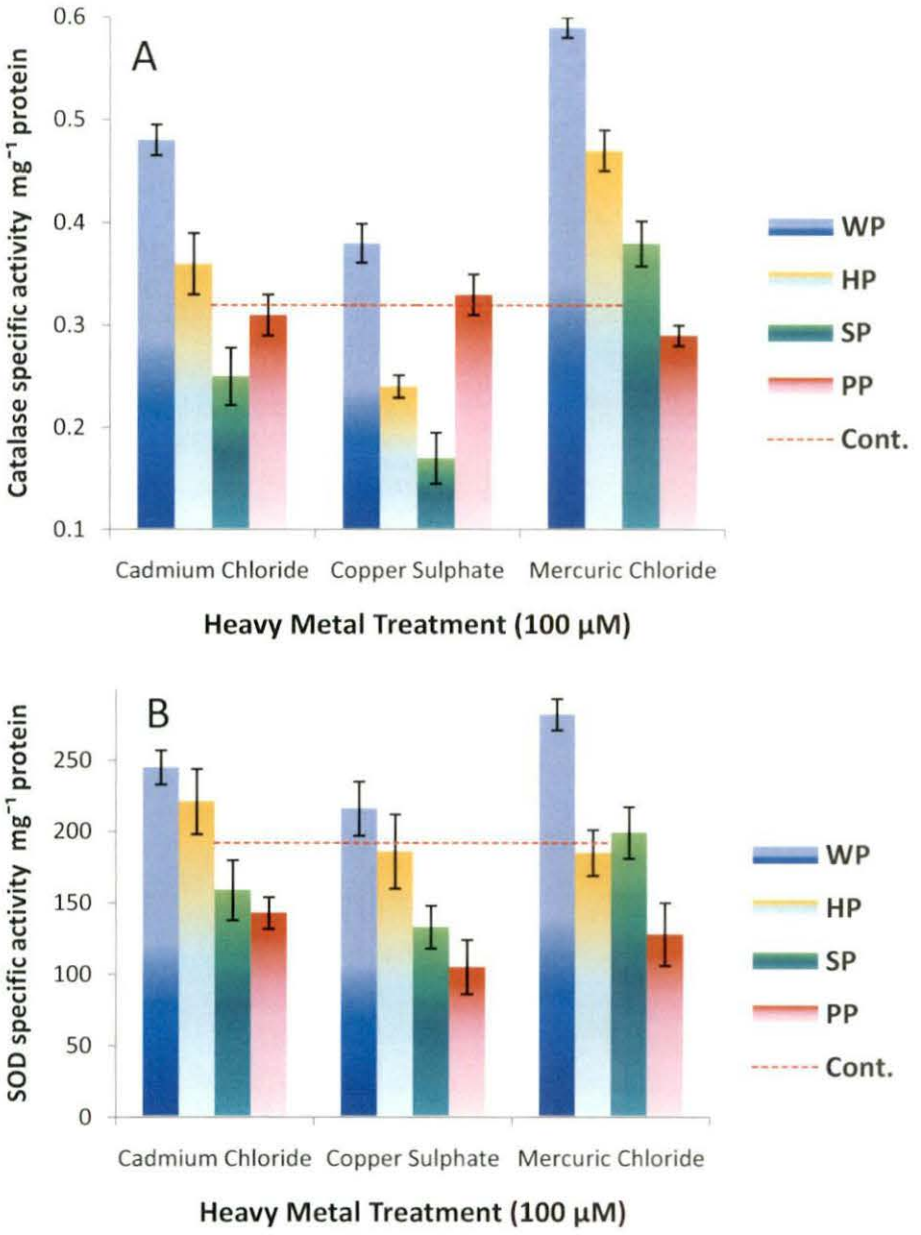

Figure 11.31 Enzymological changes associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment. (**A**) Catalase activity and (**B**) Superoxide dismutase activity

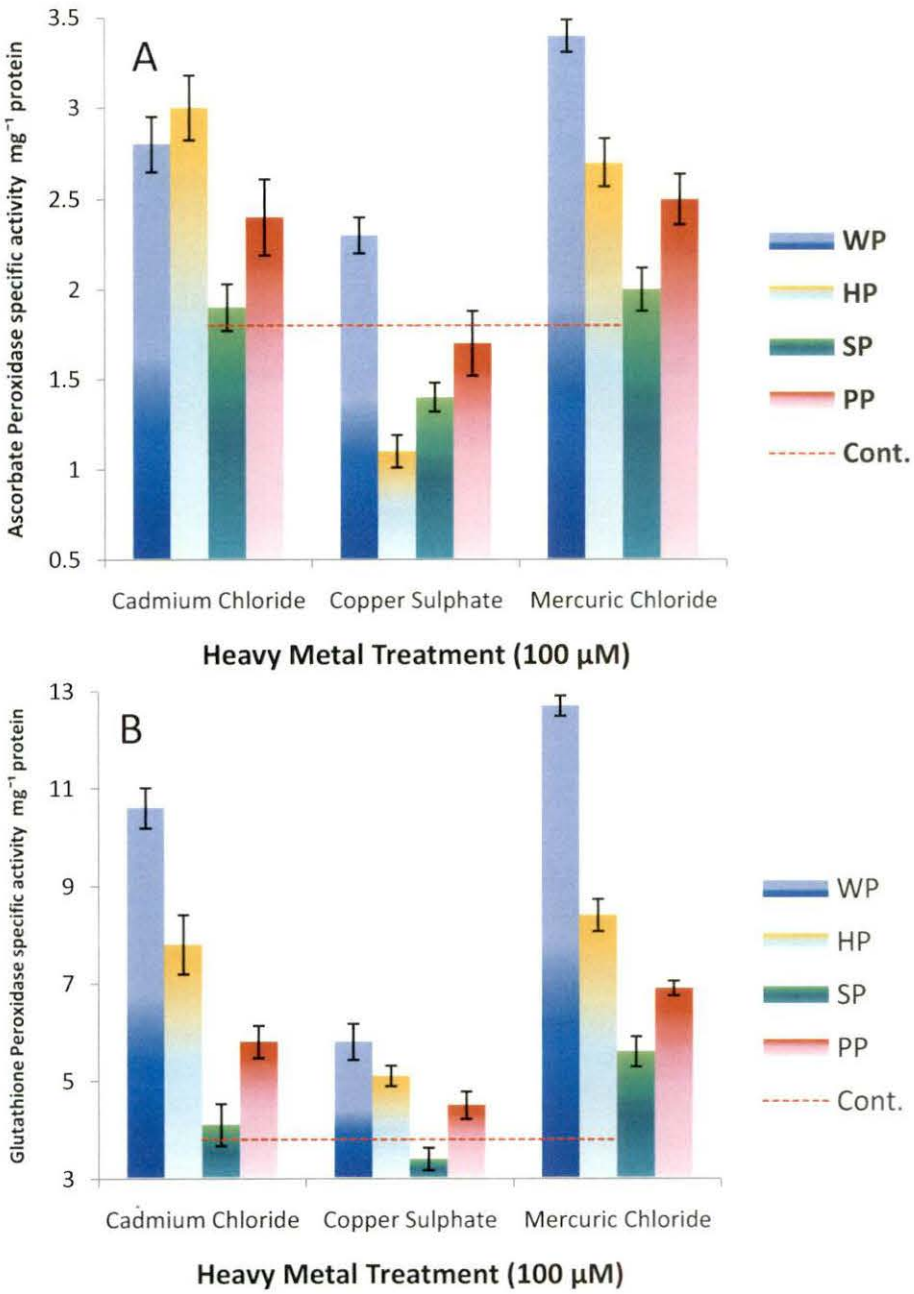

Figure 11.32 Enzymological changes associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment. (**A**) Ascorbate peroxidase activity and (**B**) Glutathione peroxidase activity

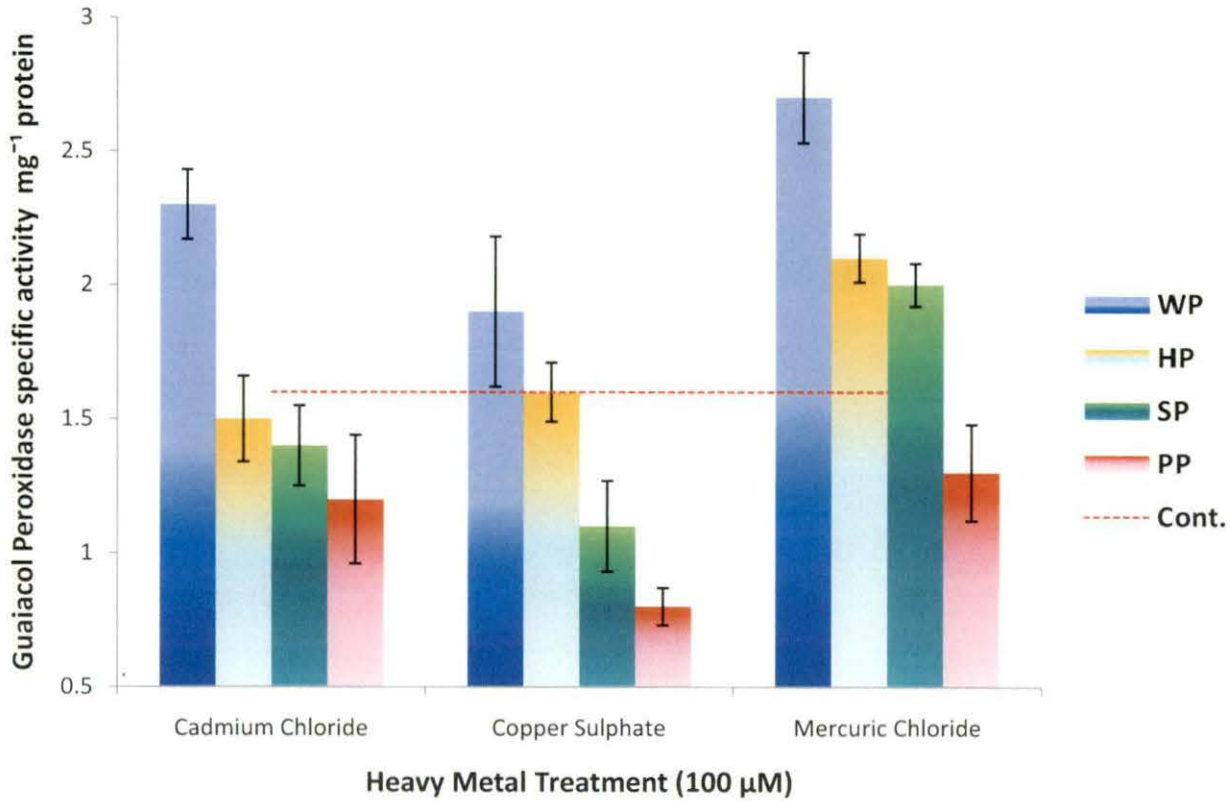

Figure 11.33 Changes of guaiacol peroxidase activity associated with heavy metal (treated with cadmium chloride, copper sulphate and mercuric chloride) stress: Before treatment, seeds were either non-primed (WP) or primed with water (HP i.e. hydropriming), salicylic acid (SP) or peptides isolated from heavy metal induced seedlings (PP); Cont = control i.e. without heavy metal treatment.

induced phytotoxicity, soluble phenolics and ascorbic acid, which was considered as the reservoir of non-enzymatic antioxidants enhanced sharply in unprimed seedlings but these antioxidant reservoir was declined after priming (Figure 11.30 A and B), indicating that their essentiality was reduced after priming and the tolerance mechanism of seedlings was somewhat different. Generation of reactive oxygen species (ROS) is the obvious fate of heavy metal stress. The enzymes like catalase, superoxide dismutase (SOD), Ascorbate peroxidase, glutathione peroxidase and guaiacol peroxidase can scavenge this metabolically active oxygen and oxidative stress may be neutralized through this path. *Vigna radiata* seeds primed with water, salicylic acid and peptides couldn't induce catalase, peroxidase and SOD permanently, though transient stimulation of these enzymes were observed in non-primed seedlings with 100 μM Cd^{2+}, Cu^{2+} and Hg^{2+} concentrations (Figure 11.31-11.33). Ascorbate and glutathione peroxidase activity is mostly induced in hydro- and peptide primed seedlings and this induction persists with higher dosage of mercury stress (Figure 11.32 A and B). In salicylic acid primed seedlings, all peroxidase activity was reduced in respect to control during stress conditions (Figure 11.32 A and B; Figure 11.33). But most interestingly, the activity of guaiacol peroxidase and SOD was drastically reduced in respect to control after peptide priming even under sub-acute lethal dose of heavy metal toxicity (100 μM) (Figure 11.31 B and 11.33).

For determining the association among morphometric, biochemical and enzymological attributes during heavy metal induced phytotoxicity with or without priming, principal component analysis (PCA) was performed. In PCA loading plots, PC1 and PC2 explained 89.58% of the variables. Figure 11.34 illustrates that the morphometric characters (red dots) along with biochemical (green dots) and enzymological attributes (blue dots) were mainly loaded on PC1 in opposite direction and their positions were far beyond zero. This indicates that they were inversely correlated with each other, *i.e.* the reduction of morphometric characters in segment-A was mainly consistent with the increment of biochemical attributes or the activity of antioxidant enzymes, principally present in segment-F (Figure 11.34). Figure 11.35 shows the association of morphometric characters more precisely, which is actually magnified view of segment A, where representation of each dot is explained. Similarly, dots represented

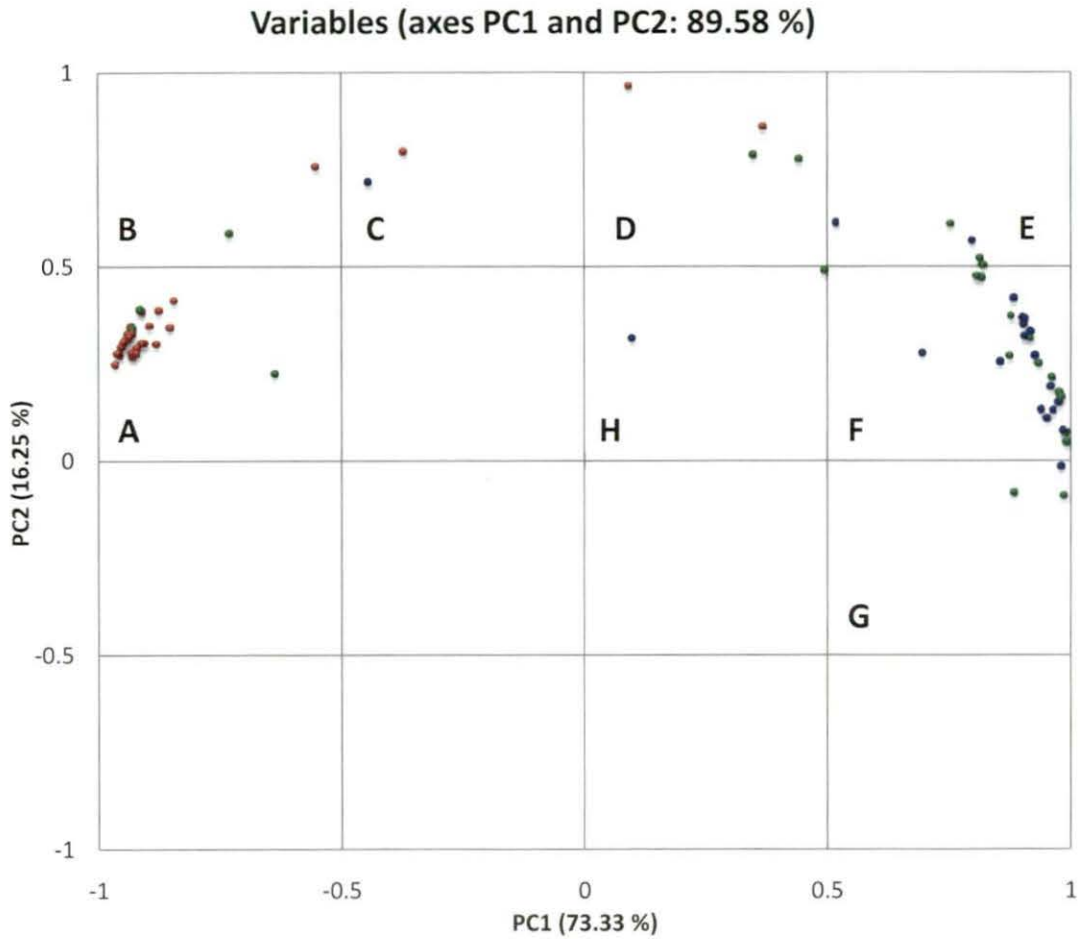

Figure 11.34 PCA factor loading plot of morphometric (red), biochemical (green) and enzymological (blue) attributes of heavy metal induced oxidative stress after priming

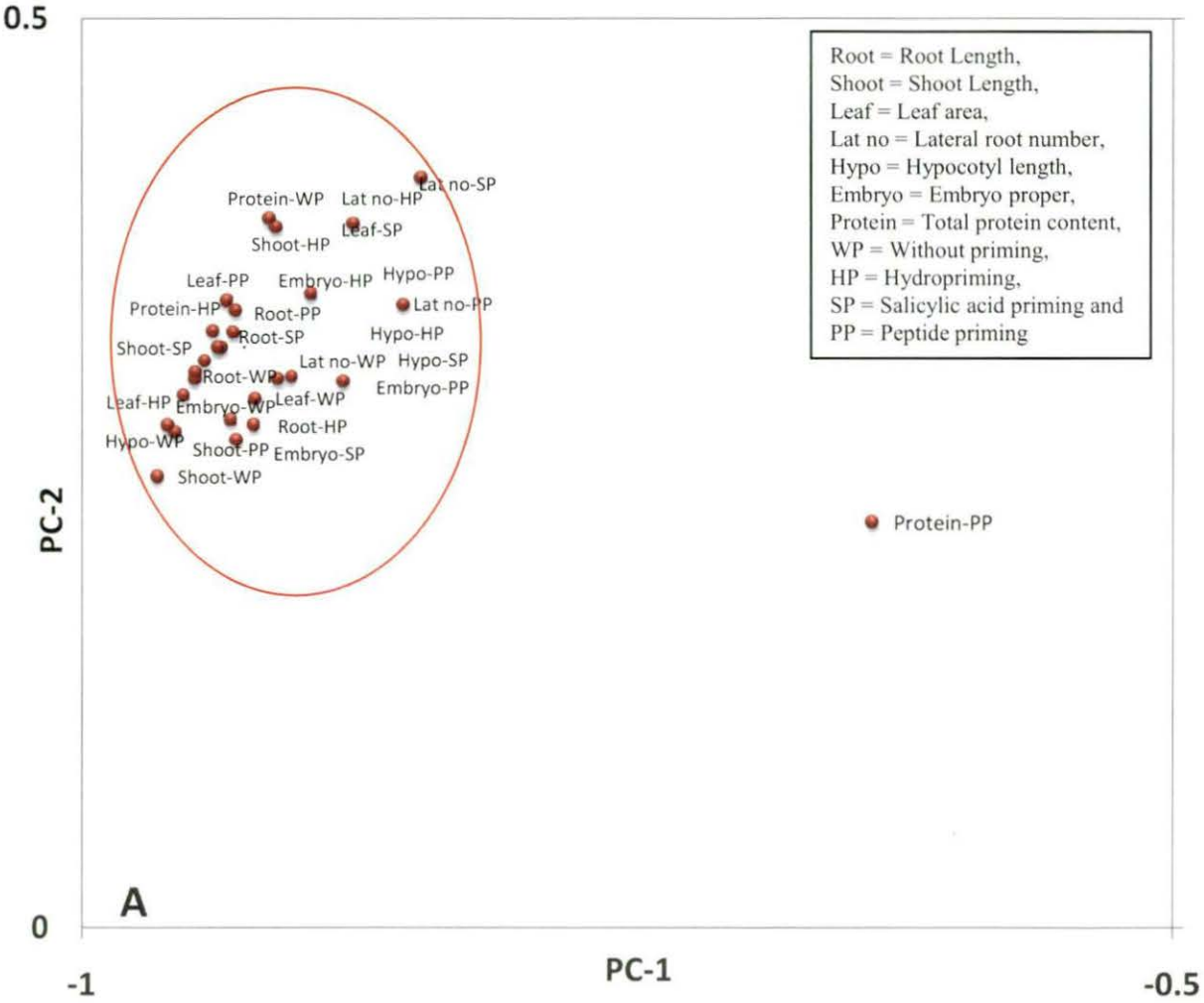

Figure 11.35 Factor loading components of 'A' block and their clustering pattern, mainly attributed with morphometric parameters

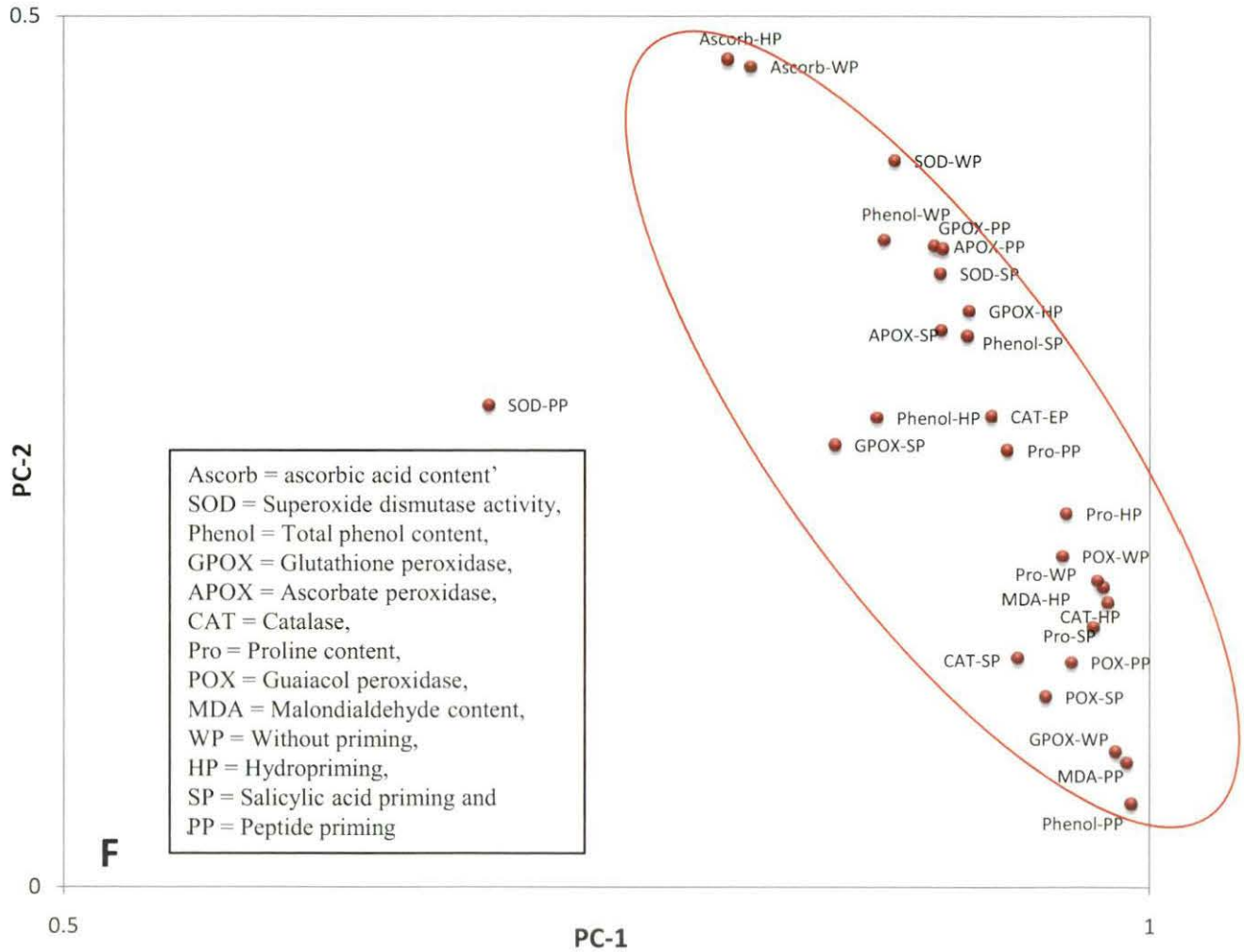

Figure 11.36 Factor loading components of 'F' block and their clustering pattern, attributed with biochemical and enzymological parameters

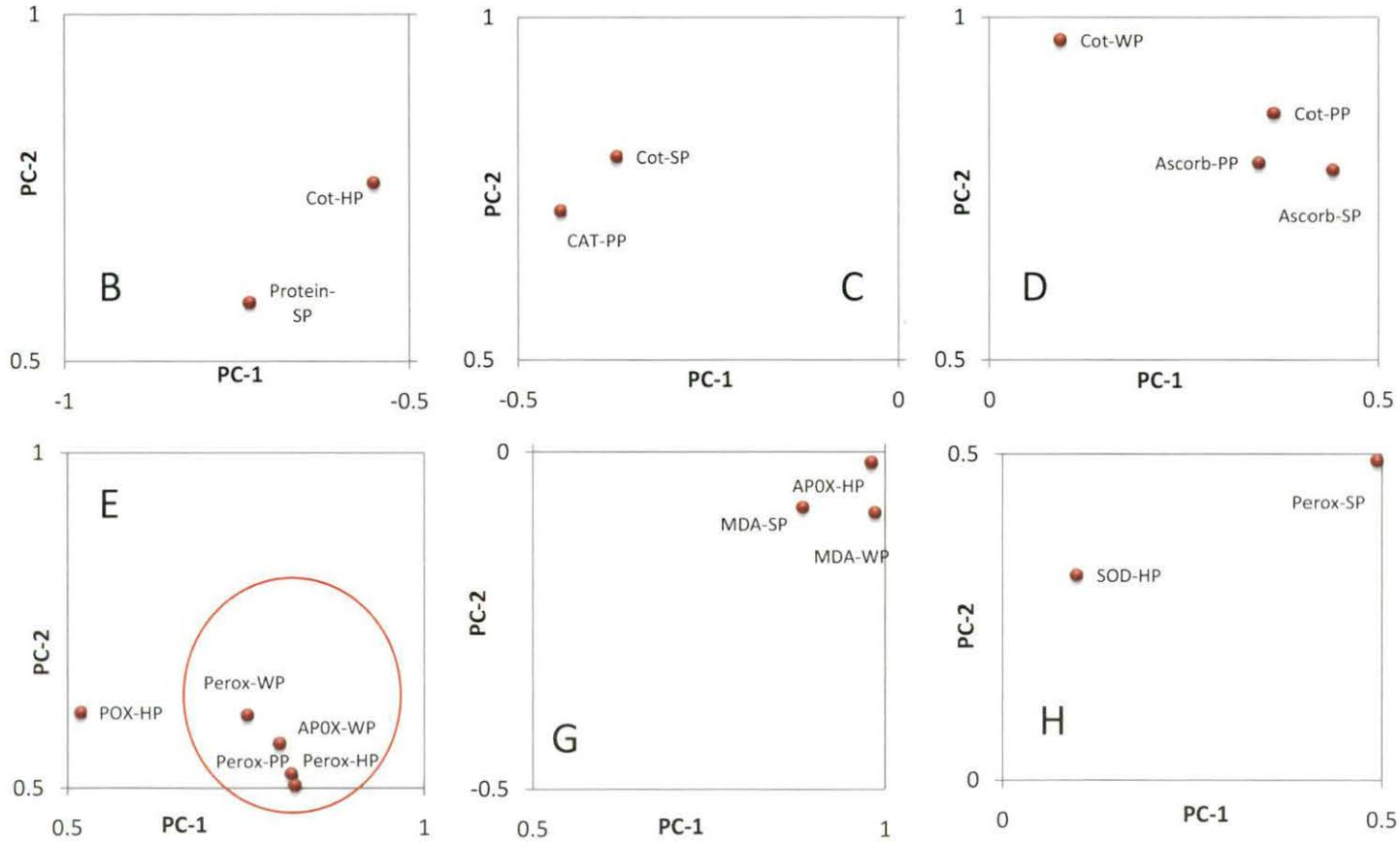

Figure 11.37 Factor loading components of 'B', 'C', 'D', 'E', 'G' and 'H' blocks and their clustering patterns, attributed with morphometric, biochemical and enzymological parameters. Cot = Cotyledon pair weight, Protein = Total protein content, CAT = Catalase activity, Ascorb = Ascorbic acid content, Perox = Peroxide content, APOX = Ascorbate peroxidase, POX = Guaiacol peroxidase, MDA = Malondialdehyde content, SOD = Superoxide dismutase activity.

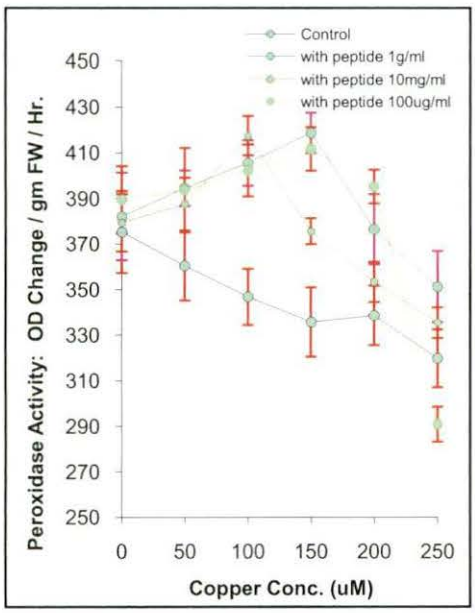

Figure 11.40 Effect of copper on peroxidase activity of cotyledon of mung bean

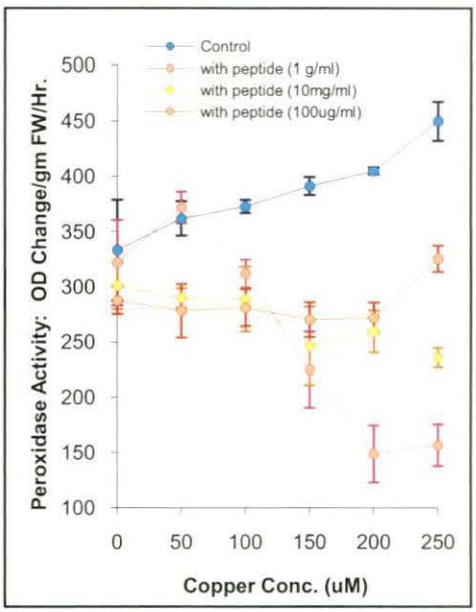

Figure 11.41 Effect of copper on peroxidase activity of embryo proper of mung bean

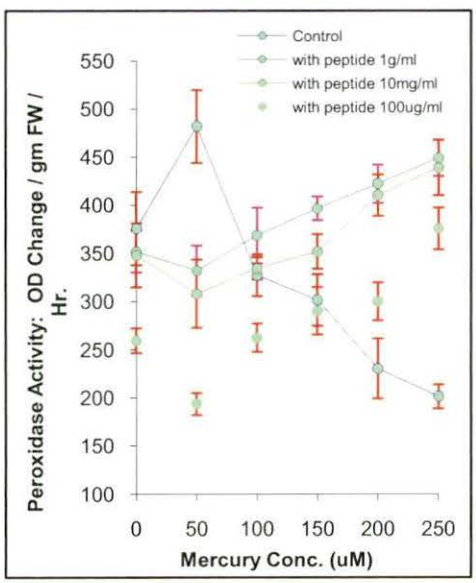

Figure 11.42 Effect of mercury on peroxidase activity of cotyledon of mung bean

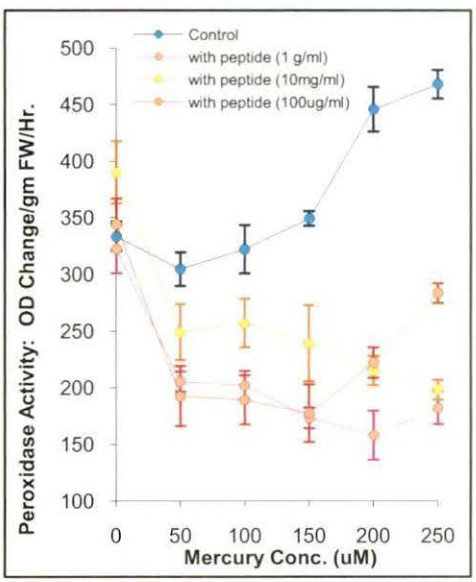

Figure 11.43 Effect of mercury on peroxidase activity of embryo proper of mung bean

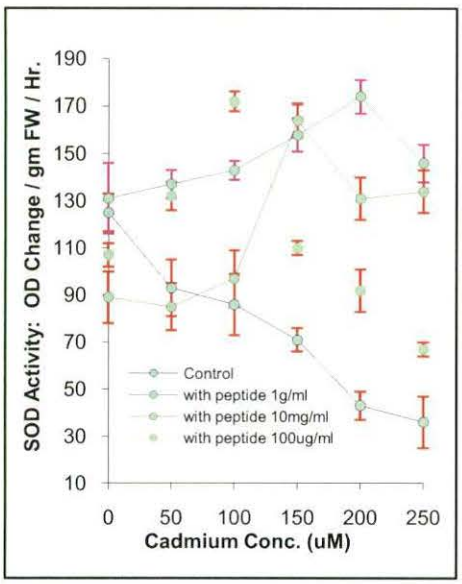

Figure 11.44 Effect of cadmium on SOD activity of cotyledon of mung bean

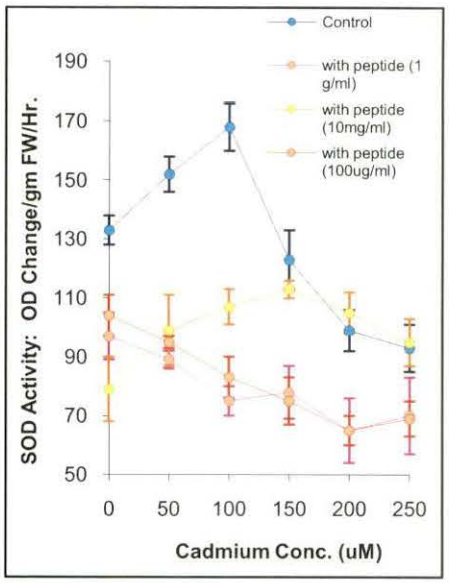

Figure 11.45 Effect of cadmium on SOD activity of embryo proper of mung bean

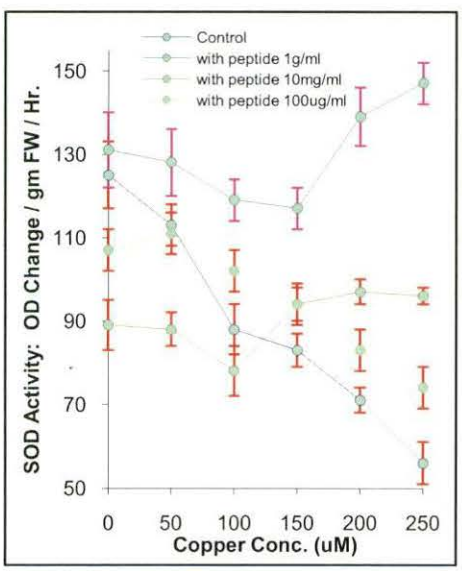

Figure 11.46 Effect of copper on SOD activity of cotyledon of mung bean

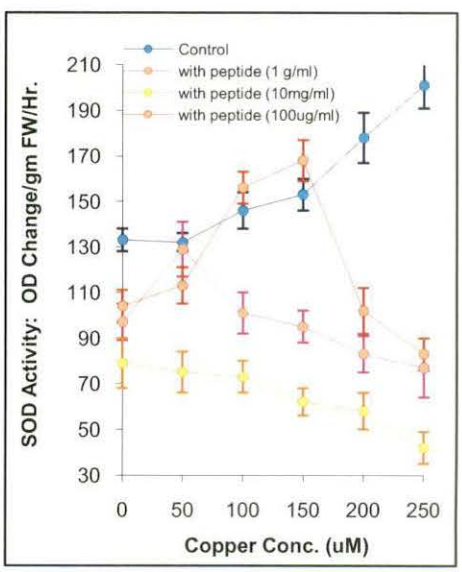

Figure 11.47 Effect of copper on SOD activity of embryo proper of mung bean

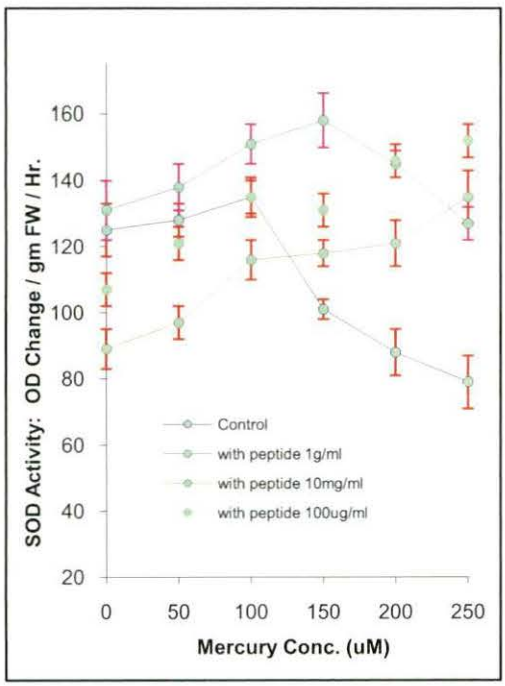

Figure 11.48 Effect of mercury on SOD activity of cotyledon of mung bean

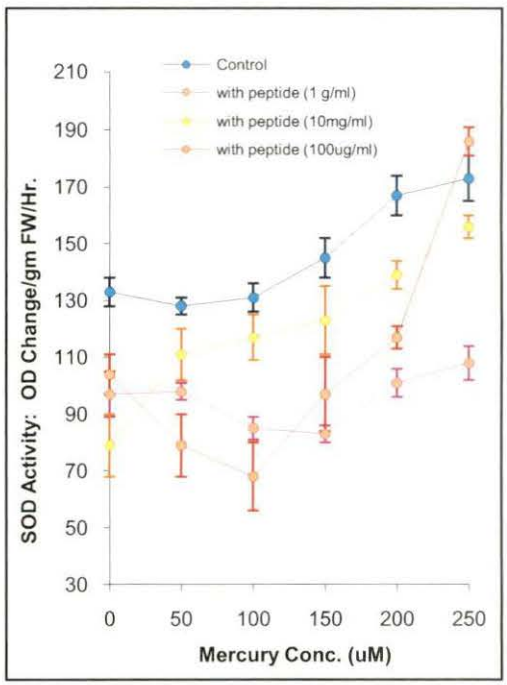

Figure 11.49 Effect of mercury on SOD activity of embryo proper of mung bean

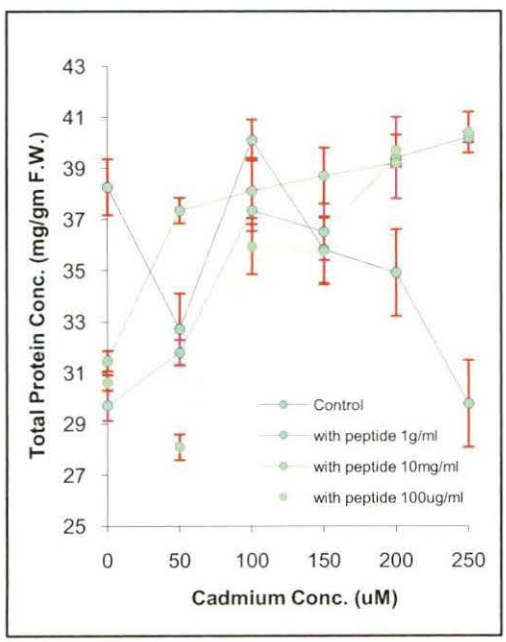

Figure 11.50 Effect of cadmium on total protein concentration of cotyledon of mung bean

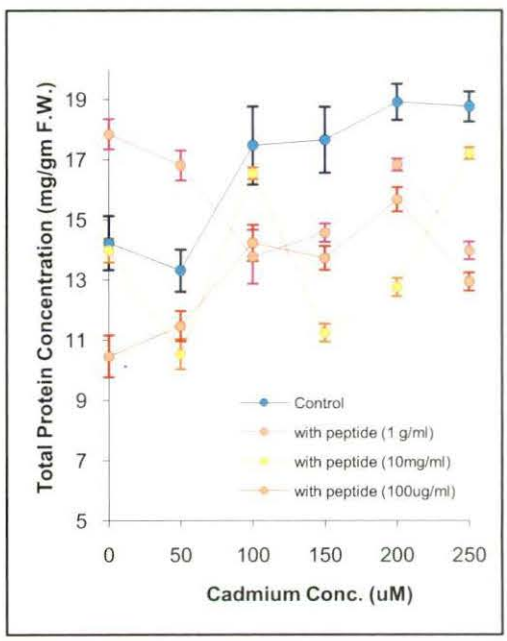

Figure 11.51 Effect of cadmium on total protein concentration of embryo proper of mung bean

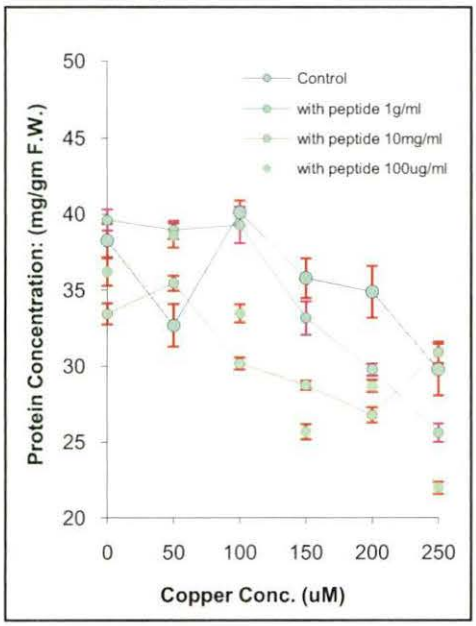

Figure 11.52 Effect of copper on total protein concentration of cotyledon of mung bean

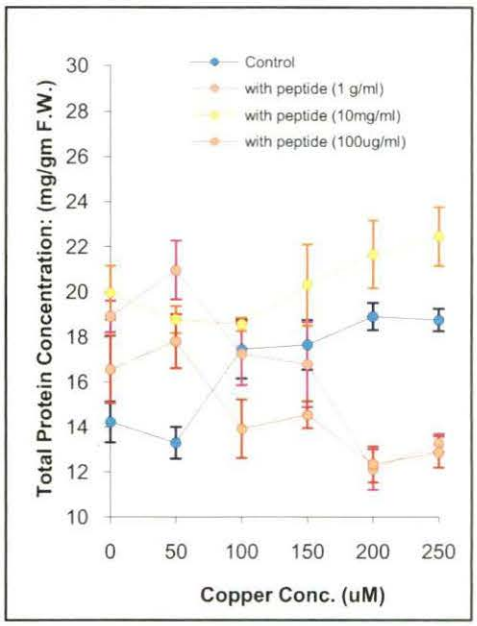

Figure 11.53 Effect of copper on total protein concentration of embryo proper of mung bean

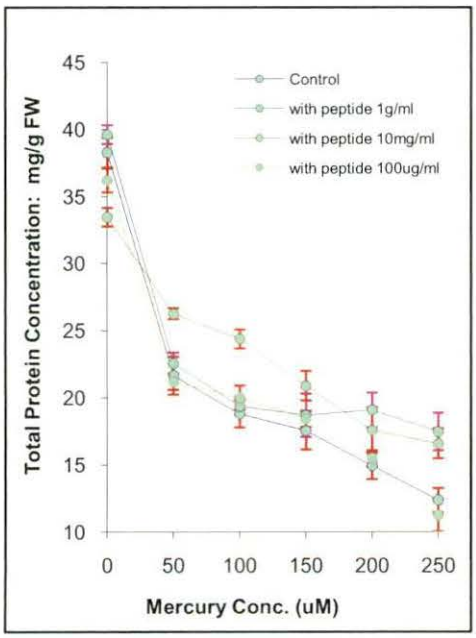

Figure 11.54 Effect of mercury on total protein concentration of cotyledon of mung bean

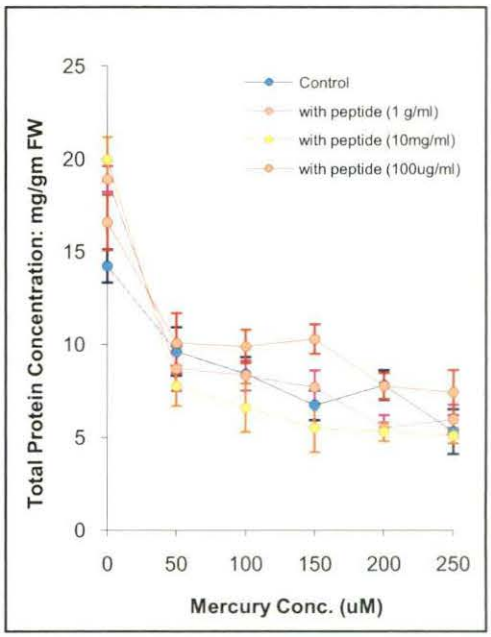

Figure 11.55 Effect of mercury on total protein concentration of embryo proper of mung bean

in segment F were clearly illustrated in Figure 11.36, where every biochemical and enzymological attributes were clustered except the dots representing SOD activity after peptide priming. Figure 11.37 illustrated the dots present in B, C, D, E, G and H. In these segments attributes were mainly scattered and have almost no relationship with heavy metal induced oxidative stress.

For detailed analysis of key-point of tolerance mechanism after peptide priming, cotyledons were separated from embryo proper and mainly two enzymes viz. guaiacol peroxidase and SOD, which exhibited unexpected low activity after priming even at sub-acute toxicity were further studied. Interestingly, both guaiacol peroxidase and SOD was not induced under heavy metal stress situation in embryo proper of peptide primed seedlings (Figure 11.39, 11.41, 11.43, 11.45, 11.47 and 11.49). Only in case of copper-induced phytotoxicity, peptide priming partially enhanced peroxidase in embryo proper up to 50 μM copper treatment (Figure 11.41). But significant enhancement of both peroxidase and SOD was noticed in cotyledons after peptide priming. Considerable induction of peroxidase was observed with lower dose of peptide pre-soaking (Figure 11.38, 11.40 and 11.42), whereas priming with higher doses exhibited better pattern of induction of SOD in mung bean cotyledons (Figure 11.44, 11.46 and 11.48). The contrasting behaviour of this induction pattern in cotyledon and embryo proper may explain differential oxidative stress response in these two organs during heavy metal stress. In untreated seedlings, the oxidative stress was equally imposed on both cotyledon and embryo proper, as revealed from higher induction of both peroxidase and SOD in these two organs. From our experiments, it can be speculated that peptide priming mitigates the oxidative stress situation of embryo proper by enhancing both peroxidase and SOD in cotyledons, which also acts as reservoir of nutrients for embryo proper during early stage of its development. The above results were also corroborated with enhancement of total protein content in cotyledons (Figure 11.50, 11.52 and 11.54) after peptide priming during metal induced toxicity and reversal of this action in case of embryo proper (Figure 11.51, 11.53 and 11.55). Peroxidase is reduced with increased dose of mercury stress in case of peptide primed seedlings, because it may be assumed that mercury can be removed from cytoplasm either by enhancing the transport of mercury into vacuoles through direct thiol based phytochelatin and/or metallothionin

mediated phytochelation system or mercury can also be excluded from cytoplasm through carrier mediated external pumps. In barley seedlings, permanent induction of catalase, guaiacol peroxidase and ascorbate peroxidase was observed after cadmium administration (Metwally *et al.,* 2003). In this study, as stated earlier, when the seedlings were directly cultured with heavy metal induced peptides in association with heavy metals, the stress enzymes like peroxidase and SOD were more active in cotyledons in respect to control whereas in embryo proper, reverse situation was recorded. This phenomenon stimulates the idea of spatial partitioning of oxidants in cotyledon and embryo proper. It may also happen that the heavy metals are mainly concentrated in cotyledons and embryo proper is secured from generation of toxicity. But increased antioxidant enzymatic defence appears not to be principally involved for the alleviation of heavy metal induced toxicity in salicylic acid and peptide treated plants. General inhibition of antioxidative enzyme defence system was observed after salicylic acid and peptide priming (Figure 11.31-11.33). Results from lipid peroxidation indicate that the malonaldehyde accumulation was lesser in primed seedlings after heavy metal stress exposure (Figure 11.28A). Also, the endogenous concentration of H_2O_2, which is considered to be a signalling intermediate in programmed cell death (Alvarez and Lamb, 1997), was comparatively lower after priming (Figure 11.29A). Reduced rate of uptake of Evans Blue by root tips in respect to control indicates that the cell death is significantly lesser in primed seedlings (Alvarez and Lamb, 1997).

Taking together these results suggest that the oxidative stress can be effectively managed through priming. More than one hypothetical explanation may account for the positive peptide priming effect on heavy metal challenged mung bean seedlings. Peptide signalling may activate heavy metal tolerance mechanism different from antioxidative defence. One mechanism is avoidance from damage and includes any mechanisms of heavy metal binding resulting in lowered plasmatic free toxic metal cations- this may be achieved through accumulation signalling of proteins like Phytochelatin and other low molecular mass metabolites and peptides like metallothionins that could be involved in heavy metal binding (Wong and Cobbett, 2009). Signalling that had passed through peptide priming could also enhance and stimulate the expression of certain ABC transporters. In case of cadmium, such transporters have been implicated in the vacuole

sequestering of the products of cadmium atom rather than cadmium itself (Rea *et al.*, 1998). During heavy metal stress, growth retardation of shoot and root is observed and this may be due to higher accumulation of H_2O_2. H_2O_2 in plant system occurs as a part of normal developmental programme and strictly controlled in committed cells during xylogenesis (Teichmann, 2001). The addition of H_2O_2 in seedlings leads to increased mechanical strength and lowers the extensibility of cell wall through oxidative cross linking of cellulose microfibrils. Such a rapid H_2O_2 mediated rigidification of cell wall would explain fast abolishing of growth that occurs within 12 hours and activation of peroxidase along with lignification by 24 hours of cadmium application (Schutzendubel *et al.*, 2001). Induction of phenyl propanoid path and activation of secondary metabolism upon heavy metal exposure has also reported before. But the processes leading to lignification must be distinguished from those leading to the production of soluble phenolics. Peptide priming enhanced the production of soluble phenolics in the cytosol (Figure 11.30A) that were induced by the heavy metals and the reaction was much faster than lignification and spread over the whole cross sections of the root. From the differences in the temporal and spatial response patterns of priming during heavy metal stress, it is clear that different signals or differences in the perceptibility of signals must have caused rapid accumulation of phenolics on one hand and delayed lignification on the other hand (as indicated from lower induction of peroxidases). Heavy metal mediated detoxification pathway induces the developmental programme leading to xylogenesis and committed cell death of protoxylem element. Lignification is the final step in this process. Phenolics may contribute together with ascorbate and proline, to H_2O_2 destruction in the so called phenol coupled ascorbate peroxidase reaction (Polle, 2001). During priming, elaborate network of low molecular weight metabolites may mitigate the oxidative stress instead of antioxidative enzymes. In many experimental observations with *Vigna radiata*, ascorbate, proline, soluble phenolics and total thiol were accumulated in higher amount in control and lowers after peptide and salicylate priming (Figure 11.28-11.30). Ascorbate and proline has been reported to accumulate with heavy metal stress signals and scavenge different free radicals in certain *in vitro* generation and detection system. Smirnoff and Cumbes (1989) demonstrated the hydroxyl radical ($OH^{\cdot}$) scavenging property of proline. $OH^{\cdot}$ radicals were commonly generated by

ascorbate/H_2O_2 and detected by hydroxylation of salicylate or by denaturation of malate dehydrogenase. Proline might react with $OH^{\cdot}$ under H^+-abstraction by forming a radical on the C-5 atom (Rustgi *et al.*, 1977). Exogenous proline in *Chlorella* was found to counteract lipid peroxidation as well as K^+ efflux observed after exposure to the heavy metals Cu, Cr, Ni & Zn. The significant metal chelation effect of proline has been proved *in vitro* in copper tolerant plants, where proline is thought to exist as a Cu-proline complex (Farago and Mullen, 1979). In an aqueous assay system, the preliminary mass spectroscopic analysis indicated the formation of proline-Cd complexes of variable masses. So definitely proline along with ascorbate plays a cardinal role in heavy metal detoxification up to a certain limit after priming.

Now if we reconstruct hypothetical framework of metabolic model, intrinsic H_2O_2 accumulation would act as a signalling molecule triggering secondary defences. In non-primed seeds, these signals would cause an untimely cell wall rigidification and lignification, thereby decreasing cellular viability and finally resulting in cell death (Schutzendubel and Polle, 2002). Priming with peptides favours free polyphenol accumulation in cytosol through which heavy metal induced stress responses are significantly buffered. It is observed from the experiment that protein concentration is generally increased in primed seedlings (except copper) with higher dose of heavy metals (Figure 11.29B). Probably it indicates the shifting of metabolic and transport pathway of non-essential, toxic heavy metals and other essential ions in different profile. The changing profile of expression of low molecular weight peptides in response to heavy metals (Figure 11.1) may support this view.

In conclusion, priming with peptides enhanced the level of tolerance of *Vigna* seedlings upon exposure to heavy metal stress and plant redox homeostasis was maintained through induction of antioxidant enzymes in appropriate organs (here cotyledons). The observed morphological and biochemical changes were the indications of beneficial effect of peptide treatment on heavy metal exposed plants.

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