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Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad)[1]

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Abstract

Pea (*Pisum sativum* L. cv. Azad) plants exposed to 4 and 40 μM of Cd for 7 d in hydroponic culture were analysed with reference to the distribution of metal, the accumulation of biomass and the metal's effects on antioxidants and antioxidative enzymes in roots and leaves. Cd-induced a decrease in plant biomass. The maximum accumulation of Cd occurred in roots followed by stems and leaves. An enhanced level of lipid peroxidation and an increased tissue concentration of hydrogen peroxide (H_2O_2) in both roots and leaves indicated that Cd caused oxidative stress in pea plants. Roots and leaves of pea plants responded differently to Cd with reference to the induction of enhanced activities of most of the enzymes monitored in the present study. These differential responses to Cd were further found to be associated with levels of Cd to which the plants were exposed. Cd-induced enhancement in superoxide dismutase (SOD) activity was more at 40 μM than at 4 μM in leaves. While catalase (CAT) prominently increased in leaves both at 4 and 40 μM Cd, ascorbate peroxidase (APX) showed maximum stimulation at 40 μM Cd in roots. Enhancement in glutathione reductase (GR) activity was also more at 40 μM than at 4 μM Cd in roots. While glutathione peroxidase (GPOX) activity decreased in roots and remained almost unmodified in leaves, glutathione *S*-transferase (GST) showed pronounced stimulation in both roots and leaves of pea plants exposed to 40 μM Cd. Increased activities of antioxidative enzymes in Cd-treated plants suggest that they have some additive function in the mechanism of metal tolerance in pea plants.

Key words: Cadmium, pea, oxidative stress, antioxidant, antioxidative enzymes.

Introduction

Cadmium (Cd^{2+}) is one of the most toxic pollutants found in air, water and soil and is non-essential for plants. Cd interacts with photosynthetic, respiratory and nitrogen metabolism in plants, resulting in poor growth and low biomass accumulation (Sanitá di Toppi and Gabbrielli, 1999). Of the various detoxification processes activated in the cells during exposure of plants to Cd, complexing of the metal by phytochelatins, compartmentalization in vacuoles, immobilization at the level of cell wall, exclusion through the action of plasma membrane, and synthesis of stress proteins play very significant roles (Sanitá di Toppi and Gabbrielli, 1999). Cd produces oxidative stress possibly by generating free radicals and active oxygen species (Hendry *et al*., 1992). These species react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, inactivation of enzymes, thus affecting cell viability. The antioxidative system of plants comprises several enzymes and low molecular weight quenchers that are principally constitutive and vary in plants at cellular and subcellular levels. Superoxide radicals generated in plant cells are converted to H_2O_2 by the action of SOD. The accumulation of H_2O_2, a strong oxidant, is prevented in the cell either by catalase or by the ascorbate–glutathione cycle where APX reduces it to H_2O. The enzymatic mechanism

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Abbreviations: APX, ascorbate peroxidase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GPOX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; GSTC, GST activity with CDNB as substrate; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid.

of detoxification involves dehydroascorbate reductase, glutathione reductase and other enzymes. Ascorbate and glutathione, other components of the antioxidative defence system, are found to increase under stress conditions. Glutathione is also a precursor of phytochelatins. The conjugation of glutathione to a variety of hydrophobic, electrophilic and cytotoxic substrates is accomplished by multifunctional enzymes glutathione transferases (GSTs). These enzymes play a regulatory role in heavy metal-induced oxidative stress. Some of the GSTs have been shown to exhibit activities of non-selenium glutathione peroxidases (GPOXs). The increased activity of GPOX in pea in the presence of copper has been presumed to be a metal detoxification response (Edwards, 1996).

Understanding the biochemical detoxification strategies that plants adopt against oxidative stress induced by accumulated metal ions is a key to manipulate heavy metal tolerance in plants. Cd produces oxidative stress in plants but variable responses with reference to an increase or a decrease in the level of antioxidant molecules and the activities of antioxidative enzymes have been reported (Shaw, 1995; Gallego *et al*., 1996; Chaoui *et al*., 1997). Most of the information on these responses pertains to leaves. The antioxidative responses of plants to heavy metals in both roots and leaves under identical conditions have not been examined critically. In the present communication the possible antioxidative mechanism that could be operational in the roots and leaves of pea plants exposed for 7 d to environmentally relevant (4 μM) as well as to marginally acute (40 μM) concentrations of Cd in hydroponic cultures is assessed.

Materials and methods

Culture conditions and Cd treatment

Pea (*Pisum sativum* L. cv. Azad) seeds procured from the local market were surface-sterilized with 0.1% $HgCl_2$ and soaked overnight in sterile water at room temperature. The seeds were germinated on perforated plastic trays (30 × 23 cm) floating on sterile water in a bigger non-perforated tray (40 × 28 cm). After 3 d of germination in the dark, the trays were transferred to light in thermostatically controlled culture room maintained at 25 ± 1 °C. The sterile water of the trough was replaced with Hoagland's solution and the seedlings were provided with photosynthetic photon flux density (PPFD) at 500 μmol m^{-2} s^{-1} by a combination of fluorescent tubes and tungsten lamps for 14 h daily. The nutrient solution was bubbled with sterile air and changed on alternate days. Subsequent to day 15, plants were subjected to three Cd treatments, (1) no Cd supply (control) except by way of water and essential nutrient salts, (2, 3), 4 and 40 μM Cd supplied as $Cd(NO_3)_2$. The nutrient solution was changed daily to avoid depletion of nutrient as well as Cd in the course of the plant's exposure to the metal. At appropriate time intervals, whole plant samples were removed and washed thoroughly with sterile distilled water to determine the fresh/dry weight and Cd accumulation.

Plant growth and total Cd content

Fresh weight of the roots and fully expanded apical leaves was taken immediately after harvesting. Dry weight of roots, stem and leaves was determined after placing samples in hot air oven at 55–60 °C till they dried to constant weight. The roots and leaves were digested in a HNO_3–$HClO_4$ (3 : 1, v : v) mixture and Cd concentration was determined by atomic absorption spectrophotometery (Perkin-Elmer, AAnalyst 300).

Assays of antioxidant enzyme activities in roots and leaves

Roots and fully expanded leaves were ground with a mortar and pestle under chilled condition in homogenization buffer specific for each enzyme. The homogenate was filtered through four layers of muslin cloth and centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was desalted with Sephadex G-25 column equilibrated with a buffer suitable for individual enzyme. Protein estimation was carried out using bovine serum albumin (BSA) as standard (Peterson, 1979).

For the estimation of CAT (EC 1.11.1.6), roots or leaves (0.5 g) were homogenized in 5 ml medium composed of 50 mM phosphate buffer, pH 7.0 and 1 mM dithiothreitol (DTT). CAT activity was assayed in 50 mM phosphate buffer, pH 7.0 by monitoring the production of dioxygen from H_2O_2 (33.5 mM), using a Clark-type oxygen electrode (Hansatech, UK) (del Río *et al*., 1977).

For the estimation of APX (EC 1.11.1.11) activity, 0.5 g plant samples were extracted in 2.5 ml homogenizing medium containing 100 mM phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.1 mM ascorbate and 2% (v/v) β-mercaptoethanol. For assay of the enzyme activity, the rate of hydrogen peroxide-dependent oxidation of ascorbic acid was determined in a reaction mixture that contained 50 mM phosphate buffer, pH 7.0, 0.6 mM ascorbic acid and enzyme extract (Chen and Asada, 1989). The reaction was initiated by addition of 10 μl of 10% (v/v) H_2O_2 and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm for 3 min.

SOD (EC 1.15.1.1) activity was assayed by using the photochemical NBT method. The assay was performed in terms of SOD's ability to inhibit reduction of nitroblue tetrazolium (NBT) to form formazan by superoxide by the method described previously (Beyer and Fridovich, 1987). The samples (0.5 g) were homogenized in 5 ml extraction buffer consisting of 50 mM phosphate, pH 7.8, 0.1% (w/v) BSA, 0.1% (w/v) ascorbate, 0.05% (w/v) β-mercaptoethanol. The assay mixture in 3 ml contained 50 mM phosphate buffer, pH 7.8, 9.9 mM L-methionine, 57 μM NBT, 0.025% (w/v) Triton X-100, and 0.0044% (w/v) riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD was defined as that being present in the volume of extract that caused inhibition of the photo-reduction of NBT by 50%.

GR (EC 1.6.4.2) was extracted from 0.5 g plant tissues in 5 ml of 100 mM phosphate buffer, pH 7.5 containing 0.5 mM EDTA. GR activity was monitored by following the increase in absorbance at 412 nm when 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was reduced by glutathione (GSH) to form TNB (Smith *et al*., 1988). The reaction mixture contained 100 mM phosphate buffer, pH 7.5, 0.5 mM EDTA, 0.75 mM DTNB, 0.1 mM NADPH, and 1 mM oxidized glutathione (GSSG). To express GR activity, the increase in absorbance was plotted against a known amount of glutathione reductase.

For the estimation of GST (EC 2.5.1.18) activity, 1 g plant samples were extracted in 5 ml medium containing 50 mM phosphate buffer, pH 7.5, 1 mM EDTA and 1 mM DTT.

The enzyme activity was assayed in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and eluate equivalent to 100 μg of protein. The reaction was initiated by the addition of 1 mM GSH and formation of *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm to calculate the GST specific activity (Li *et al*., 1995).

GPOX (EC 1.15.1.1) activity was determined from 1 g plant tissues extracted in 3 ml of 0.1 M TRIS-HCl, pH 7.5, containing 2 mM DTT and 1 mM EDTA. The enzyme activity was determined with cumene hydroperoxide as substrate using a glutathione reductase coupled assay to monitor the oxidation of GSH (Edwards, 1996). The reaction mixture constituted of 0.1 M phosphate buffer, pH 7.0, containing 0.2% (w/v) Triton X-100, 0.24 U GR, 1 mM GSH, 0.15 mM NADPH, and 1 mM cumene hydroperoxide. After addition of enzyme eluate, cuvettes were incubated at 30 °C for 10 min and NADPH was added to measure the basal rate of GSH oxidation by monitoring the absorbance at 340 nm for 3 min. The reaction was initiated by addition of cumene hydroperoxide and GPOX activity was expressed as change in absorbance at 340 nm mg^{-1} protein min^{-1}.

Quantitation of glutathione

The concentrations of reduced and oxidized glutathione were determined with an enzyme recycling assay (Griffith, 1980). The assay was based on sequential oxidation of glutathione by DTNB and reduction by NADPH in the presence of GR. Roots and leaves were extracted in 100 mM phosphate buffer, pH 7.5, containing 0.5 mM EDTA. To quantify the GSH plus GSSG and GSSG separately the extract was processed and subsequently assayed as per method given earlier (Fadzilla *et al*., 1997). The assay mixture in 1 ml contained 150 μl buffer containing 125 mM phosphate and 6.3 mM EDTA, pH 6.5, 700 μl of 0.3 mM NADPH, 100 μl of 3 mM DTNB, and 50 μl processed sample. The reaction was initiated by addition of 10 μl of GR (5 U ml^{-1}) and the change in absorbance at 412 nm was recorded. Standard curves were generated with reduced and oxidized glutathione.

Determination of H_2O_2 levels

Roots or leaves (0.3 g) were ground thoroughly in 1 ml of 200 mM perchloric acid. After centrifugation at 1200 *g* for 10 min, perchloric acid of the supernatant was neutralized with 4 M potassium hydroxide. The insoluble potassium perchlorate was removed by centrifugation at 500 *g* for 3 min. A 200 μl of supernatant was loaded on 1 ml column of Dowex 1X8-100 anion exchange resin and eluted with 800 μl of distilled water to collect 1 ml of eluate. The assay of H_2O_2 was made following Okuda *et al*. (Okuda *et al*., 1991). The assay mixture in a volume of 1.5 ml contained 1 ml extract, 400 μl 12.5 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer, pH 6.5, 80 μl 1.3 mM 3-methyl-2-benzothiazolinone hydrazone, and 20 μl (0.25 units) horseradish peroxidase. The reaction was initiated by the addition of the peroxidase and increase in absorbance at 590 nm was monitored for 3 min. For quantitation, a standard curve was generated by using graded amount of H_2O_2 in the reaction mixture.

Estimation of lipid peroxidation

The level of lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites chiefly malondialdehyde (MDA) as described previously (Heath and Packer, 1968). Plant tissues (0.2 g) were extracted in 2 ml of 0.25% TBA made in 10% TCA. Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10 000 *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation is expressed as nmol of MDA formed using an extinction coefficient of 155 mM cm^{-1}.

Statistical analysis

All values reported in this work are mean of at least three independent experiments. The mean values ± SD and exact number of experiments are given in figures and tables. The significance of differences between control and each treatment was analysed using Student's *t*-test.

Results

Pea plants grown in the presence of 4 and 40 μM Cd showed a time-dependent accumulation of this metal in their roots and shoots. Cd accumulation was more in plants exposed to 40 μM Cd than to 4 μM Cd. Cd accumulates immediately in roots, later in the stem and finally in the leaves. Maximum accumulation of Cd occurred in roots followed by stem and leaves (Table 1). The presence of 4 μM Cd in the culture medium did not cause any visible change in the growth of pea plants. An overall reduction of 21% in the dry weight of plants was observed after 7 d of growth at this concentration. Treatment with 40 μM Cd for 7 d significantly diminished the biomass accumulation as apparent from the 52% decrease in dry matter content (data not shown). While leaf area was slightly reduced in 40 μM Cd, the supply of Cd at either level did not induce any chlorosis or pigmentation of leaves.

The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. The level of MDA (one of the major TBA reactive metabolites) increased in Cd-treated pea plants. The increase in MDA content was time-dependent and significant at 40 μM Cd, and was more in roots than in leaves (Fig. 1).

SOD activity, responsible for the elimination of superoxide radicals in cells, significantly increased in the beginning in leaves (Fig. 2). The initial increase in SOD activity, however, declined by days 5 and 7 with 4 and 40 μM Cd treatment, respectively, but remained higher than the control. SOD activity in roots of Cd-treated plants was significantly lower than the control with an isolated but significant increase on day 7 of the treatment.

H_2O_2, a product of superoxide dismutase reaction, showed a significant increase that was more pronounced in leaves than in roots of the Cd-treated pea plants (Fig. 3). The concentration of H_2O_2 in roots and leaves of the control declined progressively with the growth of plants. On the other hand, the level of H_2O_2 in roots of

Table 1. *Accumulation of Cd in roots, stems and leaves of pea plants exposed for 7 d to 4 μM and 40 μM of $Cd(NO_3)_2$*

Data represent the means ± SD of three separate experiments.

Treatment	Plant organ	Cd content (μg g^{-1} DW) Duration of treatment (d)			
		1	3	5	7
Control	Roots	<0.25	<0.25	12±2.0	14±3.5
	Stem	<0.25	<0.25	2±1.2	6±2.8
	Leaves	<0.25	<0.25	2±1.2	3±1.6
4 μM Cd	Roots	88±5.6***	162±7.2***	275±8.2***	306±9.8***
	Stem	5±1.0*	31±3.3***	56±4.6***	93±4.9***
	Leaves	4±1.3	8±3.0*	16±1.7***	20±3.8**
40 μM Cd	Roots	516±12.5***	524±12.5***	582±7.2***	640±9.1***
	Stem	90±4.0***	126±5.3***	160±6.6***	212±4.6***
	Leaves	16±1.2***	25±2.9***	50±3.9***	89±4.4***

*$P<0.05$, **$P<0.01$, ***$P<0.001$.

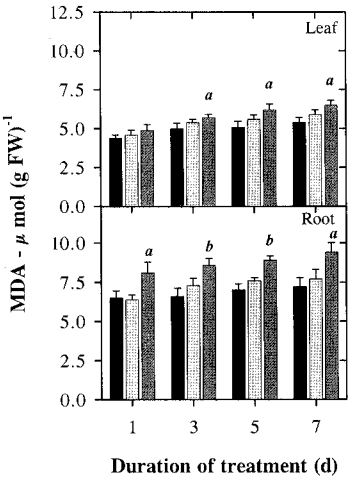

Fig. 1. Levels of lipid peroxidation expressed in terms of MDA concentration in roots and leaves of Cd-treated pea plants. (■) Control, (▯) 4 μM Cd, (▯) 40 μM Cd. Data represent the means ± SD of three separate experiments, $^aP<0.05$, $^bP<0.01$, $^cP<0.001$.

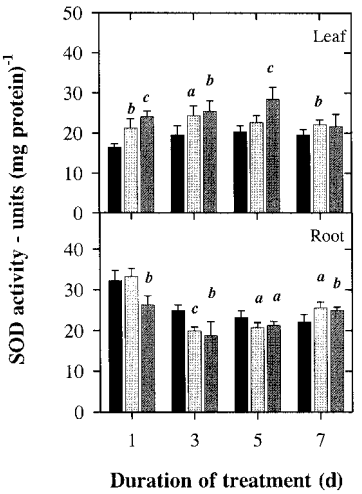

Fig. 2. SOD activity in roots and leaves of control and Cd-treated pea plants. (■) Control, (▯) 4 μM Cd, (▯) 40 μM Cd. Enzyme unit for SOD is defined as the amount of enzyme which causes a 50% decrease of the SOD-inhibitable NBT reduction. Data represent the means ± SD of five separate experiments, $^aP<0.05$, $^bP<0.01$, $^cP<0.001$.

Cd-treated plants gradually increased from 4% (at 4 μM) and 15% (at 40 μM) after 1 d to 16% (at 4 μM) and 27% (at 40 μM) over a period of 7 d. A somewhat similar trend also occurred in leaves with a significantly higher increase that ranged from 9–26% at 4 μM Cd and 25–33% at 40 μM Cd.

Excessive levels of H_2O_2 could be minimized through the activities of CAT and APX. These two enzymes increased in roots and leaves of Cd-treated pea plants. A higher induction of these enzymes occurred at a Cd concentration of 4 μM as compared to that at 40 μM. The augmentation in CAT, was lower in roots, which decreased to levels below the control with the age of plants (Fig. 4). Cd-induced increase in the activities of CAT was significantly higher in leaves (116% and 108% of the control on completion of 1 d at 4 and 40 μM, respectively). The activity gradually decreased with the duration of treatment, but remained higher than the control. APX activity in roots of plants exposed to 4 and 40 μM Cd progressively increased to 200% and 112% of the control, respectively, in the course of 7 d of treatment (Fig. 5). At these concentrations, a somewhat lower but progressive increase in APX activity occurred in leaves.

GSH, that maintains the cellular redox status and also serves as substrate for phytochelatin synthesis, showed a concentration and time-dependent decrease in its level in Cd-exposed plants (Fig. 6). As compared to the control, the decrease was more pronounced in roots (17–22% at 4 μM Cd and 29–33% at 40 μM Cd) than the leaves (7–11% at 4 μM Cd and 16–29% at 40 μM Cd). As a whole, no significant changes with respect to the control were noticed in the level of GSSG in both leaves and roots of Cd-treated pea plants (Fig. 6).

GR, that catalyses the NADPH-dependent reduction of oxidized glutathione, showed a significant increase in its activity in roots both at 4 and 40 μM exposure of

Cd (Fig. 7). The increase with reference to the control was higher in roots than in leaves. GR activity in roots of plants exposed for 1 d to 4 and 40 μM Cd increased, respectively, by 76% and 172% with respect to the control. The extent of the increase declined progressively since GR activity increased in the control with the growth of the plants. GR activity in leaves showed a significant increase at 4 μM Cd. A 50% increase in GR activity over the control was observed in leaves of plants treated with 40 μM Cd for 1 d. Thereafter GR activity dropped significantly on subsequent days to a level that was lower than that of the control.

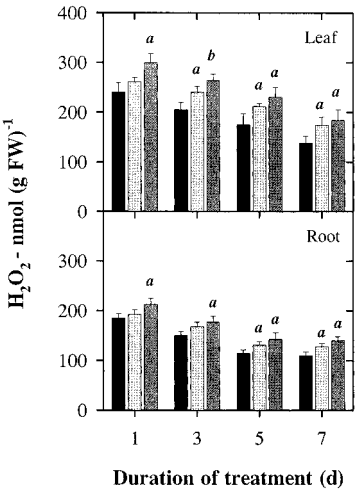

Fig. 3. Levels of H_2O_2 in roots and leaves of control and Cd-treated pea plants. (■) Control, (▒) 4 μM Cd, (▒) 40 μM Cd. Data represent the means ± SD of three separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

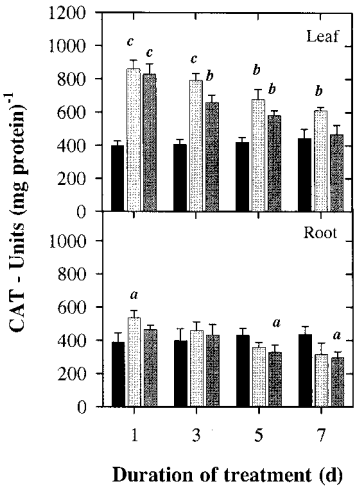

Fig. 4. CAT activity in roots and leaves of control and Cd-treated pea plants. (■) Control, (▒) 4 μM Cd, (▒) 40 μM Cd. One unit of enzyme activity is equal to 1 μmol of H_2O_2 decomposed min^{-1}. Data represent the means ± SD of three separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

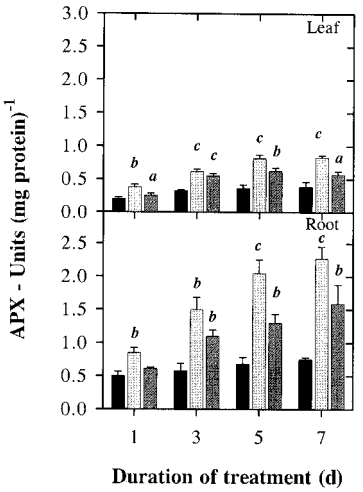

Fig. 5. APX activity in roots and leaves of control and Cd-treated pea plants. (■) Control, (▒) 4 μM Cd, (▒) 40 μM Cd. One unit of enzyme activity defined as 1 μmol of ascorbate oxidized min^{-1}. Data represent the means ± SD of three separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

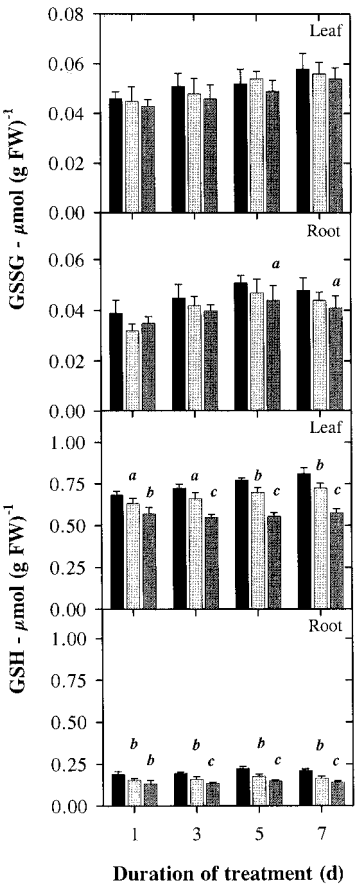

Fig. 6. Amount of GSH and GSSG in roots and leaves of control and Cd-treated pea plants. (■) Control, (▒) 4 μM Cd, (▒) 40 μM Cd. Data represent the means ± SD of five separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

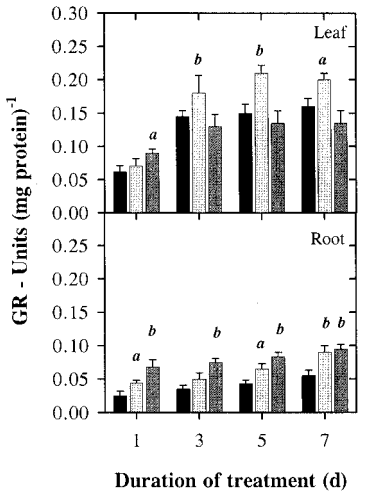

Fig. 7. GR activity in roots and leaves of control and Cd-treated pea plants. (■) Control, (▯) 4 μM Cd, (▩) 40 μM Cd. Data represent the means ± SD of three separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

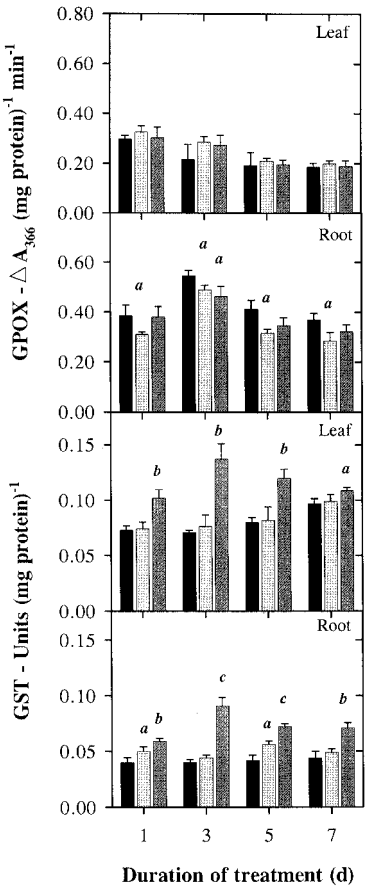

Fig. 8. GST and GPOX activities in roots and leaves of control and Cd-treated pea plants. (■) Control, (▯) 4 μM Cd, (▩) 40 μM Cd. GST activity expressed as 1 μmol of DNP-GS formed min^{-1}. GPOX activity is expressed as change in absorbance at 340 nm mg^{-1} protein min^{-1}. Data represent the means ± SD of three separate experiments, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

GST and GPOX are known to be responsive to biotic and abiotic stresses. These enzymes have not been characterized with respect to their antioxidative roles in plants. Cd treatment caused a significant increase in GST activity in both roots and leaves (Fig. 8). The highest increase in GST activity was observed at 3 d in pea plants treated with 40 μM Cd (128% and 93% over the control in roots and leaves, respectively). Over the 7 d treatment, this increase in enzyme activity declined by 76% in roots and 81% in leaves, but always remained higher than the control. Cd concentration of 4 μM was not able to induce an appreciable increase in GST activity in both roots and leaves. Unlike GST, treatment with Cd brought a reduction in GPOX activity in roots and an insignificant enhancement in leaves (Fig. 8).

Discussion

The adverse affect of Cd on growth is apparent from the reduction in biomass in Cd-treated pea plants (data not shown). An enhanced level of lipid peroxidation and increasing tissue concentration of H_2O_2 in both roots and leaves of pea plants exposed to Cd in the present study indicate that the metal caused oxidative damage to plants. Lipid peroxidation in leaves may be a consequence of generation of ROS is evident from the increased SOD activity in leaves of the Cd-treated pea plants. Both redox active (Cu and Fe) and non-redox active metal ions (Zn and Cd) are reported to increase lipid peroxidation via ROS generation in plants (Shaw, 1995; Gallego *et al*., 1996; Chaoui *et al*., 1997). In sunflower leaves exposed to Cd it was shown that Cd-induced oxidative stress is mediated by ROS since low levels of lipid peroxidation, prevention of decrease in GSH content and increase in lipooxygenase activity were observed in the presence of free radical scavengers (Gallego *et al*., 1996).

The results presented suggest that the Cd-induced increase in the levels of antioxidative enzymes may represent a secondary defensive mechanism against oxidative stress that are not as direct as the primary defensive responses such as phytochelatins and vacuolar compartmentalization (Sanitá de Toppi and Gabbrielli, 1999). Acute concentrations of Cd may adversely affect the activity of certain defence enzymes either by inhibiting their synthesis or by their inactivation and down regulation. At chronic concentrations of Cd, antioxidative mechanisms seem to operate in additive way to cope effectively with metal stress.

Low accumulation of Cd in leaves may be a strategy to protect photosynthetic function from Cd-induced oxidative stress. The significant increase in the levels of SOD, CAT, APX, and GR (Figs 2, 4, 5, 7) even before adequate accumulation of Cd took place on day 1 and 3 in leaves

(Table 1) may be indicative of a rapid signalling response. The molecules involved and the mechanism of this long-range signal transduction from roots to leaves are not precisely known but it may involve stress ethylene (Mehlhorn, 1990). Recent studies also envisage H_2O_2 and glutathione as central components of signal transduction in both environmental and abiotic stresses in plants (Foyer *et al*., 1997).

The repression as well as induction of enzyme activity as a function of Cd concentration applied was evident in both roots and leaves. APX at 4 μM Cd while GR and GST at 40 μM Cd exhibited maximum induction in roots (Table 2; Figs 5, 7, 8). Despite the transient increase, SOD, CAT and GPOX activity was usually found inhibited both at 4 and 40 μM Cd in roots (Figs 2, 4, 8). In the leaves, all the enzymes excepting GR were activated to varied levels at both 4 and 40 μM Cd (Table 2; Figs 2, 4, 5, 8). GR activity mostly remained lower than the control at 40 μM Cd in leaves (Fig. 7). Induction of enzymes as a function of duration of Cd treatment did not show a definite trend. In roots, the induction response was transient for SOD and CAT (Figs 2, 4), and with the exception of GPOX that displayed lower activity than the control throughout, APX, GR and GST activities remained higher than the control throughout the course of Cd treatment (Figs 5, 7, 8). By contrast, in leaves, activities of most of the enzymes showed a time-dependent increase or the initial increase was followed by a decline but the decreased activity remained higher than the control at the end of 7 d Cd treatment (Figs 2, 4, 5, 7, 8). These results show differential responses of anti-oxidative enzymes to Cd in roots and leaves and suggest that, depending on the enzyme examined, Cd can become inhibitory above a given concentration and/or after a given period of exposure.

SOD activity in Cd-exposed pea plants increased significantly in leaves while it mostly remained lower than the control in roots. Previous reports showed a varying response of an increase or decrease in SOD activity in plants exposed to different metals including Cd (Chongpraditnum *et al*., 1992; Somashekaraiah *et al*., 1992; Luna *et al*., 1994; Gallego *et al*., 1996; Okamoto *et al*., 1996; Schickler and Caspi, 1999). A reduction in SOD activity in the metal-treated plants has been attributed to an inactivation of the enzyme by H_2O_2 that is produced in different cellular compartments where SOD catalyses the disproportionation of superoxide radicals. H_2O_2 can also be produced by a number of non-enzymatic and enzymatic processes in cells. While mitochondria and chloroplasts are the major sources of H_2O_2 in the cells, peroxisomes and glyoxysomes also contain SOD as well as APX which are responsible, respectively, for H_2O_2 production and its scavenging (Yamaguchi *et al*., 1995; Jiménez *et al*., 1997). In the present study, the increase in level of H_2O_2 was concentration dependent and was more in leaves than in roots of the Cd-treated plants. APX and CAT, two potent scavengers of H_2O_2, maintain its level because an uncontrolled export of this toxic species from organelles to cytosol may have an adverse effect due to the formation of hydroxyl radicals by the reaction of H_2O_2 with superoxide radicals through metal catalysed Haber–Weiss reaction. Increased activities of APX in both roots and leaves and of CAT only in the roots show that they were functioning concurrently to remove H_2O_2. The inadequate response of CAT activity to Cd in roots was compensated by the increased activity of APX in this part of plant.

GR, another enzyme of the ascorbate–glutathione cycle was activated relatively more in roots than in leaves of the Cd-treated pea plants. This enzyme has been shown to be activated under different types of stress (Foyer *et al*., 1994). Metal ions such as Zn and Ni stimulate, while Cu and Fe cause a decrease in GR activity in leaves (Gallego *et al*. 1996, Chaoui *et al*., 1997, Schickler and Caspi, 1999). Cd induces an increase in GR activity in the genus *Alyssum* (Schickler and Caspi, 1999) and decreases the activity of this enzyme in *Helianthus annuus* (Gallego *et al*., 1996). A crucial role for GR in combating oxidative stress in leaves has been suggested previously (Foyer *et al*., 1991). Transgenic plants overexpressing the gene for glutathione reductase showed greater resistance to oxidative stress (Creissen *et al*., 1994). The enzyme reduces GSSG to GSH that is an essential anti-oxidant and also a substrate for phytochelatins. Various levels of metal-induced depletion of GSH have been reported in different plant species (Rauser *et al*., 1991; De Vos *et al*., 1992; Gallego *et al*., 1996). Cd-induced depletion of glutathione has been ascribed to phytochelatin synthesis (Grill *et al*., 1985). In the present study, leaves and roots of 40 μM Cd-treated pea plants showed a maximum decrease of 29% and 34%, respectively, in GSH content. The amount of GSSG as a whole showed insignificant decrease both in roots and leaves. Rauser *et al*., however,

Table 2. *Differential increase in enzyme activity (% over the control) in roots and leaves of 15-d-old pea plants exposed to 4 μM and 40 μM Cd*

Data represent the minimum and maximum values over a period of 7 d. Each value is an average of three independent experiments excepting for SOD where it is an average of five separate experiments.

Enzymes	Roots		Leaves	
	4 μM	40 μM	4 μM	40 μM
SOD	–[a]	–	11–28	13–72
CAT	–	–	39–116	5–108
APX	62–203	17–112	72–140	28–72
GR	51–76	73–172	18–40	10–50
GST	10–33	12–128	–	12–93
GPOX	(–)[b]	(–)	7–32	2–26

[a]– Marginal and sporadic increase.
[b](–) Overall decreased activity.

found a greater net decline of GSH pools in roots than in shoots in maize seedlings treated with Cd (Rauser *et al*., 1991). DeVos *et al*. reported more than a 50% decrease in GSH level in roots of Cd-treated *Silene cucubalis* (DeVos *et al*., 1992) while Gallego *et al*. found only a 20% reduction in leaves of Cd-exposed sunflower (Gallego *et al*., 1996). The stimulated GR activity in Cd-treated pea plants in the present study presumably did not allow an abrupt fall in the GSH level because of its concomitant reduction. The decline in the level of GSH in leaves of pea plants treated with 40 μM Cd may be attributed to low GR activity or to the transport of GSH from leaves to roots in order to compensate GSH requirement for the synthesis of phytochelatins in the roots (Rennenberg, 1982). Another reason for an overall reduction in the endogenous level of GSH might be due to its utilization as a reducing substrate in the synthesis of ascorbate. GSH is also consumed and degraded in order to protect cellular membranes from lipid peroxidation. The level of GSH is reduced during chilling-induced process of photoxidation (Wise and Naylor, 1987) that also involves lipid peroxidation. The interaction of GSH with peroxyl radicals as demonstrated previously (Barclay, 1988) during peroxidation of liposomes initiated in the aqueous phase, may also be a cause of its depletion. Finally, the depletion of GSH may also be due to its conjugation with xenobiotic substrates as evident in the present study from the increased GST activity towards CDNB (GSTC) in roots and leaves of the Cd-treated pea plants. However, the identity and nature of the *in vivo* substrates of GSTs are, by and large, unknown (Edwards, 1996). GSTs also exhibit alternative activities as GSH peroxidase (Bartling *et al*., 1993). GSTs and GPOXs have been well characterized in pea (Edwards, 1996). GPOXs catalyse the reduction of H_2O_2, organic hydroperoxides and lipid hydroperoxides by GSH. In the present study, GPOX activity while diminished in roots, showed an insignificant increase in leaves of the Cd-treated pea plants. On the other hand 40 μM Cd induced a significant enhancement of GSTC activity that amounted to 93% and 128% of the control in leaves and roots, respectively. These results with Cd were in contrast to those observed with Cu in pea (Edwards, 1996) in which GSTC activity remained almost unaffected but there was concentration- and time-dependent multifold increase in the GPOX activity in roots that declined after 2 d of the Cu treatment. A lower GPOX activity was found in leaves of Cu-treated pea plants. Excess concentrations of both Cu and Cd are known to cause cellular oxidative damage and lipid peroxidation. GST binds to Zn, Cd and Cu metal ions (Tommey *et al*., 1991). Whether the Cd-induced increase in GSTC activity in the present study or the multifold increase in the GPOX activity in Cu-treated roots (Edwards, 1996) are a detoxification response is the subject of further study.

Activation of the ascorbate–glutathione cycle has been found to be essential in stressed plants to combat oxidative damage (Alscher *et al*., 1997). Although ascorbate, an oxidant and a major metabolite in plants, and enzymes involved in its metabolism were not monitored in the present study, the increase in APX and GR activities in Cd-exposed pea plants maintains ascorbate and glutathione turnover and activation of the H_2O_2 scavenging ascorbate–glutathione cycle. The concurrent induction of CAT further contributed in overcoming oxidative stress by detoxifying H_2O_2. At higher concentration of Cd, the substantial stimulation of GST activity might have played some role in the metal detoxification process in pea.

The differential responses of antioxidative enzymes to Cd in roots and leaves may be attributed to varied level of ROS generation in two functionally distinct organs of the plant. Metal ions may stimulate the generation of ROS, either by direct transfer of electrons in single-electron reactions involving metal cations, or as a consequence to metal-inactivated metabolic reactions (Dietz *et al*., 1999). Chloroplast, mitochondria, peroxisomes, and plasma membrane-linked electron transport all contribute towards generation of ROS in leaves. Being a non-photosynthetic tissue the flux of ROS is presumably low in roots. Despite the higher accumulation in roots, the level of free Cd ions in roots may remain low since most of the Cd ions are either immobilized or compartmentalized in vacuoles or form Cd-phytochelatin complexes. The induction of APX, GR and GST provide additional defence against metal toxicity and keeps the metabolic activities in roots functional. Cd accumulation in leaves is relatively low due to transport barriers and, moreover, the oxidative damage imposed by Cd is avoided with an altogether increase in the activities of antioxidative enzymes.

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