Effects of Interactions Between Cadmium and Lead on Growth, Nitrogen Fixation, Phytochelatin, and Glutathione Production in Mycorrhizal *Cajanus cajan* (L.) Millsp.

Neera Garg · Nalini Aggarwal

Abstract Heavy metals (HM) are a unique class of toxicants because they cannot be broken up into nontoxic forms. Excess HM causes stunted growth, upsets mineral nutrition, and affects membrane structure and permeability. High tolerance to HM toxicity is based on reduced metal uptake or increased internal sequestration in a genotype. Arbuscular mycorrhizal (AM) fungi are important rhizospheric microorganisms that occur in metal-contaminated soils and perhaps detoxify the potential effects of metals. The aim of this work was to study the role of the AM fungus *Glomus mosseae* in the alleviation of cadmium (Cd) and lead (Pb) toxicities in *Cajanus cajan* (L.) Millsp. (pigeonpea) genotypes. The effects of interactions between Cd (25 and 50 mg/kg) and Pb (500 and 800 mg/kg) on plant dry mass, nitrogen metabolism, and production of phytochelatins (PCs) and glutathione (GSH) were monitored with and without AM fungus in genotypes Sel-85N (relatively tolerant) and Sel-141-97 (sensitive). Cd treatments were more toxic than Pb, and their combinations led to synergistic inhibitions to growth and nitrogen-fixing potential (acetylene reduction activity [ARA]) in both genotypes. However, the effects were less deleterious in Sel-85N than in Sel-141-97. Exposure to Cd and Pb significantly increased the levels of PCs in a concentration- and genotype-dependent manner, which could be directly correlated with the intensity of mycorrhizal infection (MI). Stimulation of GSH production was observed under Cd treatments, although no obvious effects on GSH levels were observed under Pb treatments. The metal contents (Cd, Pb) were higher in roots and nodules when compared with that in shoots, which was significantly reduced in the presence of AM fungi. The results indicated that PCs and GSH might function as potential biomarkers for metal toxicity, and microbial inoculations showed bioremediation potential by helping pigeonpea plants to grow in multi-metal contaminated soils.

Keywords Arbuscular mycorrhiza · Cadmium · *Cajanus cajan* · Glutathione · Lead · Phytochelatin

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Introduction

Man's energy and chemical consumption, metal smelting, mining, and manufacturing processes often result in environmental contamination with a mixture of potentially toxic metals. The main sources of heavy-metal pollution in agricultural soils are fertilizer impurities and the use of refuge-derived compost and sewage disposal and municipal and industrial waste. Soil contamination with heavy metals has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain (Salt and others 1995; Shalaby 2003). Most of the metal precipitates readily in the soil. Metals that are immobilized are considered more hazardous to plants than those that can be dissolved. Higher levels of metal pollutants such as cadmium (Cd) and lead (Pb) can have lethal effects on plant growth. Plants respond favorably to the application of phosphate fertilizers, which often contain cadmium (Cd). Therefore, agricultural soils all over the world are slightly to moderately contaminated by Cd (Vassilev 2002; Rodriguez-Ortiz and others 2006).

Under field conditions, plants are often affected by more than one metal. As reported by Baker and others (1990), Cd never occurs in isolation in the natural environment but mostly as a "guest" metal in Pb/Zn mineralization. Cd is a nonessential element for plants, and excessive amounts in soil can result in injuries such as chlorosis and growth inhibition leading to plant death (Kahle 1993). This heavy metal is also known to affect photosynthesis, nitrogen metabolism, and water and nutrient uptake (Czuba and Kraszewski 1994; Sanita di Toppi and Gabrielli 1999; Labri and others 2002). There are several reports documenting oxidative stress following exposure to high concentrations of Cd (Smeets and others 2005; Dong and others 2006; Lin and others 2007; Lopez-Millan and others 2009). Pb toxicity leads to decreases in germination, length, and dry mass of roots and shoots (Mishra and Choudhari 1998; Obroucheva and others 1998; Munzuroglu and Geckil 2002); disturbed mineral nutrition (Walker and others 1977; Burzynski 1987; Paivoke 2002); reduction in cell division (Wierzbicka 1994; Eun and others 2000); and inhibition of photosynthetic activity (Ahmed and Tajmir-Riahi 1993; Rashid and others 1994; Poskuta and others 1996; Boucher and Carpentier 1999; Jabeen and others 2009). When Cd and Pb enter the cells, even in minute amounts, they produce various toxic effects such as growth retardation (Uveges and others 2002), changes in enzyme activity (Banaszak and others 2001), inhibition of chlorophyll biosynthesis (Miranda and Ilangiovan 1996), and disturbance of respiration (Romanowsk and others 2002) and mineral nutrition and water balance (Hagemeyer and others 1986; Ouariti and others 1997). Both these heavy metals enter plants via the root system where they are found primarily in root cap cells and mucilage as well as in the root hair zone, in the apoplast of the rhizodermis, and in five to six outer layers of the cortex cells (Seregin and others 2003). Then, these metals move from the root cortex to the stele and from there through the xylem to the shoots, though the phloem is not involved (Tudoreanu and Phillips 2004). The interactions between the two metals may be synergistic or antagonistic (Ting and others 1991; John and others 2008). Due to the strong adsorptive ability of Pb in soil, Pb remains fixed in soil, forming stable compounds that are not easily moved. The Cd replaced by Pb improves the phytoavailability of Cd. Thus, Pb is always inferior to Cd in competition, and the phytoavailability of Pb decreases with the presence of Cd (Chen and others 2007). John and others (2008) showed that there was a higher accumulation of Cd than Pb in the shoots of *Cucumis sativus*, which confirms that there is less uptake of Cd in the presence of Pb. They also indicated that exposure of *Lemna polyrrhiza* to different concentrations of Cd and Pb results in an increase in growth and in pigment, proline, protein, and sugar content at lower concentrations; at

higher concentrations these parameters decreased. The effect of Cd on the inhibition of growth and development was more significant than that of Pb. These interactions among metals obviously complicate studies of the effects of metals on plant growth and other processes and need to be investigated thoroughly.

Plants, like other organisms, have adaptive mechanisms whereby they are able to respond to both nutrient deficiencies and toxicities. One response of plants to heavy-metal stress is the induction of phytochelatins (PCs), a family of related peptides that have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (where $n > 1$) and are clearly related to the tripeptide glutathione (GSH), for which $n = 1$. PC synthesis is rapidly induced in the presence of a wide range of heavy metals (Grill and others 1987; Maitani and others 1996) and is involved in the cellular detoxification mechanism responsible for the ability to form stable metal-phytochelatin complexes. Therefore, it is the formation of stable complexes that is important for metal tolerance rather than the ability to synthesize PCs (Yen and others 1999).

Soil microorganisms are known to play a key role in the mobilization and immobilization of metal cations, thereby changing their availability to plants. Metal tolerance in soil microorganisms has been studied in the context of removing metals from polluted soils (bioremediation), but also in providing a biological understanding of adaptation of living organisms to extreme environments (Khade and Adholeya 2007). Arbuscular mycorrhizal (AM) associations are integral functioning parts of plant roots and are widely recognized as enhancing plant growth at severely disturbed sites, including those contaminated with heavy metals. They are reported to be present on the roots of plants growing on heavy-metal-contaminated soils and play an important role in metal tolerance and accumulation (Pawlowska and others 1996; Del Val and others 1999; Hildebrandt and others 1999; Gaur and Adholeya 2004; Andrade and others 2005). In mycorrhizal symbiosis, increases or reductions in metal content in the host plant have been observed depending on growth conditions as well as on the fungi and plant species involved (Weissenhorn and Leyval 1995; Joner and Leyval 1997). AM fungi can alleviate metal toxicity by enhancing nutrient supply and/or improving water relations (Meharg and Cairney 2000). Other possible metal tolerance mechanisms in the AM symbiosis include dilution of the metal ions by increased root or shoot growth, increased metal:phosphorus ratio, exclusion by precipitation of polyphosphate granules, and compartmentalization into plastids (de Andrade and others 2008; Azcón and others 2009).

With respect to PC production, Turnau and others (1993) observed high concentrations of nitrogen (N) and sulfur (S) as well as Cd in the vacuoles of an AM fungi associated with *Pteridium aquinilnum* collected from Cd-contaminated

soil, suggesting the existence of thiol-binding peptides in this class of fungi. The presence of metal-binding peptides in AM plants was reported by Galli and others (1994), who found an increase in thiol compounds related to the mycorrhizal status in response to copper (Cu) stress in maize plants (de Andrade and others 2008).

In general, it has been shown that legume crops are less tolerant to Cd toxicity compared to cereals and grasses (Inouhe and others 1994; Mazen 1995). However, there is evidence of intraspecific genetic variation in the tolerance of legumes to heavy metals (Belimov and others 2003). Although significant differences in biomass concentrations of Cd in plant biomass have been found among cultivars of peanut, soybean, and navybean (Bell and others 1997), little is known about cultivar-dependent variability in the uptake of both Cd and Pb (individual as well as combined) in legume species.

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is a short-lived perennial shrub that is traditionally cultivated as an annual crop in developing countries. Based on the vast natural genetic variability in local germplasm and the presence of numerous wild relatives, Van der Maesen (1980) and Saxena (2008) reported that India is probably its primary center of origin and has the largest area (around 3.6 M ha) under pigeonpea cultivation. Pigeonpea is a hardy, widely adapted, and drought-tolerant crop with a large temporal variation for maturity. These traits allow its cultivation in a range of environments and cropping systems. Its roots help to release soil-bound phosphorus, making it available for plant growth. With so many benefits at a low cost, pigeonpea has become an ideal crop for sustainable agriculture.

The aim of this work was to study the effects of the interactions between Cd and Pb and the AM fungus *Glomus mosseae* on growth, metal distribution, and production of PCs and GSH in *Cajanus cajan* (L.) Millsp. (pigeonpea) genotypes with different metal tolerances.

Materials and Methods

Experimental Design and Growth Conditions

Greenhouse experiments were conducted to study the relative response of two genotypes of pigeonpea [*Cajanus cajan* (L.) Millsp.] in terms of growth and phytochelatin and glutathione production under heavy-metal conditions. The seeds were obtained from Pulse Laboratory, and specific strains of *Sinorhizobium fredii* AR-4 and *Glomus mosseae* were obtained from the Department of Microbiology, Indian Agricultural Research Institute (I.A.R.I), New Delhi, India. The minimum temperature ranged from 22 to 29°C and the maximum temperature ranged from 30 to 37°C. The morning relative humidity was between 55 and 92% and afternoon relative humidity was between 42 and 81%.

Preparation of Pots

The soils were fumigated with methyl bromide under airtight plastic sheets and the fumigant was allowed to dissipate for 1 week (Al-Raddad 1991). Each pot was filled with 7 kg of soil mixture; the pH of the soil was 7.2, electrical conductivity was 1.0 dSm^{-1}, and the P content was 20 ppm. The pots were watered well before sowing to check for proper drainage from the pots.

Sowing and Raising of Plants

The seeds were washed with water and surface-sterilized by dipping in a 15% H_2O_2 solution for 8 min, then they were washed several times with sterile water to remove any trace of chemical that could interfere with seed germination. Seeds were pretreated with specific rhizobial inoculum of *Sinorhizobium fredii* AR-4 and were kept at room temperature to dry. Seeds were simultaneously inoculated with AM cultures of *Glomus mosseae*. The AM spores were applied at ten spores per seed (approximately 1500 spores/100 g of media). Seeds were inoculated by placing the AM inoculum in the hole under the seed and covering the soil. Eight seeds per pot were sown at uniform depth and distance. After the establishment of seedlings, they were thinned so that there were only three plants of uniform size in each pot.

Treatments

The pots were supplemented with heavy-metal solutions of Pb (500 and 800 mg/kg dry soil) and Cd (25 and 50 mg/kg dry soil) individually as well as in combinations 15 days after emergence (DAE). Two pots from each of the treatments were selected at random for sampling at 60 DAE. Six replicates were sampled for recording the observations.

Sampling Technique

The plants were carefully separated from the pots by gently washing out the soil with running tap water in order to avoid damage to the nodules. The sticking soil, along with the plants, were then transferred to a sieve (mesh) and the roots with attached nodules were washed clear of the adhering soil. The detached roots and nodules were collected from the sieve and combined with the rest of the plant material. For dry weight (DW) measurements, the samples were dried in an oven at 70°C for a few days until they reached constant weight.

Statistical Analysis

The significance of differences between mean values obtained from the experiments was determined by using Dunnett's multiple-range test. The standard errors were also calculated using SPSS software (SPSS, Inc., Chicago, IL, USA).

Methodology

Mycorrhizal Infection

Mycorrhizal infection (MI) was calculated according to Phillips and Hayman (1970) as follows:

$$\% \ MI = \frac{\text{Total number of infected roots}}{\text{Total number of roots observed}} \times 100$$

Metal Determination

Harvested plant samples were divided into roots and shoots, carefully washed with deionized water after rinsing with tap water, and then dried at 105°C for 20 min and then at 70°C in an oven until completely dry. The dried plant samples were ground to powder after their dry weights were determined. Soil samples were air-dried and ground using a mortar and pestle and then were sieved through a 0.149-mm sieve (Wei and Zhou 2004). The plant and soil samples were digested in a solution containing 3:1 HNO_3:$HClO_4$ solution (Wang and Zhou 2003). The concentrations of heavy metals were determined using an atomic absorption spectrophotometer (AAS, Hitachi 180-180 type) with certified reference materials for quality assurance purposes. Reagent blanks and internal standards were used to ensure accuracy and precision in Cd and Pb analysis.

Determination of Glutathione

Glutathione was estimated following the method of Anderson (1985). Fresh leaf samples (0.5 g) were homogenized in 2 ml of 5% (w:v) sulfosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000 rpm for 10 min. To 0.5 ml of supernatant, 0.6 ml of 100 mM (pH 7.0) phosphate buffer and 40 μl of 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min the absorbance was read at 412 nm on a UV–Vis spectrophotometer. A standard curve was prepared from varying concentrations of reduced glutathione.

Determination of Total Nonprotein Thiols

Total nonprotein thiols were determined as described by Del Longo and others (1993). One hundred microlitres of the supernatant (described above) was placed in a microfuge tube to which 0.5 ml of reaction buffer [0.1 M phosphate buffer (pH 7.0) and 0.5 mM EDTA] and 0.5 ml of DTNB (1 mM) were added. The reaction mixture was incubated for 10 min and absorbance was read at 412 nm using the UV–Vis spectrophotometer. Values for the absorbance were corrected by preparing a blank without extract. A standard curve was prepared from varying concentrations of cysteine to calculate nonprotein thiol content in samples. For theoretical determination of PCs, the difference between total nonprotein thiol and GSH was considered to represent PCs (Bhargava and others 2005).

Results

Percent Mycorrhizal Infection (MI)

MI was calculated for the roots of inoculated plants of both the genotypes (Sel-85N and Sel-141-97) under heavy-metal stress (Table 1). No MI was found in the uninoculated plants. A gradual reduction in MI was observed when the roots were exposed to increasing concentrations of Cd and Pb in the soil. However, the decline was greater in cases of Cd compared to Pb treatments. As the concentration of the metals increased, MI decreased. When plants were exposed to a combination of Cd and Pb, the effects were synergistic and MI further decreased. Although the results showed a decrease in MI with increasing levels of heavy-metal toxicity, it was observed that MI was relatively tolerant to heavy-metal stress and significant infection was observed even at high metal concentrations under single as well as combined applications. Mycorrhizal symbiosis was comparatively more effective in Sel-85N than in Sel-141-97.

Cadmium (Cd) and Lead (Pb) Concentrations

Plants absorbed both Cd and Pb but the absorption of Cd was less compared to Pb depending upon the concentrations of metals applied (Figs. 1, 2) The rate of accumulation of Cd and Pb was higher at lower concentrations in all the plant organs (roots, nodules, and shoots). However, at higher concentrations the absorption was almost stagnant due to the toxicity caused to the plant.

The contents of Cd and Pb were highest in roots, significantly higher in the nodules, and lowest in the shoots. The genotype Sel-85N, which was tolerant to metal stress and supported higher plant biomass, also had relatively lower metal contents than Sel-141-97. In the presence of Pb, the uptake of Cd significantly increased in all plant parts, whereas the absorption of Pb decreased. The presence of the fungal symbiont in the rooting medium significantly arrested the uptake of Cd and Pb into the root

Table 1 Effects of arbuscular mycorrhizal (AM) inoculations on dry weights of roots and shoots (g^{-1} plant), mycorrhizal infection (MI, %) of pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress singly and in combinations

Treatments	Sel-85N			Sel-141-97		
	Root weight	Shoot weight	MI	Root weight	Shoot weight	MI
Control (C)	0.865 ± 0.427	4.775 ± 0.998	0.00	0.432 ± 0.052	2.330 ± 1.344	0.00
C + AM	1.462 ± 0.319	8.975* ± 1.599	85.36 ± 3.697	0.535 ± 0.136	3.612 ± 1.123	80.15 ± 5.171
Pb$_{500}$	0.711 ± 0.100	4.199* ± 1.135	0.00	0.196* ± 0.080	1.816 ± 0.799	0.00
Pb$_{500}$ + AM	0.773 ± 0.164	4.433* ± 0.938	72.34 ± 3.407	0.228 ± 0.049	1.950 ± 0.195	60.11* ± 4.548
Cd$_{25}$	0.692 ± 0.075	3.930* ± 1.341	0.00	0.156* ± 0.067	1.589* ± 0.458	0.00
Cd$_{25}$ + AM	0.740 ± 0.117	4.191* ± 0.817	68.41* ± 4.271	0.182 ± 0.074	1.715 ± 0.418	42.81* ± 3.163
Cd$_{25}$ + Pb$_{500}$	0.632 ± 0.156	3.668* ± 1.068	0.00	0.140* ± 0.053	1.434 ± 0.746	0.00
Cd$_{25}$ + Pb$_{500}$ +AM	0.682 ± 0.151	3.910* ± 0.779	54.23* ± 3.041	0.157* ± 0.703	1.505 ± 0.418	30.74* ± 3.916
Pb$_{800}$	0.517* ± 0.089	3.247* ± 0.790	0.00	0.093* ± 0.045	1.291 ± 0.349	0.00
Pb$_{800}$ + AM	0.551* ± 0.115	3.469* ± 0.965	42.90* ± 4.604	0.104* ± 0.056	1.364 ± 0.686	23.30* ± 1.595
Cd$_{25}$ + Pb$_{800}$	0.451* ± 0.196	2.845* ± 0.488	0.00	0.072* ± 0.125	1.158 ± 0.516	0.00
Cd$_{25}$ + Pb$_{800}$ + AM	0.478* ± 0.067	3.035 ± 1.233	38.76* ± 4.011	0.082* ± 0.090	1.213* ± 0.433	20.01* ± 3.179
Cd$_{50}$	0.395* ± 0.120	2.567* ± 0.894	0.00	0.039* ± 0.015	1.016* ± 0.434	0.00
Cd$_{50}$ + AM	0.409* ± 0.074	2.696* ± 0.667	30.85* ± 2.648	0.050* ± 0.014	1.064* ± 0.597	16.25* ± 2.781
Cd$_{50}$ + Pb$_{500}$	0.338* ± 0.116	2.376* ± 0.890	0.00	0.023* ± 0.010	0.847* ± 0.111	0.00
Cd$_{50}$ + Pb$_{500}$ + AM	0.360* ± 0.083	2.416* ± 0.320	24.18* ± 3.033	0.030* ± 0.015	0.895* ± 0.137	12.20* ± 0.767
Cd$_{50}$ + Pb$_{800}$	0.291* ± 0.108	2.087* ± 0.623	0.00	0.014* ± 0.005	0.752* ± 0.247	0.00
Cd$_{50}$ + Pb$_{800}$ + AM	0.296* ± 0.121	2.146* ± 0.492	20.63* ± 3.685	0.021* ± 0.115	0.769 ± 0.177	10.30* ± 0.568

Each value is the mean of six replicates ± SE. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test

system and further translocations into the aboveground parts (shoots).

Dry Weights of Roots and Shoots (g/plant)

In general, the heavy metals Cd and Pb had significant effects on the growth of both genotypes but the effect of Cd was more detrimental than that of Pb. Heavy-metal stress significantly decreased root and shoot dry weights (Table 1). As the concentration of metals increased, dry weights of roots and shoots further decreased. Shoots seemed to withstand Cd and Pb stress more than the roots and percent decline in root biomass was significantly more than shoots. The combined treatments with both metals proved to be synergistic and plant dry weights were further reduced. Because Cd treatments were more toxic, the presence of Pb might have increased the phytoavailability of Cd, thereby inducing higher negative effects. Higher concentrations of Cd and Pb further hampered the growth of the plants. This decline was less significant in Sel-85N, indicating a higher tolerance as compared to Sel-141-97 (single-metal treatments and their combinations). Although Cd content was lower than Pb, the toxicity of Cd was much higher resulting in higher declines in plant biomass compared to that with Pb. However, with the introduction of *Glomus mosseae* in the rooting medium, there was a

significant improvement in the growth of the plants. Because mycorrhizal infection was tolerant to different concentrations of metals, their effective symbiosis might have been responsible for causing tolerance in both genotypes.

Rhizobial Symbiosis

The specific strain of *Sinorhizobium fredii* AR-4 could induce significantly higher nodulation in both genotypes. The nodule number, nodule dry weight per plant, leghemoglobin, and nitrogenase activity under control and different concentrations of heavy metal in AM-inoculated and uninoculated pigeonpea plants are presented in Table 2. In general, nodules seemed to be highly sensitive to heavy-metal stress and showed significant reduction in their numbers and dry weights, even at the lower heavy-metal concentration of 25 mg Cd/kg dry soil and 500 mg Pb/kg dry soil. In Sel-141-97, the higher heavy-metal concentration of 50 mg of Cd and 800 mg of Pb were progressively deleterious and very few nodules could be counted. However, the reduction in the nodule number per plant and dry weight in Sel-85N was much less, and a comparatively higher number of nodules and greater mass were noticed, even at the highest heavy-metal concentration of 50 mg of Cd and 800 mg of Pb. When plants were exposed to the
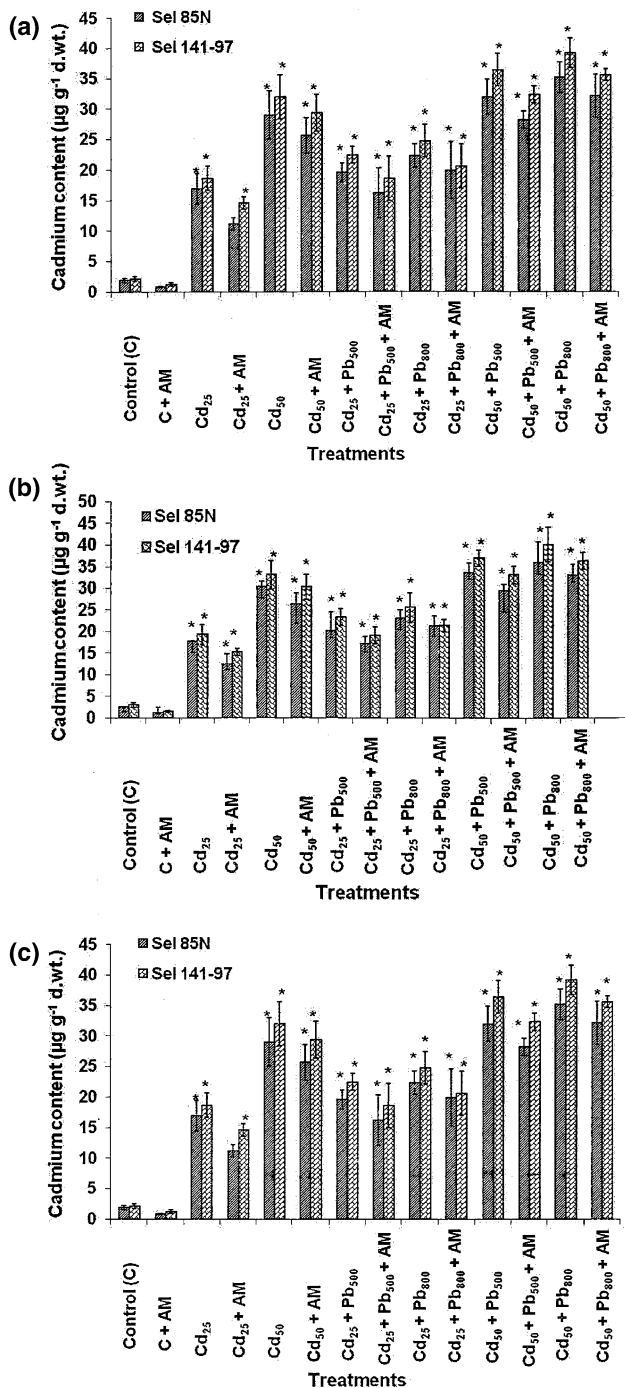
Fig. 1 Effects of arbuscular mycorrhizal (AM) inoculation on cadmium content ($\mu g\ g^{-1}$ DW) in pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations. Values are means $\pm$ SE of six replicates. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test (**a** nodules, **b** roots, **c** leaves)

Fig. 2 Effects of arbuscular mycorrhizal (AM) inoculation on lead content ($\mu g\ g^{-1}$ DW) in pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations. Values are means $\pm$ SE of six replicates. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test (**a** nodules, **b** roots, **c** leaves)

combination of Cd and Pb, root mass decreased. This led to less root hair which further decreased nodule formation, and there was less leghemoglobin content and nitrogenas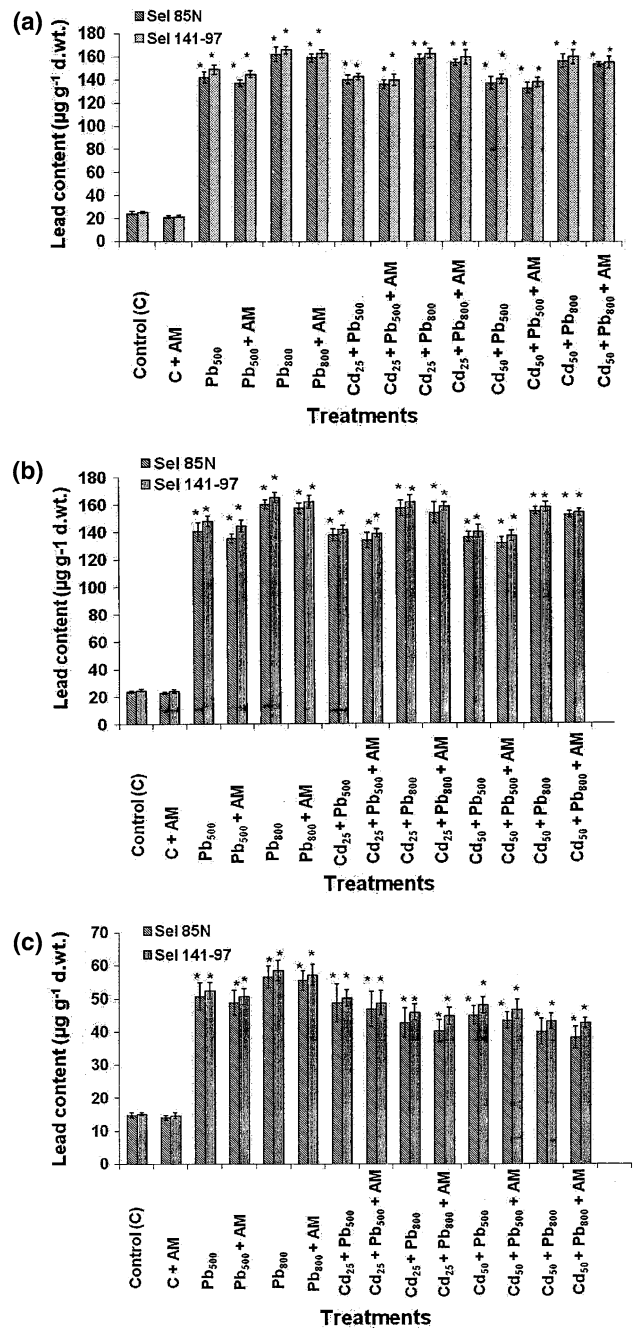e activity in the plants. The color of the nodules turned brown due to loss in leghemoglobin protein. The presence of higher levels of Cd and Pb in the nodules led to a drastic decline in the nitrogen-fixing potential of nodules (ARA). AM-inoculated plants had greater plant growth which was accompanied by less decline in nitrogen fixation potential compared to uninoculated plants. The mycorrhizal

Table 2 Effects of arbuscular mycorrhizal (AM) inoculations on nodule number per plant, nodule dry weight (g^{-1} plant), leghemoglobin content (µg g^{-1} FW), and nitrogenase activity (nmol ethylene mg^{-1} nodule DW h^{-1}) of pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations

Treatments	Sel-85N				Sel-141-97			
	Nodule number	Nodule dry weight	Leghemoglobin content	Nitrogenase activity	Nodule number	Nodule dry weight	Leghemoglobin content	Nitrogenase activity
Control (C)	25.21 ± 2.246	0.180 ± 0.088	127.77 ± 8.258	0.103 ± 0.004	15.63 ± 1.398	0.165 ± 0.010	94.40 ± 5.966	0.092 ± 0.007
C + AM	28.09 ± 1.576	0.225 ± 0.015	172.29 ± 17.576	0.143 ± 0.007	16.58 ± 0.778	0.199 ± 0.013	115.74 ± 6.527	0.118 ± 0.008
Pb$_{500}$	18.64* ± 3.630	0.150* ± 0.009	101.67 ± 10.205	0.085* ± 0.006	8.87* ± 0.583	0.085* ± 0.008	49.52* ± 6.221	0.049* ± 0.006
Pb$_{500}$ + AM	20.86 ± 1.260	0.165* ± 0.010	114.26 ± 13.323	0.096 ± 0.005	9.91* ± 0.740	0.097* ± 0.014	58.35* ± 6.060	0.057* ± 0.005
Cd$_{25}$	17.49* ± 1.914	0.146* ± 0.008	98.26 ± 8.143	0.081* ± 0.004	8.39* ± 1.416	0.072* ± 0.006	44.23* ± 5.723	0.042* ± 0.004
Cd$_{25}$ + AM	20.61 ± 0.436	0.164* ± 0.004	113.07 ± 8.070	0.094 ± 0.005	9.48* ± 0.398	0.079* ± 0.009	51.68* ± 5.519	0.048* ± 0.004
Cd$_{25}$ + Pb$_{500}$	12.89* ± 1.585	0.122* ± 0.013	80.30* ± 9.513	0.065* ± 0.004	4.71* ± 0.969	0.052* ± 0.008	32.73* ± 4.321	0.029* ± 0.006
Cd$_{25}$ + Pb$_{500}$ +AM	15.34* ± 0.503	0.136* ± 0.013	89.13* ± 6.368	0.076* ± 0.004	5.66* ± 0.642	0.061* ± 0.004	39.25* ± 4.503	0.035* ± 0.005
Pb$_{800}$	11.60* ± 1.456	0.102* ± 0.009	76.34* ± 6.283	0.051* ± 0.009	3.27* ± 0.360	0.037* ± 0.008	24.33* ± 2.462	0.021* ± 0.003
Pb$_{800}$ + AM	13.27* ± 2.082	0.115* ± 0.013	87.95* ± 4.981	0.061* ± 0.005	3.93* ± 0.549	0.043* ± 0.010	30.11* ± 2.610	0.025* ± 0.002
Cd$_{25}$ + Pb$_{800}$	10.71* ± 1.114	0.092* ± 0.010	61.02* ± 4.462	0.047* ± 0.003	2.83* ± 0.231	0.030* ± 0.008	18.71* ± 3.569	0.016* ± 0.003
Cd$_{25}$ + Pb$_{800}$ + AM	12.38* ± 0.667	0.102* ± 0.009	70.60* ± 8.115	0.054* ± 0.004	3.55* ± 0.278	0.037* ± 0.006	23.41* ± 2.373	0.019 ± 0.004
Cd$_{50}$	10.35* ± 1.011	0.079* ± 0.020	46.78* ± 10.396	0.038* ± 0.003	2.47* ± 0.312	0.023* ± 0.006	15.05* ± 2.137	0.012* ± 0.001
Cd$_{50}$ + AM	11.77* ± 1.009	0.089* ± 0.011	53.56* ± 14.221	0.041* ± 0.004	2.82* ± 0.234	0.028* ± 0.006	18.63* ± 3.490	0.015* ± 0.003
Cd$_{50}$ + Pb$_{500}$	9.26* ± 1.091	0.069* ± 0.011	41.78* ± 5.293	0.030* ± 0.002	1.59* ± 0.458	0.015* ± 0.003	10.26* ± 1.436	0.008* ± 0.001
Cd$_{50}$ + Pb$_{500}$ + AM	9.88* ± 1.225	0.074* ± 0.007	46.31* ± 7.265	0.033* ± 0.002	2.05* ± 0.085	0.019* ± 0.004	12.98* ± 2.006	0.010* ± 0.001
Cd$_{50}$ + Pb$_{800}$	8.19* ± 0.316	0.057* ± 0.005	32.44* ± 2.469	0.022* ± 0.002	0.82* ± 0.086	0.008* ± 0.002	–	–
Cd$_{50}$ + Pb$_{800}$ + AM	8.69* ± 0.570	0.059* ± 0.007	34.62* ± 5.169	0.024* ± 0.002	1.24* ± 0.100	0.012* ± 0.002	–	–

Each value is the mean of six replicates ± SE. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test

association displayed a synergistic effect on plant-*Rhizobium* symbiosis and the plants seemed more symbiotically efficient when AM fungi were present in the root system of all the plants.

Phytochelatins (PCs) and Glutathione (GSH) Production

Nonprotein thiols (NPT) increased with increasing concentrations of both Cd and Pb (Fig. 3). The levels of PCs and GSH also increased significantly with exposure to Cd, whereas exposure to lower concentrations of Pb alone had no obvious effect but at high concentrations Pb had some effect on PCs and GSH levels in both genotypes (Figs. 4, 5). As Cd content was higher in nodules as discussed earlier, PCs were also found to be higher in nodules than in roots and shoots. Although Pb treatments alone had little effect on PC production, Pb combined with Cd yielded higher PC activity compared to that from single Cd treatments, probably because of lead's role in increasing Cd availability in the soil. The degree of the effect of the combination was dependent on the concentration of both Cd and Pb. Because the presence of AM fungus reduced the absolute contents of both metals significantly, it had a direct role in further increasing the production of PCs and GSH, and the production of these biomarkers made the plants more adaptable to the stress conditions.

Discussion

The data from studies have clearly shown that Cd and Pb strongly accumulate in roots, more so than in nodules, but substantially lower amounts were observed in leaves (An 2004; Lima and others 2006; Bidar and others 2007). It was also found that accumulation of Pb in roots was greater than Cd. These results were in agreement with the data obtained by other authors for different plant species (Burzynski 1987; Trivedi and Erdei 1992; Burzynski and Buczek 1998; Kim and others 2002). It was proposed by Brennan and Shelley (1999) that a very high accumulation of Pb in roots might be connected with precipitation of the metal at the root surface as amorphous Pb-phosphate. It was noteworthy that transport of Cd to shoots was higher compared to Pb. These data suggest that the mechanism of translocation of Pb and Cd from roots to shoots is different (Kim and others 2002; Brekken and Stennes 2004; Duman and others 2007; Bidar and others 2009).

The constant increase of lead and cadmium in different plant parts resulted in a general reduction in the growth of *Cajanus cajan* genotypes. Greater decline in plant biomass was observed in the presence of Cd than Pb, although the uptake of the former was significantly less than that of
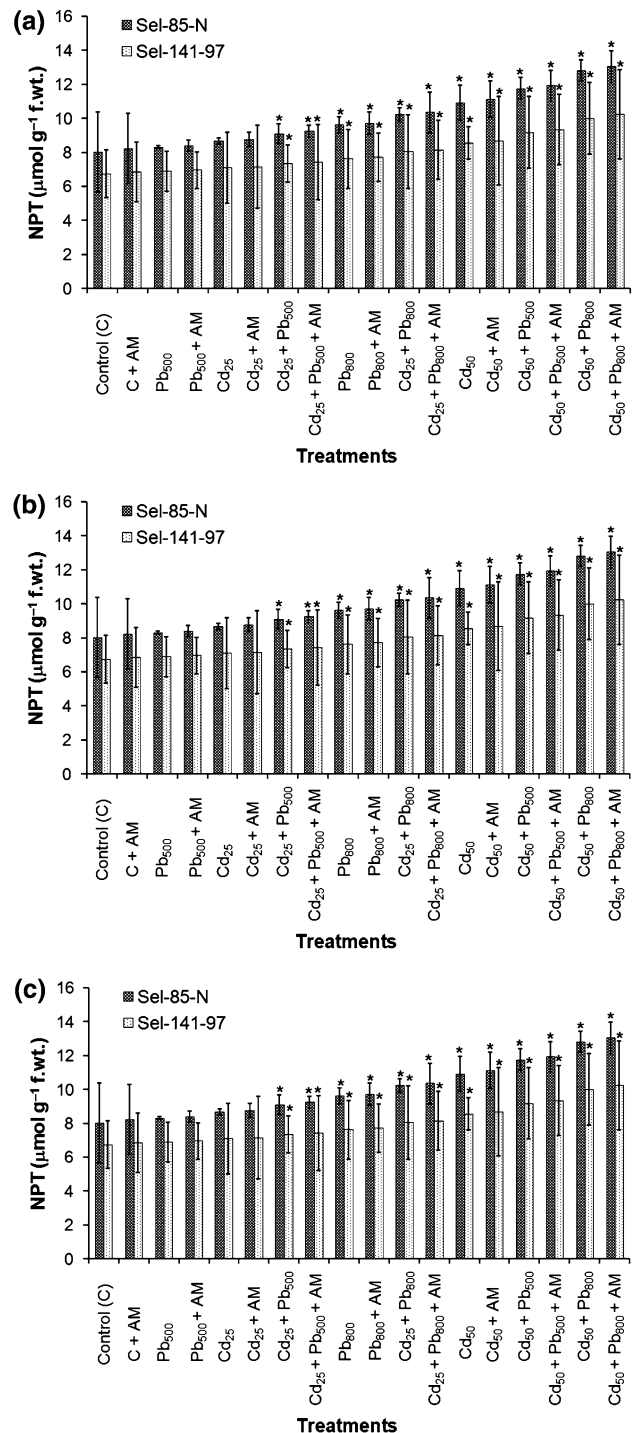
Fig. 3 Effects of arbuscular mycorrhizal (AM) inoculation on nonprotein thiols (µmol g^{-1} FW) in pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations. Values are means ± SE of six replicates. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test (**a** nodules, **b** roots, **c** leaves)

the latter. The reduction in growth of *C. cajan* provided evidence that elements like Cd or Pb, if present in excess, are responsible for producing toxic effects that reduce
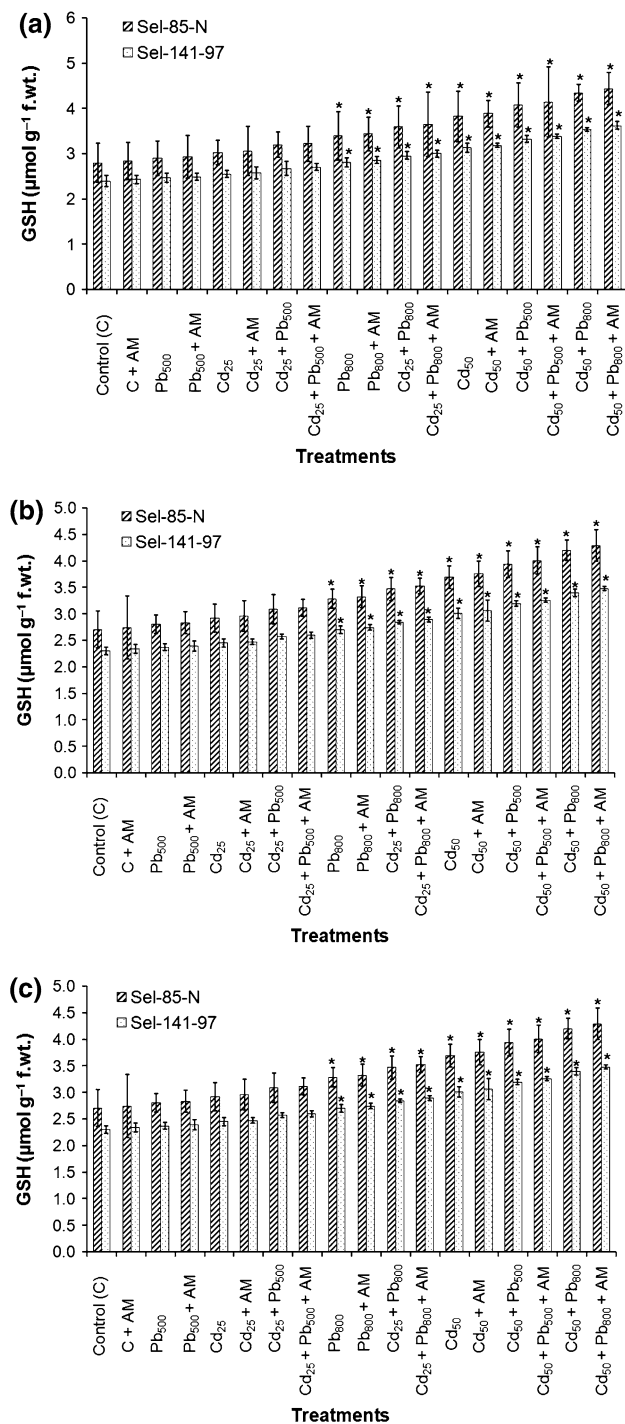
Fig. 4 Effects of arbuscular mycorrhizal (AM) inoculation on glutathione production (μmol g^{-1} FW) in pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations. Values are means $\pm$ SE of six replicates. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test (**a** nodules, **b** roots, **c** leaves)

Fig. 5 Effects of arbuscular mycorrhizal (AM) inoculation on phytochelatin production (μmol g^{-1} FW) in pigeonpea genotypes under cadmium (Cd) and lead (Pb) stress, singly and in combinations. Values are means $\pm$ SE of six replicates. *Asterisk* denotes significant difference between exposed and control plants ($p < 0.05$) as determined by Dunnett's multiple-comparison test (**a** nodules, **b** roots, **c** leaves)

plant growth and development (Dixit and others 2001; Vange and others 2004; Hassan and others 2005; Mobin and Khan 2007; Anjum and others 2008). This growth
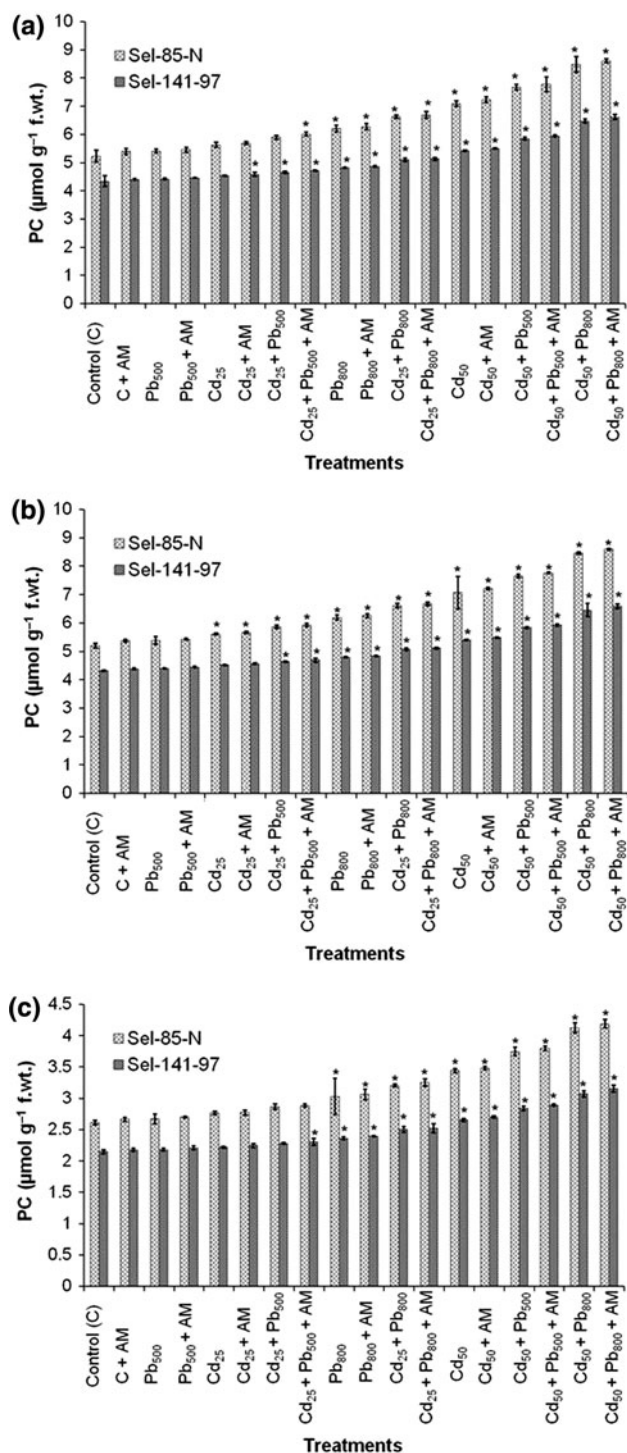
reduction might be due to competition for the same uptake systems between Cd and Pb and other divalent ions required for plant development (Alcantara and others 1994;

Lozano-Rodriguez and others 1997; Rivetta and others 1997). Also, Cd and Pb affect plant growth by inhibiting cell division (Paradiso and others 2008).

Legumes and their associated rhizobial bacteria are important components of the biogeochemical cycles in agricultural and natural ecosystems. Nitrogen fixation in nodules is sensitive to heavy-metal stress that affects soil biological activity and plant metabolism. The presence of Cd in the growth media reduces nodule formation and impairs nodule functioning in several legumes (Zornoza and others 2002; Carpena and others 2003; Chen and others 2003; Younis 2007). The results of the present study also revealed that nodule differentiation was affected under Cd and Pb stress. This differentiation was manifested by the appearance of brown nodules due to loss in leghemoglobin pigment. Senescence of legume root nodules is a complex process that implies the structural breakdown of both the bacteroides and the host cells. A positive correlation exists between leghemoglobin content and nitrogenase activity (Dakora 1995; Comba and others 1998). The inhibition of ARA by heavy metals could result from the effects of the heavy metals on bacteroid oxygen uptake because bacteroid respiration provides the energy and reducing power that nitrogenase needs for efficient nitrogen fixation (Balestrasse and others 2004, 2006; LiQin and others 2008; Shvaleva and others 2010).

The results of the present study showed the induction of PCs by both Cd and Pb, although Cd was found to be a stronger inducer of the production of PCs than Pb probably because of the higher toxic effect of Cd observed in the plants. Phytochelatin synthase is one of the key enzymes in PC synthesis and is responsible for the metal-regulated synthesis of PCs. This enzyme requires free metal ions such as Cd^{2+} and Pb^{2+} for activity (Chen and others 1997; Li and others 2006; Mishra and others 2006; Pal and Rai 2010). Therefore, the results obtained in the present study further demonstrate that the synthesis of PCs is closely linked to metal uptake and accumulation. This observation is consistent with reports on plants exposed to unequal concentrations of excess Cd or Pb (Zenk 1996; Gisbert and others 2003; Kang and others 2007; Jabeen and others 2009; Pal and Rai 2010). When Cd and Pb were combined, reciprocal synergistic effects of Cd and Pb with respect to the production of PCs were observed. The synergistic effects observed in the present study are likely to be connected with the effects of intracellular metals simultaneously activating the catalytic activities of PCs in the cytoplasm. Furthermore, the present results obtained using different concentrations of Cd and Pb in combination suggests that there are interactive effects of Cd and Pb with respect to the production of PCs.

Glutathione (GSH) is a major low-molecular-weight thiol compound in most plants (Kunert and Foyer 1993;

Foyer and others 1997). It is a major nonprotein thiol in plant cells and also represents a storage form of reduced sulfur (S). It is not only a central compound in S metabolism but also a major component of the cellular antioxidative defense system against heavy-metal toxicity (May and others 1998a, b; Gong and others 2005; Pitschke and others 2006; Blum and others 2007; Szalai and others 2009). Glutathione acts as a disulfide reductant to protect thiol groups on enzymes (Noctor and others 2002). Sulfate assimilation pathways provide plants with cysteine which is further used for protein synthesis and as a source of reduced sulfur for the biosynthesis of a number of S-containing compounds, including GSH. It has previously been shown that cysteine synthesis is improved with the addition of S sources and that the surplus of Cys limits the synthesis of GSH (Saito and others 1994; Nikiforova and others 2003). Therefore, as observed in the present study, GSH synthesis of plant cells is dependent on and regulated by the S supply of the plants. It has been suggested that GSH acts as a first line of defense against metal toxicity by complexing/influxing metals before the induced synthesis of PCs arrives at the effective level (Singhal and others 1987; Freedman and others 1989). Furthermore, GSH is the direct precursor/substrate for the synthesis of PC (Anjum and others 2008; Singh and others 2008). It has also been reported that elevated levels of GSH synthesis appear to be an intrinsic response of plants to stress and may be involved in signal transduction of environmental stress (May and others 1998a). Based on these results, GSH may serve as a biomarker for metal toxicity in plants, especially for Cd. This indicates that under metal stress, pigeonpea plants have a positive responsive mechanism to regulate GSH synthesis with an adaptation to metal stress and for PC synthesis. The results suggest that GSH may have a dual role in response to metal stress.

Pigeonpea plants developed chlorosis and necrosis when grown in heavy-metal-contaminated soil not inoculated with AM fungi, but these plants resisted the adverse soil conditions when they were inoculated with AM fungi. High amounts of heavy metals in the soil can decrease plant growth and nutrient uptake and it is known that AM fungi protect plants against the toxicity of heavy metals (Rabie 2005; Abou-Shanab and others 2008). Extrapolation of the data shows that nodule formation and percentage of mycorrhizal infection in pigeonpea plants were greatly reduced with increasing Cd and Pb concentrations (Wang and others 2007). Nevertheless, Cd was more toxic than Pb to inoculated plants. This finding is in line with results of Hildebrandt and others 1999 and Vogel-Mikus and others (2005) who reported that sensitivity of AM symbionts to heavy-metal-contaminated soil was expressed as a reduction in spore germination, hyphal growth, or root colonization, while sensitivity of N_2-fixing bacteria was

expressed as decreased nodule formation and bacterial numbers (Andrade and others 2004). AM symbiosis is known to support nitrogen fixation by providing legumes with phosphorus (P) and other immobile nutrients such as copper and zinc that are essential for nitrogen fixation (Clark and Zeto 2000; Scheublin and van der Heijden 2006). However, reports on the role of AM fungi in improving nodulation and nitrogen metabolism under Cd and Pb stress are scarce. The present study showed that the presence of AM fungi enhanced the number of nodules, their dry weights, leghemoglobin content, and nitrogenase activity in both genotypes. There are a few reports on the effects of mycorrhizal fungi on Cd and Pb uptake by plants. We recorded less Cd and Pb in the shoots of mycorrhizal plants compared with nonmycorrhizal plants at the highest Cd and Pb levels. Galli and others (1994) suggested a possible retention of heavy metals by fungal mycelia involving adsorption to cell walls, thereby minimizing metal translocation to the shoots. This hypothesis was corroborated by Joner and others (2000) who demonstrated that AM mycelia had a high metal sorption capacity. However, the lower Cd and Pb concentrations found in mycorrhizal plants compared to those in nonmycorrhizal plants may be a consequence of the dilution effects caused by their greater growth, as suggested by Plenchette and others (1983), Jamal and others (2002), Yu and others (2005), and Vosatka and others (2006). AM attenuates the toxic effect of metals, retaining them in the fungal structure with the subsequent restriction of metal transfer to the plant (Joner and Leyval 1997); Vivas and others 2003). We noticed a qualitative and quantitative increase in plant organ phytochelatin synthesis that correlated with fungal presence. Heavy-metal stress has recently been shown to increase the transcription of a phytochelatin synthetase gene in mycorrhizal pigeonpea roots, in which the expression of genes was also enhanced (Rivera-Becerril and others 2002, 2005). In general, it is currently accepted that the presence of heavy metals in the soil induces changes in the pattern of plant gene and protein expression (Robinson and others 1994; Prasad 1995; Hajduch and others 2001). Recent findings by Repetto and others (2003) showed that mycorrhizal symbioses also modulate the expression of several plant proteins. The significance of these changes and their relation to plant tolerance are not clear yet.

Conclusion

Specific intraspecific variation was found between the tested genotypes of Cajanus cajan in their tolerance to Cd and Pb in the presence of toxic Cd and Pb concentrations when the plants were grown in contaminated soil. The differential effects between Cd and Pb tolerance, heavy-metal concentrations, and more efficient biomass production indicate the existence of independent genetic controls of these traits and represent a useful model for studying mechanisms of heavy-metal tolerance in pigeonpea genotypes.

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