Lead-induced oxidative stress and metabolic alterations in *Cassia angustifolia* Vahl.

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Abstract

Forty-five-days old plants of Indian senna (*Cassia angustifolia* Vahl.) were subjected to 0 - 500 μM lead acetate (Pb-Ac) in pot culture. Changes in contents of thiobarbituric acid reactive substances (TBARS), ascorbate, glutathione, proline, sennosides (*a*+*b*), and activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) were studied at pre-flowering (60 d after sawing, DAS), flowering (90 DAS) and post-flowering (120 DAS) stages of plant development. Compared with the controls, the Pb-Ac treated plants showed an increase in contents of TBARS, dehydroascorbate, oxidized and total glutathione at all stages of growth. However, sennoside yield and contents of ascorbate and reduced form of glutathione declined. Proline content increased at 60 DAS but declined thereafter. Activities of SOD, APX, GR and CAT were markedly increased. Sennoside content was higher at 60 and 90 DAS but lower at 120 DAS, compared to the control.

Additional key words: antioxidants, antioxidative enzymes, lead acetate, senna, sennosides.

Introduction

Pb-contaminated soils cause sharp decreases in crop productivity and thus pose a serious problem for agriculture. The primary effect of lead toxicity in plants is a rapid inhibition of root growth, probably due to inhibition of cell division in root tips (Eun *et al.* 2000). Secondarily, it may induce oxidative stress (Ruley *et al*. 2004, Reddy *et al*. 2005) that damages cell and its components such as chloroplasts, and alter the concentration of different metabolites including soluble protein, proline, ascorbate and glutathione, and the enzymatic antioxidants including superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Van Assche *et al.* 1988, Kastori *et al.* 1992). Expression levels of these antioxidants are altered so as to combat adverse effects of the stress (Alscher *et al.* 2002, Qadir *et al.* 2004).

The leaves, pods and seeds of Indian senna (*Cassia angustifolia* Vahl.) contain the anthroquinone glycosides, mainly sennoside *a* and sennoside *b* (Laemli *et al.* 1985), and are used in pharmaceutical industry.

There are only few reports about the effect of abiotic stresses on the functioning of secondary metabolism, particularly on the concentration of secondary metabolites of therapeutic properties (Singh *et al.* 1999, Arshi *et al.* 2002, 2004). Moreover, the effects of heavy metals and oxidative stress and their interactions on secondary metabolism are yet to be understood. The present study evaluates the impact of lead and the lead-induced oxidative stress on TBARS content, proline content and antioxidants in Indian senna with special reference to the concentration and yield of sennosides.

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase, DAS - days after sowing; GR - glutathione reductase; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances.
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Materials and methods

Plants and growth conditions: Seeds of Indian senna (*Cassia angustifolia* Vahl.) were procured from Medicinal and Aromatic Plant Project, Gujarat Agriculture University, Anand, Gujarat, India. Seeds were sown in pots, each containing 12 kg of sandy-loam soil (pH 6.8 ± 0.2), in June (of two consecutive years) when average minimum and maximum temperatures lay around 28 and 40 °C, respectively, and relative humidity around 75 - 85 %. Plants were treated with various concentrations of lead acetate (Pb-Ac; control = 0 μM, T_1 = 100 μM, T_2 = 250 μM and T_3 = 500 μM) at 45 d after sowing (DAS; vegetative stage). Pots were treated/watered keeping in view the water-holding capacity of the soil in the pot and, whenever rain was imminent, pots were covered with a clear polyethylene film. Treatments were arranged in a completely randomized block design with 3 replications. Sampling of leaves was done at pre-flowering (60 DAS), flowering (90 DAS) and post-flowering (120 DAS) stages.

Thiobarbituric acid reactive substances: TBARS, considered as oxidative damage products, were determined in leaf samples by the method of Heath and Packer (1968) with slight modifications. One gram of fresh tissue was ground in 10 cm^3 0.1 % trichloroacetic acid (TCA) and centrifuged at 7826 *g* for 5 min. The mixture of 1 cm^3 of supernatant with 4.0 cm^3 of 0.5 % thiobarbituric acid (TBA) was heated at 95 °C for 30 min, cooled and centrifuged at 1957 *g* for 5 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity after subtraction from the value obtained at 600 nm. TBARS was then quantified using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Proline content in leaf samples was estimated by the method of Bates *et al.* (1973). 0.5 g of fresh leaf was homogenized in 10 cm^3 of 3 % sulphosalicylic acid and centrifuged at 7826 *g* for 10 min. The mixture of 2 cm^3 of supernatant, 2 cm^3 of acid ninhydrin reagent and 2 cm^3 of glacial acetic acid was boiled at 100 °C in a water bath for 30 min, reaction stopped in an ice bath and then 4 cm^3 of toluene was added to each sample. The toluene (upper) layer was read at 520 nm on a UV-Vis spectrophotometer (*Model DU-640B*, *Beckman*, CA, USA). The corresponding concentration of proline was determined against the standard curve of L-proline.

Ascorbate (Asc), dehydroascorbate (DAsc) and total ascorbate (Asc + DAsc) were estimated by a modified method of Law *et al.* (1983). Fresh leaf tissue (0.5 g) was ground in 2 cm^3 of 0.1 M Na-phosphate buffer (pH 7) and 1 mM EDTA and centrifuged at 7826 *g* for 10 min. The supernatant was distributed in two separate sets for the assay of total ascorbate and ascorbate (Asc). To each sample (0.4 cm^3), 0.2 cm^3 of 10 % TCA was added. After 5 min, 0.01 cm^3 of 5 M NaOH was added, mixed and centrifuged for 2 min. To 0.2 cm^3 of the supernatant 0.2 cm^3 of Na-phosphate buffer (150 mM, pH 7.4) and 0.2 cm^3 of double distilled water (DDW) were added. For determination of Asc, another 0.2 cm^3 of supernatant was used with 0.2 cm^3 of Na-phosphate buffer and 0.1 cm^3 of 10 mM dithiothritol (DTT) and, after a thorough mixing, was left at room temperature for 15 min. 0.1 cm^3 of 0.5 % N-ethylmaleimide was then added to each of the tubes and incubated at 24 °C for >30 s. Further, 0.4 cm^3 of 10 % TCA, 0.4 cm^3 of 44 % H_3PO_4, 0.4 cm^3 of 4 % bipyridyl and 0.2 cm^3 of 3 % $FeCl_3$ were added. After being vortex-mixed, samples were incubated at 37 °C for 60 min and absorbance was recorded at 525 nm.

Glutathione content: Reduced (GSH), oxidised (GSSG) and total glutathione (GSH + GSSG) were determined by the glutathione recycling method of Anderson (1985). Fresh leaf (0.5 g) was homogenized in 2 cm^3 of 5 % sulphosalicylic acid at 4 °C. The homogenate was centrifuged at 7826 *g* for 10 min. To a 0.5 cm^3 of supernatant, 0.6 cm^3 of reaction buffer (0.1 M Na-phosphate, pH 7, 1 mM EDTA) and 0.04 cm^3 of 0.15 % 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were added and read at 412 nm after 2 min. To the same, 0.04 cm^3 of 0.4 % NADPH and 0.002 cm^3 of glutathione reductase (GR; 0.5 enzyme unit) were added and reaction was run for 30 min at 25 °C. The samples were again read at 412 nm to determine the total glutathione.

Enzyme assays: The method of Dhindsa *et al.* (1981) was followed with slight modification for estimating SOD activity. Fresh leaf material (0.2 g) was homogenized in 2.0 cm^3 of extraction mixture containing 0.5 M Na-phosphate buffer, pH 7.3, 3 mM EDTA, 1 % PVP, 1 % Triton X 100 and centrifuged at 11 269 *g* at 4 °C. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The assay mixture, consisting of 1.5 cm^3 reaction buffer containing 0.1 M Na-phosphate buffer, pH 7.5, 1 % PVP, 0.2 cm^3 of L-methionine, 0.1 cm^3 enzyme extract with equal amount of 1 M $NaHCO_3$, 2.25 mM NBT solution, 3 mM EDTA, 60 μM riboflavin and 1.0 cm^3 of DDW was incubated under 15 W inflorescent lamp at 28 °C. 50 % reduction of NTB was considered as one unit of enzyme activity.

APX activity was estimated by the method of Nakano and Asada (1981). Fresh leaf material (1 g), ground in 5 cm^3 of extraction buffer (0.1 M K-phosphate, pH 7, 3 mM EDTA, 1 % PVP, 1 % Triton X 100), was centrifuged at 7826 *g* for 10 min at 4 °C. APX activity was determined in supernatant by the decrease in absorbance of ascorbate at 290 nm, due to its enzymatic

breakdown. 1 cm^3 of reaction buffer contained 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.05 cm^3 of extract containing enzyme. The reaction was run for 5 min at 25 °C. APX activity was calculated by using coefficient of absorbance 2.8 mM^{-1} cm^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of ascorbate per min.

GR activity was determined by the method of Foyer and Halliwell (1976) modified by Rao (1992). Fresh leaf material (0.5 g), ground in 2 cm^3 of extraction buffer (0.1 M Na-phosphate, pH 7.0, 3 mM EDTA, 1 % PVP, 1 % Triton X 100) was centrifuged at 7826 *g* for 10 min. The supernatant was immediately assayed for GR activity through glutathione-dependent oxidation of NADPH at 340 nm. 1 cm^3 reaction mixture containing 0.2 mM NADPH, 0.5 mM GSSG and 0.05 cm^3 of enzyme extract was kept for 5 min at 25 °C. Corrections were made for any GSSG oxidation in the absence of NADPH. The activity was calculated using coefficient of absorbance of 6.2 mM^{-1} cm^{-1}. One unit of enzyme determines its amount necessary to decompose 1 μmol of NADPH per min.

CAT activity was determined by the method of Aebi (1984). Fresh leaf material (0.5 g), ground in 5 cm^3 of extraction buffer (0.5 M Na-phosphate, pH 7.3, 3 mM EDTA, 1 % PVP, 1 % Triton X 100) was centrifuged at 7826 *g* for 20 min at 4 °C. CAT activity in supernatant was determined by monitoring the disappearance of H_2O_2, measuring a decrease in absorbance at 240 nm. Reaction was run in a final volume of 2 cm^3 of reaction buffer (0.5 M Na-phosphate, pH 7.3) containing 0.1 cm^3 3 mM EDTA, 0.1 cm^3 of enzyme extract and 0.1 cm^3 of 3 mM H_2O_2 for 5 min. CAT activity was calculated by using coefficient of absorbance of 0.036 mM^{-1} cm^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of H_2O_2 per min.

Sennoside content: The sennosides (*a*+*b*) were extracted using the method of (Laemli *et al*. 1985). 100 mg of dried (60 °C for 72 h) leaves was extracted 3 times with 20, 20 and 10 cm^3 of DDW. The extract was kept in boiling water for 15 min, cooled, filtered through *Whatman* filter paper No. 1 and made to a final volume of 50 cm^3 by adding DDW. 5 cm^3 of cooled extract with 10 cm^3 of 15 % $FeCl_3$, was incubated at 80 °C for 20 min. Thereafter, 0.1 cm^3 of concentrated HCl was added and extracted three times with 20, 20 and 10 cm^3 of ether using a separating funnel. The aqueous layer was collected and its volume was made up to 50 cm^3 by adding HPLC grade water. HPLC (Model *Delta 600*, *Waters*, MA, USA) was employed for the quantitative analysis of sennoside *a* and *b* using the method described by (Srivastava *et al*. 1983a,b). The solvent used in HPLC was a mixture of tetrahydrofurane with 2 % glacial acetic acid and HPLC water (1:3), flow rate was 1 cm^3 min^{-1} through the column C_{18}. The sennoside standards of *Sigma* (St. Louis, USA) were used.

Statistical analysis: Statistical analyses of the data obtained were carried by the two-way *ANOVA* (Cochran and Cox 1957) to evaluate whether the values were significantly different.

Results

The amount of leaf TBARS increased with age in control plants. Lead treatments further increased the TBARS content in a dose-dependent manner up to 154, 211 and 116 % under the highest Pb concentration used (500 μM Pb-Ac) at 60, 90 and 120 DAS, respectively (Fig. 1).

In control plants, maximum proline content was observed at 90 DAS. Lead-treated plants showed a dose-dependent increase of proline content at 60 DAS but a decrease (up to 37 and 36 %) over their controls, respectively, at 90 and 120 DAS (Fig. 2).

In control plants, the maximum contents of Asc [9.8 mmol g^{-1}(f.m.)], DAsc [1.1 mmol g^{-1}(f.m.)] and total

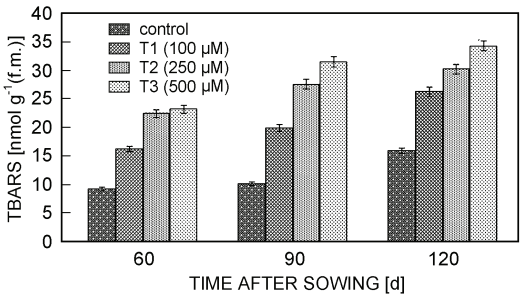

Fig. 1. Changes in thiobarbituric acid reactive substances (TBARS) content in leaves of *Cassia angustifolia* as influenced by lead acetate (0 - 500 μM) analysed at 60, 90 and 120 d after sowing (DAS) after being treated at 45 DAS.

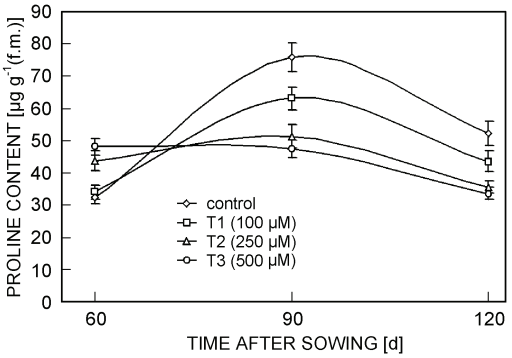

Fig. 2. Changes in proline content in leaves of *Cassia angustifolia* as influenced by lead acetate (0 - 500 μM) analysed at 60, 90 and 120 DAS after being treated at 45 DAS.

ascorbate were observed at 90 DAS. In treated plants, the Asc and total ascorbate contents declined in a dose-dependent manner but DAsc content increased. The decline in Asc content, over the control, ranged from 81 % (at 60 DAS) to 88 % (at 120 DAS) under 500 μM Pb-Ac (Fig. 3*A*).

In control plants, maximum contents of GSH [561 nmol g^{-1}(f.m.)], GSSG [271 nmol g^{-1}(f.m.)] and total glutathione were observed at 90 DAS. Treated plants showed a rapid decline in GSH content, ranging from 23 % (T_1, 60 DAS) to 33 % (T_3, 120 DAS) over their controls (Fig. 3B). On the other hand, the GSSG and

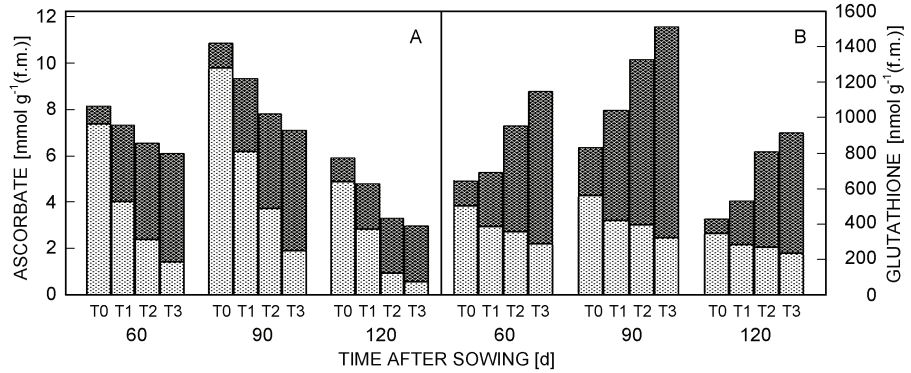

Fig. 3. Changes in ascorbate (*light columns*), dehydroascorbate (*dark columns*) and total (ascorbate + dehydroascorbate) ascorbate content (*A*) and GSH (*light columns*), GSSG (*dark columns*) and total (GSH + GSSG) glutathione content (*B*) in *Cassia angustifolia* as influenced by various concentrations (T0 - 0, T1 - 100, T2 - 250, T3 - 500 μM) of lead acetate.

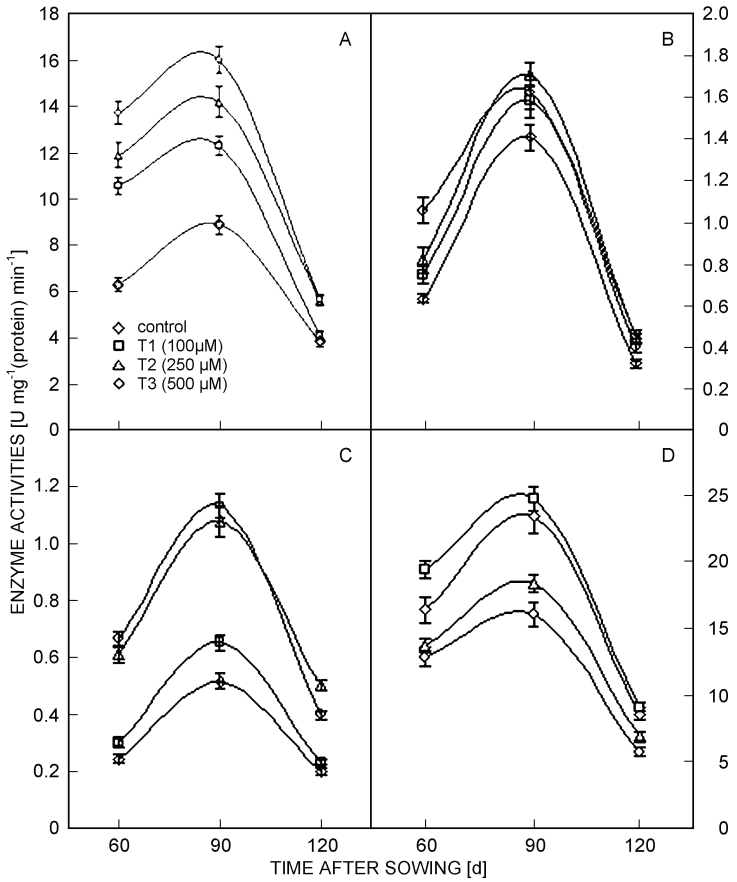

Fig. 4. Changes in activities of SOD (*A*), APX (*B*), GR (*C*) and catalase (*D*) in leaves of *Cassia angustifolia* as influenced by lead acetate (0 - 500 μM) analysed at 60, 90 and 120 DAS after being treated at 45 DAS.

total glutathione contents increased rapidly in a dose-dependent manner; the increase was also time dependent for total glutathione content (Fig. 3*B*).

Lead-treated plants showed a dose-dependent increase in SOD activity. The increase was up to 118 % (T_3, 60 DAS) but remain only 49 % higher than in the controls at 120 DAS (Fig. 4*A*). Treated plants showed a dose-dependent increase in APX activity, up to 68 % (T_3, 60 DAS) over the control, though it was only 24 % higher at 120 DAS; the least increase by Pb treatment was noted at 90 DAS (Fig. 4*B*). Plants treated with Pb had a dose-dependent increase in GR activity (171, 120 and 100 % increase over the controls at T_3; 60, 90 and 120 DAS, respectively) (Fig. 4*C*). CAT activity in control plants was maximum at 90 DAS. Lead treatment T_3 caused a decline of it up to 22, 32 and 34 % over the controls at 60, 90 and 120 DAS, respectively. However, application of a lower lead concentration (T_1) increased the CAT activity by 18, 6 and 6 % than their controls at 60, 90 and 120 DAS, respectively (Fig. 4*D*).

The sennoside (*a*+*b*) content in the leaves increased under all concentrations of lead, up to 46 % (60 DAS) and 39 % (90 DAS) under T_3 (Fig. 5). However, a decline (39 %) after 120 DAS was observed under T_3. Lead-treated plants showed a dose- as well as time-dependent decrease in yield of sennosides (Fig. 5).

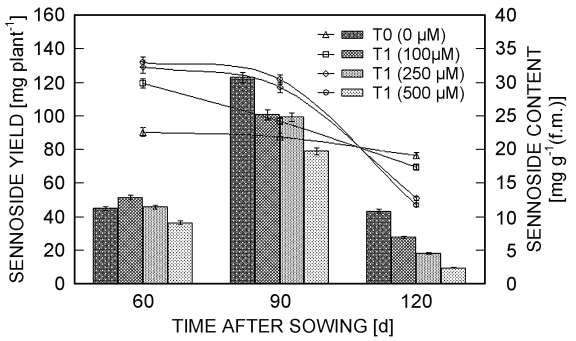

Fig. 5. Changes in sennoside (*a*+*b*) content (*lines*) and sennoside yield (*columns*) in leaves of *Cassia angustifolia* as influenced by lead acetate (0 - 500 µM) analysed at 60, 90 and 120 DAS after being treated at 45 DAS.

Discussion

Oxidative damage is often exclusively associated with peroxidation reactions in membrane lipids. Thus, membrane lipid peroxidation is an indicator of oxidative damage resulting in the loss of cell membrane lipids caused by free radicals and hydroperoxides (Thompson 1988, Smirnoff 1993). Ultimately, a reduction in biomass is caused by heavy metal stress (Mazen 2004) and also a decreased yield of metabolites as also observed in this study. Accumulation of TBARS, the decomposition product of polyunsaturated fatty acids of biomembranes, certain amino acids and DNA, in tissues of Pb-treated plants indicates that Pb is capable of inducing oxidative stress in the plant system and accelerates the senescence rate. This induction of oxidative stress by heavy metal might be due to blockage of electron flow in photo-system 2 (Kato and Simizu 1985). Lipid peroxidation can be enhanced by higher activity of iron-containing enzyme lipoxygenase (Thompson *et al.* 1987). Moreover, some routes of H_2O_2 generation other than through dismutation of superoxide might be accelerated by Pb^{2+} treatment (Choudhuri 1988).

Proline accumulates rapidly and more frequently than any other amino acid under unfavourable environmental conditions (Kishor *et al.* 1995, Lutts *et al.* 1999). It could be involved in stress-resistance mechanisms by acting as an osmoprotectant thereby facilitating osmoregulation, protection of enzymes, stabilization of protein-synthesis machinery, and regulation of cytosolic acidity as suggested in case of Cd (Alia and Saradhi 1991). However, we have observed considerable accumulation of proline only at 60 DAS and it content subsequently declined. It suggests that physiological dryness might be an initial consequence of lead stress, and later Pb either stimulated degradation of proline or inhibited its synthesis.

Ascorbate is known as a major primary antioxidant (Nijs and Kelley 1991), reacting directly with hydroxyl radical, superoxide and singlet oxygen (Buettner and Jurkiewiez 1996). It also acts as a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol (Padh 1990) and the proline residues (Liso *et al.* 1985) and has a role in cell elongation, cell vacuolization and cell wall development (Kerk and Feldman 1995). Generated oxidative stress appears to correlate with decline in the ratio of ascorbate to dehydroascorbate. In our study, lead stress caused a reduction in the overall ascorbate pool, involving a rapid increase in dehydro-ascorbate and a decrease in ascorbate contents thus indicating a change in redox balance in the antioxidant system in response to heavy metals as reported by Piqueras *et al.* (1999).

GSH helps in Asc regeneration (Noctor and Foyer 1998), regulates the protein thiol-disulfide exchange reactions (Rennenberg 1982), increases stress tolerance and seems to be an important signal molecule (May *et al.* 1998). Regeneration of GSH from GSSG is mediated by the activity of GR (Noctor and Foyer 1998). The increased GSH content in stressed plants in our study may reflect, at least partially, its increased demand as a substrate for enzymes participating in the detoxification of membrane-lipid peroxidation, such as glutathione-S-transferase (Maars 1996) and phospholipid hydroperoxide glutathione peroxidase (Gueta-Dahan 1997). Also, the increased glutathione redox state in stressed plants may serve as a signal affecting the expression of defense genes

(Foyer *et al.* 1997).

GR, a crucial enzymatic component of the cellular resistance mechanism usually operates in cycle with APX. The activity of GR suggests that the GSH/GSSG ratio is kept high under normal conditions. Under oxidative stress, an increased GR activity could be required to supply GSH to the ascorbate-glutathione (Asc-Glu) cycle. The observed increase in GR activities in the present study substantiates some earlier reports of GR upregulation during oxidative stress (Reddy *et al*. 2005).

In chloroplasts where catalase is absent, its role is played by an ascorbate-specific peroxidase which uses ascorbic acid as a hydrogen donor to break down hydrogen peroxide (Asada 1994). That we have noted an increase in APX activity in response to enhanced production of AOS, conforms to some earlier findings (Alscher *et al.* 1997, Mittova *et al.* 2002, Simonovičová *et al.* 2004) shown upregulation of APX.

Upregulation of SODs is essential for combating the oxidative stress and catalyzing the dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2. The role of SOD under oxidative stress is evident from studies on transgenic plants which have shown a 4 to 40-fold increase in various isoforms of SOD (Sen Gupta *et al.* 1993, McKersie *et al.* 1996). Under Pb stress, SOD acts as an enzymatic protector (antioxidant) against peroxidation (Ruley *et al*. 2004, Reddy *et al*. 2005). Pb treatments in our study increased the SOD activity in plants, the extent of increase being dose-dependent. The increase is attributed to *de novo* synthesis of enzymatic protein (Allen *et al.* 1997). SOD activity is of more relevance in metal stress studies for the maintenance of the overall defense system of plants subjected to oxidative damage (Slooten *et al.* 1995).

Upregulation of activities and levels of antioxidants in response to oxidative treatments is evident by a slow increase in the activities of SOD and APX and in the contents of GSH, which reached their maximum about 90 DAS on exposure to lead stress. Absence of rapid increase in the level of transcripts or active forms of antioxidant enzymes is related to the role of AOS in signal transduction, which would be most effective if the oxygen radical scavenging systems were not drastically increased as an immediate response to oxidative stress. Whether and how a slow increase in antioxidative response is related to the role of AOS in signal transduction in the stressed plants remains a question. However, increased contents of SOD, APX and GR prevent plant damage from $O_2^{\bullet -}$ and H_2O_2 to a great extent.

Stress conditions possibly cause a depletion of catalase activity by reducing the rate of protein turnover (Hertwig *et al.* 1992). The major function of CAT is to metabolise the peroxide liberated in the peroxisome following the conversion of glycollate during photorespiration. We observed a decrease in CAT activity with lead treatment except at lower concentration (100 μM Pb-Ac). Decline in CAT activity is regarded as a general response to many stresses supposedly due to the inhibition of enzyme synthesis or change in assembly of the enzyme sub-units (MacRae and Ferguson 1985). Its activity declined with progress of oxidative stress as observed under water-stress conditions (Zhang and Kirkham 1994). The decrease in CAT activity could indicate its inactivation by accumulation of H_2O_2 induced by lead.

An increase in sennoside (anthraquinone glycosides) content at a particular time is indicative of a more conversion of primary metabolites or immediate precursors to the synthesis of sennoside/secondary metabolites under abiotic stresses (Bilia *et al.* 1993, Qureshi *et al.* 2005). Plants growing on lead-contaminated soil experience oxidative stress (Ruley *et al*. 2004, Reddy *et al*. 2005) with excessive AOS generation. AOS could mainly be responsible for increase in sennoside content through triggering the conversion of sennoside precursors to sennosides. Reduction in sennoside content after a particular time might be due to a consistently declining activity of shikimate pathway which is the main route of anthraquinone biosynthesis in plants (Han *et al.* 2002), and/or due to interference of Pb^{2+} ions with enzymes associated with sennoside biosynthetic pathway. A time dependent accumulatiom of Pb in plant parts has earlier been reported with generation of oxidative stress (Reddy *et al*. 2005). In our studies, the decrease in most of the parameters at later stages (120 DAS) in *C. angustifolia* was due to senescence which was further accelerated by lead treatment.

In summary, our results suggests that oxidative stress induced by lead treatment was directly proportional to the concentration of Pb. The stress accelerated senescence processes as measured in terms of oxidative damage. It also evoked cellular antioxidative system, as observed in terms of glutathione content and upregulation of superoxide dismutase, ascorbate peroxidase and glutathione reductase, to protect the plant from oxidative damage caused by AOS. Catalase activity was, however, inhibited except at low Pb concentration. The oxidative stress also altered the levels and yield of secondary metabolite (sennosides) probably by converting the precursors of sennosides to sennosides or by interfering with its biosynthetic pathway.

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