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Molecular Performance and Antioxidative Manners of Garden Cress under Heavy Metal Toxicity

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lead is a potential pollutant that readily accumulates in soils and sediments. Although lead is not an essential element for plants, it gets easily absorbed and accumulated in different plant parts. Oxidative stress indices such as thiobarbituric acid reactive species (TBARS), as well as, superoxide anion radical production rate were strongly and rapidly induced under higher lead concentrations (400 and 600 ppm), whereas induction was delayed under lower concentrations (100 and 200 ppm). A great variation in isoforms of different antioxidant enzymes, e.g. superoxide dismutase (SOD; EC1.15.1.1), catalase (CAT; EC1.11.1.6.) and ascorbate peroxidase (APx; EC1.11.1.11) were detected in response to lead treatments. Lead toxicity increased lipid peroxidation and reactive oxygen species (ROS) generation especially at higher concentrations, but the increase was significantly lower at lower concentrations. The results obtained indicate that the pattern of antioxidant isozymes are affected upon exposure of *L. sativum* seedlings to lead and the variation are concentration dependent. Within such response patterns, gene expression is a valuable stress marker in ecophysiological studies.

Key words: Pollution, Garden cress, Molecular performance, Antioxidant, Oxidative stress.

Soils contaminated with lead cause sharp decreases in crop productivity thereby posing a serious problem for agriculture. Among the metals, lead has become a particularly important cosmopolitan environmental pollutant (Sharma & Dubey, 2005). It is commonly used in gasoline to improve the efficiency of fuel, but when released through vehicle exhaust pipes it substantially pollutes the environment particularly in urban areas. Lead causes two types of unfavorable processes in plants. First, lead inactivates several enzymes by binding with their SH-groups (Rauser, 1995). Secondly, lead ions, similarly as those of other heavy metals, can intensify the processes of reactive oxygen species (ROS) production leading to oxidative stress (Prasad *et al*., 1999; Cuypers *et al*., 1999). Most recently, it has been reported that heavy metals cause a series of three waves of reactive oxygen species (ROS) generation, first with the NADPH oxidase-dependent accumulation of hydrogen peroxide, followed by the accumulation of superoxide anions in mitochondria, and finally, fatty acid hydroperoxide, as detected in tobacco cells (Garnier *et al*., 2006). An enhanced level of lipid peroxidation and hydrogen peroxide concentration in both roots and shoots are the major indicators of heavy metal induced oxidative stress in plants (Dixit *et al*., 2001). These processes, which destructively affect cell structure

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and metabolism, are mutually connected and stimulate each other, which may result in a decreased efficiency of oxidation-reduction enzymes or the electron transport system leading to fast production of ROS in the cell (Stroinski & Kozlowska, 1997). Plants are equipped with antioxidative defense system that includes enzymatic and non-enzymatic components. The synchronous action of enzymatic antioxidant components such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) are able to scavenge ROS and also control their generation. Superoxide anion radicals produced in different compartments of plant cells are rapidly converted into H_2O_2 in a reaction catalyzed by SOD (Noctor & Foyer, 1998; Gupta *et al*., 1999). SODs represent a group of multimeric metalloenzymes catalyzing the disproportionation of superoxide free radicals, generated by univalent reduction of molecular oxygen to H_2O_2 and O_2 in different cellular compartments (Fridovich, 1989). In eukaryotic organisms SOD occurs in three isoforms: mitochondrial Mn-SOD, cytosolic Cu,Zn-SOD and extracellular EC-SOD (Cu,Zn-SOD) (Scandalios, 1993). Genes encoding different SOD isoforms respond in a varied way to metabolic and environmental signals (Alscher, 1997). Genetic studies with SOD loci have been performed in several plant species soybean (Griffin &Palmer, 1989), maize (Baum & Scandalios, 1982). Isozymes of SOD have been reported to show dimeric (Bowler *et a*l., 1994), tetrameric (Baum and Scandalios, 1982) or monomeric (Rajora *et al*., 1991) in response to abiotic stresses. Catalases are involved in scavenging H_2O_2 generated during the photo-respiration and ²-oxidation of fatty acids (Morita *et al* ., 1994) . Peroxidases are heme containing proteins that utilize H_2O_2 in the oxidation of various organic and inorganic substrates (Asada, 1994). The protective function of CAT is limited due to its localization mainly in peroxisomes (Foyer *et al*., 1994). Antioxidative enzymes occur in cells in many isoforms, often exhibiting different properties (Noctor &Foyer, 1998). Regulation of the level of antioxidative enzymes gives plants an additional protective ability against oxidative stress (Foyer *et al*., 1994). The molecular mechanisms of Pb^{2+} toxicity are scarcely reported and poorly understood. Hence an attempt has been made to evaluate the influence of Pb^{2+} on the superoxide anion radical production rate, lipid peroxidation and gene expression of the antioxidant enzymes isoforms, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) for Pb^{2+} detoxification in garden cress (*Lepidium sativum* L.) leaves.

MATERIALS AND METHODS

Plant materials and treatments

Seeds of *L. sativum* L. collected from different localities of Saudi Arabia were screened for germination response to Pb^{2+} (data not shown). *L. sativum* obtained from Abha was the most lead tolerant species. Seeds were surface sterilized by immersing in 0.1 % $HgCl_2$ for two min and washed with five changes of sterile distilled water and soaked in continuously aerated distilled water for 24 h in darkness. Thirty seeds were sown in each pot (15 cm diameter x 20 cm height), filled with pre acid washed sand. All pots were placed in a growth chamber under 70-80% RH with 16/8h light/ darkness cycle and controlled temperature of 28/ 25° C. Light intensity was 500 ¼mol m^{-2} s^{-1} at the top of plants supplied by a mixture of fluorescent and incandescent lamps. Each pot was irrigated with 250 mL distilled water at first, then occasionally with a certain amount of distilled water in order to keep the soil water content constant. Pots were irrigated with half strength Hoagland solution every two days to reach 80% of water holding capacity throughout the experimental period.

Experimental design

Twenty five-days-old *L. sativum* seedlings of a uniform size were carefully taken from the pots to avoid any injury to the roots and placed in sponge support collars. Collars were then fitted into holes in the tops of glass bottles containing 500 mL continuous aerated Hoagland solution supplemented with various concentrations of Pb^{2+} for 10 days. Individual Pb^{2+} treatments were a control, with Hoagland nutrient solution (0 ppm Pb^{+2}), and four Pb^{2+} concentrations of 100, 200, 400 and 600 ppm using lead acetate. These concentrations were chosen on the basis of preliminary experiments, the lowest one being below the toxicity threshold and the highest one above. The pH of the nutrient solution was buffered to pH 5.0 and kept constant during the

experiment. All solutions were changed every 3 days during 10 days of experiment to maintain the metal concentrations. All bottles were placed in a growth chamber under the same conditions.

Estimation of lipid peroxides

The thiobarbituric acid reactive species (TBARS) level of lipid peroxidation products was estimated following the method of Dhindsa and Matowe, (1981). Fresh leaf samples (200 mg) were ground in 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath and centrifuged at 10000×g for 10 min. The absorbance of the supernatant was read at 532 nm and correction for unspecific turbidity was done by subtracting the absorbance of the same at 600 nm. A total of 0.25% TBA in 10% TCA served as blank. The concentration of lipid peroxides together with the oxidative modified proteins of plants were quantified and expressed as total TBARS in terms of nmol g^{-1} fresh weight using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of superoxide anion radical (O_{2}^{-}) production rate

As it was described in earlier reports (Navari-Izzo *et al*., 1999), leaves were placed in a test tube and poured over with a solution containing 0.05 M potassium phosphate buffer (pH 7.8), 0.05% nitro blue tetrazolium (NBT), and 10 mM NaN_3. After 5 min incubation in the dark, 2 ml of the solution was taken up from the tubes and heated at 85°C for 15 min. The samples were cooled and absorbance was measured at 580 nm.

The amount of accumulated lead was determined according to Wolf's (1982) method. Fresh samples were surface sterilized with 1M HCl and then with 1 mM Na_2EDTA to resolve excess surface bound Pb^{2+} and then dried in oven at 70 °C for 2-3 days. Dried samples were ground to a fine powder in a mortar and pestle and digested with conc. H_2SO_4. Digested samples were dissolved in deionized distilled water and the contents of Pb^{2+} was measured by atomic absorption spectrophotometer (Perkin Elmer 2380 Atomic absorption spectrometer) using an air-acetylene flame, in terms of µg g^{-1} dry wt. of sample.

Extraction of antioxidant enzymes

Leaves were extracted 15 min at 4°C in 5 ml of cold extraction buffer (100 mM potassium phosphate) pH 7.8, 300 mg polyvinylpyrrolidone, 1% (v/v) Triton X-100 (Schwanz *et al*., 1996). The extract was centrifuged (30 min 20,000 g, 4°C and equilibrated with 100 m M potassium phosphate, pH 7.8. For stabilization of ascorbate peroxidase (APx) the elution buffer contained 5 mM ascorbate. The supernatant was used for the enzyme assays.

Enzymes assay

Superoxide dismutase (SOD) was assayed according to Stewart and Bewely (1980). One unit of SOD activity was the amount of enzyme activity that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme. *Ascorbate peroxidase (APX)* activity was assayed according to Asada (1992). The reaction was initiated by the addition of H_2O_2. One unit of APX was the amount of enzyme that oxidized 1 µmol of ascorbate minute at room temperature. *Catalase (CAT)* activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm (Asada, 1992). One unit of enzyme activity is equal to 1 mmol of H_2O_2 decomposed per min.

Isoenzyme profile of some antioxidant enzymes

Antioxidant enzymes were extracted from leaves and polyacrylamide gel electrophoresis was performed in vertical slab gel following the method of Davis at 4°C (Davis, 1964). Tris-glycine (pH 8.3) was used as electrode buffer and 7.5% running and 3.5% stacking gels were used. Enzyme samples corresponding to 25 mg protein mixed with glycerol were layered on top of the stacking gel and electrophoretic run was completed using a current of 20 mA per slab. For detection of isoforms, gels were soaked in the appropriate staining solution for each antioxidant enzyme according to Eduardo Vallejos (1983).

RESULTS

The intensity of Pb^{2+} accumulation increased mainly in the root during the subsequent 10 days treatment with different concentrations of Pb^{2+} as compared to the control seedlings. With higher concentration of Pb^{2+}, (400 and 600 ppm), a considerable amount of Pb^{2+} was observed accumulated in the leaves after 10 days of treatment (Table 1).

After 10 days of treatment of In *L. sativum* seedlings with different concentrations of Pb^{2+},

the level of lipid peroxides, measured in terms of TBARS increased with increase in the concentration of Pb^{2+} in the growth medium (Table 1). A 400 and 600 ppm Pb^{2+} level led to about 160-220 % increase in TBARS level in leaves after 10 days treatment relative to the control (Table 2). The superoxide anion radical production rate was slightly increased upon reducing the concentration of metal, representing only 1.1 fold increase at 100 ppm Pb-treated seedlings, while in subsequent higher concentration its amount was nearly twice as high as in the last two Pb^{2+} concentrations, representing 1.86 and 2.96 fold increase at 400 and 600 ppm Pb^{2+} treatment compared to the control (Fig. 1).

Superoxide dismutase activity was significantly increased under the effect of lower concentrations of Pb^{2+}, whereas a highly significant decrease was shown in response to the higher concentrations especially at 400 and 600 ppm, with 35 and 65% decrease, respectively (Fig. 2). Catalase activity was enhanced significantly upon exposure to all Pb^{2+} concentrations except the highest concentration (600 ppm). The CAT activity of *L. sativum* roots after exposure to 400 ppm Pb^{2+} for 10 days was 189% more than the control. On the other hand the corresponding value for 600 ppm treatment was 45% less than the control. Ascorbate peroxidase activity was enhanced significantly under all treatments (Fig. 2).

Among the genes involved in antioxidative response, the SOD transcript remained unchanged within lower concentrations treatment up to 200 ppm Pb^{2+} in comparison to control, while the expression of SOD was strongly down-regulated by Pb^{2+} and its level also decreased significantly in response to the higher Pb^{2+} concentrations. CAT expression was raised significantly in response to lower concentrations of lead compared to the control, and their expression was observed in most treatments. The highest lead concentration (600 ppm) resulted in a highly significant decrease in CAT expression and no detectable isoform bands appeared. Similar results were observed for the APX expression; lower and moderate Pb^{2+} concentrations (up to 200 ppm) resulted in a significant increment in APX expression. On the other hand a great depression in its expression was observed with the highest Pb concentrations.

The data in Table 1 showed that all plants are significantly affected by Pb toxicity and they enhanced lipid peroxidation in a dose-dependent manner. Thus, the results of TBARS obtained have shown that Garden cress treated with higher concentration of Pb (400 and 600 ppm) has significantly higher lipid peroxidation (TBRAS) in comparison with other lower concentrations. However, at higher concentrations all plants showed almost the same ability for lipid peroxidation.

DISCUSSION

The translocation of metals from the roots into the shoot is a controversial issue. As roots remain completely immersed and fully exposed to higher metal concentrations in the growth medium, majority of the metals become sequestered in the roots (Bibi *et al*., 2005). In our experiment the uptake of Pb^{2+} by *L. sativum* roots and their translocation into the shoot when exposed to Pb^{2+} at the concentrations of 100-600 ppm seemed to be strongly correlated with the external metal

Table 1. Effect of increasing concentration of Pb2+ in the growth medium on lead accumulation in shoot and root and total lipid peroxide of Lepidium sativum roots after 10 days treatment. Each value is the mean of three replicates (SD). Values carrying asterisk are significantly different at P 0.05

Treatments Pb^{2+} (ppm)	Pb^{2+} (µg g^{-1} D.W)		Total lipid peroxide TBARS (nmol g^{-1} F.W.)
	Shoot	Root	
C	0.00	0.00	80.24± 4.23
100	0.08±0.010	0.28±0.019	91.56± 4.83
200	0.24±0.03m	0.90±0.17m	107.87±5.23m
400	0.38±0.08m	1.94±0. 13m	128.76 ± 6.23m
600	0.85±0.10m	3.18±0.91m	176.43± 7.23m

concentrations (Table 2). It was previously reported that Pb^{2+} become more sequestered in the roots than in the leaves (Burzynski & Grabowski, 1984; Yruela, 2005). A similar situation exists in our finding, whereby there was 90 and 318 % higher Pb^{2+} accumulation roots treated with 100 and 600 ppm Pb^{2+}, while there was only 8 and 85 % Pb^{2+} accumulation in leaves after the same concentrations.

Both elevated lipid peroxidation and superoxide anion radical production rate are characteristic features of oxidative stress caused

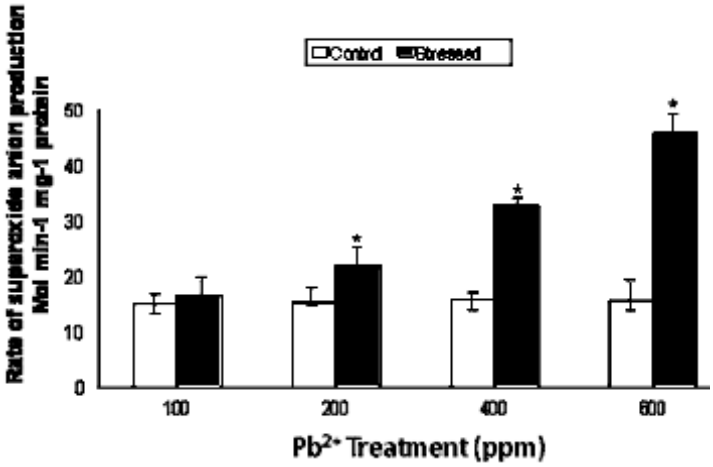

Fig.1. Changes in the rate of superoxide production rate in roots of untreated and plants of *Lepidium sativum* subjected to various concentrations of Pb^{2+} for 10 days. The control for these measurements are non-treated roots. Each value represents the mean ±SE of five replicates

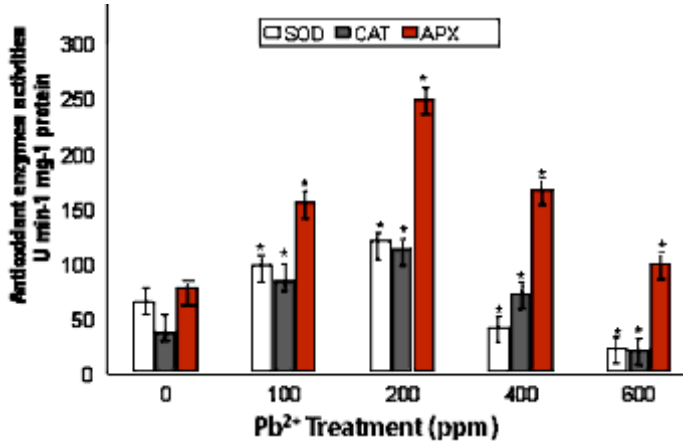

Fig. 2. Effect of increasing concentration of Pb^{2+} in the growth medium on superoxide dismutase (SOD); catalase (CAT) and ascorbic peroxidase (APX) activities in roots of *Lepidium sativum* after 10 days treatment. Values are mean±/S.D. Based on three replicates and bars carrying asterisk are significantly different at P £ 0.05

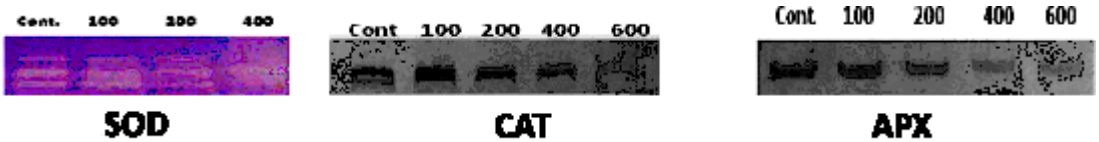

Fig. 3. Isoenzyme profile of superoxide dismutase (SOD), A; catalase(CAT), B; ascorbic peroxidase (APX), C in enzyme preparations from leaves of 25 days grown seedlings of *Lepidum sativum*. Seedlings were raised for 10 days under different concentrations of Pb^{2+} in the growth medium. (C) Control; (100), 100 ppm Pb^{2+}; (200), 200 ppm Pb^{2+}; (400), 400 ppm Pb^{2+} ; (600), 600 ppm Pb^{2+}

by Pb^{2+} toxicity. It has been suggested that a slight increase in lipid peroxide and anion radical pools are characteristic of moderate levels of Cd^{2+} stress, while its significant increment shows severe stress (Lima *et al*., 2006). *Lepidium sativum* responded to Pb^{2+} treatment with elevated level of both lipid peroxide as well as production of superoxide anion radical and their levels were correlated with Pb^{2+} concentrations, suggesting that more important mechanisms are involved in Pb^{2+} detoxification (Metwally *et al*., 2005). An almost twofold higher lipid content was detected in leaves treated at 600 ppm Pb^{2+} after 10 days treatment, compared with control. The corresponding value for anion radical production was 2.96 fold higher, compared to the control (Table 1).

The unexpected increase in SOD activity under lower concentrations of Pb2+ which scavenge superoxide radicals and protect the biomolecules from such radical was accompanied by steady production of anion radical. The increase in superoxide dismutase, CAT and APX enhanced particularly the capacity for oxygen radical scavenging and maintenance of the integrity of cellular membranes and all sub-cellular structures. The present results on changes in SOD activity are supported by Chen et al. (2002). There was a loss of enzymes isoform with the expression of low band intensity in Pb^{2+} stressed roots. Such loss of SOD isozyme could be due to oxidative inactivation during lead stress. Pb^{2+} concentration treatment resulted in the appearance of isoforms with high band intensity even with high concentration, which may be related to the relative enhancement of enzymes transcription which confirm their important role in stress tolerance.

Expression of genes involved in antioxidative response suggested the appearance of oxidative stress in Pb-treated leaves. An alteration of gene expression induced by heavy metals in plants has been reported by Finkemeier *et al*. (2003), Metwally *et al*. (2003) and Sharma *et al*. (2004). Our results demonstrate that all the analyzed genes for antioxidant enzymes involved in scavenging ROS are up-regulated in *Lepidium sativum* leaves but differentially based on the metal concentrations. Each was up-regulated in the presence of 100 and 200 ppm Pb^{2+} representing the lower and moderate stress, while in the presence of higher concentrations, elevated expression was detected only at 400 ppm for CAT and APX (Fig. 1). This is most likely associated with the developmentally increased expression of these genes in *L. sativum* leaves, which was observed by analyzing their expression in leaves of control (untreated plants). Alternatively, the significant depression of gene expression for SOD started at 200 ppm Pb^{2+} and expression was not detected at 600 ppm (Fig. 1). Finkemeier *et al*.(2003), and Sharma *et al*. 2005 suggested that CAT, APX and GPX were up-regulated, but dehydro-ascorbate reductase DHAR expression did not show any significant change after Cd treatment. However, they analyzed whole roots of 10-day-old seedlings and found that GR was up-regulated only in the presence of Pb^{2+} and Cd^{2+} with simultaneous nitrogen deficiency.

Deleterious effects caused by ROS resulting from oxidative stress can be prevented by the defense mechanism in plants, i.e. the antioxidative system, which is composed not only of the low-molecular components but also of enzymatic components such as SOD, CAT, and APX. Our results demonstrate that prolonged stress induced by Pb^{2+} concentrations, can result into the activation of antioxidative enzymes and also enhance the gene expression of these antioxidant enzymes. Subsequently, the up-regulation of gene expression may cause the appearance and maintenance of antioxidative enzyme isoforms.

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