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Research Article

BIOCHEMICAL CHANGES AND PROTEIN PROFILING BY SDS-PAGE OF CAJANUS CAJAN DURING THE EARLY STAGES OF SEEDLING GROWTH IN RESPONSE TO CADMIUM STRESS

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A B S T R A C T

Pigeonpea is an important pulse crop in India. Seedlings of pigeonpea (*Cajanus cajan* L. Mill.) grown on treated with different cadmium (Cd) concentrations representing 0, 0.02, 0.04 and 0.06 mM were used in three pigeonpea cultivars, LRG30, LRG41 and ICPL85063 on protein, amino acid, free organic acids, proline and leaf storage protein profiles using SDS-PAGE were studied. Total protein content was exhibited higher levels in cv.LRG30 and lower levels of total free amino acid content when compared to LRG41 and ICPL85063 in response to Cd stress among the three pigeonpea cultivars. The changes in protein and amino acid contents in different organs of pigeonpea seedlings suggest that Cd affected the mobilization, hydrolysis and translocation of the storage substances to the growing axis from the cotyledons. The accumulation of free organic acids was relatively less in cv.LRG30 than in LRG41 and ICPL85063. The pigeonpea cultivars, LRG41 and ICPL85063 registered lower values of proline content when compared to LRG30 in response to Cd ions supplied indicating that LRG30 possesses better tolerance with more proline content. A change in leaf protein pattern was expressed differently among the three cultivars of pigeonpea. Among the three cultivars, LRG30 showed sharp and thick band pattern, where as thin and light coloured bands were observed in cv.LRG41 and ICPL85063 when compared to their controls.

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INTRODUCTION

Heavy metal pollution is of considerable importance and relevant to the present scenario due to the increasing levels of pollution and its obvious impact on human health through the food chain (Hadjiliadis, 1997; Wojas *et al*., 2008; Dai *et al*., 2011). Due to diverse human activities, such as mining and smelting, metal pollution is becoming a major risk to many ecosystems. Heavy metals have been considered as major environmental pollutants and their phytotoxicity is well established (Ross, 1994; Prasad *et al*., 2001; Prasad and Srzalka, 2002). Among the pollution-producing metals like As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn, cadmium (Cd) is regarded as one of the most toxic metal pollutant which is a nonessential element with no known physiological functions. Cadmium interferes with the functional activities of enzymes involved in various synthetic and metabolic activities of the plant system (Van Assche and Clijsters, 1990; Reddy and Prasad, 1992a).

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is one of the most important and major pulse crop, which is considered to be a profitable and popular crop of India.

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Its seed protein is about 21% which is on par with any other legume. The seeds constitute principal source of vegetable protein which forms part of daily dietary of Indian homes and therefore needs much attention. During seed germination, seed proteins are extensively hydrolysed and the released amino acids are translocated to embryonic axis for the synthesis of protein and other nitrogenous compounds. Presence of excess amounts of heavy metal suppresses the normal mobilization of storage proteins and ribonucleic acids. It also affects even the translocation of amino acids and ribonucleotides that are formed to certain extent to the growing embryonic axis of *Pisum sativum* (Mittal and Sawhney, 1990). One of the causes for amino acid accumulation in Cd-treated lettuce plants might be the consequence of the reduced functioning of respiration mostly due to membrane damage (Vazquez *et al.*, 1992a). The interaction of Cd with SH groups may increase the levels of methionine and cysteine (Reese and Winge, 1988; Rauser, 1990) and consequent reduction of protein synthesis. Altogether, reduced protein synthesis and/or enhanced protein hydrolysis may result in reduction of protein content under the influence of Cd (Melnichuk *et al.*, 1982).

Amino acids are the primary products of inorganic nitrogen assimilation. In addition, free amino acids may also be formed by protein hydrolysis. Heavy metal toxicity causes the generation of ROS and its reaction with lipids, pigments, proteins and amino acids, resulting in membrane damage,

inhibition of photosynthesis and enzyme inactivation (Stoeva *et al*., 2003; Wang *et al*., 2008). Due to its high mobility and water solubility, Cd readily enters the roots through the cortical tissue and can reach the xylem via an apoplastic and/or symplastic pathway, complexed to organic acids or phytochelatins (Salt *et al.*, 1995). The principal classes of intracellular metal binding ligands include organic acids, metallothioneins (MTs) and sulphydryl rich phytochelatins (PCs) (Grill *et al.*, 1985; Robinson *et al.*, 1988; Tomsett and Thurman, 1988). Complexation of heavy metal ions to organic acids was assumed to be a supporting device to maintain low cytoplasmic concentrations. Organic acids such as malate and citrate form complexes within the vacuoles (Verkleij and Schat, 1990; Manara, 2012).

Proline plays important roles in osmoregulation, protection of enzymes (Nikolopoulos and Manetas, 1991), stabilization of the machinery of protein synthesis (Kadpal and Rao, 1985), regulation of cytosolic acidity (Venekemp, 1989), and scavenging of free radicals (Smirnoff and Cumbes, 1989). It also acts as an effective singlet oxygen quencher (Alia *et al*., 2001). Higher plants exhibit preferential accumulation of proline under stress conditions in seedlings of certain crop plants treated with Pb, Cd, Co and Zn where the proline content increased proportionally with an increasing metal concentration. It is reported that the presence of proline can reduce the levels of free radicals being generated by chloroplasts (Bassi and Sharma, 1993, Talanova *et al.*, 2000; Sharma and Dietz, 2009). Importantly, proline reduces Cd stress not by sequestering Cd but by reducing Cd-induced free radical damage and maintaining a stringent reducing environment (higher GSH levels) within the cell. In seeking additional supporting evidence for this, exogenous proline induces Cd tolerance by maintaining a higher GSH level and GSH metabolizing enzymes in mung bean (*Vigna radiata* L.) (Hossain *et al.*, 2010). Huang *et al.* (2010) studied the physiological and biochemical responses in the leaves of two mangrove plant seedlings (*Kandelia candel and Bruguiera gymnorrhiza*) exposed to multiple heavy metals (Cd, Pb and Hg) and concluded that proline, GSH and PC's-SH in *K. candel* may play a more important role in ameliorating the effect of heavy metal toxicity than in *B. gymnorrhiza*.

Among biochemical techniques, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is widely used due to its validity and simplicity for describing genetic structure of crop germplasm (Javid et al., 2004). SDS-PAGE is practically a reliable method because seed and leaf storage proteins are largely independent of environmental fluctuation (Iqbal et al., 2005; Nisar et al., 2007; Hameed et al., 2009) and their profiling using SDS-PAGE is particularly considered as a consistent tool for economic characterization of germplasm (Javid et al., 2004; Jinghui et al., 2006).

Currently, environment contamination and plant exposure to heavy metals is a growing problem throughout the world. Toxic levels of heavy metals were reported to affect a variety of processes in plants (Sanitá di Toppi *et al*., 2001), the information on heavy metal stress is limited and requires detailed investigations. The current study was achieved to evaluate pigeonpea responses to Cd stress through biochemical parameters and protein profiling with the following particular objectives: (i) to compare the pigeonpea cultivars in terms of their variation in reaction to varied concentrations of Cd stress i.e. various 0.02, 0.04 and 0.06 mM; (ii) to determine the biochemical parameters; (iii) to investigate the changes in profile of proteins in tolerant and less tolerant genotypes in relation to cadmium tolerance including compartmentation involving different organs of germinating seeds.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of three cultivars of pigeonpea (*Cajanus cajan* (L.) Millspaugh) namely LRG30 (Long duration, 180-300 days), LRG41 (Medium duration, 150-180 days), and ICPL85063 (Short duration, 100-150 days) obtained from ICRISAT, Patancheru and LAM, Guntur, Andhra Pradesh, India were used for the present investigation. These varieties are grown around the Visakhapatnam and its surrounding villages.

The seeds of healthy and uniform size were selected and surface sterilized with 0.001 M mercuric chloride for 2 min, washed thoroughly with glass-distilled water and then soaked in distilled water for 2 h. The soaked seeds were then spread over plastic trays (approximately 50 seeds per tray) lined with two-layered whatman No.1 filter paper containing different concentrations of cadmium. Cadmium as cadmium chloride: $CdCl_2$ H_2O was used in three concentrations of metal representing 0.02, 0.04 and 0.06 mM for cadmium. These concentrations were selected on the basis of preliminary experiments in which the concentrations less than 0.02 mM for cadmium. The seeds raised in distilled water served as controls. Twenty five ml of each test solution was added separately to each tray and the filter papers were replaced on every alternate day during the study period. The seeds of the three cultivars were allowed to germinate at 30 ± 2°C for 8 days under a photoperiod of 12 h and at a photosynthetic photon flux density (PPFD) of 195 µmol $m^{-2}s^{-1}$. The analyses were made in different parts of the seedling viz. root, shoot and cotyledons separated prior to start of each experiment. Five replicates were used for each treatment.

Total proteins

Total protein content was estimated by adopting the method of Lowry *et al*. (1951). The author considers it as more suitable to pigeonpea seedlings than the method of Bradford (1976).

Extraction: Five hundred mg of a sample was ground and macerated thoroughly by using a suitable volume of 10% (w/v) trichloroacetic acid. The homogenate was centrifuged at 5000 xg for 20 min and the supernatant was discarded. This process was repeated twice. The pellet was suspended in 5 ml of 0.1 N NaOH solution and was used for the estimation of proteins.

Estimation: One ml of protein extract was taken into a clean dry test tube. To this 5.0 ml of reagent `C´ was added, mixed thoroughly and allowed to stand at room temperature for 10 min. Then 0.5 ml of reagent `D´ was added rapidly with immediate mixing and allowed to incubate for 30 min at room temperature. The colour developed was read at 660 nm using Systronics 112 spectrophotometer. A similarly treated blank was used for zero setting. The protein content was calculated from a standard curve prepared from bovine serum albumin.

Preparation of reagents

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH.
Reagent B: 0.5% $CuSO_4$ in 1% sodium potassium tartarate solution.

Reagent C: It is a mixture of 50 ml of reagent A + one ml of reagent B.
Reagent D: 1 N Folin-ciocalteus phenol reagent.

Total free amino acids

Total free amino acids were determined according to the method of Moore and Stein (1948).

Extraction: 100 mg of a plant sample was homogenised in 80% ethanol. The supernatant after centrifugation was evaporated in a boiling water bath and the left over residue was dissolved in 15 ml citrate buffer pH 2.2. The supernatant after centrifugation was neutralized to the end point of methyl red with 1 N NaOH.

Estimation: To one ml of amino acid extract, 2 ml of ninhydrin reagent was added. After keeping the aluminium caps, the test tubes were kept shaking for 2 or 3 min and were heated in vigorous boiling water bath for 20 min. The colour intensity was read at 570 nm by Shimadzu (UV-240) spectrophotometer using the mixture of neutralised citrate buffer and ninhydrin reagent as blank. Standard curve was prepared by using leucine.

Preparation of ninhydrin reagent*:* The ninhydrin reagent was prepared directly in the brown bottles. Forty seven ml of ethylene glycol was poured into the bottle. To it, 1.25 g of carefully weighed ninhydrin was added. The solution was shaken until the ninhydrin was completely dissolved. To this solution, 1% stannous chloride in acetate buffer (pH 5.1 ± 0.03) was added. The ninhydrin reagent was ready for use 2 to 3 h after preparation.

Total free organic acids

Total free organic acid content of the different parts of control and treated seedlings of three pigeonpea cultivars were determined according to the method of Ting and Dugger (1968). One gram of the samples were taken, chopped into fine slices and boiled for 30 min with glass distilled water free of carbon dioxide. The homogenate was centrifuged at 5000 xg for 15 min and the supernatant was made up to a known volume. Ten ml of aliquots of the supernatant were titrated against 0.01 N NaOH using phenolphthalein as indicator and the results were expressed as milliequivalents of acid per organ as well as per g fresh weight.

Proline

Proline content was estimated by the acid ninhydrin method of Troll and Lindsley (1955) as modified by Tully *et al*. (1979). The extraction of proline was carried by grinding 200 mg of plant material in 5 ml of water and heating at 100°C for 30 min sealed tubes. After cooling the content was centrifuged and the supernatant was made up to a known volume.

For the estimation of proline, 5 ml of the extract was taken; 5 ml of glacial acetic acid and 5 ml of ninhydrin reagent were added and heated in a water bath for 1 h in test tube with plastic screw cap. The solution was cooled to room temperature and the colour was extracted with 5 ml of toluene by shaking them vigorously for 5 min in a separating funnel. The phases were allowed to separate. The toluene phase was transferred to a cuvette and the absorbance was determined at 515 nm in Systronics 112 spectrophotometer. Standard curve was prepared by using known quantities of proline.

Preparation of ninhydrin reagent: The desired amount of the reagent was prepared using the proportions of 125 mg of ninhydrin in 3 ml of glacial acetic acid and 2 ml of 6 M phosphoric acid and heating to 70°C. The reagent is stable for atleast 24 h.

Total protein extraction and SDS-PAGE analysis

In order to perform SDS-PAGE analysis of the three pigeonpea cultivars, total protein was extracted using method developed by Goggin *et al*., (2011). About 2.5 g of leaves were ground to a powder in liquid nitrogen, and then placed in a centrifuge tube with two volumes of extraction buffer, 0.1M phosphate buffer, pH 7.4 containing 8 M urea, 2% (v/v) Triton X-100 and 5 mM DTT. After 20 min incubation on ice with gentle rocking, the tubes were centrifuged at 12,000 g for 10 min and the pellet was collected.

SDS-PAGE was carried by 12% acrylamide separating gel and 5% acrylamide stacking gel with 0.5 mm spacers. The separating gel contained 6.68 ml of MQ water, 5 ml of 1.5 M Tris-HCl, pH 8.8, 200 µl of 10% (w/v) sodium dodecylsulphate (SDS), 8 ml of 30% acrylamide, 10 µl of TEMED and 100 µl of 10% (w/v) ammonium persulphate. The stacking gel consisted of 1.2 ml of MQ water, 0.5 ml of 0.5 M Tris-HCl, pH 6.8, 20 µl of 10% (w/v) SDS, 300 µl of 30% acrylamide, 2 µl of TEMED and 10 µl of 10% (w/v) ammonium persulphate. 50 µg of extracted protein pellet was mixed in a microtube with 2X sample buffer (1M Tris-HCl, pH 6.8, 10% SDS, Glycerol, β-mercaptoethanol, 1% Bromophenol blue) and incubated at 95°C for 3 min and a brief cooling on ice. 10 µl protein samples were loaded per lane and electrophoresed at 20 mA for 5 hours with 1X SDS-PAGE running buffer (100 mM glycine, 25 mM Tris and 0.1% (w/v) SDS). After electrophoretic run, the gels were stained overnight with Coomassie Brilliant Blue R-250 solution [40% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R-250]. The gels were destained [in 40% (v/v) methanol, 10% (v/v) acetic acid] at room temperature. Gel image was digitalized at 600 dpi using a GS-800 Calibrated Densitometer (Bio-Rad).

Statistical analysis: The data collected were computed and analysed by using statistical analysis IBM-SPSS (Version 21.0). Means and standard errors (SE) were calculated along with Analysis of variance (ANOVA) test for comparing the significance of the differences between means ($P < 0.05$). The data was used to determine whether treatment and cultivar differences were statistically significant by comparing the growth performance of three pigeonpea cultivars in terms of their mean root and shoot lengths between increasing concentrations of Cd supplied.

RESULTS

Total proteins

The total protein content of the roots of the three pigeonpea cultivars increased with increasing seedling age in all the Cd treatments. However, the Cd treated ones showed values lower than the corresponding controls. The decrease becomes more conspicuous with increasing concentrations of externally supplied Cd (Fig.1a, b, c). The total protein content of the shoots of the pigeonpea seedlings exhibited a trend similar to the roots with increasing age of the seedling as well as with increasing concentrations of externally supplied Cd (Fig.2a, b, c).

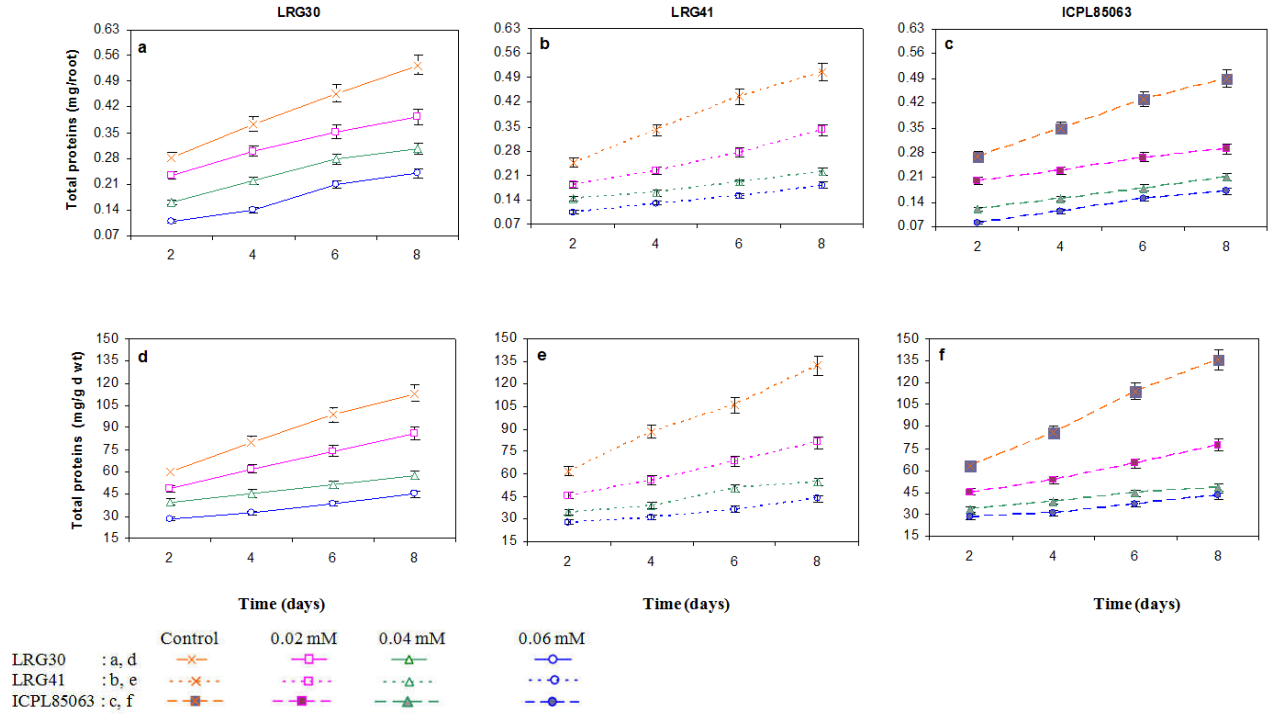


Fig 1 Total protein content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

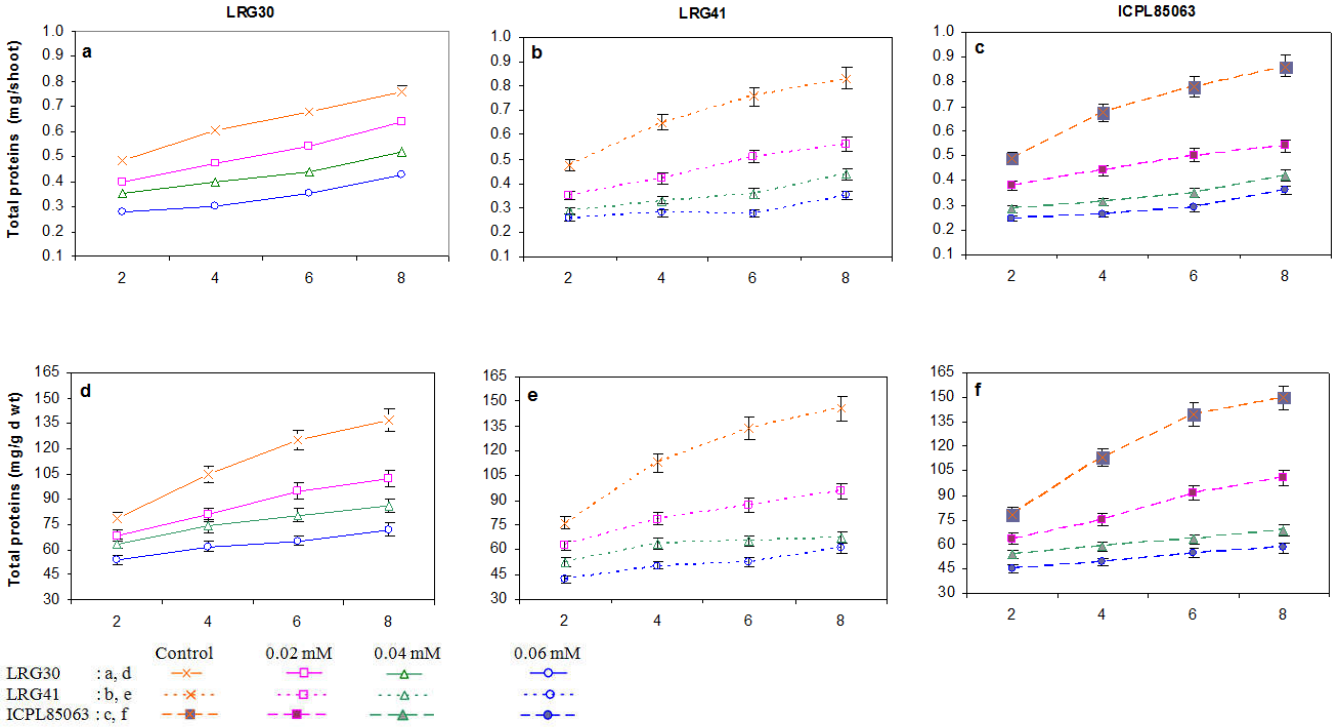


Fig 2 Total protein content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

On the other hand, the decline in the total protein content of the cotyledons of the three pigeonpea cultivars affected considerably by retaining greater quantities in them with increasing concentrations of externally supplied Cd when compared to their controls (Fig.3a, b, c). On the whole the total protein content of the cotyledons of the Cd treated germinating seeds decreased with increasing age of the seedling.

The per cent decrease in the total protein content of the roots of the 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentrations were 23.92, 39.13 and 54.35% in LRG30; 36.37, 56.82 and 65.91% in LRG41 and 37.21, 58.14 and 65.12% in ICPL85063 respectively in relation to their controls. The per cent decrease in the total protein content of the shoots of the respective Cd treatments were 20.59, 35.30 and 48.53% in LRG30; 32.90, 52.64 and 63.16% in LRG41 and 35.90, 55.13 and 62.83% in ICPL85063 when calculated in relation to their controls. The per cent retention in the total protein content of the cotyledons of the respective Cd treated germinating seeds of pigeonpea were 32.14, 51.78 and 67.14% in LRG30; 49.04, 65.60 and 77.71% in LRG41 and 47.51, 59 and 75.46% in ICPL85063 over their corresponding controls.

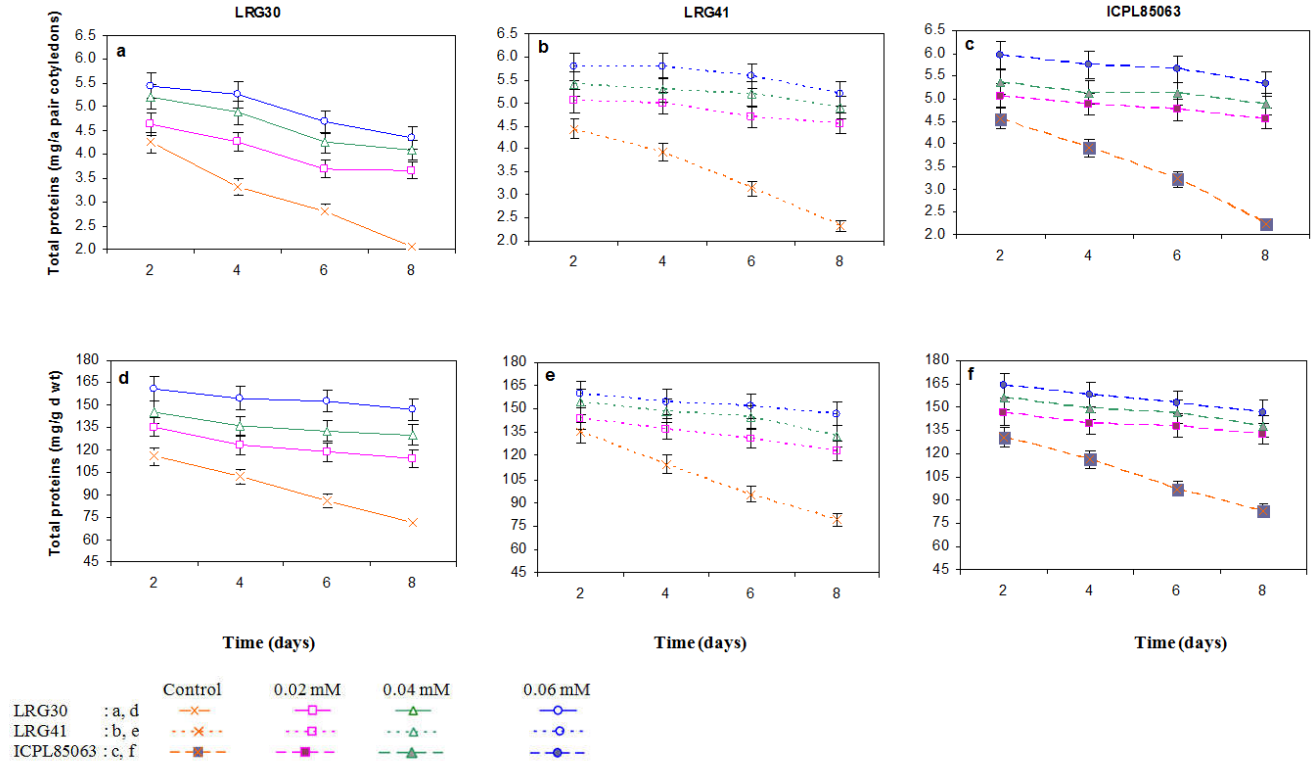


Fig 3 Total protein content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

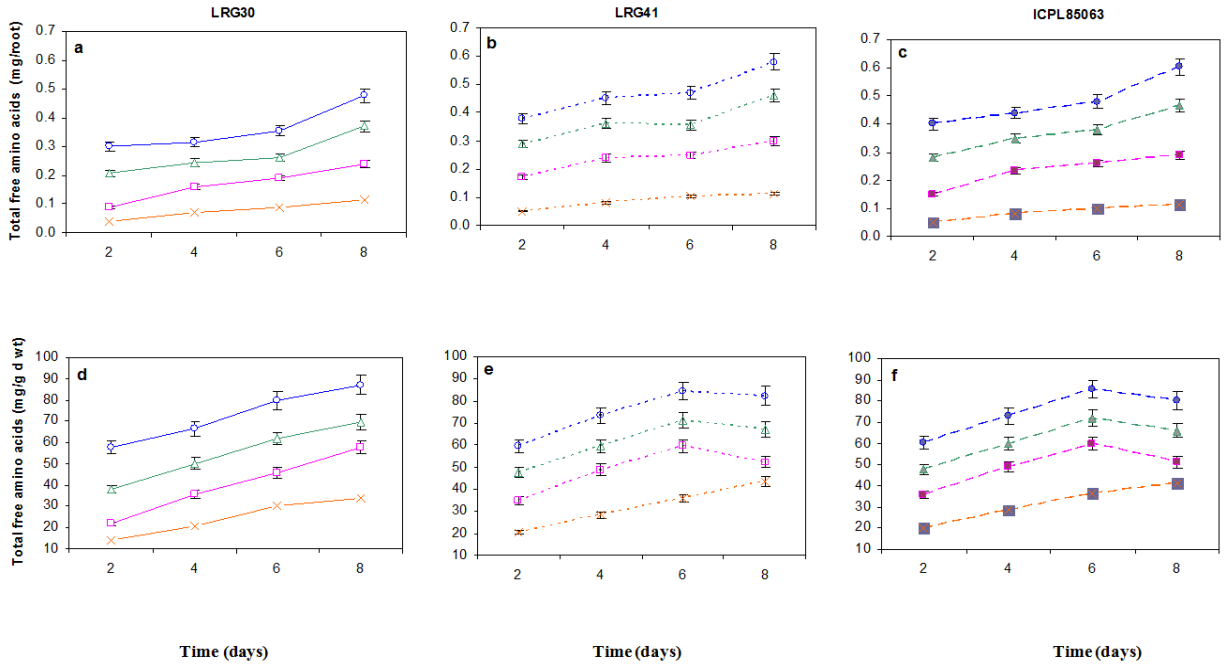
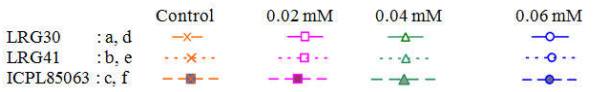


Fig 4 Total free amino acid content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).



Basing on the per cent reduction values, the pigeonpea cv.LRG30 exhibited higher levels of total protein content than LRG41 and ICPL85063 in response to Cd stress.

On dry weight basis, the changes in the total protein content of root, shoot and cotyledons of the three pigeonpea cultivars showed a trend similar to units expressed per organ basis both with increasing seedling age and with increasing concentrations of externally supplied metal ions (Fig.1d, e, f; 2d, e, f and 3d, e, f). The total protein content of the roots showed 0.05 level of significance in cv.LRG30 and 0.01 level of significance in cv.LRG41 and ICPL85063.

Shoots of the three pigeonpea cultivars showed 0.01 level of significance with the external concentrations of Cd supplied. On the other hand, the total protein content of the cotyledons of the three pigeonpea cultivars, established a significant level of 0.01 in response to increasing concentrations of externally supplied Cd (Table-1).

Total free amino acids

The accumulation of total free amino acids in the roots of the three pigeonpea cultivars increased with increasing age of the seedling as well as with increasing concentrations of externally supplied Cd (Fig.4a, b, c). The total free amino acid content of the roots of the Cd treated pigeonpea cultivars registered higher values when compared to their controls.

The total free amino acid content of the shoots of the Cd treated pigeonpea cultivars exhibited a trend similar to that observed for roots (Fig.5a, b, c). The accumulation of total free amino acids in the cotyledons of the three pigeonpea cultivars increased from 2 to 8 days of seedling growth. However, the amino acids of the cotyledons of the pigeonpea seedlings decreased in the order of increasing concentrations of externally supplied Cd and always registered lower values when compared to their controls (Fig.6a, b, c).

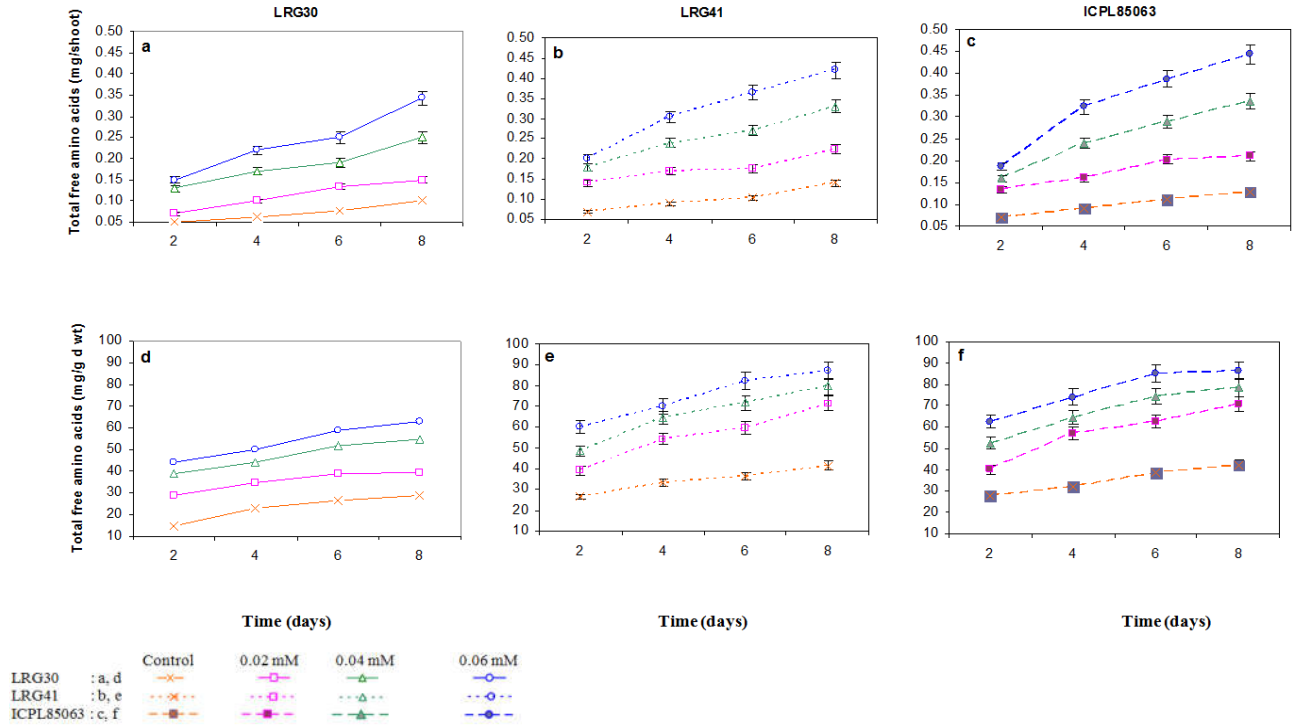


Fig 5 Total free amino acid content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

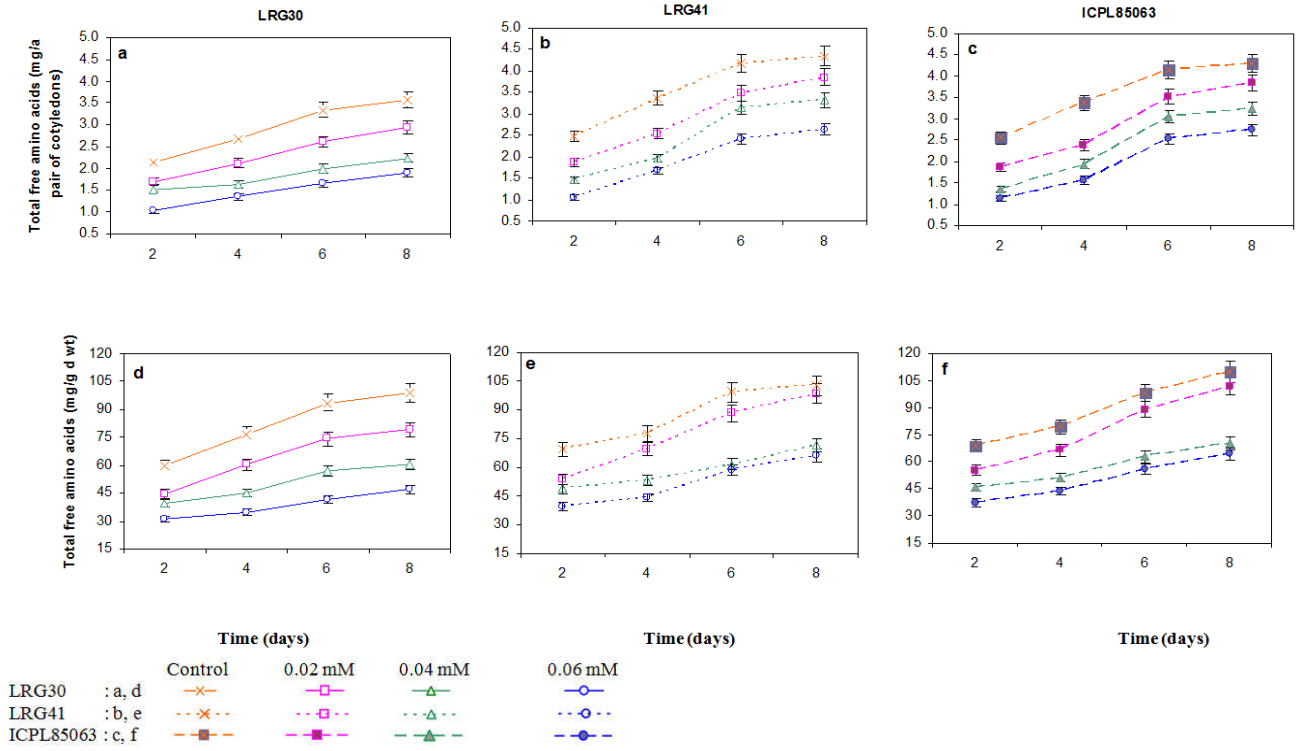


Fig 6 Total free amino acid content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

The accumulation of total free amino acids content in the roots of 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentrations showed an increase of 2.11, 2.89 and 3.88 folds in LRG30; 2.50, 3.60 and 4.70 folds in LRG41 and 2.60, 3.80 and 4.80 folds in ICPL85063 respectively when compared to their controls. The free amino acid content of the shoots of the respective Cd treatments showed an increase of 1.62, 2.37 and 3.12 folds in LRG30; 1.80, 2.70 and 3.70 folds in LRG41 and 1.82, 2.63 and 3.54 folds in ICPL85063 when compared to their corresponding controls. However, the total free amino acid content of the cotyledons of the respective Cd-treated germinating seeds showed a reduction of 21.56, 40.12 and 50.60 folds in LRG30; 16.75, 24.89 and 42.11 folds in LRG41 and 14.98, 26.58 and 38.65 folds in ICPL85063 when calculated in relation to their controls. The pigeonpea cultivars, LRG41 and ICPL85063 registered higher values of total free amino acids when compared to LRG30 in response to Cd treatment.

On dry weight basis the accumulation of total free amino acids in the roots of the three pigeonpea cultivars increased up to 6 days of seedling growth and thereafter showed a slight decline in response to Cd treatments (Fig.4d, e, f). However, the total free amino acid content of the root of the pigeonpea cultivar, LRG30 grown in different concentrations of Cd exhibited a continuous increase with increasing seedling growth (Fig.5d, e, f and 6d, e, f). The total free amino acid content of the roots and shoots of pigeonpea cultivars showed 0.05 level of significance in cv.LRG30 and 0.01 level of significance in LRG41 and ICPL85063 with external concentrations of Cd supplied. On the other hand, the total free amino acid content of the cotyledons showed 0.05 level of significance in the three pigeonpea cultivars with external concentrations of Cd ions (Table-1).

Table-1 The significant values between treatments and control of biochemical parameters and increasing concentrations of externally supplied Cd or dry weight of the different parts of 6-day old seedlings of the different parts of three pigeonpea cultivars, LRG30, LRG41 and ICPL85063 were statistically evaluated by one-way ANOVA.

	LRG30	LRG41	ICPL85063
		ROOTS	
Protein	.014*	.004**	.001**
Amino acids	.030*	.008**	.008**
Proline	.001**	.220@	.105@
		SHOOTS	
Protein	.006**	.003**	.003**
Amino acids	.017*	.006**	.006**
Proline	.012*	.002**	.002**
		COTYLEDONS	
Protein	.008**	.001**	.000**
Amino acids	.019*	.035*	.046*
Proline	.012*	.001**	.009**

**1% Level of Significant (P <0.01)

*5% Level of Significant (P <0.05)

@ Not Significant

Total free organic acids

The total free organic acid content of the roots of pigeonpea cultivars germinated and grown in different concentrations of Cd, increased from 2 to 8 days of seedling growth. An increasing trend was observed in cultivars exposed to external Cd and reached maximum levels at the higher concentrations of Cd availability (Fig.7a, b, c). The total free organic acid content of the shoots and cotyledons of the pigeonpea seedlings treated with different concentrations of Cd exhibited a trend similar to that observed for roots (Fig.8a, b, c and 9a, b, c). However, the total free organic acid content of the cotyledons decreased with increasing seedling growth.

The accumulation of total free organic acids in the roots of 6-day old seedlings of the three pigeonpea cultivars exhibited an increase of 1.28, 1.48 and 1.74 folds in LRG30; 1.47, 1.76 and 1.91 folds in LRG41 and 1.46, 1.75 and 1.93 folds in ICPL85063 in response to 0.02, 0.04 and 0.06 mM Cd concentrations respectively over their corresponding controls. The increase in the accumulation of total free organic acid content of the shoots of respective Cd treated germinating seeds were 1.04, 1.33 and 1.58 folds in LRG30; 1.24, 1.50 and 1.67 folds in LRG41 and 1.24, 1.49 and 1.68 folds in ICPL85063 over to their corresponding controls. The increase in the total free organic acid content of the cotyledons of the respective Cd treated germinating seeds were 1.21, 1.53 and 1.82 folds in LRG30; 1.14, 1.40 and 1.67 folds in LRG41 and 1.13, 1.40 and 1.66 folds in ICPL85063 over their appropriate controls.

The accumulation of total free organic acid content was relatively more in the pigeonpea cultivars, LRG41 and ICPL85063 than in LRG30. On fresh weight basis, the changes in the total free organic acid content of the root, shoot and cotyledon of the three pigeonpea cultivars exhibited a trend similar to that observed on per organ basis (Fig.7d, e, f; 8d, e, f and 9d, e, f).

Proline

A steep rise in the accumulation of proline content in the roots of the Cd treated germinating seeds of the three pigeonpea cultivars was noted from 2 to 8 days of seedling growth. Moreover, the proline content of the roots increased with increasing concentrations of externally supplied Cd and registered higher values at all stages of seedling growth when compared to their respective controls. Eventhough the increasing concentrations of externally supplied Cd led to increased accumulation of proline content, the 0.06 mM Cd concentrations exhibited relatively lower levels of proline content in the roots of 6-and 8-day old seedlings of the three pigeonpea cultivars when compared to the 0.04 mM Cd treatment (Fig.10a, b, c). The proline content of the shoots and cotyledons increased steadily with increasing seedling growth as well as with increasing concentrations of externally supplied Cd and registered higher values when compared to their corresponding controls (Fig.11a, b, c and 12a, b, c).

The accumulation of proline content in the roots of 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentrations showed an increase of 85.81, 110.98 and 94.96 folds in LRG30; 47.90, 74.25 and 59.88 folds in LRG41 and 45.56, 76.92 and 60.35 folds in ICPL85063 respectively over their corresponding controls. The per cent increase in the proline content of the shoots of the respective Cd treatment were 21.63, 39.46 and 66.67 folds in LRG30; 14.40, 23.80 and 39.67 folds in LRG41 and 15.53, 23.30 and 38.84 folds in ICPL85063 over their respective controls.

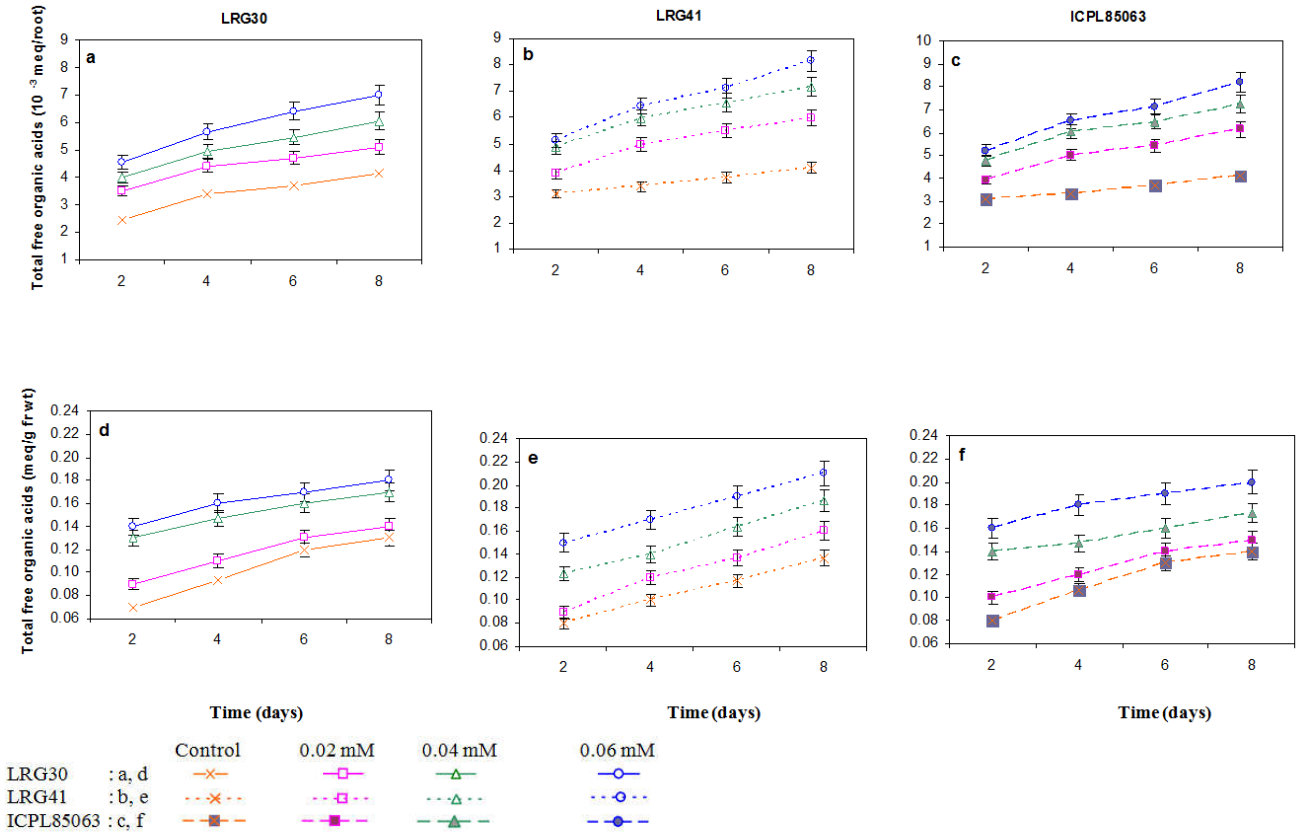


Fig 7 Total free organic acid content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

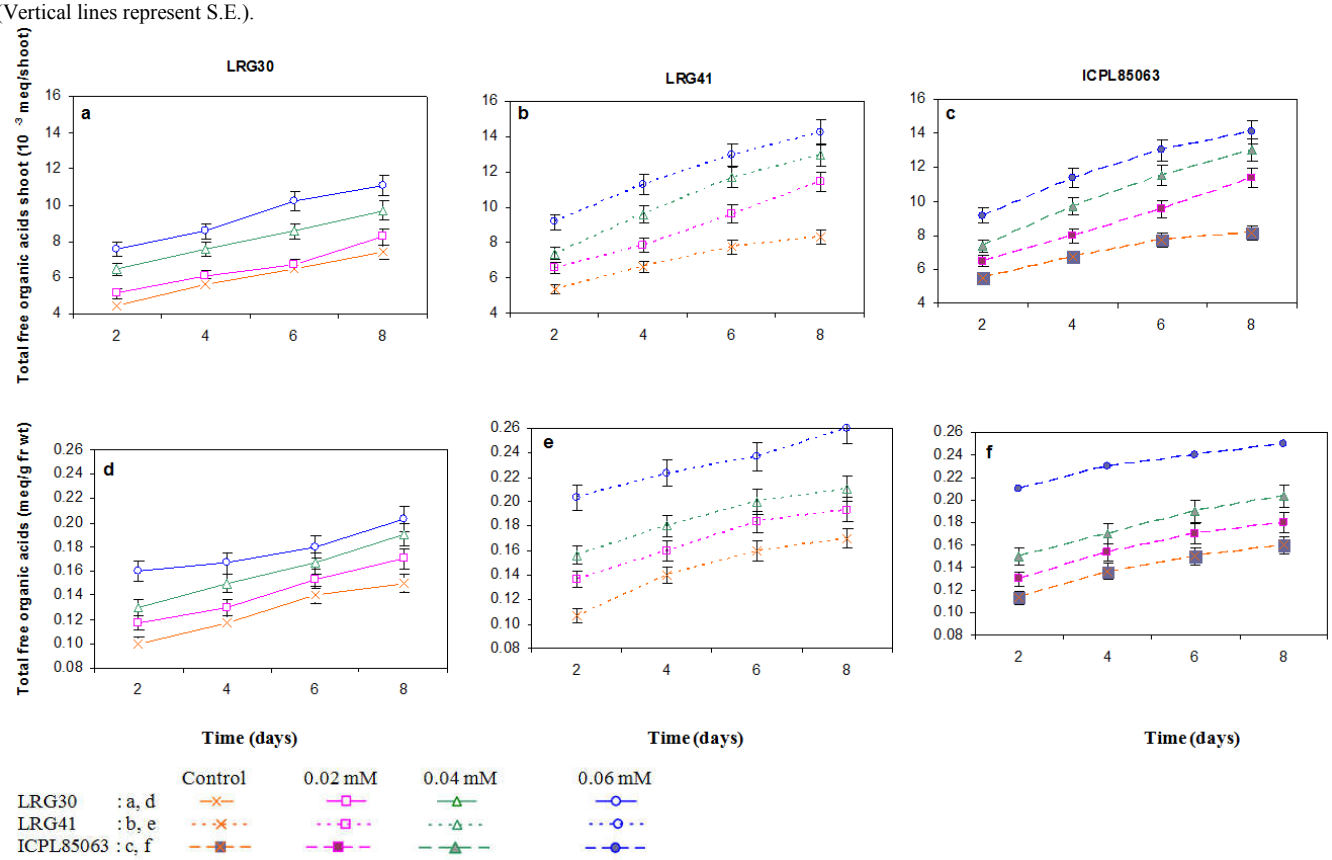


Fig 8 Total free organic acid content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

The per cent increase in the proline content of the cotyledons of the respective Cd treated germinating seeds of pigeonpea were 25.08, 38.29 and 48.91 folds in LRG30; 18.01, 27.94 and 33.66 folds in LRG41 and 18.81, 28.38 and 33.78 folds in ICPL85063 over their appropriate controls. Among the three cultivars of pigeonpea, LRG30 registered greater values of proline content than LRG41 and ICPL85063 in response to Cd treatment.

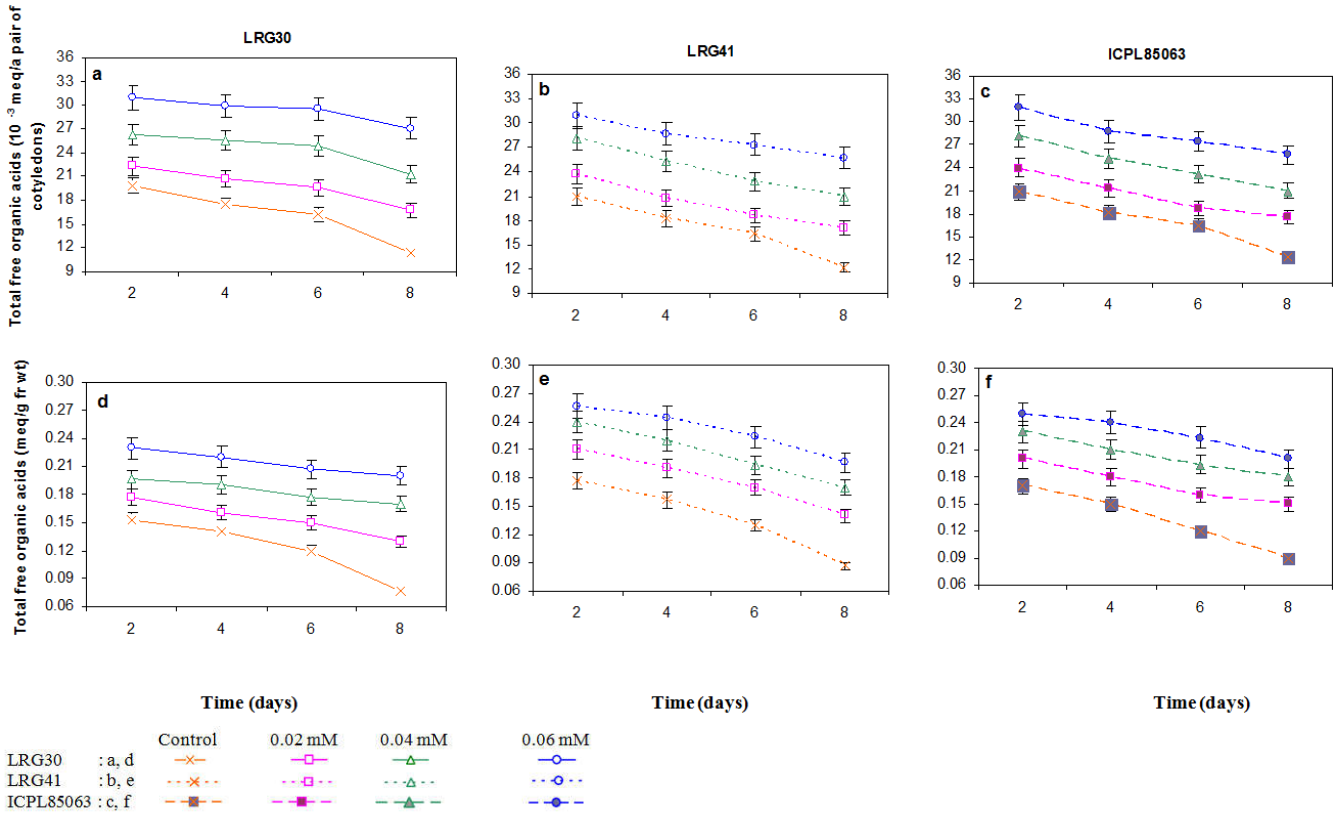


Fig 9 Total free organic acid content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

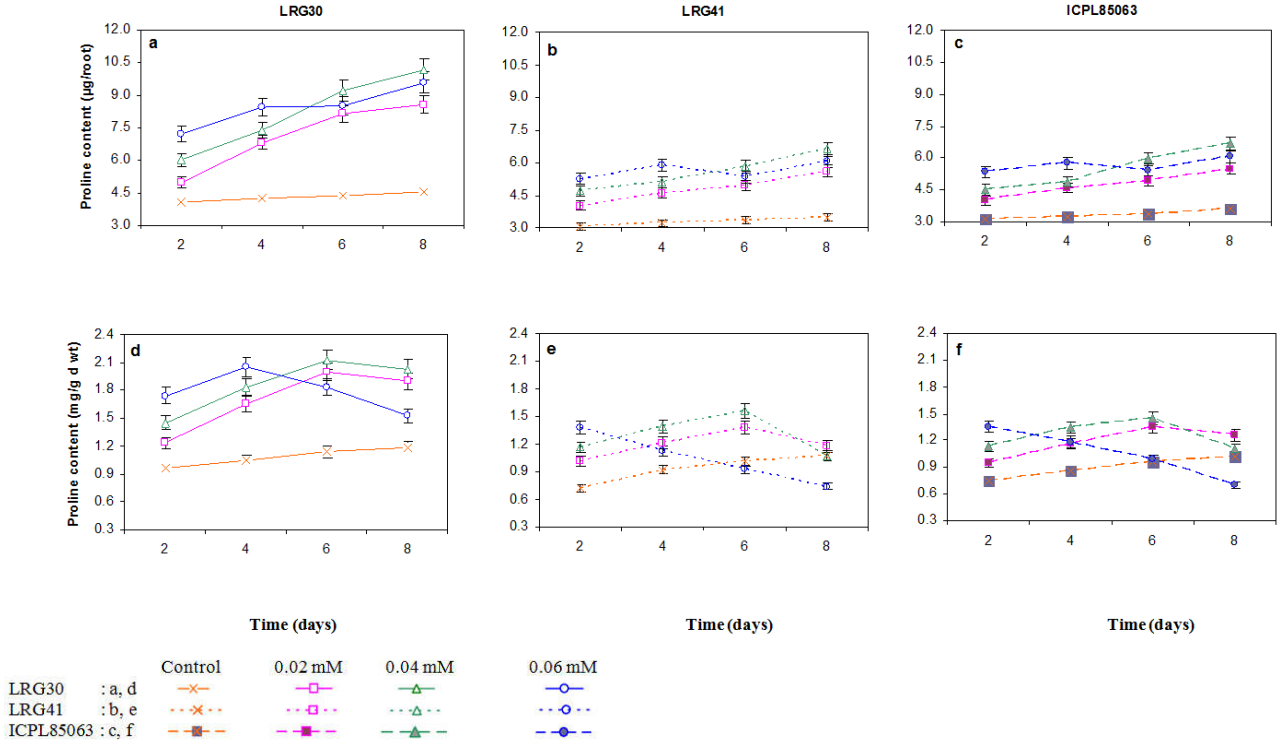


Fig 10 Proline content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

On dry weight basis, the proline content of the roots of the three pigeonpea cultivars increased up to 6 days of seedling growth followed by a slight decline in response to 0.02, 0.04 and 0.06 mM Cd concentrations supplied. The pigeonpea cv.LRG30 exposed to 0.06 mM Cd concentration exhibited a slight increase in the proline content of the roots up to 4 days of seedling growth whereas in cv.LRG41and ICPL85063 respective Cd concentration exhibited a progressive decline in the proline content of roots. However, the proline content of the roots of Cd treated germinating seeds of the three pigeonpea cultivars registered higher values when compared to their controls except in the roots of 8-day old seedling of LRG41 and ICPL85063 grown in 0.06 mM Cd concentration (Fig.10d, e, f). On dry weight basis, the changes in the accumulation of

proline content in the shoots and cotyledons of the three pigeonpea cultivars exhibited a trend similar to per organ basis both with increasing seedling growth as well as with increasing concentrations of externally supplied metal ions (Fig.11d, e, f and 12d, e, f).

The proline content of the roots showed 0.01 level of significance in cv.LRG30 and not significant in LRG41 and ICPL85063 with external concentrations of Cd.

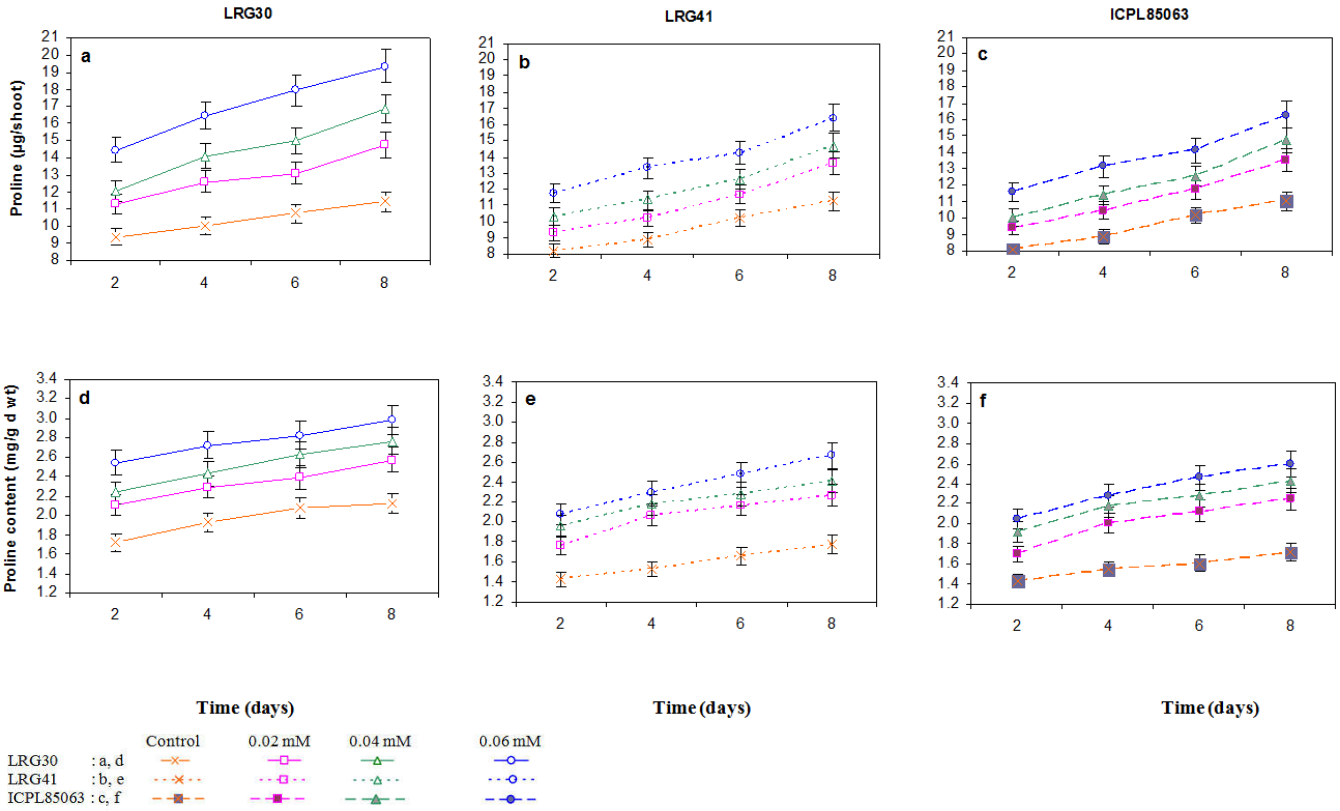


Fig 11 Proline content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

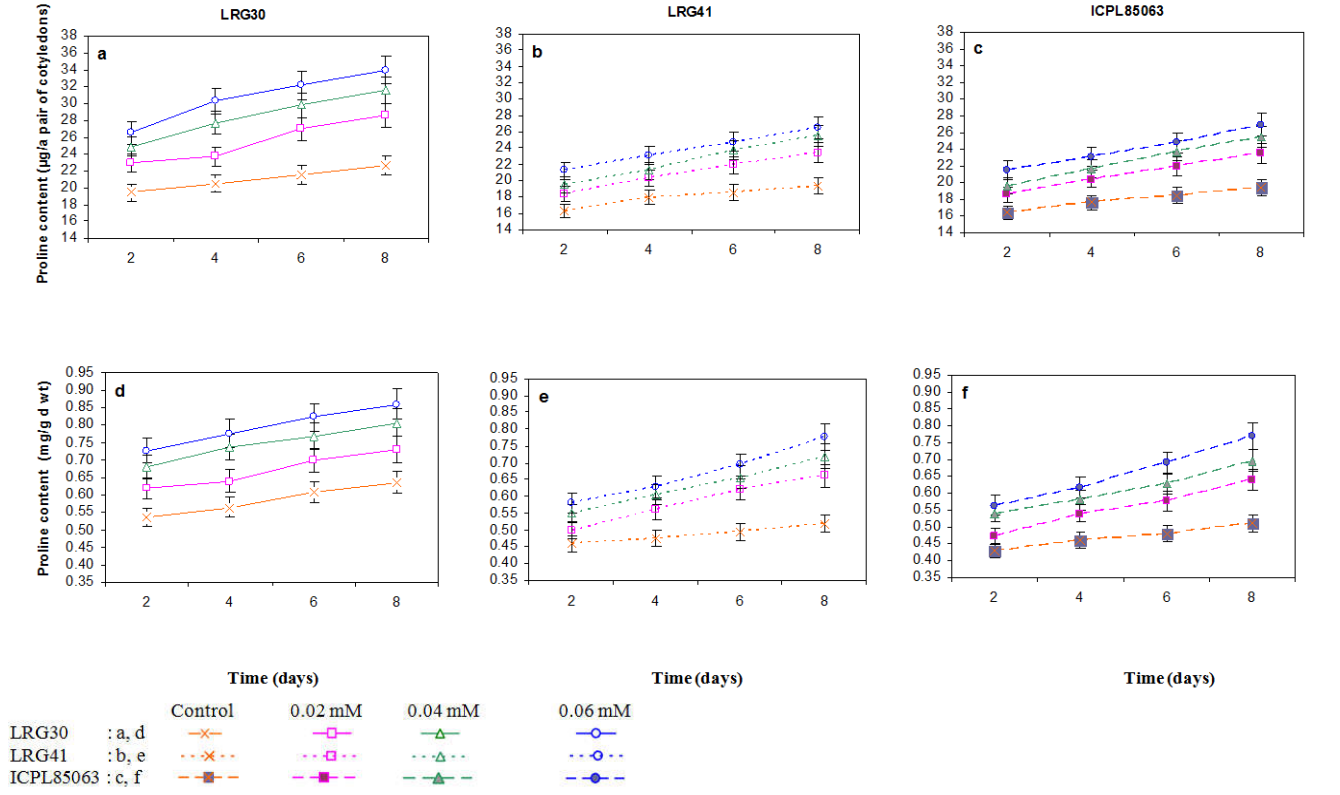
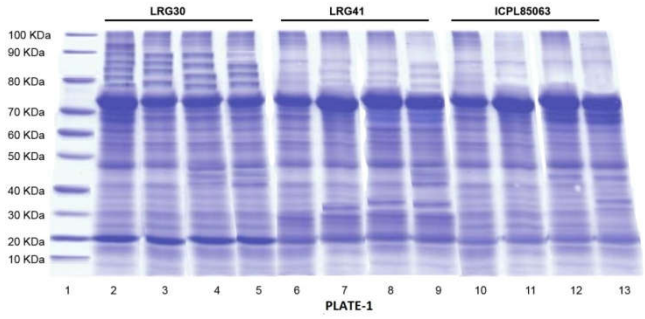


Fig 12 Proline content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

The proline content of the shoots showed 0.05 level of significance in cv.LRG30 and 0.01 level of significance in LRG41 and ICPL85063 with the external concentrations of Cd. On the other hand, the proline content of the cotyledons showed 0.05 level of significance in cv.LRG30 and 0.01 level of significance in LRG41 and ICPL85063 with the increasing concentrations of externally supplied Cd (Table-1).

SDS-PAGE analysis

SDS-PAGE analysis was performed to evaluate the changes in protein profile among the three cultivars of pigeonpea LRG30, LRG41 and ICPL85063 (Plate-1).



Legends for Plate 1 Leaf storage protein profiles using SDS-PAGE of three pigeonpea cultivars, LRG30, LRG41 and ICPL85063 in response to cadmium stress.

	Control	0.02 mM	0.04 mM	0.06 mM
Ladder:	1			
LRG30:	2	3	4	5
LRG41:	6	7	8	9
ICPL85063 :	10	11	12	13

The pigeonpea protein profiles were composed of protein bands ranging from 20-95 kDa. Clear differences in the band pattern were observed in protein changes between tolerant and less tolerant genotypes on the polyacrylamide gels. The protein band pattern varied among three genotypes with intensity of protein expression. A 22 kDa protein band in cv.LRG30 showed 5 fold more intensity than LRG41 and ICPL85063. Similarly, protein bands ranging from 78-95 kDa showed the same pattern of expression in cv.LRG30 than LRG41 and ICPL85063. A 75 kDa protein band with strong and sharp blue band was expressed in all the three cultivars with respect to control and Cd treated ones. The results showed that the protein expression in tolerant genotype, LRG30 started to decrease with the increase of Cd concentration from 0.02 to 0.06 mM. Likewise, the less tolerant genotypes, cv.LRG41 and ICPL85063 also showed the similar pattern in protein expression was observed when compared with control ones.

DISCUSSION

The mobilization of storage proteins is one of the most important post-germinative events in the growth and development of seedling. During germination period, the storage proteins are degraded by a variety of proteases which convert the insoluble storage proteins into soluble peptides and free amino acids. These are mobilized to the embryonic axis to support its growth and also provide energy by oxidation of the carbon skeleton after deamination (Okamato and Minamikawa, 1998; Muntz *et al*., 2001; Schlereth *et al*., 2001). The total protein content of the roots and shoots of Cd-treated germinating seeds of three pigeonpea cultivars recorded lower values when compared to their controls. The decrease in protein content of roots and shoots became more conspicuous with increasing concentrations of externally supplied metal ions (Fig.1 and 2). However, the total free amino acid content of the roots and shoots of the three pigeonpea cultivars, increased with increasing concentrations of externally supplied Cd ions (Fig.4 and 5). The increased accumulation of amino acids in the roots and shoots may be due to the reduced protein synthesis under the influence of heavy metals and thus caused the decrease in total protein content of the roots and shoots (Chien and Kao, 2000; Hsu and Kao, 2003, 2005). On the other hand, the free amino acid content of the cotyledons of the Cd-treated germinating seeds of pigeonpea cultivars decreased in contrast to the higher retention of protein content (Fig.3 and 6). Among the three cultivars of pigeonpea, LRG30 exhibited higher levels of total protein content and lower levels of total free amino acid content when compared to LRG41 and ICPL85063 in response to Cd stress. Cadmium causes the denaturation of proteins (Jungmann *et al*., 1993; Singh and Tewari, 2003; Piotrowska *et al*., 2010). It is the task of stress proteins to restore the native conformation of proteins denatured by Cd or to decompose them. Chettri *et al*. (2004) reported that when heavy metal toxicity crosses the threshold limit, the protein level decreases and this might be due to the breakdown of protein synthesis mechanism at toxic level of heavy metals or due to reduced incorporation of free amino acid into protein.

EI-Shintinawy and EI-Ansary (2000) monitored the changes in amino acid metabolism in soybean seedlings exposed to toxic concentrations of Cd in order to measure the ability of the seedlings to tolerate the heavy metal. The increase of different amino acids in Cd treated pigeonpea seedlings may be attributed to individual or combined effect of the following possibilities. It might be caused by the malfunctioning of respiratory activity mostly due to membrane damage (Vazquez *et al*., 1992b), resulting in the accumulation of several krebs cycle compounds such as 2-oxogluterate and pyruvate which may promote the synthesis of specific amino acids. It may also results due to the complexion of Cd ions with sulfhydryl groups. Further, it may be due to reduced protein synthesis or increased protein breakdown (Rauser, 1990).

Organic acids play an important role in cell metabolism, mainly through respiratory metabolism, by providing carbon skeleton for the synthesis of amino acids and other cellular constituents (Towers and Mortimer, 1954). The organic acid also acts as intermediates of a number of metabolic products and also helps to maintain cellular pH. Any changes in their metabolism would therefore affect the physiology of the pigeonpea seedlings. The total free organic acids increased in all parts of the three pigeonpea cultivars in response to increasing concentrations of externally supplied Cd (Fig.7-9). However, the accumulation of free organic acids was relatively more in the pigeonpea cultivar, LRG41 and ICPL85063 than in cv.LRG30. Organic acids and amino acids can bind heavy metals and may therefore be deployed in response to metal toxicity (Rauser, 1999).

Proline, an amino acid, is well known to get accumulated in wide variety of organisms ranging from bacteria to higher plants on exposure to abiotic stress (Saradhi *et al*., 1993; Ahmad *et al*., 2006). Proline accumulation in shoots of *Brassica juncea*, *Triticum aestivum* and *Vigna radiata* in response to cadmium toxicity was demonstrated by Dhir *et al*.

(2004) but they found that proline accumulation decreased with the exposure to cadmium in hydrophytes (*Ceratophyllum*, *Wolffia* and *Hydrilla)*. It has been often suggested that proline accumulation may contribute to osmotic adjustment at the cellular level (Perez-Alfocea *et al*., 1993) and stabilizes the structure of macromolecules and organelles. Proline also acts as a major reservoir of energy and nitrogen, which can be used in resuming the growth after the stress removal. Various environmental stresses such as heavy metals, drought and temperature have been caused to increase the level of proline (Chandrashekhar and Sandhyarani, 1996; Rai *et al*., 2004; Claussen, 2005). Hayat *et al*. (2007) determined that proline level increased the physiological drought stress generated by cadmium. Although the mechanism of accumulation of proline in plants or plant parts exposed to stress is still unknown. It is suspected to be due to a decrease in the activity of the electron transport system leading to accumulation of NaDH and H^+. Proline accumulation (presumably through synthesis from glutamic acid) might be an adaptive mechanism for reducing (a) the level of accumulated NADH and (b) the acidity; ($2NADH + 2H^+$) is used for synthesizing each molecule of proline from glutamic acid (Sawhney *et al*., 1990; Alia *et al*., 1993).

Seedlings of the three pigeonpea cultivars raised in a range of Cd concentrations showed a significant increase in the level of proline. The extent of enhancement in the level of proline content of the different parts of the three pigeonpea cultivars increased with increasing concentrations of externally supplied Cd ions as well as with increasing age of the seedlings (Fig.10-12). Proline, a universal protectant of various stresses, has been reported in seedlings of certain crop plants treated with Cd, Co, Zn and Pb, where the proline accumulation level increased proportionately with an increase in metal concentration (Meenakshi *et al*., 2007; John *et al*., 2008; Yasemin and Kutbay, 2011). Increased proline content was observed due to increased Cd concentration probably due to increasing intracellular osmotic pressure (Claussen, 2005). However, a proportionate rise in the proline content of roots was observed only up to 0.04 mM concentration of Cd beyond which there was a decline in the proline content of the roots of the three pigeonpea cultivars. High levels of heavy metal concentrations perhaps suppress the metabolic processes including proline formation (Bassi and Sharma, 1993). Of the three cultivars of pigeonpea, LRG30 registered greater values of proline content than LRG41 and ICPL85063 in response to Cd treatment. The Cd ions were known to affect the integrity of membranes (Reddy and Prasad, 1992) and changes in the permeability of membranes lead to water stress like conditions resulting in an increase in proline level (Pesci and Reggiani, 1992). However, Kastori *et al*. (1992) reported that the increased proline concentration in leaf discs incubated in heavy metal solutions showed an increase in proline concentration in the presence of heavy metals even in fully turgid plant cells. Thus the effect of heavy metals on proline concentration may not completely depend on their effect on water relations.

The SDS-PAGE is considered to be a practical and reliable method for determining and identification of protein expression (Ghafoor et al., 2002). In the present study, SDS-PAGE analysis was performed to compare leaf protein profiles among the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 (Plate-1). The protein band pattern varied among the three cultivars with intensity of protein expression. Intensity and weak protein band differences were observed between the three cultivars. Expression of sharp and thick 22 kDa protein band in cv.LRG30 than the other cultivars suggests that the earlier expression of this category of proteins may have a role in tolerance response. Absence or presence of some bands may also indicate a functional involvement in stress response. The protein expression in tolerant cultivar, LRG30 started to decrease with the increasing concentration of Cd from 0.02 to 0.06mM as well as, the less tolerant cultivar, cv.LRG41 and ICPL85063 also showed the same pattern in protein expression. It seems that tolerance reaction might be due to more rapid synthesis or less degradation of responsive proteins to heavy metal stress. SDS-PAGE is reported by several researchers in comparing the several plant genotypes (Gept, 1989; Das and Mukarjee, 1995; Kakaei et al., 2010a, 2010b, 2010c).

CONCLUSIONS

The protein content of the roots and shoots of Cd treated germinating seeds of three pigeonpea cultivars increased with increasing seedling growth and recorded lower values when compared to their corresponding controls. The protein content of the cotyledons declined gradually with increasing seedling growth and retained higher amounts over their controls. In the three pigeonpea cultivars, LRG30 registered higher values of protein content in response to Cd treatment. The accumulation of total free amino acids in different parts of the three pigeonpea cultivars increased with increasing seedling growth as well as with increasing concentrations of externally supplied Cd. The amino acid content of cotyledons decreased with increasing concentrations of metal ions supplied. The pigeonpea cultivars, LRG41 and ICPL85063 registered higher values of total free amino acids when compared to LRG30. Cadmium treatment showed the accumulation of free organic acids in different parts of the three pigeonpea cultivars. The accumulation was relatively more in the pigeonpea cultivars, LRG41 and ICPL85063. A steep rise in the accumulation of proline content in different parts of the Cd-treated germinating seeds of the three pigeonpea cultivars was observed with increasing seedling growth as well as with increasing concentrations of externally supplied metal ions. Eventhough, the increasing concentrations of externally supplied Cd led to increased accumulation of proline content, in roots the higher concentrations were specially associated with lower levels of proline content. A greater level of proline content was observed in cv.LRG30. Generally, tolerance in heavy metal stress condition might be due to more rapid accumulation of proteins with low and high molecular weights and coordinated pattern changes in biochemical parameters. Furthermore, according to less tolerant genotype response in this study, expression of different protein band patterns were observed in tolerant cultivar, LRG30 when compared to the less tolerant cultivars, cv.LRG41 and ICPL85063, irregular changes in protein profile and inability to rapid accumulation of responsible proteins might be the possible cause for susceptibility in heavy metal stress condition. These findings provide a framework for future investigations in order to highlight the biological impact of cadmium, as well as to study the role of stress induced proteins in the cadmium response pathways.

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