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CADMIUM PHYTOTOXICITY INDUCES LIPID PEROXIDATION AND ALTERS THE ANTIOXIDANT ENZYME ACTIVITIES IN *CAJANUS CAJAN* SEEDLINGS

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ABSTRACT

The response of three cultivars of pigeonpea (Cajanus cajan (L.) Millspaugh) plants were evaluated after exposure to $CdCl_2$. In the present investigation 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 various non-enzymatic antioxidants like ascorbic acid, SH-compounds, total glutathione and lipid peroxidation were studied. Cadmium induced lower levels of ascorbic acid content, it may be presumed that better detoxifying mechanisms with respect to Cd is available in pigeonpea. Cadmium treatment resulted in the accumulation of higher levels of SH-compounds. Among the three cultivars of pigeonpea, LRG30 registered higher values of SH-content in response to Cd treatment. The pigeonpea cultivar, LRG30 registered higher values of total glutathione content than LRG41 and ICPL85063 in response to Cd treatment. Increased lipid peroxidation was expressed more in cv. LRG41 and ICPL85063 in response to Cd treatment indicating heavy metal sensitivity of cv. LRG41 and ICPL85063.

KEY WORDS

Ascorbic acid content, Cadmium, total glutathione content, lipid peroxidation, pigeonpea cultivars.

INTRODUCTION

Biological membranes play a crucial role in various plant processes and are sensitive to cellular environment. The plasma membrane is directly exposed to the edaphic environment in which the metal ions like Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+} Ni^{2+}, Co^{2+} and if present at elevated concentrations may cause disruption in normal cellular and whole plant functions (De Vos *et al.*, 1991; Cumming and Taylor, 1990). Cadmium (Cd) phytotoxicity triggers stress induces responses by generation of free radicals and Reactive oxygen species (ROS) derived from oxygen that has implications in numerous developmental and adaptive responses due to macromolecular damage to plants cells. Reactive oxygen species are regarded as highly cytotoxic and their level within plant cells must be controlled by antioxidant defense systems (Shah *et al*., 2001; Yurekli and Porgali, 2006; Michael and Krishnaswamy, 2011) and cause lipid peroxidation, membrane damage and inactivation of enzymes (Sanitá di Toppi and Gabrielli, 1999; Skórzyńska-Polit *et al*., 2003/2004; Zhang *et al*., 2003; Monterio *et al*., 2009; Sharma *et al.,* 2012). The malondialdehyde (MDA) (product of lipid peroxidation) level is regarded as a biochemical marker for injury mediated by ROS (Palma *et al*., 2002; Verma and Dubey, 2003; Sinha *et al*., 2005). In order to study oxidative stress in response to heavy metals, a plethora of experiments have been carried out, using different plant species, doses of metals, and exposure times (Schützendübel and Polle, 2002). A variety of macromolecules including proteins, lipids, polysaccharides and nucleic acids can be oxidatively modified, and the manifestations of this damage are multifarious, running the gamut from altered

membrane fluidity and permeability attributable to lipid peroxidation, through loss of conformation and enzyme activity to genomic damage arising from scission of DNA (Szuster-Ciesielska *et al.,* 2000; Davies, 2003). Therefore, ROS production and removal must be efficiently controlled. To minimize the damaging effects of ROS, the plant cells possess evolved non-enzymatic and enzymatic antioxidative defense mechanism.

A metal induced decline in the pool of glutathione has been observed in many plants and fungi (Tukendorf and Rauser, 1990; Meuwly and Rauser, 1992; Bergmann and Rennenberg, 1993; Klapheck *et al.*, 1994; Tukendorf, 1996). In intact maize seedlings, the glutathione content in roots being depleted more rapidly than in shoots after exposure to Cd (Berger *et al.*, 1989; Steffens, 1990). Therefore, the present investigation is aimed at understanding to analyze the Cd toxicity induced oxidative stress, changes in redox pool and responses of cellular antioxidants and powerful ROS detoxifying pathways and to map the damage to macromolecules (lipids, phospholipids, proteins) as a result of Cd induced oxidative stress in pigeonpea. The basis of Cd toxicity is still not completely understood, but it might result from its high affinity for sulfydryls e.g. threefold higher than Cu ions, (Schützendübel and Polle, 2002). This metal by binding to sulfydryl groups of structural proteins and enzymes leads to misfolding, inhibition of activity and/or interference with redox enzymatic regulation (Hall, 2002).

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.

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 (2,6-DCIP) by ascorbic acid (AA) from its pink colour in the acid medium to the colourless leucoform. One-gram samples of treated and control pigeonpea seedlings were macerated thoroughly and rapidly with 10 ml of 5% (w/v) metaphosphoric acid using a mortar and pestle. The homogenate was filtered through filter paper and the filtrate was made up to 20 ml with 5% metaphosphoric acid. Duplicate samples of 10 ml aliquots were titrated with 2,6-DCIP reagent until a pink end point which persists for 15 secs was obtained. The quantity was calculated using the formula:

$$L \times S \times \frac{D}{A} \times \frac{1}{W} = \text{mg of AA per 1 g material}$$

Where, L = ml of DCIP reagent used in the titration; S = mg of AA reacting with 1 ml of reagent.

D = volume of the extract in ml; A = volume of the aliquot titrated in ml; W = weight of the sample in g

Preparation of reagents

a) **5% metaphosphoric acid:** 50 ml of metaphosphoric acid were dissolved on a shaker in glass distilled water and made up to 1 litre.

b) **Ascorbic acid standard solution:** 50 mg of ascorbic acid (AR) was dissolved in 250 ml glass distilled water. One ml of this solution contains 0.2 mg of ascorbic acid. It was prepared immediately before use for standardization of the DCIP reagent.

c) **0.025% 2,6-DCIP reagent:** 50 mg of 2,6-dichlorohenol indophenol was dissolved in 150 ml of

glass distilled water, warmed gently until dissolved and 42 mg of sodium carbonate was added. The solution was cooled and decanted into 200 ml volumetric flask and was made up to required volume with distilled water.

d) **Standardization of the dye:**

5 ml of standard ascorbic acid solution containing 0.2 mg per ml was taken in a Erlenmeyer flask and was titrated with 2,6-DCIP reagent until a pink end point that persists for 15 sec was obtained. As 1 mg of ascorbic acid was oxidized by so much amount of DCIP reagent, 1 mg of ascorbic acid divided by the number of milliliters used in the titration gives the value(s) of the reagent in milligrams per ml (i.e., mg of ascorbic acid reacting with 1 ml of reagent).

Water soluble SH-compounds

The DTNB reactive SH-compounds were determined according to the method of Ellman (1959) and Grill *et al*. (1979). Two g of plant material was homogenized with 20 ml of water containing 0.15% ascorbic acid. The homogenate was centrifuged at 20,000 xg for 10 min. To 2 ml of the clear supernatant, 2 ml DTNB reagent was added. The yellow colour developed was measured in 1 cm cuvette at 412 nm Schimadzu (UV-240) spectrophotometer against a blank consisting of 2 ml phosphate buffer giving the absorbance E_1. In a second experiment, the absorbance of 2 ml H_2O + 2 ml DTNB reagent was measured against 2 ml H_2O + 2 ml phosphate buffer, giving the absorbance E_2. The SH-content of the supernatant (extract) was calculated according to the equation:

(E_1-E_2) x 2/13,600 = mole SH per 1-liter extract.

Preparation of DTNB reagent: It was prepared by dissolving 3.96 mg of 5,5'-dithiobis (2-nitrobenzoic acid) in 10 ml of 0.2 M phosphate buffer (pH 7.5).

Total glutathione (GSH+GSSG) content

Total glutathione content was 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-nitrobenzoic acid) (DTNB) to give 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 monitored at 412 nm and was proportional to the sum of GSH and GSSG present in the samples.

Extraction: Two hundred mg of different parts of pigeonpea seedlings were homogenised separately in 1.5 ml of 2% metaphosphoric acid containing 2 mM EDTA and 1 g/ml PVPP (polyvinylpolypyrrolidone) by using a pre-cooled mortar and pestle and then centrifuged at 17,000 xg for 10 min. The pH of the extract was brought to 5.5 with 10% sodium citrate.

Estimation: Three working solutions namely (i) 0.3 mM NADPH (ii) 6 mM DTNB and (iii) approximately 50 units of glutathione reductase per ml were prepared by using 125 mM sodium phosphate buffer (pH 7.5) containing 6.3 mM Na-EDTA (it helps in chelating the metals which would otherwise interfere with the recycling enzyme, glutathione reductase). All the solutions were stored at 0 °C and were stable for at least two weeks. Total glutathione content was estimated by mixing 700 µl of solution (i) 100 µl of solution (ii) and the sample extract of 200 µl to give a final volume of 1.0 ml, directly in a cuvette with 1 cm light path and equilibrated to 30 °C. To this solution, 10 µl of solution (iii) was added and then the absorbance was monitored at 412 nm continuously by using Schimadzu (UV-240) spectrophotometer. The total glutathione content present in the aliquot was evaluated by comparison of the rate observed to a standard curve generated with known amounts of glutathione.

Lipid peroxidation

Lipid peroxidation in different parts of control and treated 6-day old pigeonpea seedlings was determined by estimating the malondialdehyde content following the method adopted by Heath and Packer (1968) with a slight modification. One g of plant material was macerated in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 10,000 xg for 5 min. To one ml of the aliquot of the supernatant, 4 ml of 20% TCA containing 0.5% thiobarbutaric acid was added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice bath. The mixture was centrifuged at 10,000 xg for 15 min and the absorbance of the supernatant was measured at 532 nm by using Systronics 112 spectrophotometer. Measurements were corrected for unspecific turbidity by substracting the absorbance at 600 nm. The concentration of malondialdehyde was

calculated by using extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Statistical analysis

The data collected were computed and analyzed 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 between increasing concentrations of Cd supplied.

RESULTS

Ascorbic acid

The ascorbic acid content of the roots of the Cd-treated germinating seeds of pigeonpea increased continuously from 2-8 days of seedling growth. However, the values always remained lower than the respective controls. Further the decrease becomes more conspicuous with increasing concentrations of externally supplied Cd (Fig.1a, b, c). The ascorbic acid content of the shoots of the three pigeonpea cultivars exhibited a trend similar to that observed for roots both with increasing seedling growth as well as with increasing concentrations of Cd (Fig.2a, b, c). On the other hand, the ascorbic acid content of the cotyledons of the Cd treated germinating seeds of three pigeonpea cultivars decreased with increasing seedling growth and the values remained higher at any particular stage of seedling growth when compared to their respective controls (Fig.3a, b, c).

The per cent reduction in the ascorbic acid 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 16.43, 31.75 and 45.80% in LRG30; 35.80, 50.24 and 62.28% in LRG41 and 35.87, 52.50 and 62.79% in ICPL85063 respectively when compared to their controls. The per cent reduction in the ascorbic acid content of the shoots of the corresponding Cd-treated pigeonpea cultivars were 9.16, 20.97 and 33.20% in LRG30; 24.48, 40.56 and 51.03% in LRG41 and 21.36, 40.54 and 51.51% in ICPL85063 in relation to their controls. The per cent retention in the ascorbic acid content of the cotyledons of the respective Cd treated germinating seeds of pigeonpea were 47.41, 73.51 and 106.62% in LRG30; 66.97, 97.06 and 153.76% in LRG41 and 61.21, 97.06 and 145.08% in ICPL85063 over their appropriate controls. Among the three pigeonpea cultivars, the ascorbic acid content of the different parts of LRG30 registered higher values when compared to LRG41 and ICPL85063 in response to Cd treatment.

On dry weight basis, the ascorbic acid content of the roots and shoots of the three pigeonpea cultivars decreased with increasing seedling growth and with increasing concentrations of Cd (Fig.1d, e, f and 2d, e, f). On dry weight basis, the changes in the ascorbic acid content of the cotyledons of the three pigeonpea cultivars exhibited a trend similar to organ basis both with increasing growth of the seedling as well as with increasing concentrations of externally supplied metal ions (Fig.3d, e, f).

The ascorbic acid content of the roots showed a 0.05 level of significance in cv. LRG30 and 0.01 level of significance in LRG41 and ICPL85063 and shoots of the three pigeonpea cultivars showed 0.05 level of significance with the increasing concentrations of externally supplied Cd. The ascorbic acid content of the cotyledons of the three pigeonpea cultivars showed 0.05 level of significance with the external concentrations of Cd supplied (Table-1).

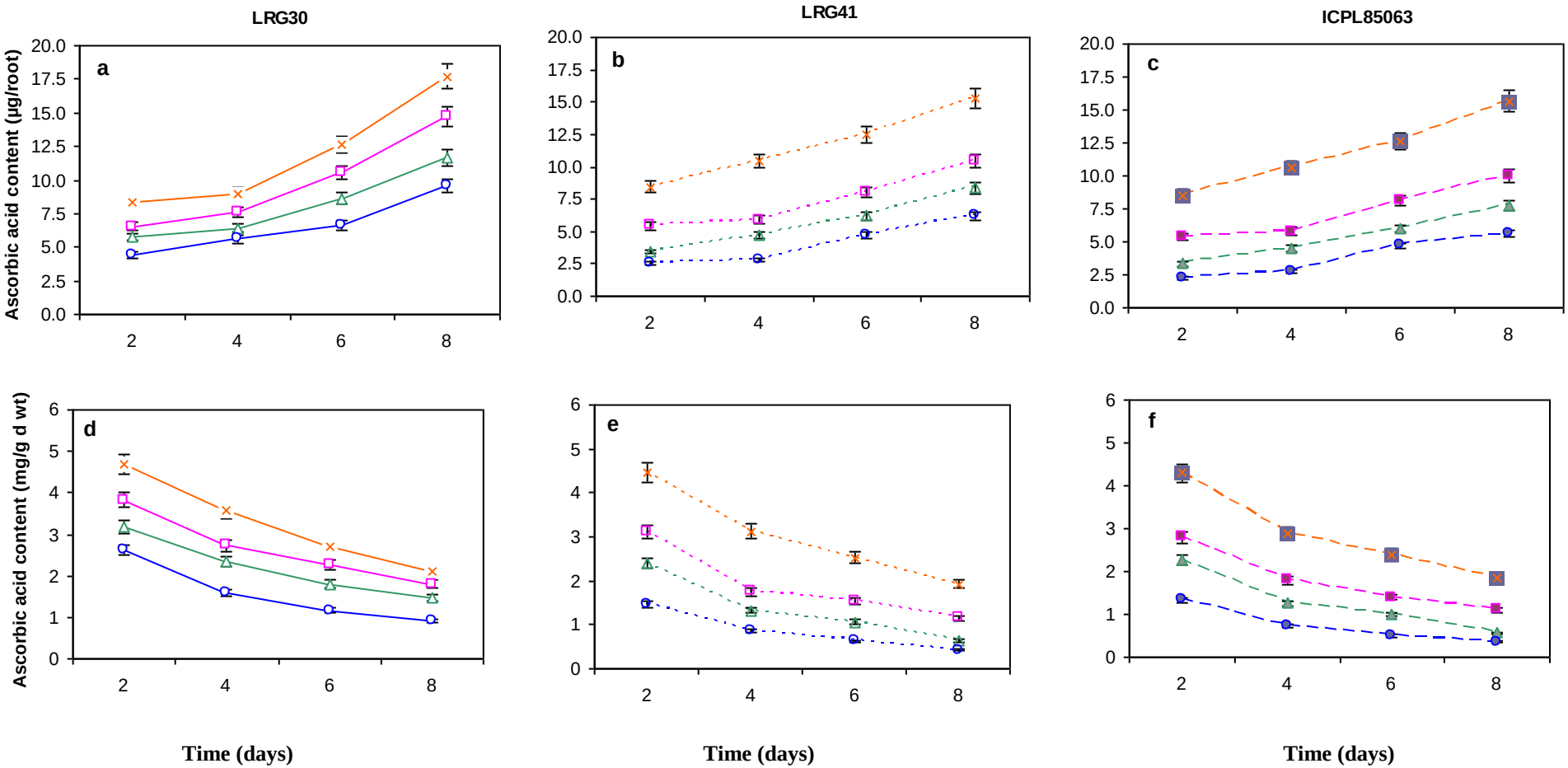

Fig. 1 - Ascorbic acid content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

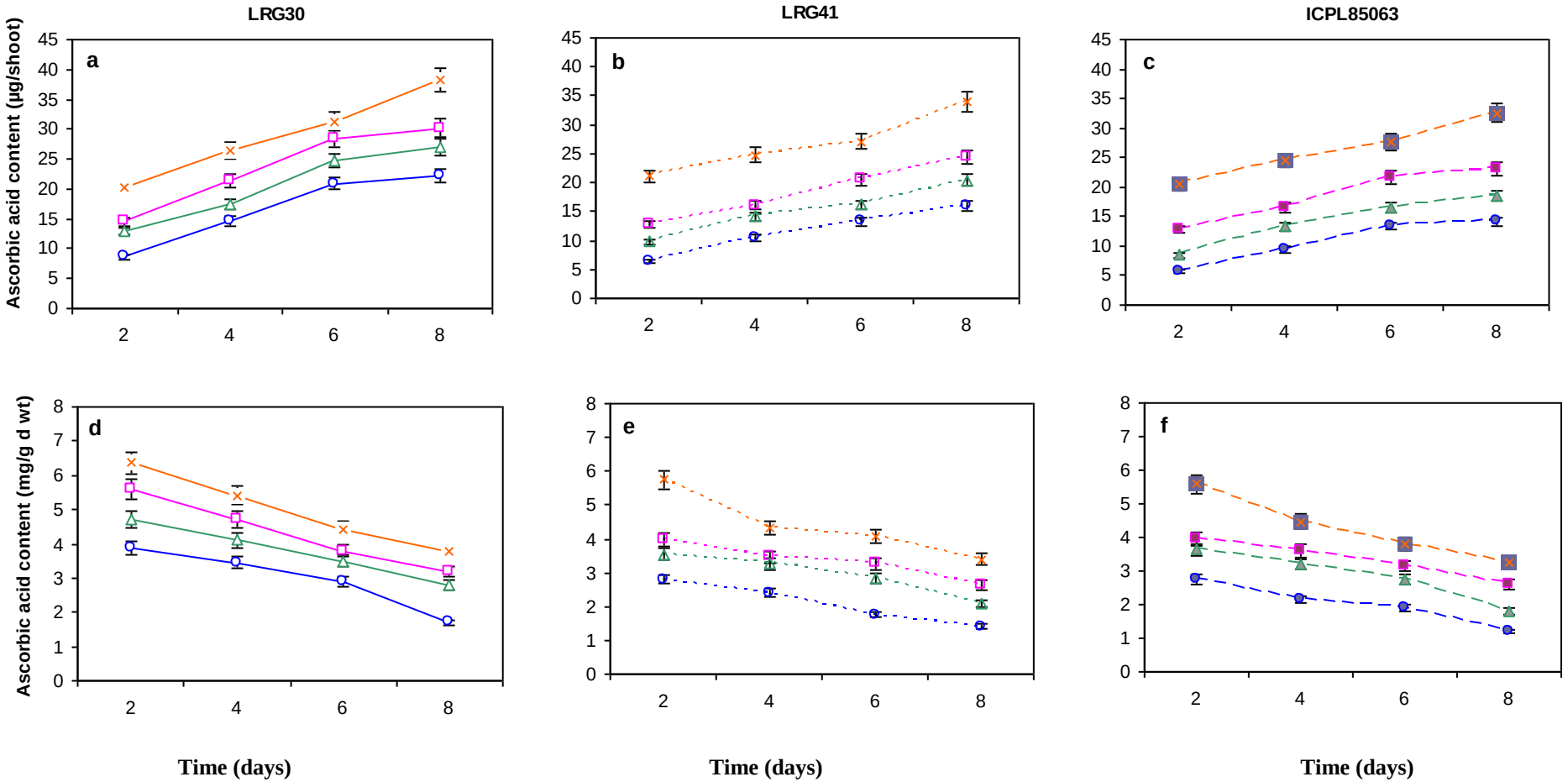

Fig. 2 - Ascorbic acid content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

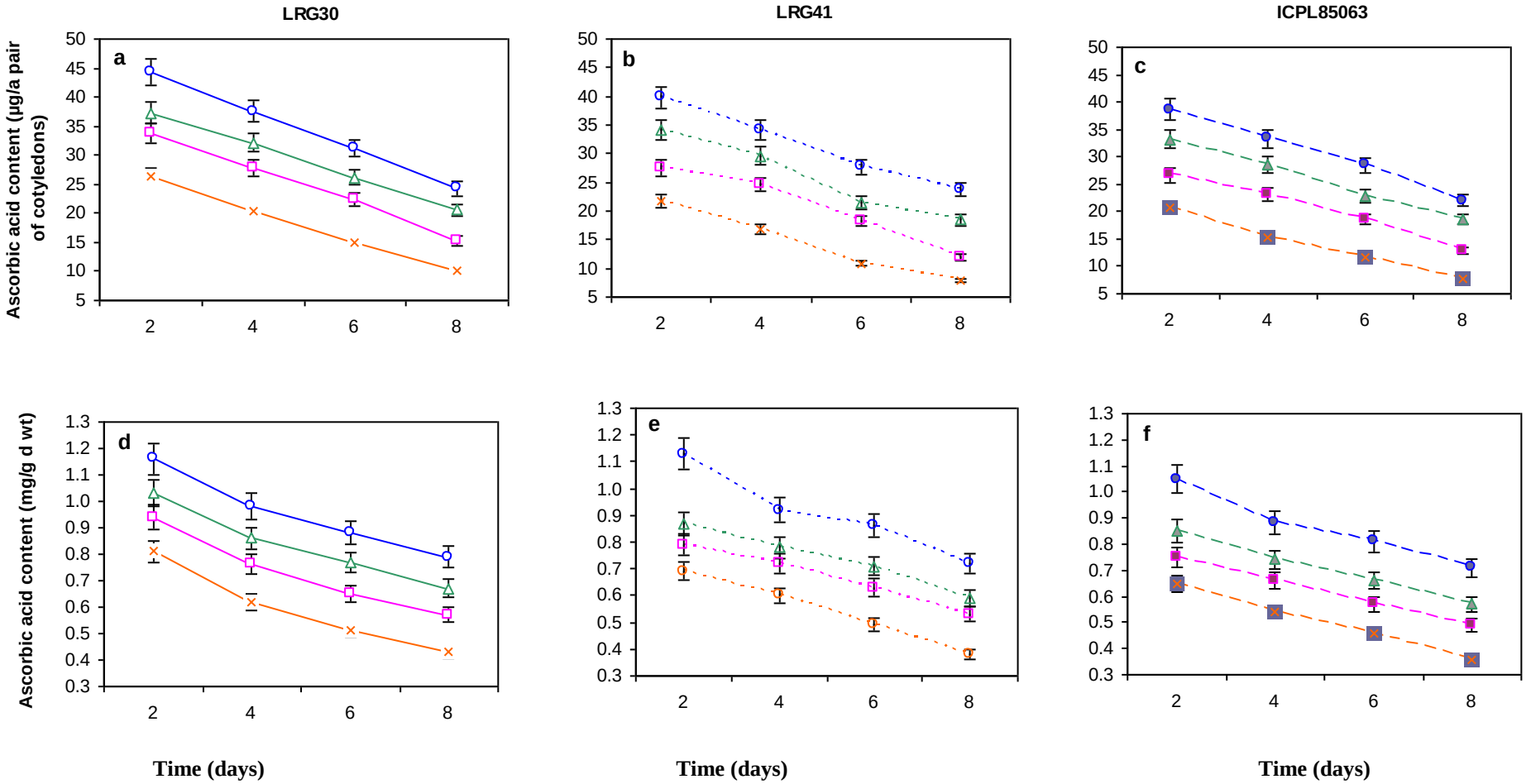

Fig. 3 - Ascorbic acid content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

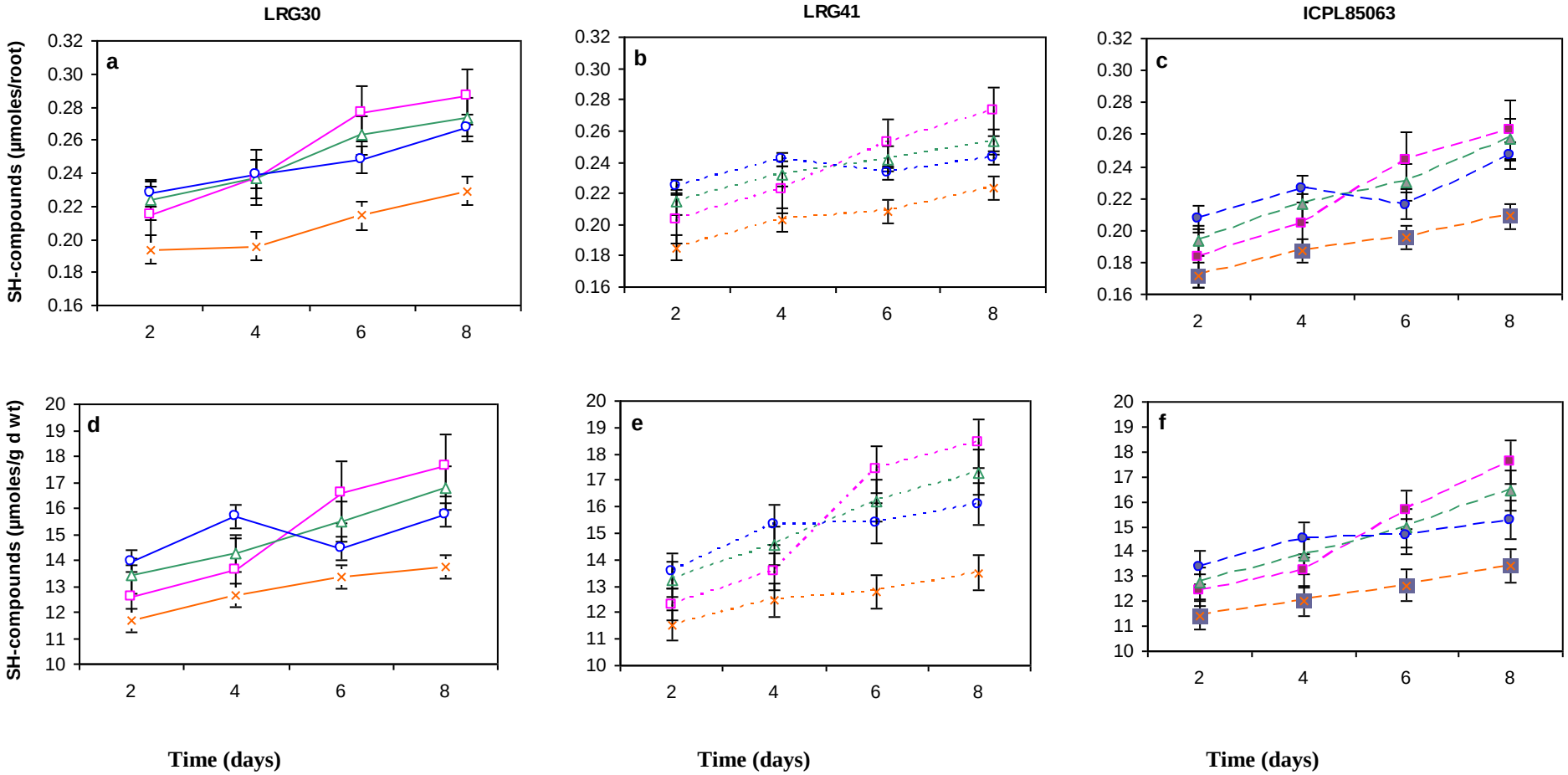

Fig. 4 - Sulphydryl (SH) compounds of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

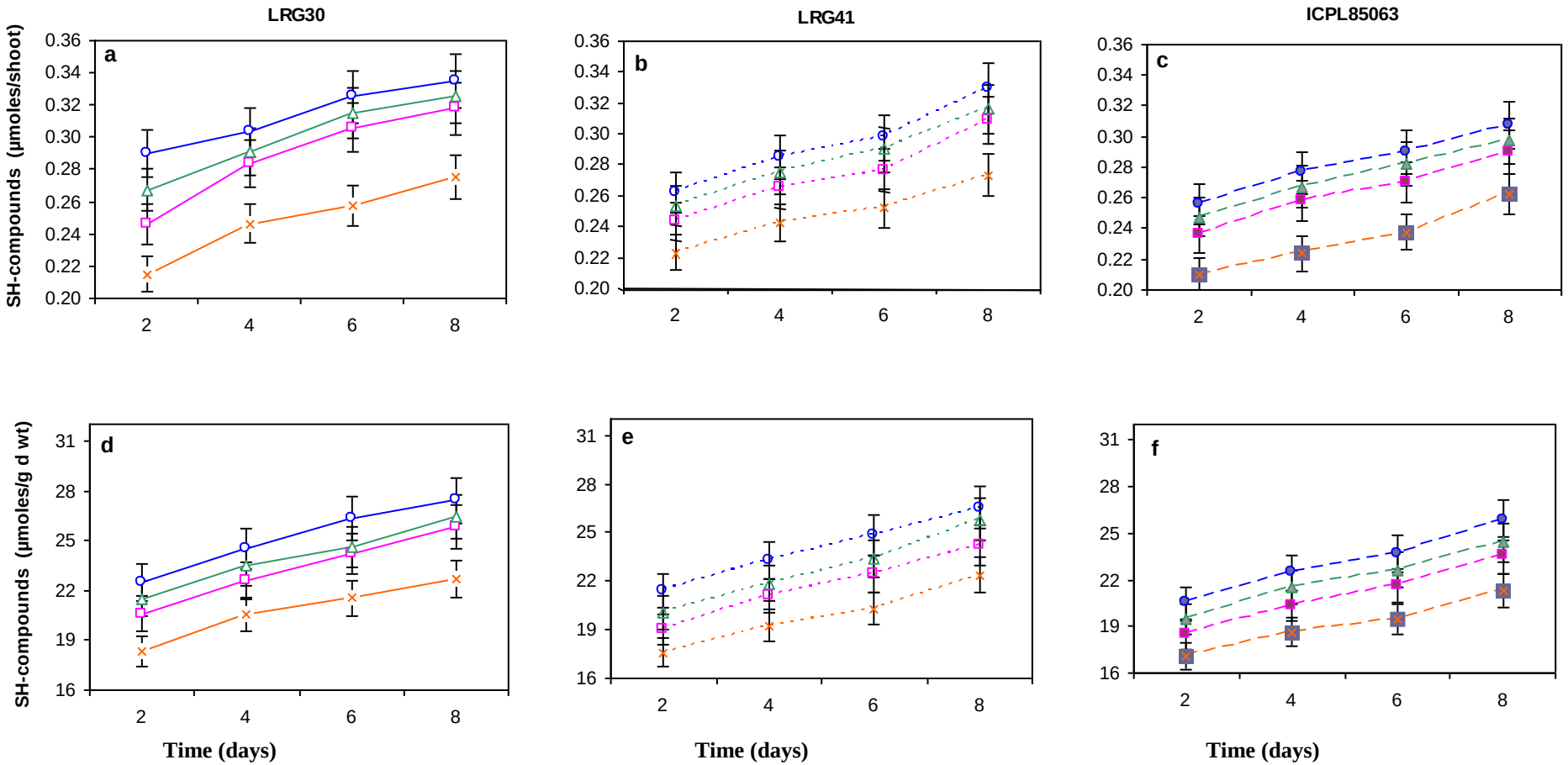

Fig. 5 - Sulphydryl (SH) compounds of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

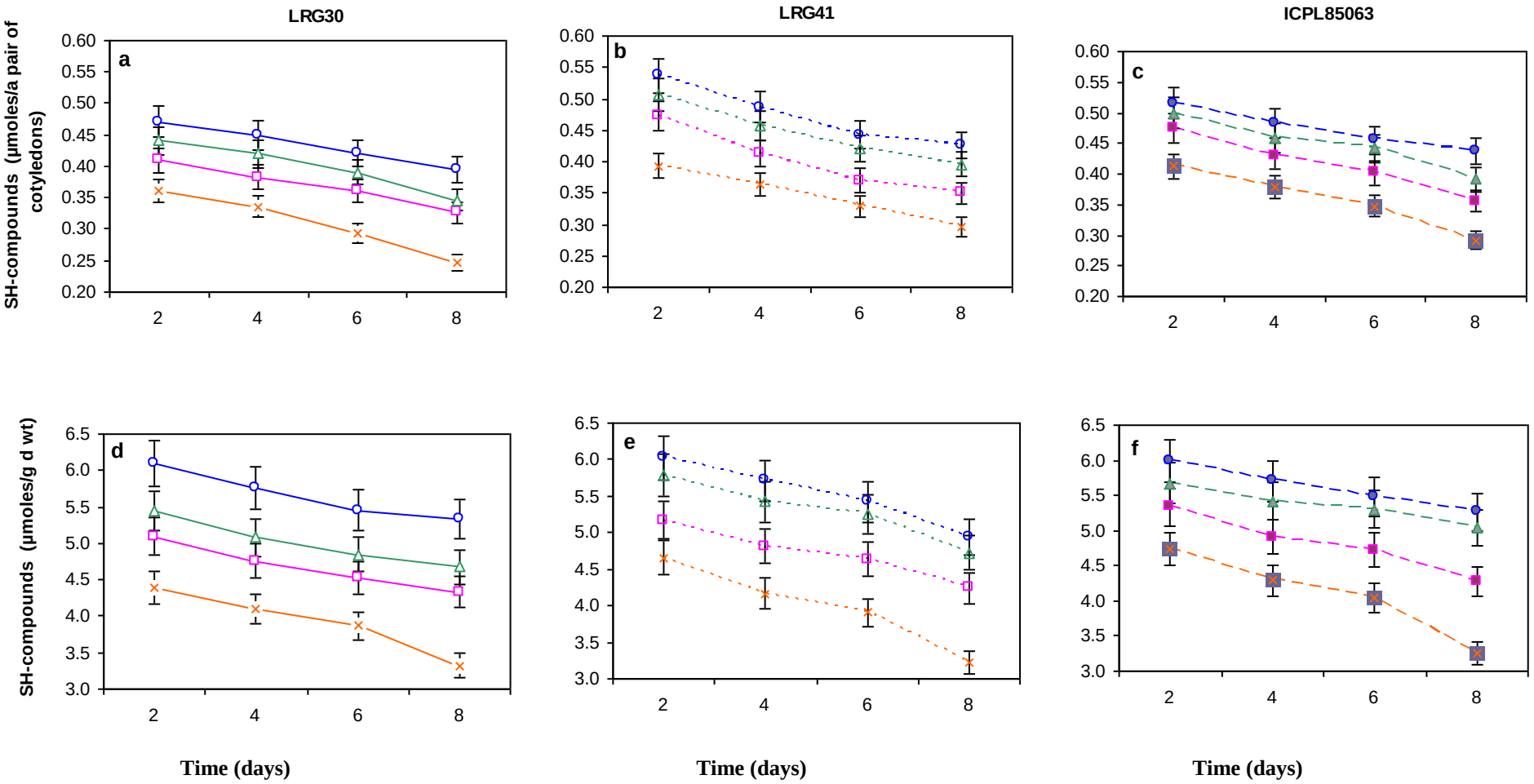

Fig. 6 - Sulphydryl (SH) compounds of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

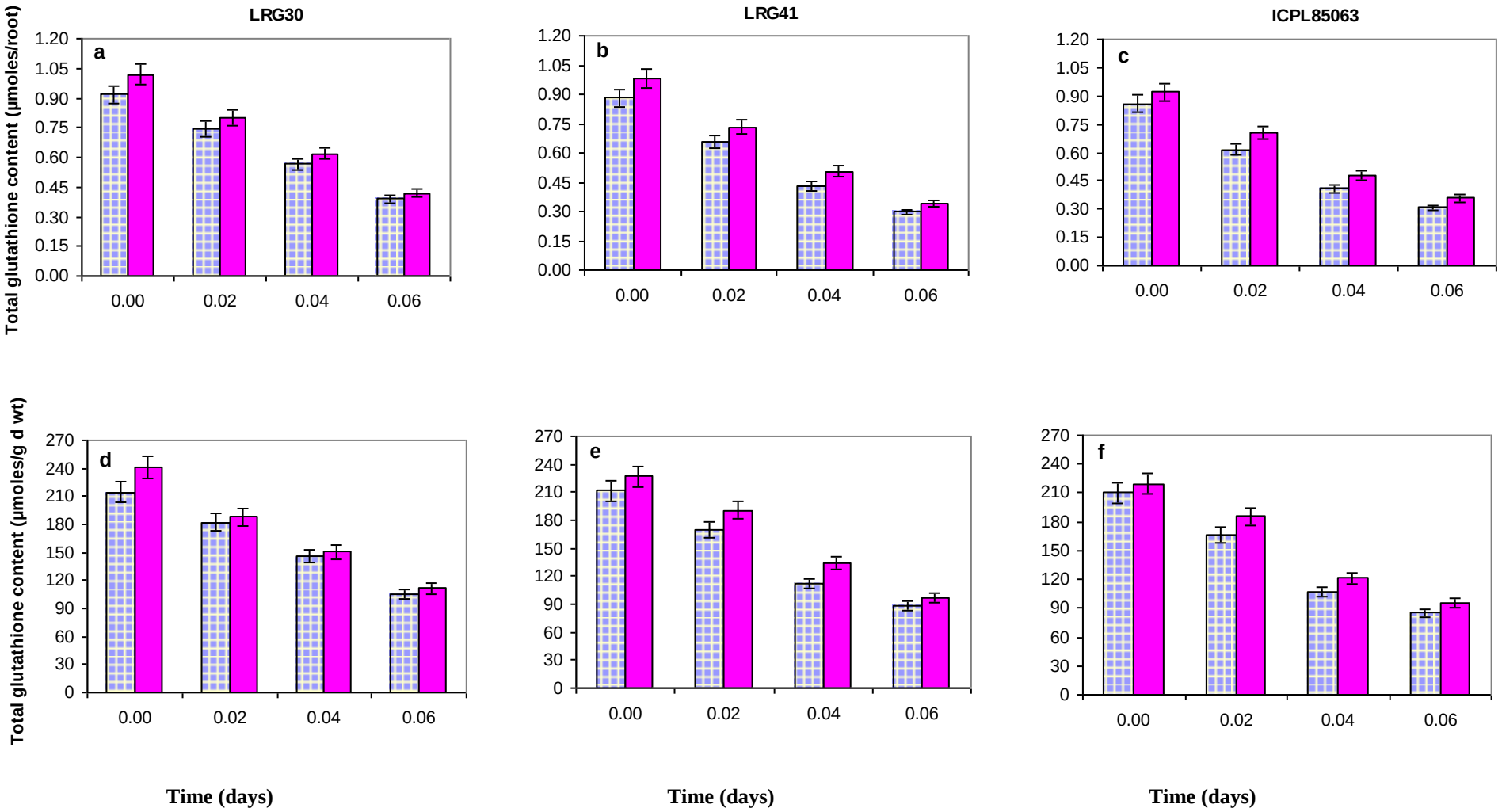

Fig. 7 - Total glutathione content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

LRG30:a, d ; LRG41:b, e; ICPL85063:c, f -

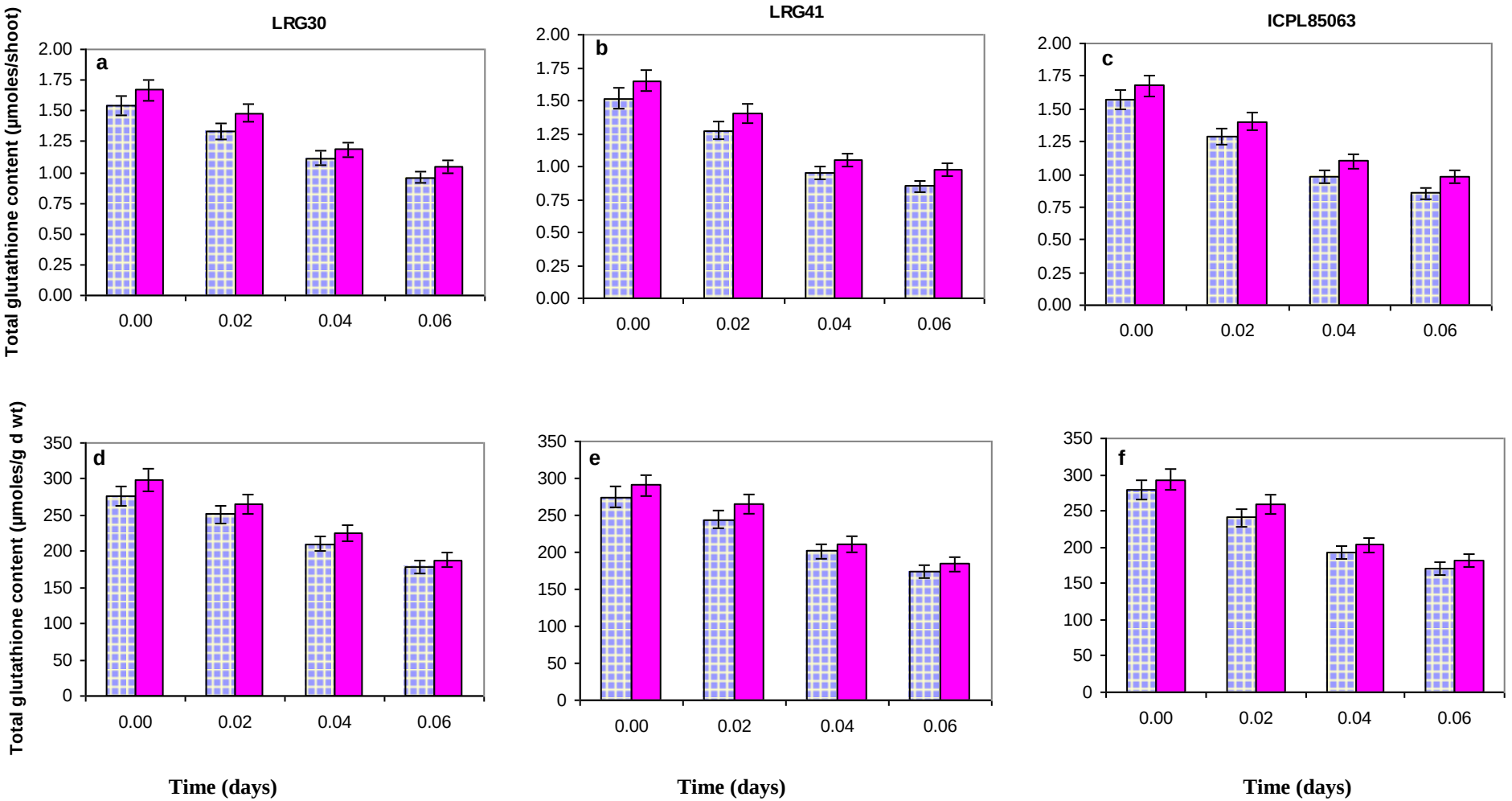

Fig. 8 - Total glutathione content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

LRG30:a, d ; LRG41:b, e; ICPL85063:c, f -

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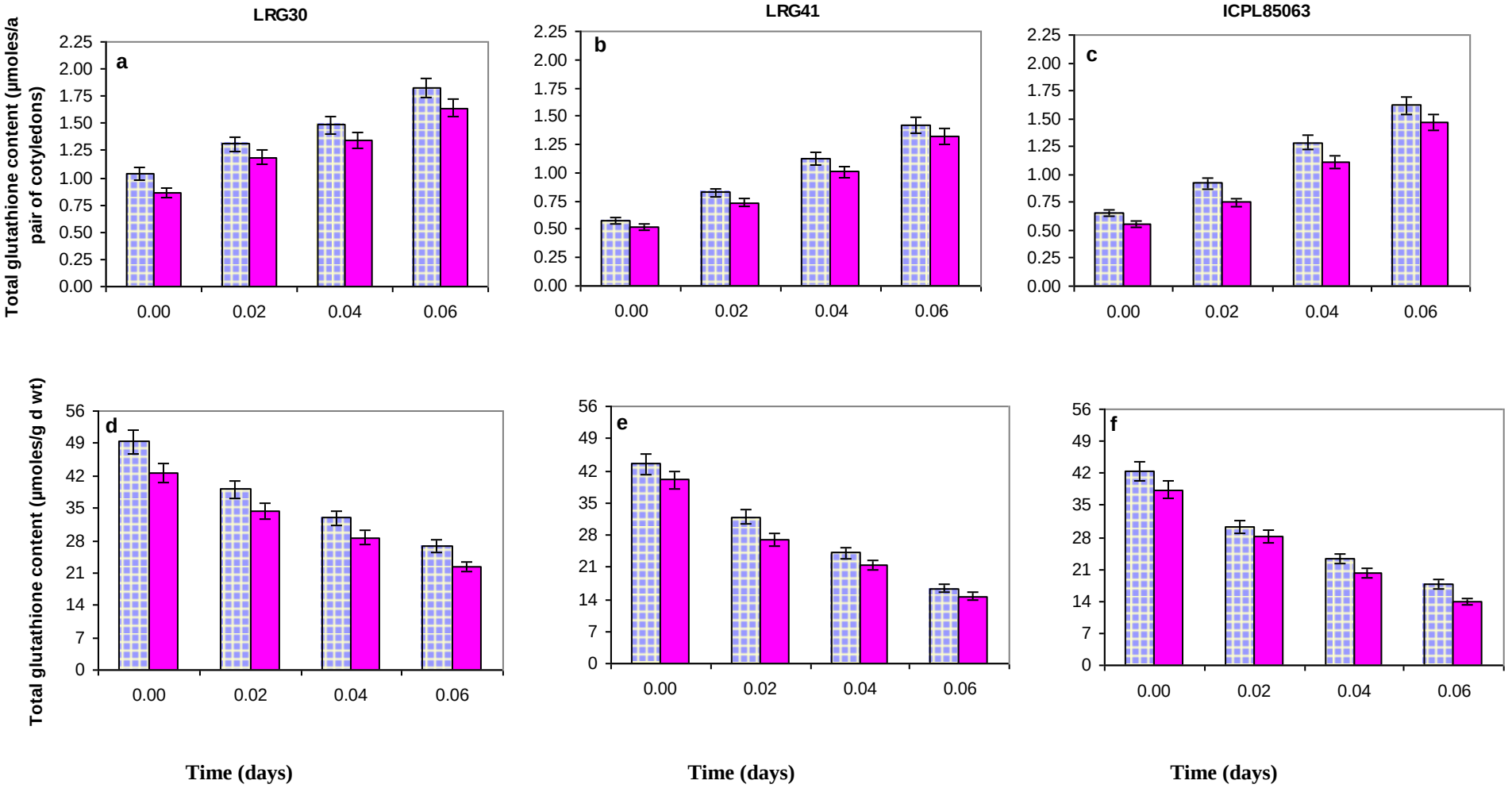

Fig. 9 - Total glutathione content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

LRG30:a, d ; LRG41:b, e; ICPL85063:c, f -

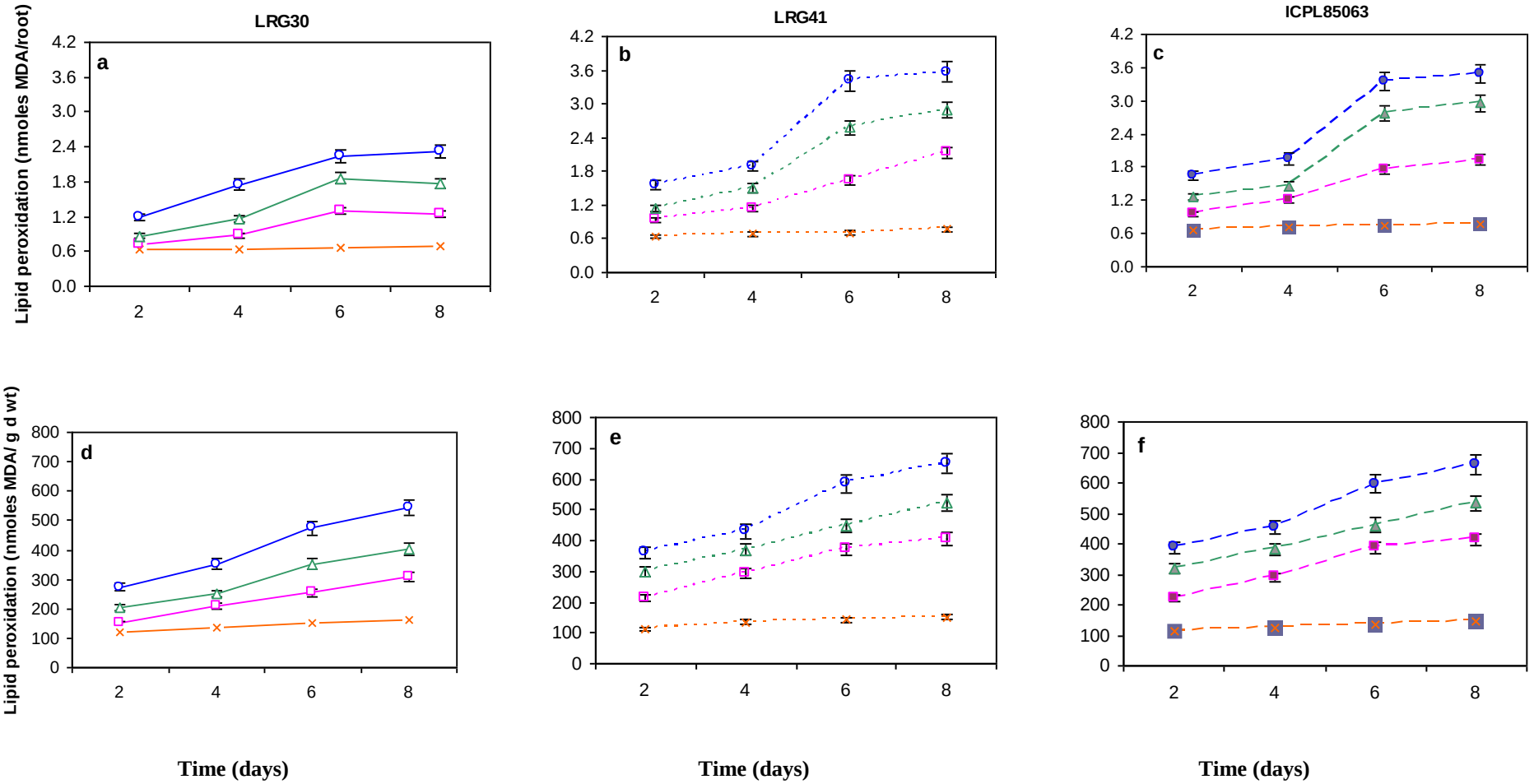

Fig. 10 - Malondialdehyde content of roots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

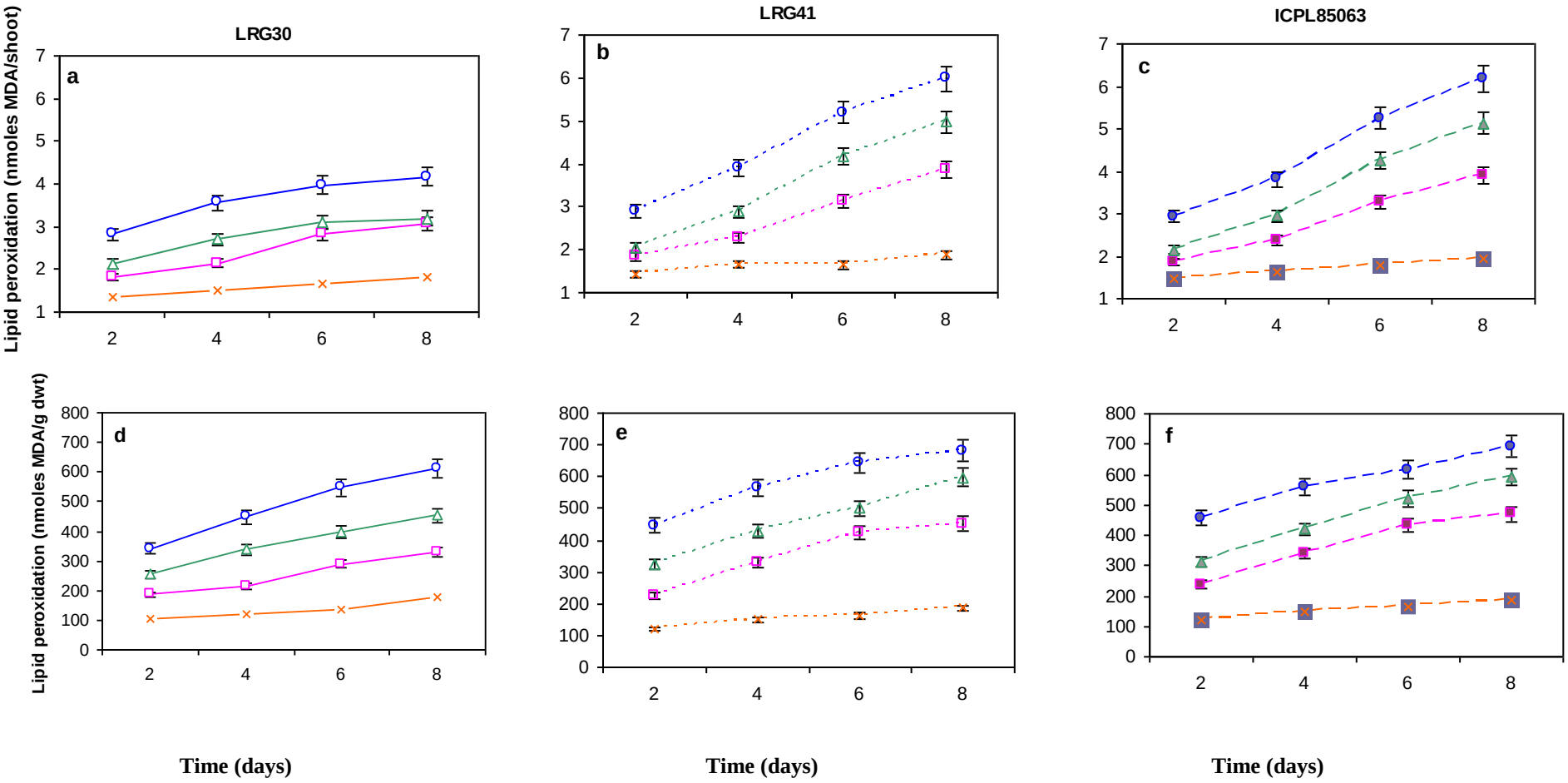

Fig. 11 - Malondialdehyde content of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

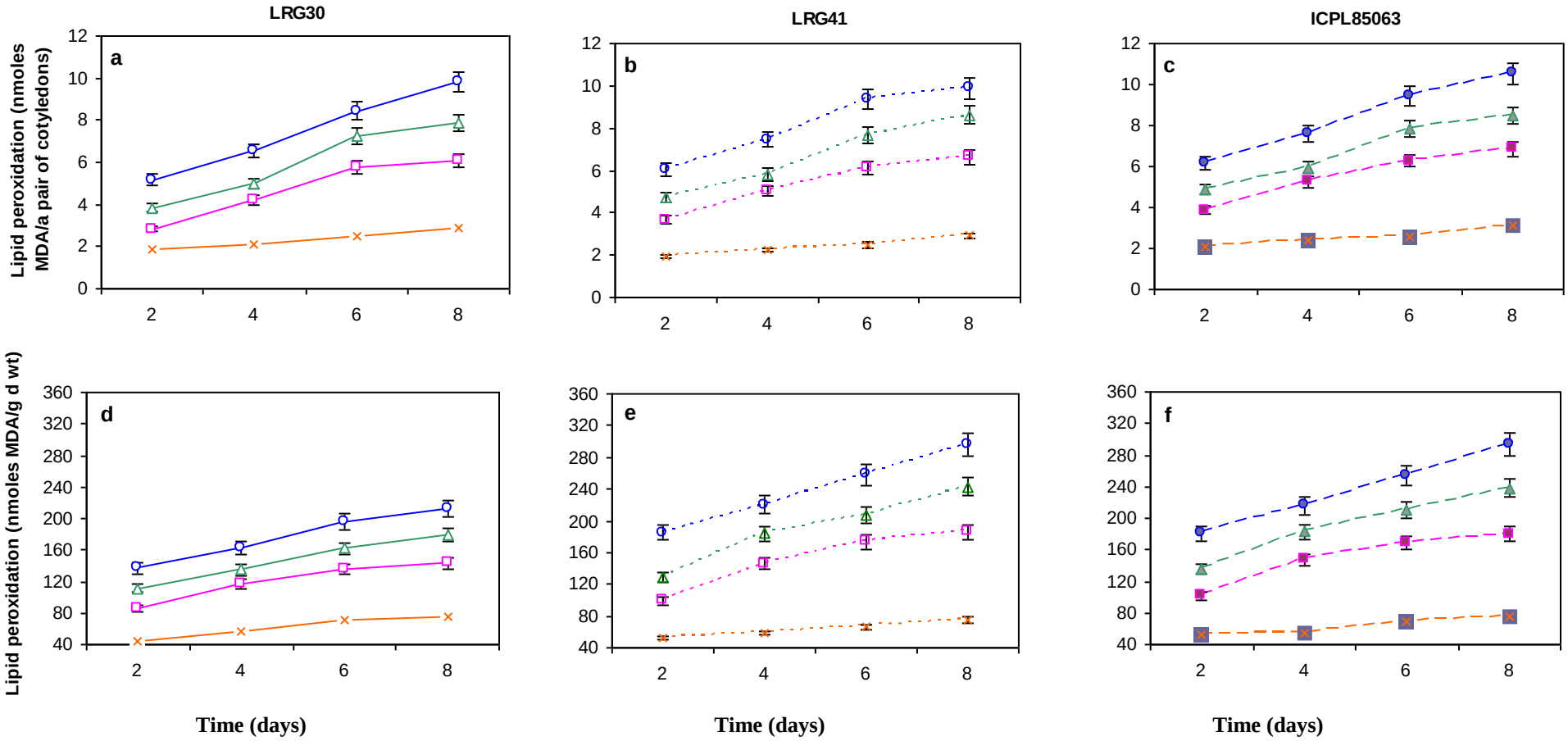

Fig. 12 - Malondialdehyde content of cotyledons of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.).

SH-Compounds

The content of water soluble sulphydryl (SH) compounds of the roots of the three pigeonpea cultivars increased in all the treatments with increasing seedling age from 2 to 8 days. In relation to the increasing concentrations of externally supplied Cd, the content of SH-compounds of the roots of three pigeonpea cultivars increased in the initial stages of seedling growth (2-and 4-day old seedlings) followed by decrease in the latter stages of seedling growth (6-and 8-day old seedlings). However, the content of the SH-compounds in the roots of the Cd treated germinating seeds of the three pigeonpea cultivars registered higher values at all stages of seedling growth when compared to their appropriate controls (Fig.4a, b, c). The content of SH-compounds in the shoots of the three pigeonpea cultivars increased steadily with increasing seedling growth as well as with increasing concentrations of externally supplied Cd and registered higher values when compared to their controls (Fig.5a, b, c). The content of the SH-compounds in the cotyledons of the three pigeonpea cultivars decreased with increasing seedling growth (Fig.6a, b, c). However, the retention in the content of the SH-compounds of the cotyledons increased with increasing concentrations of Cd and registered higher values at all stages of seedling growth when compared to their appropriate controls.

The per cent increase in the content of SH-compounds of the roots of 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentrations were 33.33, 23.80 and 19.05% in LRG30; 19.04, 14.28 and 11.90% in LRG41 and 20, 15 and 12.50% in ICPL85063 respectively over their controls. The per cent increase in the content of the SH-compounds of the shoots of the three pigeonpea cultivars grown in the respective Cd concentrations were 19.23, 23.07 and 25% in LRG30; 11.2, 18 and 20% in LRG41 and 12.50, 17.50 and 20.83% in ICPL85063 over their appropriate controls. The per cent retention in the content of the SH-compounds of the cotyledons of the respective Cd treated germinating seeds were 24.13, 34.48 and 44.82% in LRG30; 12.12, 27.28, and 33.34% in LRG41 and 14.3, 25.71 and 31.43% in ICPL85063 over their corresponding controls. Among the three cultivars of pigeonpea studied, LRG30 registered higher values of SH-compounds than LRG41 and ICPL85063 in response to Cd treatment.

On dry weight basis, the changes in the content of the SH-compounds of the roots, shoots and cotyledons of the three pigeonpea cultivars exhibited a trend similar to per organ basis both with increasing seedling growth and with increasing concentrations of externally supplied metal ions (Fig.4d, e, f; 5d, e, f and 6d, e, f).

The content of the SH-compounds in the roots showed a 0.05 level of significance in cv. LRG30 and 0.01 level of significance in LRG41 and ICPL85063 and the shoots of the three pigeonpea cultivars showed a 0.01 level of significance and the cotyledons exhibited 0.05 level of significance in cv. LRG30 and 0.01 level of significance in LRG41 and ICPL85063 with the external concentrations of Cd supplied (Table-1).

Total glutathione content

The studies on the total glutathione content of the three pigeonpea cultivars were confined to 6-day and 8-day old seedlings only. Roots of the Cd treated germinating seeds of the three pigeonpea cultivars exhibited an increasing trend from 6-8 days of seedling growth. However, the total glutathione content of the roots decreased with increasing concentrations of externally supplied Cd and registered lower values when compared to their controls (Fig.7a, b, c). The total glutathione content of the shoots of the three pigeonpea cultivars exhibited a trend similar to roots both with increasing seedling age and with increasing concentrations of externally supplied Cd (Fig.8a, b, c). The total glutathione content of the cotyledons of the three pigeonpea cultivars decreased from 6 to 8 days of seedling growth. However, the cotyledons of the Cd-treated germinating seeds of pigeonpea retained higher values of total glutathione content when compared to their respective controls and the degree of retention in the total glutathione content was more with increasing concentrations of Cd (Fig.9a, b, c).

The per cent reduction in the total glutathione content of the roots of the 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd 19.57, 38.05 and 57.61% in LRG30; 26.14, 51.14 and 65.91% in LRG41 and 27.91, 52.33 and 63.96% in ICPL85063 respectively when compared to controls. The per cent reduction in the total glutathione content of the shoots of the Cd treated pigeonpea cultivars were 12.99, 27.28 and 37.66% in LRG30; 16.45, 37.50 and 44.08% in LRG41 and 17.84, 37.58 and 45.23% in ICPL85063 with respect to their controls. The per cent retention in the total glutathione content of the

cotyledons of the Cd-treated germinating seeds of pigeonpea were 25.96, 42.30 and 75.96% in LRG30; 41.38, 93.10 and 144.83% in LRG41 and 41.53, 96.92 and 149.23% in ICPL85063 over their corresponding controls. Of the three cultivars of pigeonpea, LRG30 registered higher values of total glutathione content than LRG41 and ICPL85063 in response to Cd treatment. On dry weight basis, the changes in the total glutathione content of the roots and shoots of the three pigeonpea cultivars exhibited a trend similar to organ basis (Fig.7d, e, f and 8d, e, f). However, on dry weight basis, the total glutathione content of the cotyledons of the three pigeonpea cultivars decreased with increasing seedling growth as well as with increasing concentrations of externally supplied metal ions and registered lower values when compared to their respective controls (Fig.9d, e, f).

The total glutathione content of both roots and shoots showed a 0.05 level of significance in the three pigeonpea cultivars and the cotyledons exhibited 0.05 level of significance in cv. LRG30 and LRG41 and 0.01 level of significance in ICPL85063 with the increasing concentrations of Cd supplied (Table-1).

Table-1: The significant values between treatments and control of different parameters of antioxidative system and with increasing concentrations of externally supplied Cd or dry weight 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			
Ascorbic acid	.040*	.005**	.005**
SH-compounds	.025*	.004**	.002**
Glutathione	.035*	.023*	.020*
Lipid peroxidation	.029*	.007**	.005**
SHOOTS			
Ascorbic acid	.018*	.032*	.033*
SH-compounds	.006**	.009**	.006**
Glutathione	.042*	.030*	.021*
Lipid peroxidation	.021*	.005**	.002**
COTYLEDONS			
Ascorbic acid	.018*	.023*	.035*
SH-compounds	.016*	.007**	.008**
Glutathione	.010*	.013*	.007**
Lipid peroxidation	.006**	.004**	.004**

****1% Level of Significant (P <0.01)**
***5% Level of Significant (P <0.05)**
@ Not Significant

Lipid peroxidation

The formation of malondialdehyde is a measure of lipid peroxidation. Therefore, lipid peroxidation was determined in relation to the amount of malondialdehyde formed in all parts of the pigeonpea seedlings grown in the presence of externally supplied Cd ions. The enhancement in the levels of malondialdehyde content was observed in the roots of the three pigeonpea cultivars with increasing age of the seedling as well as with increasing concentrations of the Cd (Fig.10a, b, c). Moreover, the malondialdehyde content of the roots of the Cd treated germinating seeds of pigeonpea registered higher values when compared to their controls (Fig.11a, b, c). The changes in the levels of lipid peroxidation product of the shoots and cotyledons of the Cd treated pigeonpea cultivars showed a trend similar to the roots both with increasing

seedling growth and with increasing concentrations of externally supplied metal ions (Fig.12a, b, c).

The malondialdehyde content of 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 1.94, 2.78 and 3.36 folds in LRG30; 2.31, 3.63 and 4.80 folds in LRG41 and 2.36, 3.76 and 4.53 folds in ICPL85063 respectively over their corresponding controls. The malondialdehyde content of the shoots of the Cd treated germinating seeds showed an increase of 1.70, 1.88 and 2.38 folds in LRG30; 1.90, 2.55 and 3.17 folds in LRG41 and 1.84, 2.40 and 2.96 folds in ICPL85063 over their respective controls. The malondialdehyde content of the cotyledons of the Cd treatments exhibited an enhancement in its content of 2.33, 2.92 and 3.41 folds in LRG30; 2.47, 3.10 and 3.77 folds in LRG41 and 2.45, 3.06 and 3.68 folds in ICPL85063 over their appropriate controls. In between the three pigeonpea cultivars, the lipid peroxidation of membranes was more active in LRG41 and ICPL85063 than in LRG30 in response to Cd treatments.

On dry weight basis the pattern of changes in the malondialdehyde contents of the root, shoot and cotyledons of the three pigeonpea cultivars exhibited a trend similar to that observed for organ basis both with increasing age of the seedling and with increasing concentrations of externally supplied metal ions (Fig.10d, e, f; 11d, e, f and 12d, e, f).

The malondialdehyde content of both roots and shoots showed 0.05 level of significance in cv. LRG30 and 0.01 level of significance in LRG41 and ICPL85063, the cotyledons of the three pigeonpea cultivars showed 0.01 level of significance with the increasing concentrations of Cd supplied (Table-1).

DISCUSSION

Plants have different capacity to accumulate metals, which depends on the plant species, plant age, the metal type and exposure time. In plants, metals in the soil can enter the roots through symplastic or apoplastic pathways before entering the xylem and being translocated to the shoot (Lux et al., 2011). The aerial part of tobacco plants accumulated the greater Cd content with the highest Cd concentration used, according to the reported high mobility in the phloem (Mendoza-Cózatl et al., 2011). Cd can accumulate in all plant parts and can trigger a series of changes that can lead to phytotoxicity (Kopittke et al., 2010; Lux et al., 2011). Heavy metals can induce oxidative stress directly or indirectly, by interaction with biochemical redox processes (Van Assche and Clijsters, 1990) through the production of reactive oxygen species (ROS). Plant cells are protected against ROS by their antioxidant defense systems. The plants have developed protective mechanisms of mitigating and repairing the ROS damages to survive against Cd stress (Overmyer *et al*., 2003; Edreva, 2005). These are specific but complex mechanisms involving morphological changes, physiological and biochemical adaptations and so on. Mainly, the ROS-scavenging mechanisms are non-enzymatic system consisting of glutathione (GSH) and ascorbic acid (Yin *et al*., 2008).

Ascorbic acid, a well-known antioxidant plays a prominent role together with glutathione in scavenging free oxyradicals. Ascorbic acid acts as a detoxifier reduces the toxicity of the heavy metals to protection to the cell from expansion or abnormalities in their structural features (Smith *et al*., 1989). Cadmium treatment decreases the ascorbic acid content of the roots and shoots of the three pigeonpea cultivars (Fig.1 and 2). The reactive free oxygen radicals promote the oxidation of ascorbic acid to dehydroascorbic acid, leading to reduction in the ascorbic acid content of plants (Fridovich and Handler, 1961). The decrease in ascorbic acid content was relatively more in cv. LRG41 and ICPL85063 than in LRG30.

Plant tissues contain considerable amounts of non-protein sulphydryl (SH) compounds, which may be mostly glutathione or in some species homoglutathione (Rennenberg, 1982; De Kok, 1990). Treatments of the three pigeonpea cultivars with Cd led to considerable increase in SH-compounds (Fig.4-6). Among the three cultivars of pigeonpea studied, LRG30 registered higher values of SH-compounds due to the Cd treatment led to the accumulation of higher levels of SH-compounds.

Cadmium treatment led to a decrease in the total glutathione (GSH + GSSG) content of the roots and shoots of the three pigeonpea cultivars. In all the treatments, the glutathione content of the roots and shoots increased with increasing seedling growth and decreased with increasing concentrations of externally supplied metal ions (Fig.7 and 8). In intact maize seedlings, the glutathione content in roots being depleted more rapidly than in shoots after exposure to Cd (Berger *et al*., 1989; Steffens, 1990). Further, glutathione plays a prominent role in the defense

mechanism against oxygen free radicals and the antioxidant property of the glutathione (GSH) depends on the oxidation of SH group of the tripeptide (GSH) to disulphide form (GSSG) (Smith *et al*., 1989; Zopes *et al*., 1993). However, ions of several metals such as Zn^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, and Hg^{2+} form very stable complexes with glutathione, disturbs the inter conversion of oxidised and reduced glutathione which results in a lowering of the levels of available antioxidants in the cells (Christie and Costa, 1984). Of the three cultivars pigeonpea, LRG30 registered higher values of total glutathione content than LRG41 and ICPL85063 in response to different concentrations of Cd. The decline of glutathione content in plants may result from several causes. One of them being that the toxic metal ions may have inhibited one or more of the enzymes involved in glutathione synthesis and metabolism (Rauser, 1987). The decline in total glutathione content of plants under heavy metal exposure may also be due to utilisation of glutathione in the biosynthesis of metal-binding peptides (Robinson and Jackson, 1986; Grill *et al*., 1985, 1987).

The plant cell membranes are considered as primary sites of heavy metal injury, and the membrane destabilization is frequently attributed to lipid peroxidation (Singh *et al*., 2006). In cellular level, lipid peroxidation is the most significant damage caused by ROS. The malondialdehyde (MDA) (product of lipid peroxidation) level is regarded as a biochemical marker for injury mediated by ROS (Palma *et al*., 2002; Verma and Dubey, 2003; Sinha *et al*., 2005; Monterio *et al*., 2009). Increased production of MDA by Cd exposure has been observed in pea (Chaoui *et al*., 1997; Metwally *et al*., 2005), rice (Shah *et al*., 2001) and sunflower seedlings (Gallego *et al*., 1996). Under heavy metal stress, H_2O_2 and $O_2^{\bullet -}$ via the Haber-Weiss reaction, are converted into highly reactive $OH^{\bullet}$ radical and this causes lipid peroxidation (Apel and Hirt, 2004). The peroxidation of cell membranes severely affects its integrity. The content of MDA was significantly increased in all parts of the pigeonpea seedlings by treatment with Cd indicating increased lipid peroxidation in metal exposed plants (Fig.10-12). The level of MDA was low in shoots when compared to roots. The increased lipoxygenase activity of Cd-treated plants may lead to the production of free radicals from dioxygenation of membrane lipids and unsaturated fatty acids which in turn may damage the membranes and cellular constituents such as proteins, DNA and chlorophyll (Summerfield and Tappel, 1984). The results obtained in the present study indicated that Cd toxicity induce oxidative stress in pigeonpea cultivars, as was evident by increased accumulation of lipid peroxidation products in all organs of the stressed seedlings. In between the three pigeonpea cultivars, the lipid peroxidation was more active in LRG41 and ICPL85063 than in LRG30 in response to Cd treatments. The increased lipid peroxidation observed in the present study was probably due to the harmful effect of excessive levels of H_2O_2 or its ROS derivatives in the cellular compartments. Excessive levels of ROS may have resulted in damage to cell organelles including the photosynthetic apparatus, ultimately leading to severe cellular damage and chlorosis of the leaves (Bowler *et al*., 1992; Wang *et al*., 2008).

CONCLUSION

The ascorbic acid and total glutathione contents of the roots and shoots of the Cd-treated germinating seeds of pigeonpea cultivars increased with increasing seedling growth. However, they decreased with increasing concentrations of metal ions supplied. The ascorbic acid and total glutathione contents of the cotyledons decreased with increasing seedling growth, but their values were retained higher when compared to their controls. The water-soluble SH-content of the roots and shoots of the Cd treatments increased with increasing age of the seedlings and registered higher values at all the stages of seedling growth when compared to their controls. The SH-content of the cotyledons decreased with increasing seedling growth and retained higher values over their respective controls. Lipid peroxidation in terms of malondialdehyde formation increased in the three pigeonpea cultivars with increasing age of the seedlings as well as increasing concentrations of externally supplied Cd ions. In between the three pigeonpea cultivars, lipid peroxidation was more active in cv. LRG41 and ICPL85063.

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REFERENCES

Apel K and Hirt H., 2004. Reactive oxygen species: metabolism oxidative stress and signaling transduction. *Ann. Rev.Plant Biol.,* 55:373-399.

Berger JM, Jackson PJ, Robinson NJ, Lujan LD and Delhaize E., 1989. Precursor-product relationships of poly (γ-glutamylcysteinyl) glycine biosynthesis in *Datura innoxia*. *Plant Cell Rep.*, 7:632-635.

Bergmann L and Rennenberg H., 1993. Glutathione metabolism in plants. In Sulfur Nutrition and Assimilation in Higher Plants, L.J. De Kok, ed (The Hague, The Netherlands: SPB Academic Publishing bv), pp. 109-123.

Bowler C, Van Montagu M and Inze D., 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 43:83-116.

Chaoui A, Mazhoudi S, Ghorbal MH and Ferjani EE., 1997. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). *Plant Sci*., 127:139-147.

Christie NT and Costa M., 1984. In vitro assessment of the toxicity of metal compounds. IV. Deposition of metals in cells: interactions with membranes, glutathione, metallothionein and DNA. *Biol. Trace Elem. Res*., 6:139-158.

Cumming JR and Taylor GJ., 1990. Mechanism of metal tolerance in plants: physiological adaptations for exclusion of metal ions from the cytoplasm. In: Alscher, R.G., Cumming, J.R. (Eds.), Stress Response in Plants: Adaptation, and Acclimation Mechanism. Wiley-Liss, New York, pp. 329-356.

Davies MJ., 2003. Singlet oxygen-mediated damage to proteins and its consequences. *Biochem. Biophys. Res. Commun*., 305:761-770.

De Kok LJ., 1990. Sulfur metabolism in plants exposed to atmospheric sulfur. In: Rennenberg H, Brunold C, De Kok LJ, Stulen I (eds) Sulfur Nutrition and Sulfur Assimilation in Higher Plants: Fundamental Environmental, Agricultural Aspects. SPB Academic Publishing, The Hague, pp. 111-130.

De Vos CHR, Schat H, DeWaal MAM, Vooijs R and Ernst WHO., 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Physiol. Plant*., 82:523-528.

Edreva A., 2005. Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach. *Agr. Ecosyst. Environ*., 30:119-133.

Ellman GL., 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys*., 82:70-77.

Fridovich I and Handler P., 1961. Detection of free radicals generated during enzymic oxidation by the initiation of sulphite oxidation. *J. Biol. Chem*., 236:1836-1840.

Gallego SM, Benavides MP and Tomaro ML., 1996. Effects of heavy metal ion excess on sunflower leaves: evidences for involvement of oxidative stress. *Plant Sci.,* 121:151-159.

Griffith OW., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem*., 106:207-212.

Grill D, Esterballer H and Klosch U., 1979. Effect of sulphur dioxide on glutathione in leaves of plants. *Environ.Pollut*., 17:187-194.

Grill E, Winnaker EL and Zenk MH., 1985. Phytochelatins: the principal heavy metal complexing peptides of higher plants. *Science*, 230:674-676.

Grill E, Winnaker EL and Zenk MH., 1987. Phytochelatins: a class of heavy metal binding peptides from plants are functionally analogous to metallothioneins, *Proc. Natl. Acad. Sci.* U.S.A. 84:439-443.

Hall JL., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.,* 53:1-11.

Heath RL and Packer K., 1968. Leaf senescence; corrrelated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J. Exp. Bot.,* 32:93-101.

Klapheck S, Fliegner W and Zimmer I., 1994. Hydroxy methyl-phytochelatins [(γ-glutamylcysteine)$_n$-serine] are metal-induced peptides of the Poaceae. *Plant Physiol*., 104:1325-1332.

Kopittke, P.M., Blamey, F.P.C., Menzies, N.W., 2010.Toxicity of Cd to signal grass (*Brachiaria decumbens* Stapf.) and Rhodes grass (*Chloris gayana* Kunth.). Plant Soil 330:515-523.

Lux, A., Martinka, M., Vaculík, M., White, P.J., 2011. Root responses to cadmium in the rhizosphere: a review. J. Exp. Bot. 62:21-37.

Mendoza-Cózatl, D.G., Jobe, T.O., Hauser, F., Schroeder, J.I., 2011. Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. Curr. Opin. Plant Biol. 14: 554-562.

Metwally A, Safronova VI, Belimov AA and Dietz KJ., 2005. Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. *J. Exp. Bot*., 56:167-178.

Meuwly P and Rauser WE., 1992. Alteration of thiol pools in roots and shoots of maize seedlings exposed to cadmium: adaptation and developmental cost. *Plant Physiol*., 99:8-15.

Michael PI and Krishnaswamy M., 2011. The effect of zinc stress combined with high irradiance stress on membrane damage and antioxidative response in bean seedlings. *Environ. Exp. Bot*., 74:171-177.

Monterio MS, Santos C, Soares AMVM and Mann RM., 2009. Assessment of biomarkers of cadmium stress in lettuce. *Ecotoxicol. Environ. Safety,* 72:811-818.

Overmyer K, Brosché M and Kangasjärvi J., 2003. Reactive oxygen species and hormonal control of cell death. *Trends Plant Sci.,* 8:335-342.

Palma JM, Sandalio LM, Corpas FJ, Romero-Puertas MC, McCarthy I and del Rio LA., 2002. Plant proteases, protein degradation and oxidative stress: role of peroxisomes. *Plant Physiol. Biochem.,* 40:521-530.

Rauser WE and Ackerley CA., 1987. Localization of cadmium in granules within differentiating and mature root cells. *Can. J. Bot*., 65:643-646.

Rennenberg H., 1982. Glutathione metabolism and possible biological roles in higher plants. *Phytochem.,* 21:2771-2781.

Robinson NJ and Jackson PJ., 1986. "Metallothionein like" metal complexes in angiosperms, their structure and function. *Physiol. Plant*, 67:499-506.

Roe JH., 1964. Chemical determination of ascorbic, dehydroascorbic and diketogluconic acids, in: D. Glick (Ed.), Methods of Biochemical Analysis I, Interscience, New York, pp. 115-139.

Sanita di Toppi L and Gabbrielli R., 1999. Response to cadmium in higher plants. *Env. Exp. Bot*., 41:105-130.

Schützendübel A and Polle A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J. Exp. Bot.,* 53:1351-1365.

Shah K, Kumar RG, Verma S and Dubey RS., 2001. Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. *Plant Sci*., 161:1135-1144.

Sharma P, Jha AB, Dubey RS and Pessarakli M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot*., 2012:1-26.

Singh N, Ma LQ, Srivastava M and Rathinasabapathi B., 2006. Metabolic adaptations to arsenic-induced oxidative stress in *Pteris vittata* L. and *Pteris ensiformis* L. *Plant Science*, 170:274-282.

Sinha S, Saxena R and Singh S., 2005. Chromium induced lipid peroxidation in the plants of *Pistia stratiotes* L., role of antioxidants and antioxidant enzymes. *Chemosphere,* 58:595-604.

Skórzyńska-Polit E, Drazkiewicz M and Krupa Z., 2003/4. The activity of antioxidative system in cadmium-treated *Arabidopsis thaliana. Biol. Plant,* 47:71-78.

Smith IK, Vierheller TL and Thorne CA., 1989. Properties and functions of glutathione reductase in plants. *Physiol. Plant*., 77:449-456.

Steffens JC., 1990. The heavy metal-binding peptides of plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 41:553-575.

Summerfield FW and Tappel AL., 1984. Effects of dietary polyunsaturated fats and vitamin E on ageing and peroxidative damage to DNA. *Arch. Biochem. Biophys*., 282:408-416.

Szuster-Ciesieslska A, Stachura A, Sbotwinska M, Kamibska T, Sniezka R, Paduch R, Abramczyk D, Filar J and Kandefer-Szerszen M., 2000. The inhibitory effect of zinc on cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures. *Toxicology,* 145:159-171.

Tietze F., 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissues. *Anal. Biochem*., 27:502-522.

Tukendorf A and Rauser WE., 1990. Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium. *Plant Sci*., 70:155-166.

Tukendorf A., 1996. Phytochelatin synthesis in maize seedlings in response to excess zinc. *Biol. Plant,* 38:137-140.

Van Assche F and Clijsters H., 1990. Effects of metals on enzyme activity in plants. *Plant Cell Environ*., 13:195-206.

Verma S and Dubey RS., 2003. Lead toxicitiy induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. *Plant Sci.,* 164:645-655.

Wang Z, Zhang Y, Huang Z and Huang L., 2008. Antioxidative response of metal-accumulator and non-accumulator plants under Cd stress. *Plant soil*., 310:137-149.

Yin XL, Jiang L, Song NH and Yang H., 2008. Toxic reactivity of wheat (*Triticum aestivum*) plants to herbicide isoproturon. *J. Agri. Food Chemistry*, 56(12):4825-4831.

Yurekli F and Porgali ZB., 2006. The effects of excessive exposure to copper in bean plants. *Acta. Biol. Cracov. Bot*., 48(2):7-13.

Zhang F, Shi W, Jin Z and Shen Z., 2003. Response of antioxidative enzymes in cucumber chloroplasts to cadmium toxicity. *J. Plant Nut.,* 26:1779-1788.

Zopes S, Klapheck L and Bergmann, 1993. The function of homoglutathione and hydroxymethyl glutathione for the scavenging of hydrogen peroxide. *Plant Cell Physiol*., 34:515-521.

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