Effect of Enhanced Lead and Cadmium in soil on Physiological and Biochemical attributes of *Phaseolus vulgaris* L

Priti Bhardwaj[1], Ashish K. Chaturvedi[1*] and P. Prasad[1]

[1]High Altitude Plant Physiology Research Centre
H.N.B. Garhwal University, Srinagar, Garhwal
Uttarakhand, India
bhardwajpriti@gmail.com, *ashi_spc@rediffmail.com, ppratti@gmail.com

Abstract: *Phaseolus vulgaris* L. plants were grown in soil supplemented with different Pb and Cd concentrations (2, 4, 6, 8 gKg^{-1} for Lead and 1.5, 2.0, 2.5, 3.0 gKg^{-1} for Cadmium). Germination % was remained unaffected at low concentration of both metals as compared to control plants but at higher concentration of Cd i.e. 3g/Kg soil, germination was completely inhibited. Growth was also decreased as concentration of metals was increased as compared to control plants. Photosynthetic pigments, total soluble sugar, starch content as well as soluble protein content decreased as concentration of metals was increased in comparison of control plants. However, total free amino acid content and lipid peroxidation were increased with increasing concentration of heavy metals. Electrophoretic studies revealed that Acid phosphatase, Peoxidase and Esterase isoenzyme activities were increased with increasing concentration of heavy metals. Electrical conductivity of tissue leachates of leaves of *P. vulgaris* L. was recorded minimum in control plants while maximum was recorded in 8g Pb and in 2.5g/kg Cd treatments. Activity of antioxidative enzymes as ascorbate peroxidase (APX; EC 1.11.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2) was increased while Catalase (CAT; EC 1.11.1.6) activity decreased with increasing concentration of heavy metals. [Nature and Science. 2009;7(8):63-75]. (ISSN: 1545-0740).

Key words: Pb toxicity, Antioxidant enzymes, Physiological changes, biochemicals.

1. Introduction

Environmental deterioration has generated an increase of stress in all forms of life. Of these, stress on agricultural crops is of prime importance since agriculture is lifetime of global society. Abiotic stresses like water stress, salinity stress, and high temperature stress are known to adversely affect growth and grain yield of paddy (Pareek et al., 1999). Along these stresses, toxic heavy metal stress is an emerging and more dangerous stress for major crops.

Bioaccumulation of toxic heavy metals by various crop plants has been reported by number of workers and is a matter of serious health hazard (Mishra and Singh, 2000).Lead has long residence time in soil due to low solubility and strong binding capacity with soil colloids. In soil it accumulates and enters into food chains. Lead stress causes multiple direct & indirect effects on plant growth and metabolism (Balsberg, 1989).

The Reactive Oxygen Species (ROS) are produced in the young senecing leaf cells excessively under stressful conditions and are removed by complex non-enzymic (ascorbate, glutathione,α-tocopherol) and enzymic (CAT, APX, GPX, SOD, GR etc) antioxidant systems (Prochazkova et al., 2001). Plants have evolved antioxidant pathways that are usually sufficient to protect them from oxidative damage during periods of normal growth and moderate stress (Hauptmann and Cadenas, 1997).When severely stressed, however, the production of reactive oxygen species (ROS) can exceed the capacity of the antioxidant system to neutralize them and oxidative damage can occur. Heavy metals are known to induce free radical formation (Aust et al., 1985) and a consequent oxidative damage in senescing leaf cells under light (Panda and Patra, 2000). The aim of present study is to assess the effect of different concentrations of Lead and Cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris* L. (French bean).

2. Materials and Methods

For the present study local cultivar of *Phaseolus vulgaris* L. (French bean) was selected to study the effect of selected heavy metals Lead and Cadmium. For treatments, nitrate salts of lead & Cadmium Viz. Cadmium nitrate [Cd $(NO_3)_2$ $4H_2O$] and Lead nitrate [Pb $(NO_3)_2$] were used. Soil was collected from the field situated in the backyard of High Altitude Plant Physiology Research Centre. Soil, FYM (Farm Yard Manure) and sand were dried & thoroughly mixed at the ratio of 1:1:1 followed by thorough mixing of calculated amount metal salt. The amended soil was filled in polythene bags of size of 16 cm diameter and 13 cm

height. Each bag was filled with 1 Kg of amended soil. The control set is comprised of un-amended soil.

2.1 Toxic metals Treatments

The different concentration of heavy metals chosen were 2, 4, 6, 8 gKg^{-1} for Lead and 1.5, 2.0, 2.5, 3.0 gKg^{-1} for Cadmium. For each treatment five replicates were maintained. After allowing bags filled with amended and unamended soil to stand for 7 days, seeds of *Phaseolus vulgaris* were sown. Watering was done at regular interval. The whole experiment was conducted in the glass house condition. Morphological and biochemical changes were studied at 15 days interval upto one and half months after initiation of heavy metal stress. Enzyme activity and changes in the polypeptides and isoenzymes were studied at final harvest.

2.2 Morphological observations

Morphological observations like root length, shoot length, leaf area, fresh and dry weight were recorded after each harvest. Leaf area was calculated using a graph paper.

2.3 Biochemical estimations

Fresh leaves were used for the estimation of chlorophyll and carotenoid contents (Holm, 1954), soluble sugars and starch (McCready et al., 1950), soluble proteins (Bradford, 1976) and total free amino acids (Moore and Stein, 1954).

2.4 Lipid Peroxidation

Lipid peroxidation in leaves was measured in terms of Malanodialdehyde (MDA), a product of lipid peroxidation content determined by the thiobaribituric acid (TBA), according to the method of Heath and Packer (1968) as modified by Dhindsa et al., (1981).

2.5 Electrical conductivity

For the estimation of leakage of cellular electrolytes, 100 mg of leaf tissue were incubated in 10 ml of deionised water for 6 h. The electrolyte in the tissue leachates were determined by measuring the electrical conductivity (EC) in a conductivity meter (Systronics) and the EC of tissue leachate was expressed in terms of $mS^{-1}g^{-1}$ dry mass (d.m.).

2.6 Electrophoretic analysis of polypeptides and Isoenzymes

Polypeptide pattern was analyzed on 10% SDS polyacrylamide gels using a BIO- RAD MiniPROTEAN II system. Isoenzymes esterase, peroxidase and acid phosphatase were analyzed on 10 % Polyacrylamide slab gels using a BIO- RAD MiniPROTEAN II system.

(i) Esterase: Esterase isozymes were detected on gels by the method described by Bhadula and Sawhney, 1987.

(ii) Peroxidase: Peroxidase isoenzymes were detected on gels by the method as described by Welter (1982).

(iii) Acid Phosphatase: Detection of acid Phosphatase isozymes on gels was done by method of Bhadula and Sawhney, 1987.

2.7 Spectrophotometric estimation of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2) activities

2.7.1 Preparation of enzyme extract

Fresh leaves of 0.1 g fresh weight was homogenized at 4^{o} C in 5 ml extraction buffer [50mM potassium phosphate buffer (pH 7.0), 1% Triton X-100 and 7 mM 2-mercaptoethanol] with mortar and pestle. The homogenate was centrifuged at 25000 rpm for 20 min. and the supernatant was used as the crude extract for the APX, GPX and GR assay. However, for the estimation of CAT activity the extraction buffer was 0.05 M tris- HCl buffer (pH 7.5) 3 mM $MgCl_2$ and 1 mM EDTA. Protein content was determined using bovine serum albumin as a standard, according to the method of Bradford (1976).

2.7.2 Enzyme essay

CAT activity was assayed by measuring the rate of disappearance of H_2O_2 using method of Maehly and Chance (1959). **APX** activity was determined according to the method Chen and Asada (1989). **GPX** activity was determined according to Upadhyaya et al., (1985). **GR** activity was assayed by measuring the decrease in absorbance at 334 nm due to the oxidation of NADPH (Klapheck et al., 1990). All the enzyme activities were calculated and expressed as enzyme units per milligram of protein per minute.

3. Results and discussion

3.1Growth and Morphological changes

The germination-percentage was affected with different concentrations of Pb & Cd as compared with controls one. The results in relation to the effect of different concentrations of Pb & Cd on germination performance measured in terms of per cent germination were shown in Table I. Germination declined with increasing concentration of both heavy metals in a concentration dependent manner. But at the highest concentration of Cd i.e. 3g/kg soil, germination was completely inhibited. For control plants percent germination was 67.71% (Table I and Figure 1).

During seed germination a number of hydrolyzing enzymes become active. These include amylases, proteases and lipases that hydrolyse polysaccharides, proteins & lipids respectively into their monomers. The

stored materials in the cotyledons & the endosperm are hydrolysed and transferred to the growing embryo. This involves the activation & synthesis of several hydrolyzing enzymes (Bose et al., 1982). Inhibition of seed germination, hydrolyzing enzymes (amylase and proteases) and seedling growth treatment of a number of toxic salts, viz. Pb, Hg, Cu, Ni, Co etc. have been reported in many plants (Sharma et al., 1995; Jain et al., 1998).

Excess supply of heavy metals such as cobalt sulphate, nickel sulphate, sodium molybdate and sodium dichromate with nutrient solution caused depressed germination, length of radicles, lowered the mobilization of reserved materials from the cotyledons to the developing embryo axis and adversely affected a no. of respiratory enzymes in the case of green-gram (Kumar and Brisht, 1986).

Growth is the best indices for evaluating plant response to environmental stress Morphological studies in the present study had shown that minimum growth occurred in the plants in which Pb concentration was 8g/kg soil and for Cd it was 2.5g/kg soil when compared with control plants. Growth was not observed at the highest concentration of Cd i.e. 3g/kg soil. With increase in Pb and Cd concentrations, there was a gradual decrease in plant height. At different concentrations of lead, i.e. 2,4,6,8 g Pb / kg of soil, shoot length decreased by 37.98%, 55.81%, 61.24%, and 70.93% respectively. Root length was decreased 28.43%, 42.74%, 65.24%, and 87.73% respectively as compared to control. For different concentrations of cadmium i.e. 1.5, 2.0, 2.5 g Cd / kg of soil, shoot length decreased by 67.05%, 70.54%, 78.68% and root length by 46.83%, 57.06%, and 69.33% respectively. The more severe decrease in the length of roots may be due to their direct contact with lead and cadmium polluted soil. Similar observations have been observed on *Triticum sativum* and *Lens esculanta* by Mesmar and Jaber (1991). Seedling biomass (Fresh wt. and Dry wt.) also declined proportionately with increasing concentrations of both the heavy metals. Fresh weight decreased by 28.43%, 42.74%, 65.24%, 87.73% and dry weight by 26.67%, 46.67%, 53.33%, 87.78% respectively for different concentrations of Pb.

Xiong (1997) also reported progressive decline in plant dry weight with increasing concentrations of Pb in soil. Fresh weight of seedling at different concentrations of Cd decreases 54.09%, 62.07%, 82.76% and dry weight 75.56%, 78.89%, 81.11% respectively. Leaf area showed significant decline with increase in concentrations Pb and Cd. For different concentrations of Pb it was 16.87%, 56.27%, 68.62%, 79.78% and for Cd it is 22.96%, 63.47%, and 84.91% respectively. (Table I)

Table I. Morphological changes in *P. vulgaris* grown under different metal concentrations

3.2 Biochemical changes

Treatments	Percent germination (%)	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)	Leaf area (cm^2)
Control	67.71	25.8	4.89	4.64	0.9	52.83
2g $Pb(NO_3)_2$	66.66	16	3.5	3.24	0.66	43.92
4g $Pb(NO_3)_2$	64.66	11.4	2.8	1.56	0.48	23.1
6g $Pb(NO_3)_2$	53.33	10	1.7	1.04	0.42	16.58
8g $Pb(NO_3)_2$	52.32	7.5	0.6	0.98	0.11	10.68
1.5g$Cd(NO_3)_2$	63.33	8.5	2.6	2.13	0.22	40.7
2.0g$Cd(NO_3)_2$	59.99	7.6	2.1	1.76	0.19	19.3
2.5g$Cd(NO_3)_2$	53.33	5.5	1.5	0.8	0.17	7.97
3.0g $Cd(NO_3)_2$	0	0	0	0	0	0

3.2.1Photosynthetic pigments

Along with the growth of plants under increasing concentration of heavy metals, the entire metabolic activity of the plants under metal stress was affected resulting in reduced metabolic activities. Under the metal stress, the levels of photosynthetic pigments, namely Chl. 'a' and Chl 'b' and Carotenoids decreases as the concentrations of Pb and Cd in soil increases. Chl. 'a' was more affected in comparison to carotenoids. At 2, 4, 6, 8 g Pb/kg in soil, Chl 'a' showed a decrease of 25.73%, 43.08%, 54.45%, and 68.23%, Chl 'b'; 21.62%, 41.98%, 49.64%, 64.76% and carotenoids 8.52%, 65.90%, 70.16%, 72.13% respectively. Same decreasing pattern showed with different concentrations of Cd. At 1.5, 2.0, 2.5 g Cd/kg soil, Chl 'a' decreases 21.25%, 40.71%, and 67.26%; Chl 'b' decreases 16.27%, 39.14%, and 63.12%; carotenoids 35.41%,52.46%,70.49% respectively (Figure 2). The decline in the levels of these pigments clearly showed the metal interference with pigment metabolism. Similar observations were made by Mukherji and Maitra (1976) in rice where Pb toxicity resulted in lowering Chl a/b ratio. Lead was found to inhibit δ amino levulinic acid dehydratase activity in mung bean resulting in a decrease in Chl. Content (Prasad and Prasad, 1987). Pb also distorts the membrane structure of chloroplasts, which ultimately leads to decrease in Chl. Content.

3.2.2 Carbohydrates

Soluble carbohydrate contents in plants decreased with increasing concentration of heavy metals. At the highest concentration of Pb i.e. 8g and Cd 2.5 g /kg soil, the carbohydrate content was minimum and maximum carbohydrate content was observed in control.

Soluble sugar: Effect of both heavy metals, Pb and Cd on the sugar content of leaves showed that there was a decrease of 1.85%, 14.68%, 17.43% and 26.45% in sugar content at different concentrations of Pb whereas 2.50%, 13.82% and 18.29% decrease at different concentration of Cd respectively (Figure 3).

Starch: Starch content of leaves decreases 7.09%, 27.90%, 34.98% and 50.22% at different concentration of Pb and 41.93%, 46.48%, and 50.85% at different concentrations of Cd (Figure 4).

3.2.3 Soluble protein content

Similar results as in the carbohydrate content were found for soluble protein content as minimum protein content was recorded in plants at the highest concentration of both heavy metals. Toxicity of Pb & Cd altered the protein content of leaves. There was a decrease of 3.56%, 26.92%, 33.69% and 57.69% at Pb concentrations and 14.48%, 18.62% and 23.17% at Cd concentrations (Figure 5). Kastori et al., (1992) reported in *Helianthus annus* that content of soluble proteins decreased with high concentration of heavy metals. Protein content under heavy metal influence may be affected due to: (I) Enhanced protein hydrolysis resulting in decreased concentration of soluble proteins (Melnichuk et al., 1982), (ii) Catalytic activity of lead (Bhattacharya and choudhuri, 1997); (iii) Protein synthesis becoming reduced under all stress condition.

3.2.4 Total free amino acids and electrical conductivity

Total free amino acid and electrical conductivity at the increasing concentration of heavy metals increased in the plants. Total free amino acid increased in stressed plant as the metal stress applied. At different concentrations of Pb i.e. 2, 4, 6 and 8 g/kg soil amino acid content increases 40.61%, 42.58%, 46.59% and 65.81% respectively. For different concentrations of Cd i.e. 1.5,2 and 2.5 g/kg soil amino acid content increases 46.77%, 52.30%, 54.08 respectively (Figure 6). Electrical conductivity of tissue leachates of leaves at different concentrations of Pb and Cd was shown in Figure 7. In general, conductivity increases from control to higher concentration of metals. At different concentrations of Pb, there was an increase of 10.26%, 18.60%, 20.45% and 23.61% while for Cd it was 21.35%, 24.73% and 28.57% respectively (Figure 7).

Several studies with heavy metals support the view of changes in membrane architecture and permeability (Jensen and Adal-Steinsson, 1989). Various workers (Vangronsveld and Clijsters, 1994) suggested oxidation of cross linking of protein thiols, inhibition of plasma membrane ATPase as the mechanism of heavy metal induced membrane damage. Green et al., (1980) have suggested interaction of heavy metals with membrane phospholipids or the displacement of membrane-bound Calcium as a cause of heavy metal-induced changes in membrane structure.

3.2.5 Lipid peroxidation

Changes in lipid per oxidation in leaves of *P. vulgaris* at different treatments were shown in fig 10. As the concentration of metal increases, lipid per oxidation increases. For different concentrations of Pb it was 11.61%, 15.2%, 14.81% and 24.19% increase and for Cd it was 17.74%, 19.58% and 21.18% increase respectively (Figure 8).

Excess of Pb and Cd promoted lipid peroxidation with excessive production of MDA content over untreated control in concentration – regulated manner in plants of *P. vulgaris*, Lead & Cadmium – induced membrane damage seems to be largely due to enhanced membrane lipid-peroxidation, corroborating well with the data of Gallego *et al.,* (1999). Membrane lipid peroxidation is also mechanically important from the

perspective of production of oxyfree radicals like OH and HO_2 that further causes enhanced oxidative injury (Cakmak and Horst, 1991).

3.2.6 Antioxidant enzymes activities (APX, GPX and GR)

Development of oxidative stress in plants exposed to heavy metals (Weckz and Clijesters, 1996) is largely ascribed to heavy metal induced disbalance between the generations of toxic oxygen radicals and their scavenging through the anti-oxidative defense mechanism. The latter provides an efficient system for detoxification and scavenging of the toxic oxygen species through an adaptive mechanism involving upregulation of anti-oxidative enzymes such as SOD, CAT, POD, APX and GR (Foyer et al., 1994) and enhance accumulation of cellular antioxidants such as ascorbate glutathione cycle these reactions also down regulate the conversion of the super oxide ions to the highly reactive and genotoxic hydroxyl (OH) ions.

With increase in the concentrations and time of treatment of heavy metals, there was decrease in activities of antioxidative enzymes but in the present study with the increase of heavy metals increase the activity of anti-oxidative enzymes APX, GPX and GR in the leaves of *P. vulgaris* while there was a corresponding decrease in the CAT activity as compared to control plants. In the present study, increase in the concentrations of Pb & Cd increase in the activity of APX, GPX and GR in leaves of *P. vulgaris* as compared to the control plants shown in Figure 11-14 while activity of CAT decreases as the concentration of Pb and Cd increases (Figure 9-12). From the result it has been clear that the concentration of heavy metals which was given to the plants do not impose any kind of oxidative stress because the activity of antioxidant enzymes was higher. But from all other parameters which were studies, it is clear that increasing concentrations of heavy metals significantly affect on the physiology and biochemistry of plants. As we have compared between both the metals, Cd was more toxic than the Pb because the lower concentration of Cd have almost same effect as the higher concentration of Pb.

3.2.7 Polypeptides and isoenzyme patterns:

The SDS-PAGE pattern of polypeptides extracted from leaves of *P. vulgaris* at different concentrations of Pb and Cd were analyzed using 10% SDS gel shows that the polypeptides decrease as the concentration of both the metals increase compared with control (Figure 13).

Acid phosphate (APase, EC 3.1.3.2) is widely distributed in plants it has long been recognized that Apase activity in plants typically increased when plants become phosphorus (Pi) deficient the increase Apase activity correlates with a low level of Pi in numerous species and plant parts (Ueki and Sato, 1997). Salt, water and osmotic stresses have also been reported to increase APase activity (Szabo-Nagy et al., 1992).

Isoenzyme activity of Esterase, Peroxidase & Acid phosphatase was studied through electrophoresis. Isoenzyme variation in leaves of *P. vulgaris* at different concentrations of Pb and Cd was shown in Figure 14, 15 and 16 respectively.

In the present study, it was observed that the APase activity in plants was maximum at the highest concentration of Pb and Cd in comparison with control plants (Figure 16). Esterase activity was also increased slightly as the concentration of heavy metal increased (Figure 14). Peroxidase activity in leaves of plants increased with increasing concentration of heavy metal (Figure 15).

An increase in peroxidase activity probably represents an induced protective reaction delaying senescence (Birecka et al., 1977). Since, as we know the importance of peroxidase isoenzyme to catalyze the reaction that protects the plants, against damage by free radicals. Populations showing low peroxidase activity indicated that it may not adapt them at wider range because plants may lose the permeability of membrane and proceed toward the end of life due to the harmful action of free radicals. Lipid of membranes where a peroxidation of unsaturated fatty acids takes place is main cellular components susceptible to damage by free radicals (Monk et al., 1989).

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Correspondence to:
Ashish K. Chaturvedi
High Altitude Plant Physiology Research Centre
H.N.B. Garhwal University, Srinagar, Garhwal
Uttarakhand, India
Phone No. +911346 253760
Fax +911346 252070
ashi_spc@rediffmail.com

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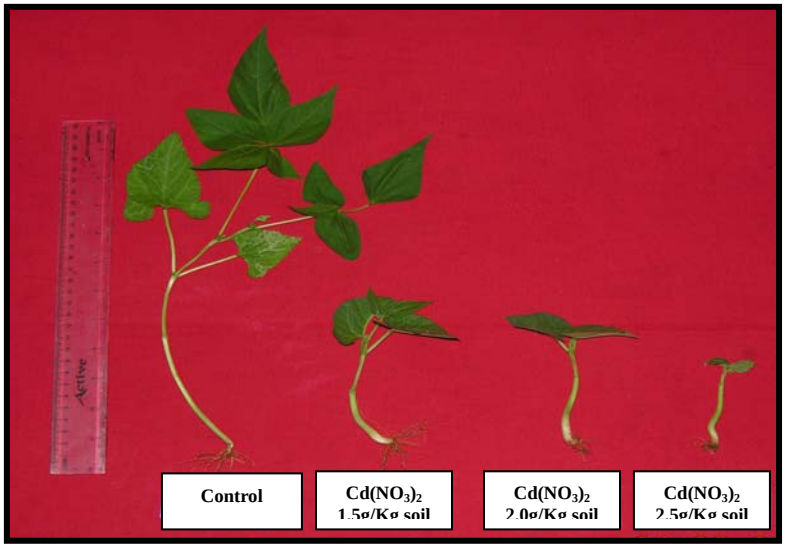


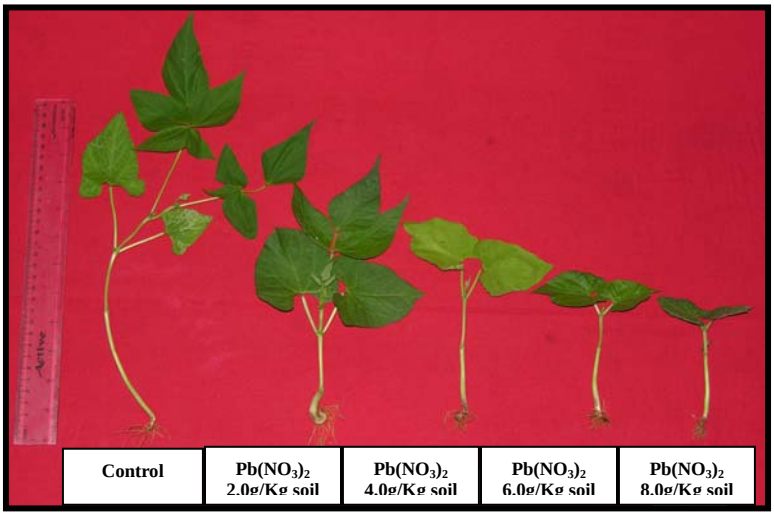


Figure 1.Comparative morphology of *P. vulgaris* seedlings grown under different metals (after 20 days growth)

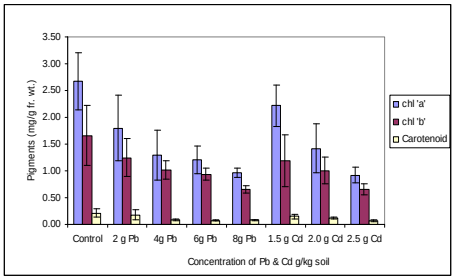


Figure 2 Changes in the leaf pigment contents of *P. vulgaris* grown under different concentrations of Pb & Cd

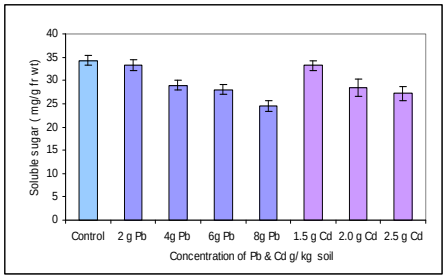


Figure 3. Changes in the soluble sugar content of *P. vulgaris* grown under different concentrations of Pb & Cd

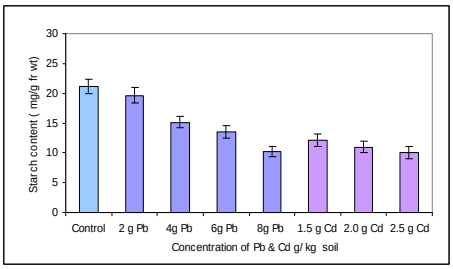


Figure 4. Changes in the starch content of *P. vulgaris* grown under different concentrations of Pb & Cd

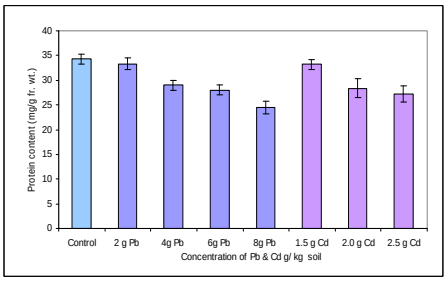


Figure 5. Changes in the soluble protein content of *P. vulgaris* grown under different concentrations of Pb & Cd

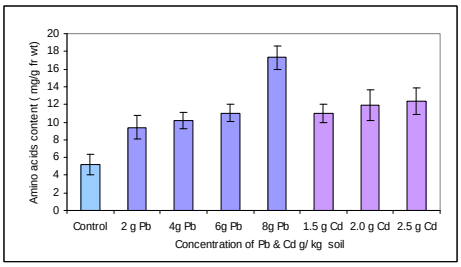


Figure 6. Changes in the leaf free amino acid content of *P. vulgaris* grown under different concentrations of Pb & Cd

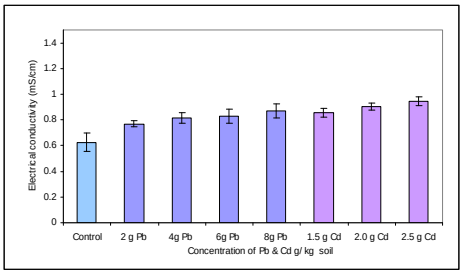


Figure 7. Changes in the electrical conductivity of leaf tissue leachate of *P. vulgaris* grown under different concentrations of Pb & Cd

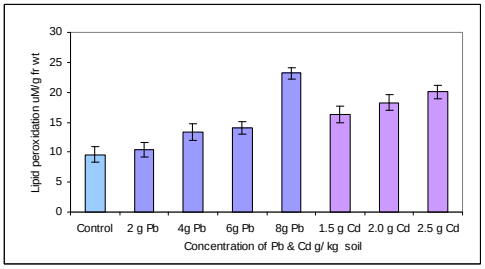


Figure 8. Changes in the leaf lipid peroxidation content of *P. vulgaris* grown under different concentrations of Pb & Cd

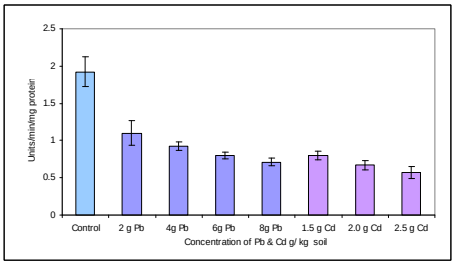


Figure 9. Changes in the leaf catalase activity of *P. vulgaris* grown under different concentrations of Pb & Cd

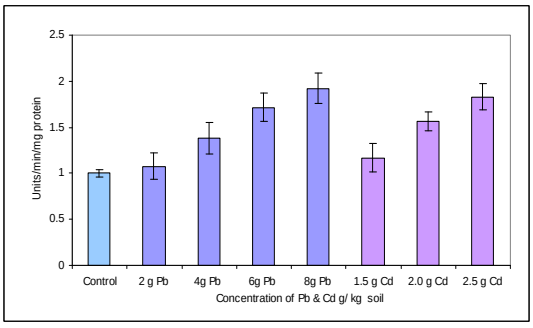


Figure 10. Changes in the leaf APX activity of *P. vulgaris* grown under different concentrations of Pb & Cd

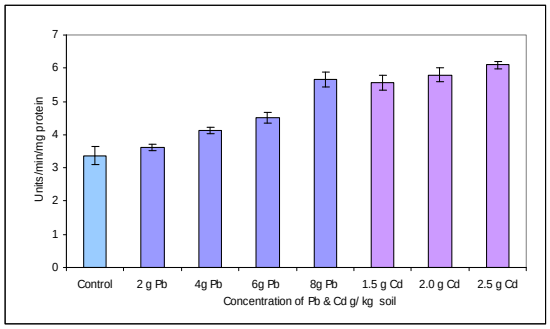


Figure 11. Changes in the leaf GPX activity of *P. vulgaris* grown under different concentrations of Pb & Cd

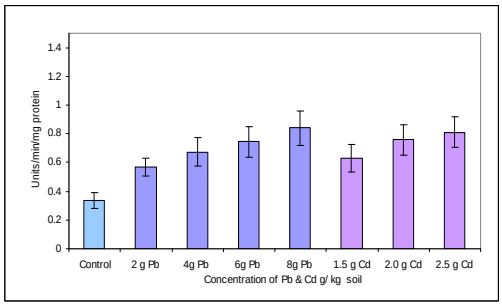


Figure 12. Changes in the leaf GR activity of *P. vulgaris* grown under different concentrations of Pb & Cd

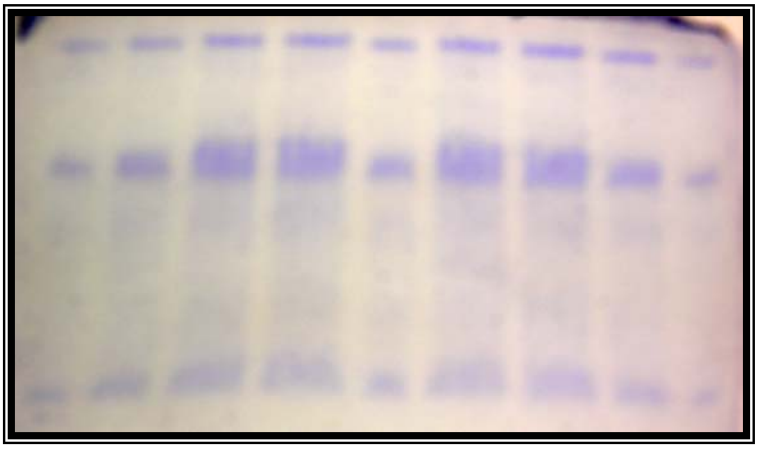
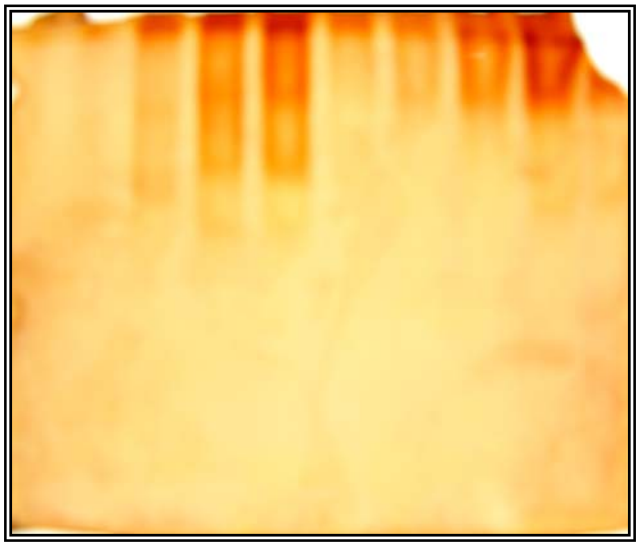


Figure 13. Changes in leaf polypeptide banding pattern of *P. vulgaris* grown under different metal concentrations

(C=control, 2Pb=2 g Pb/ kg soil, 4Pb=4 g Pb/ kg soil, 6Pb=6 g Pb/ kg soil, 8Pb=8 g Pb/ kg soil, 1.5 Cd=1.5 g Cd/kg soil, 2 Cd=1.5 g Cd/kg soil, 2.5 Cd=2.5 g Cd/kg soil)

1.5 Cd 2 Cd 2.5Cd C 2 Pb 4Pb 6 Pb 8 Pb

Figure 14. Changes in Esterase activity of *P. vulgaris* grown under different metal concentration



1.5 Cd 2 Cd 2.5Cd C 2 Pb 4Pb 6 Pb 8 Pb

Figure 15. Changes in Peroxidase activity of *P. vulgaris* grown under different metal concentration

Figure 16. Changes in Acid phosphatase activity of *P. vulgaris* grown under different metal concentration

(C=control, 2Pb=2 g Pb/ kg soil, 4Pb=4 g Pb/ kg soil, 6Pb=6 g Pb/ kg soil, 8Pb=8 g Pb/ kg soil, 1.5 Cd=1.5 g Cd/kg soil, 2 Cd=1.5 g Cd/kg soil, 2.5 Cd=2.5 g Cd/kg soil)