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Alteration of gene expression, superoxide anion radical and lipid peroxidation induced by lead toxicity in leaves of *Lepidium sativum*

Mohamed M. Ibrahim[1*] and Sameera O. Bafeel[2]

[1]Science Department (Biology section), Teacher's College, King Saud University, Riyadh, Saudi Arabia; [2]Biology Department, College of Science, King Abdul Aziz University, Jeddah, Saudi Arabia
sbafil@kau.edu.sa and mibrahim@ksu.edu.sa

Corresponding author e-mail: m_ibramim2004@yahoo.com

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Heavy metals, *Lepidium sativum*, gene expression, antioxidant isozymes, ecophysiology

1 SUMMARY

A rapid change in gene expression in plants in response to stress is important for environmental adaptation. An mRNA differential display technique was used in this study to analyze alterations in gene expression in *Lepidium sativum* in response to different concentrations of lead. The relationship of differential expression patterns to the identification of cDNAs induced during lead toxicity was determined. Polymorphism was detected under lead toxicity and the differentially expressed genes in stressed seedlings were strongly and rapidly induced under higher lead concentrations (400 and 600 ppm), whereas induction was delayed and transcripts accumulated to a lower level 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 variations are concentration dependent. Within such response patterns, gene expression is a valuable stress marker in ecophysiological studies.

2 INTRODUCTION

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 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 systems that include 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, e.g. soybean (Griffin & Palmer, 1989), maize (Baum & Scandalios, 1982). Isozymes of SOD have been reported to show dimeric (Bowler *et a*l., 1994), tetrameric (Baum & Scandalios, 1982) or monomeric (Rajora *et al*., 1991) structures in response to abiotic stress.

Catalases are involved in scavenging H_2O_2 generated during the photorespiration and β-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), and catalase (CAT) for Pb^{2+} detoxification in garden cress (*Lepidium sativum* L.) leaves.

3 MATERIALS AND METHODS

3.1 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 (15cm diameter x 20cm 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 µmol 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.

3.2 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 continuously 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.

3.3 Estimation of lipid peroxides: The 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 10 000×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 oxidatively 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}.

3.4 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% nitroblue 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.

3.5 Determination of lead content: The amount of accumulated lead was determined according to Wolf (1982). 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.

3.6 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).

3.7 Semi-quantitative RT-PCR: Total RNA was isolated from leaf segments from appropriately treated seedlings using RNeasy Plant Kit (Qiagen) and cDNA was synthesized from DNase-treated total RNA with Omniscript RT Kit (Qiagen) using oligo-dT-primer. Primers were prepared according to sequences published by Metwally *et al.* (2003) for CAT and APX; by (Rajora *et al*., 1991) for SOD. Cycle numbers were optimized to assure that the amplification reaction was tested in the exponential phase and the gene expression product was also detectable in control leaves. The PCR products were applied to 5% PAGE and silver stained.

4 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

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of Pb^{2+} was observed accumulated in the leaves after 10 days of treatment (Table 1).

In *L. sativum* seedlings, after 10 days treatment 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 concentrations 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 withinlower concentrations treatment up to 200 ppm Pb^{2+} in comparison to the 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 (when 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.

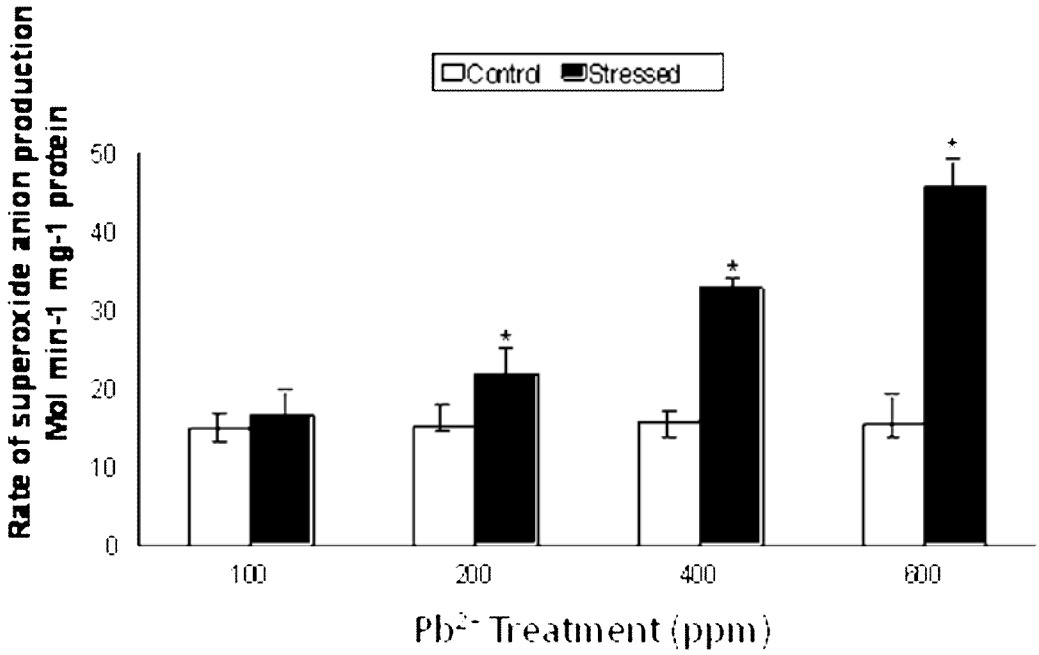

Figure 1: Changes in the rate of superoxide production rate in roots of untreated and plants of *Lepidium*

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.

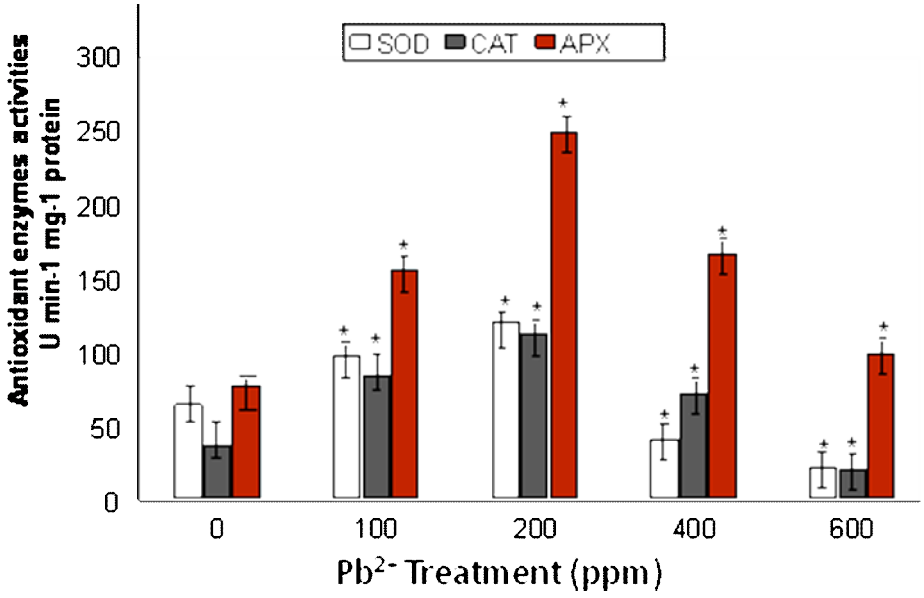

Figure 2: Effect of increasing concentration of Pb^{2+} in the growth medium on superoxide dismutase (SOD); catalase (CAT) and ascorbic peroxidase (APX) activities in roots of *Lepidium sativum* after 10 days treatment. Values are mean± S.D. Based on three replicates and bars carrying asterisk are significantly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

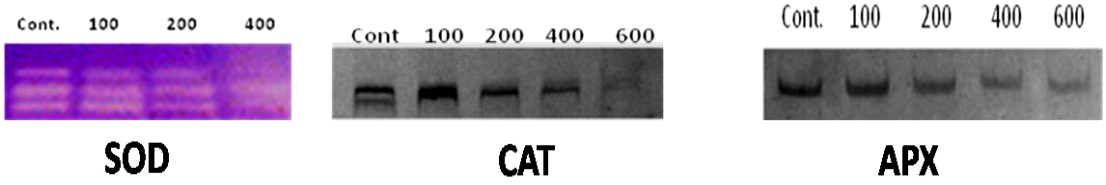

Figure 3: Isoenzyme profile of superoxide dismutase (SOD), A; catalase(CAT), B; ascorbic peroxidase (APX), C in enzyme preparations from leaves of 25 days grown seedlings of *Lepidum sativum*. Seedlings were raised for 10 days under different concentrations of Pb^{2+} in the growth medium. (C) Control;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

Table 1: Effect of increasing concentration of Pb^{2+} in the growth medium on lead accumulation in shoot and root and total lipid peroxide of *Lepidium sativum* roots after 10 days treatment. * = sign. At $P<0.05$.

Treatments Pb^{2+} (ppm)	Pb^{2+} ($\mu g\ g^{-1}$ D.W.). Shoot	Root	Total lipid peroxide TBARS (nmol g-1 F.W.)
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±0.03*	0.90±0.17*	107.87±5.23*
400	0.38±0.08*	1.94±0. 13*	128.76 ± 6.23*
600	0.85±0.10*	3.18±0.91*	176.43± 7.23*

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5 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 at the concentrations of 100-600 ppm seemed to be strongly correlated with the external metal concentrations (Table 2). It was previously reported that Pb^{2+} becomes 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 in roots treated with 100 and 600 ppm Pb^{2+}, while there was only 8 and 85 % Pb^{2+} accumulation in leaves after treatment with the same concentrations.

Both elevated lipid peroxidation and superoxide anion radical production rate are characteristic features of oxidative stress caused 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 after 10 days treatment, compared to the 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 Pb2+ stressed roots. Such loss of SOD isozyme could be due to oxidative inactivation during lead stress. Pb2+ 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 *L. 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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