REGULAR ARTICLE

ANTIOXIDATIVE METABOLISM IN THE SEEDLINGS OF PIGEONPEA IN RESPONSE TO LEAD AND CADMIUM STRESS

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SUMMARY

Effects of Pb and Cd stress on antioxidative systems were studied in pigeonpea (*Cajanus cajan* (L.) Millspaugh) cultivars T21 and LRG30. The seeds were grown under different concentrations of Pb and Cd for 8-days and activities of enzymes including superoxide dismutase, peroxidase, catalase, glutathione reductase, malate dehydrogenase were assayed. In addition, quantitative estimations of ascorbic acid and glutathione were also carried out in control and Pb and Cd treatments of pigeonpea seedlings. The activities of antioxidative enzymes showed a marked increase in response to heavy metal stress. However the ascorbic acid and glutathione content were decreased by increasing heavy metal concentrations. This study revealed that varietal differences play an important role in the plant response to heavy metals.

Key words: Antioxidants, cadmium, heavy metal stress, lead, pigeonpea.

1. Introduction

Heavy metals make a significant contribution to environmental pollution as a result of human activities such as mining, smelting, electroplating, intensive agriculture, sludge dumping and military operations (Nedel koska and Doran, 2000). They present a risk for primary and secondary consumers and ultimately humans (Zeller and Feller, 1999). Excess accumulation of these micronutrients and other heavy metals like Cd, Pb and Ni in plants operate as stress factors causing physiological constraints leading to decreased vigour and plant growth (Ouzounidou, 1993; Hall, 2002). They generally interfere with the activities of a number of enzymes essential for normal metabolic and developmental processes (Stoyanova and Tschakalova, 1993; Moustakes et al., 1994).

Plants develop certain intracellular tolerance mechanisms for modulating the free metal concentrations (Rauser, 1990). Certain heavy metals like copper and iron can be toxic by their partcipation in redox cycles producing hydroxyl radicals ($^{.}OH$) which are extremely toxic to living cells (Stochs and Bagchi, 1995). By contrast with those metals, Cd and Pb are non-redox metals unable to participate in Fenton-type reactions. The enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidases are involved in the detoxification of $O_2^{.-}$, and H_2O_2 respectively, thereby preventing the formation of $^{.}OH$ radicals. Ascorbate peroxidase (APX) and glutathione reductase (GR), as well as glutathione are important components of the ascorbate-glutathione cycle responsible for the removal of H_2O_2 in different cellular compartments (Jimenez et al., 1997). Glutathione is also the substrate for the biosynthesis of phytochelatins, which are involved in heavy metal detoxification (Zenk, 1996).

Cadmium induces changes of the antioxidant status in some animal tissues either by increasing superoxide radical production and lipid peroxidation or by decreasing the enzymatic and non-enzymatic antioxidants (Stochs and Bagchi, 1995). However, less information is available in plants. In *Phaseolus vulgaris*, *Phaseolus aureus* and *Helianthus annus* the toxicity of Cd has been related with the increase of lipid peroxidation and alterations in antioxidant

systems (Somashekaraiah et al., 1992; Shaw, 1995 and Gallego et al., 1996).

In this work the effect of Pb and Cd on pigeonpea seedlings of two cultivars in different physiological parameters and enzymatic antioxidants was studied in order to know the possible involvement of Pb and Cd in the generation of oxidative stress.

2. Materials and Methods

Two cultivars of pigeonpea (*Cajanus cajan* (L.) Millspaugh) namely T21, a medium duration and LRG30, a long duration variety obtained from ICRISAT, Patancheru, India were used for the present investigation.

Pb and Cd treatments

The seeds of uniform size were selected and soaked in distilled water for two hours and were surface sterilized with 0.01M Sodium hypochlorite for 2 min. The washed seeds were then spread over trays lined with two layered filter papers containing 0.5, 1.0 and 1.5 mM concentrations of Pb (lead acetate: $(CH_3COO)_2Pb.3H_2O$) and $CdCl_2$ (cadmium chloride: $CdCl_2$ $2.5H_2O$). Seeds germinated and seedlings raised in distilled water (zero concentration) served as controls. Twenty-five seeds were taken in each tray. The seeds were allowed to germinate at $30\pm2°C$ for 8-days under a photoperiod of 12 h and at 195 μ mol m^{-2} s^{-1} PPFD. Then the seedlings were collected for biochemical and enzymatic analysis.

Ascorbic acid content

Ascorbic acid content was estimated according to the method of Roe (1964) which is based on the reduction of the dye, 2, 6-dichlorophenol, indophenol (DCIP) by ascorbic acid (AA) from its pink colour in the acid medium to the colourless leuco-form.

Ascorbic acid oxidase

Ascorbic acid oxidase activity was determined according to the method of Povolotskaya and Sedenka (1956) as modified by Gopalachari (1963).

Total Glutathione (GSH+GSSG) content

Total glutathione content was conveniently assayed by enzymatic recycling procedure of Tietze (1969) as followed by Griffith (1980). In this method GSH (reduced form) was sequentially oxidized by 5, 5-dithiobis (2-nitro benzoic acid) (DTNB) to gave GSSG (oxidized form) with stiochiometric formation of 5-thio-2-nitrobenzoic acid (TNB). The oxidized form of glutathione (GSSG) was then reduced by the action of glutathione reductase and NADPH. The extent of TNB formation was monitered at 412 nm and was proportional to the sum of GSH and GSSG present in the samples.

Glutathione reductase (E.C. 1.6.4.2.)

The assay of glutathione reductase in the crude tissue homogenates, was carried out according to Smith et al., (1988) using 5, 5-dithiobis (2-nitro benzoic acid) (DTNB) which is based on the change in absorbance at 412 nm due to the formation of 5-thio, 2-nitrobenzoic acid (TNB).

Superoxide dismutase (E.C.1.15.1.1)

The activity of the superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium, adopting the method of Beauchamp and Fridovich (1971).

Catalase (E.C.1.11.1.6)

Catalase activity was estimated by the permanganate method of Povolotiskaya and Sedenka (1956) as followed by Gopalachari (1963) with slight modification.

Peroxidase (E.C.1.11.1.7)

Peroxidase activity was estimated by the method followed by Kar and Mishra (1976).

3. Results

Ascorbic acid content of the control seedlings of two cultivars of pigeonpea increased in seedling axes and decreased in the cotyledons with increasing age. Although the seedling axes of Pb and Cd treatments exhibited a trend similar to controls, they always registered lower values of ascorbic acid content in both the cultivars of pigeonpea (Fig 1.A). Ascorbic acid oxidase of the seedling axes and cotyledons of control seedlings of two pigeonpea cultivars exhibited a continuous decrease of ascorbic acid oxidase activity with increasing age. Although the seedling axes of the Pb and Cd treatments also exhibited a trend similar to

that of controls, the values recorded were greater at the early stages of the seedling growth followed by a decline (Fig 1.B). The cotyleldons of the two cultivars of pigeonpea seedlings always showed lower values of ascorbic acid oxidase activity when compared to their respective controls.

Fig-1. A. Ascorbic acid content and **B.** Ascorbic acid oxidase activity of seedlings of pigeonpea, cv.T21 and cv.LRG30 in response to Pb and Cd stresses (Vertical lines represent S.E.).

    Seedling axis : a and c
    Cotyledon : b and d
    Lead : a and b
    Control: O - O; 0.5: mM Δ- Δ ; 1.0: mM □-□ ; 1.5: mM ◊-◊
    Cadmium: c and d

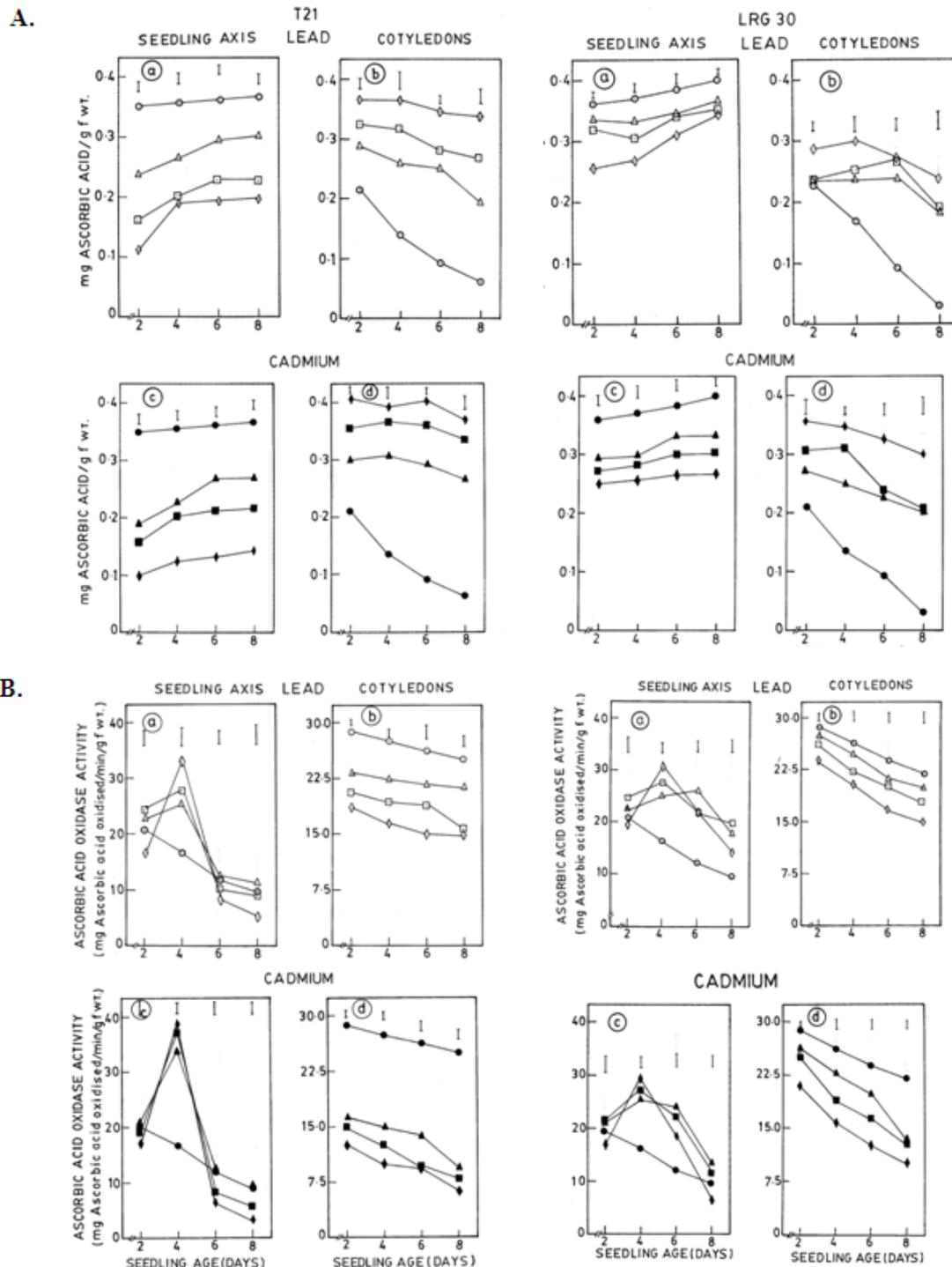Control: ●-● ; 0.5: mM ▲-▲ ; 1.0: mM ■-■; 1.5: mM ◆-◆

Fig-2.

A. Total Glutathione **B.** Glutathione reductase activity of 6 -day old seedlings of pigeonpea, cv.T21 and cv.LRG30 in response to Pb and Cd stresses (vertical lines represent S.E.).
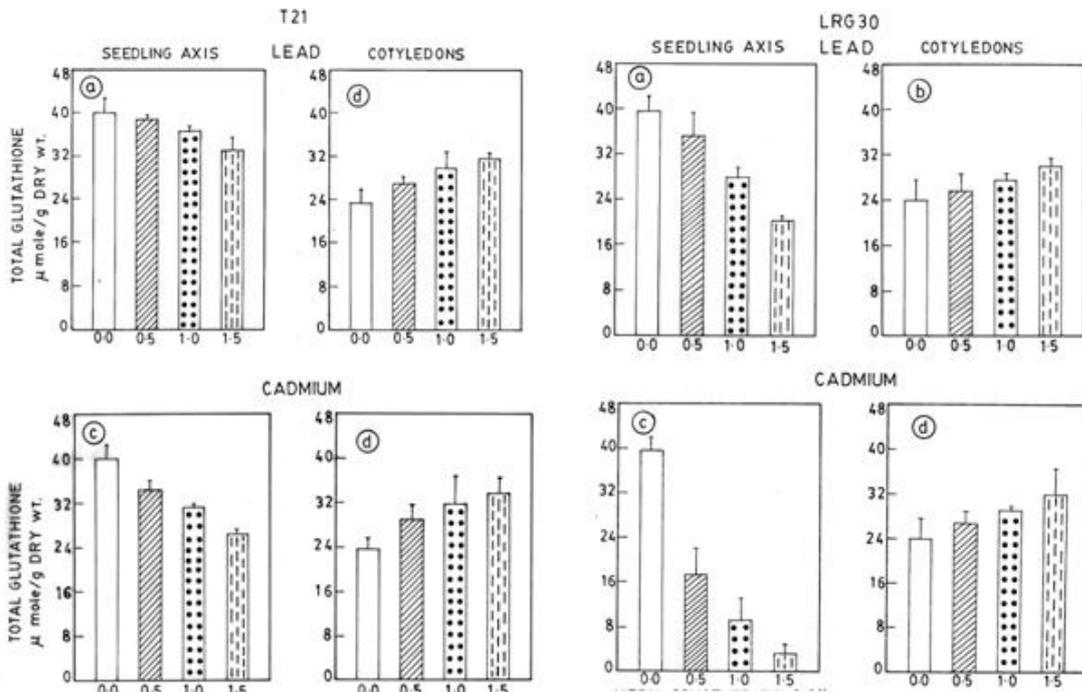
Seedling axis   : a and c

cotyledon   : b and d

Lead   : a and b

Cadmium   : c and d

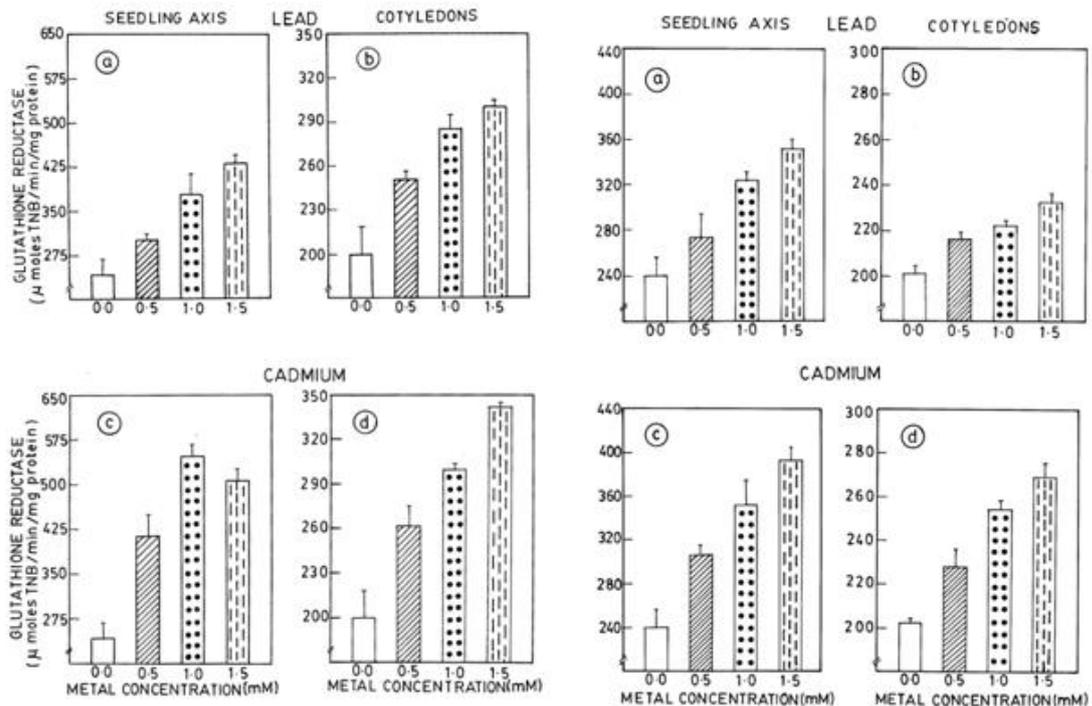Control   : ☐ ; 0.5 mM : ▨ ; 1.0 mM : ⬚ ; 1.5 mM: ⬚

Fig-3.

A. Superoxide dismutase activity B. Catalase activity C. Peroxidase activity of seedlings of pigeonpea, cv.T21 and cv.LRG30 in response to Pb and Cd stresses (Vertical lines represent S.E.)

Seedling axis   : a and c

Cotyledons   : b and d
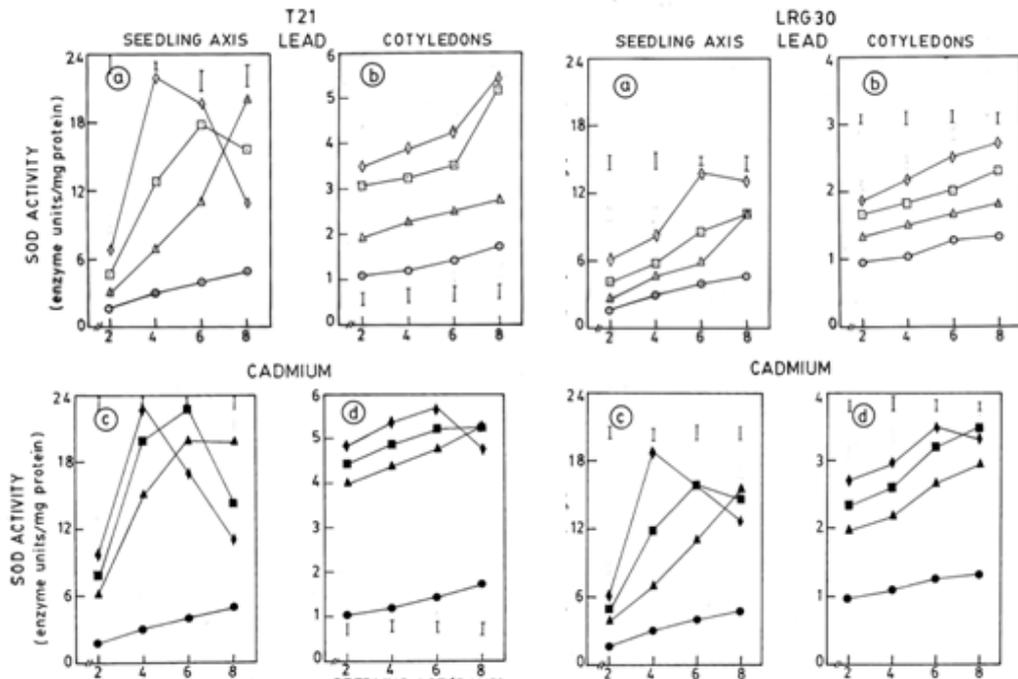
Lead   : a and b

Control   : 0-0 ; 0.5: mM ; Δ -Δ ; 1.0mM: □-□ ; 1.5mM: ◊-◊

Cadmium   : c and d

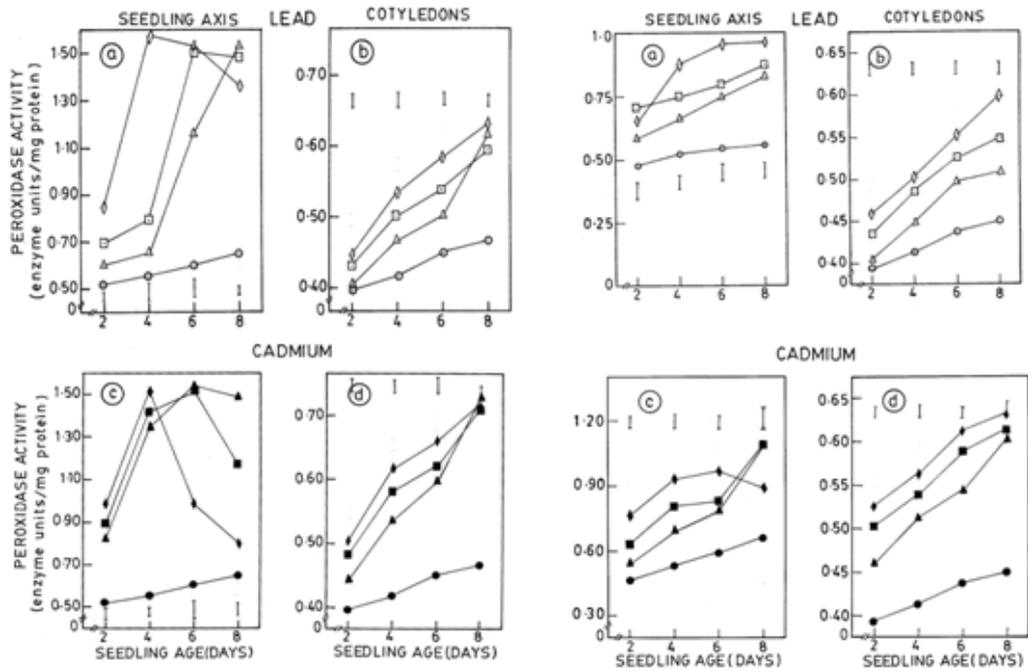Control   : ●-● ; 0.5: mM; ▲-▲ ; 1.0: mM; ■-■; 1.5: mM; ◆-◆

A.

B.
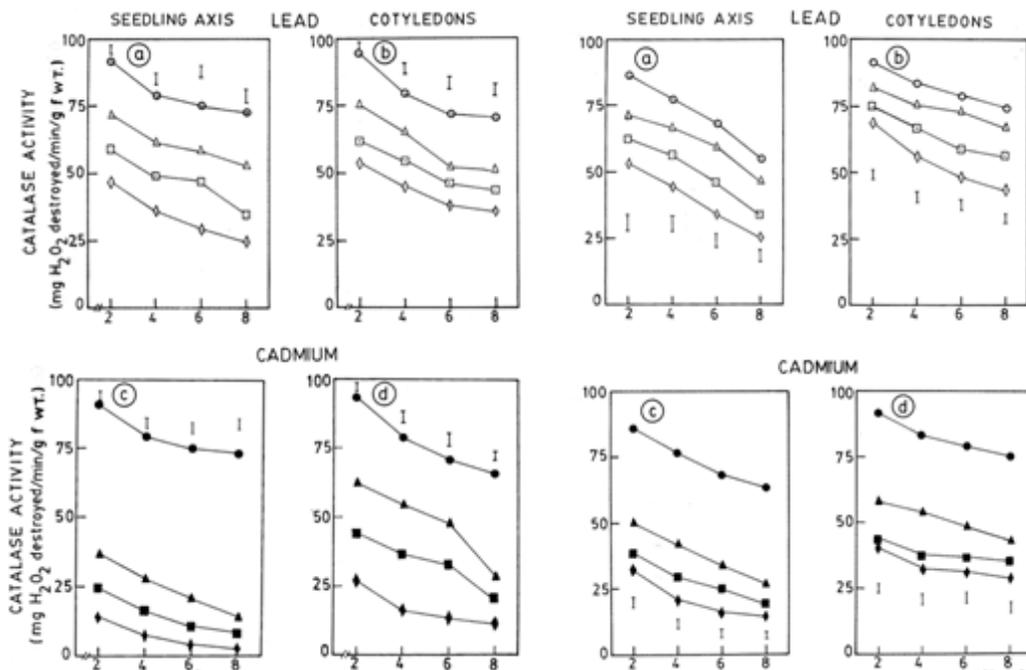
Total glutathione content of the seedling axes decreased with increasing concentrations of externally supplied Pb and Cd ions when compared to their respective controls. However the studies on the changes in total glutathione content were confined to 6-day old pigeonpea seedlings only. Glutathione content remained more in the cotyledons of the Pb and Cd treated seedlings when compared to their respective controls (Fig 2. A). Interestingly cv.LRG30 recorded lower levels of glutathione content in seedling axes than the cv.T21. The glutathione content in the cotyledons were more in cv.T21 than cv.LRG30. Glutathione reductase activity was studied on 6-day old seedlings only. The glutathione reductase activity of both the cultivars of pigeonpea increased with increasing concentrations of Pb and Cd supplied and registered higher values when compared to their controls (Fig 2. B). The glutathione reductase activity in the seedling axes of cv.T21 exposed to 1.5 mM cadmium showed slight decrease when compared to 1.0 mM treated seedlings.

Superoxide dismutase activity of the seedlings of control in both the cultivars of pigeonpea increased in seedling axes and in cotyledons with increasing age. Although the superoxide dismutase activity of all treatments of both the cultivars of pigeonpea exhibited a trend similar to that of controls, the greater levels of superoxide dismutase activity were recorded with increasing concentrations of Pb and Cd treatments. In cv.T21 sudden increase in superoxide dismutase activity of seedling axes was observed at four days of seedling growth followed by a decline. However at 0.5 mM Pb and Cd concentrations superoxide dismutase activity showed gradual increase throughout the period of investigation. Further in cv.LRG30 gradual increase of superoxide dismutase activity were observed. However at 1.5 mM Pb concentration and 1.0mM and 1.5 mM Cd concentrations slight decrease was observed at 8-day of seedling growth. The cotyledons of the Pb and Cd treated seedlings always registered more levels of superoxide dismutase activity when compared to their respective controls (Fig 3.A). The catalase activity of the two cultivars of pigeonpea decreased in seedling axes and in their cotyledons with increasing age of the control seedlings. Although the catalase activity of the Pb and Cd treatments exhibited a trend similar to that of controls, they always recorded lower values when

compared to controls and the decrease was more evident with increasing concentrations of Pb and Cd supplied. When cv.T21 and cv.LRG30 were compared, the decrease in catalase activity becomes more conspicuous in cv.T21 (Fig 3.B). Peroxidase activity of the seedlings of controls of both the pigeonpea cultivars increased with increasing age of the seedlings. Although the peroxidase activity of Pb and Cd treatments exhibited a trend similar to that of controls, the values recorded were always greater than their controls (Fig 3.C).

4. Discussion

Ascorbic acid is universally present in plants as a constituent of oxidation – reduction systems and is actively involved in the plant growth, differentiation and development (Chinoy et al., 1971). Ascorbic acid acts as potent antioxidant and the highly reactive oxyradicals promote the oxidation of ascorbic acid to dehydroxy ascorbic acid, leading to the reduction of ascorbic acid (Nakavo and Asada, 1980). The retention of ascorbic content in the cotyledons may be due to its reduced translocation. Ascorbic acid oxidase is unlikely to be involved in scavenging system, since the reaction consumes molecular oxygen, rather than hydrogen peroxide (Lin and Varner, 1991). Greater activity of ascorbic acid oxidase was observed in cv.T21 than cv.LRG30 in the 4-day old seedling axes of pigeonpea seedlings in response to Pb and Cd treatments (Fig.1.B). Wakiuchi et al., (1971) reported that the ascorbic acid oxidase activity is very active when the cell structure has been disrupted.

Glutathione plays an important role in Pb and Cd detoxification mechanisms (Howden and Cobbett, 1992). Among the two pigeonpea cultivars interestingly the decrease in total glutathione content was more in seedling axes of cv.LRG30 than cv.T21. It may be due to the more efficient detoxification mechanisms operated by the seedlings of cv.LRG30 against the free oxyradicals generated under Pb and Cd toxicity and/or the utilization of glutathione in the biosynthesis of metal binding peptides. Robinson and Jackson (1986) and Grill et al., (1987) observed that phytochelatin formation

almost equal to the level of glutathione consumed during cadmium treatment in different plant species. Glutathione reductase activity is considered to be involved indirectly in the scavenging of free radicals (Asada and Thakahashi, 1987). Glutathione reductase activity showed higher levels in cv.T21 than cv.LRG30. Glutathione reductase activity in cadmium exposed sensitive plants of *Silene vulgaris* reached significantly higher levels than in tolerant ones (Knecht et al., 1995). However, at higher concentrations of cadmium, the seedling axes of the pigeonpea cv.T21 showed lower values of glutathione reductase compared to their respective controls. It may be presumably due to the binding of cadmium ions with the functional sulphhydryl groups of the glutathione reductase activity in cadmium treated seedlings of both the cultivars of pigeonpea.

Enzymes such as catalase and superoxide dismutase have been identified as the protective enzymes against peroxidation reactions (Monk et al., 1989). Superoxide dismutase and catalase provide an efficient mechanism for the removal of free radicals from the cells (Manohar and Balasubramanian, 1986). Greater values of superoxide dismutase activity were recorded in seedling axes and in cotyledons with increasing concentrations of Pb and Cd ions (Fig 3.A). It was observed that increasing concentration of cadmium coincided with increasing superoxide dismutase activity in *Alyssum argentums* and *Alyssum maritinum* (Schickler and Caspi, 1999). It has been reported $O_2^{\cdot-}$ that superoxide dismutase acts as the first enzyme in detoxifying process and converts radical to H_2O_2 at very fast rate (Polle and Renenberg, 1994). Accumulation of hydrogen peroxide is less toxic than hydroxy radical. However accumulation of H_2O_2 in higher quantities may be cytotoxic and therefore the hydrogen peroxide formed due to the activity of the superoxide dismutase may be degraded by catalase and peroxidase resulting in the formation of water and oxygen (Scott et al., 1987). Therefore concurrent increase in catalase, a predominant utiliser of hydrogen peroxide may become necessary. However, the cv.T21 showed lower levels of catalase activity than

the cv.LRG30 in response to Pb and Cd treatments (Fig 3.B). Thus it could be assumed that there could be more cellular damage due to the accumulation of hydrogen peroxide in the cv.T21 than in cv.LRG30 with Pb and Cd treatments. The hydrogen peroxide accumulation may result from the increased activities of superoxide dismutase and reduced activities of catalase. The end product, hydrogen peroxide will in turn may be toxic to the pigeonpea seedlings.

The catalase activity is involved in the protection from oxidative stress. However its protective role is limited because of its localization in the peroxisomes, its relatively poor affinity for its substrate and its sensitivity to light induced inactivation (Foyer et al., 1994). The decline in catalase activity under Pb and Cd treatments resulted in considerable accumulation of H_2O_2 and consequent cellular damage. For this reason cv.LRG30 exhibited relatively better seedling growth than cv.T21 of the treated seedlings. Peroxidase activity also plays an important role in eliminating excess H_2O_2 accumulated in the cells under various stress conditions (Bowler et al., 1992). It has been postulated by several investigators that the action of peroxidase located in cell walls would be to confer rigidity to the cell wall and prevent later expansion involved in growth (Fry, 1986; Goldberg et al., 1986). Comparatively low peroxidase activity was noted in cv.LRG30 which exhibited a better seedling growth than cv.T21 treated seedlings which are characterized by high level of peroxidase activity (Fig 3.C).

In conclusion the generation of oxidative stress could be a characteristic of the mechanism of Pb and Cd toxicity in plants. In between two cultivars LRG30 appears relatively less sensitive than the T21. Among the metals adopted for this study, the same concentration of Cd seems to cause more phytotoxicity than Pb. The findings are in agreement with our hypotheses stated earlier. However more information is needed at the subcellular and molecular levels in order to get deeper insights into the mechanistic explanations of Pb and Cd toxicity.

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