INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
09–378/MSA/2010/12–2–205–210
http://www.fspublishers.org

Full Length Article

Lead Toxicity Induced Growth and Antioxidant Responses in *Luffa cylindrica* Seedlings

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ABSTRACT

In vitro culture experiment was conducted to investigate the growth and antioxidant changes in activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and phenylalanine ammonium-lyase (PAL) in *Luffa cylindrica* seedlings exposed to lead toxicity. The fresh weights in the cotyledons, hypocotyls and radicles decreased gradually with increasing lead concentrations between 200 and 800 μM. SOD activity in the cotyledons, hypocotyls and radicles increased significantly with increasing lead concentrations up to 400, 200 and 800 μM, respectively. POD activity in the cotyledons, hypocotyls and radicles reached peaks at lead concentrations of 400, 200 and 400 μm, respectively. CAT activity in the cotyledons, hypocotyls and radicles showed the highest increments at lead concentrations of 400, 800 and 800 μM, respectively. PAL activity in the cotyledons, hypocotyls and radicles increased significantly with the rising lead concentrations up to 800, 800 and 200 μM. Electrophoresis anaylsis showed that different patterns of POD isoenzymes depend on lead concentrations and tissue types, and the staining intensities of the isoenzymes are well consistent with the changes of the activity assayed in solutions. Our results showed that increased SOD, POD, CAT and PAL activity may be associated with the tolerance capacity of *L. cylindrical* to protect the plant from oxidative damage. © 2010 Friends Science Publishers

Key Words: Lead; *Luffa cylindrica*; ROS-scavenging enzymes; Isoenzyme pattern

INTRODUCTION

Heavy metal pollution in the air, water and agricultural soils is of major ecological concern due to its impact on human health through the food chain and its high persistence in the environment (Valko *et al.*, 2005). Lead (Pb) is one of the most abundant, ubiquitous toxic elements posing a critical concern to human and environmental health. It can cause multiple direct and indirect effects on plant growth and metabolism, along with visible symptoms including stunted growth and small leaves, as well as leading to membrane disorganization and reduced photosynthesis (Sharma & Dubey, 2005; Ahmad *et al.*, 2008). In addition, it is generally accepted that toxic levels of heavy metal can affect a variety of physiological processes in plants. One of the major consequences is the production of large quantities of reactive oxygen species (ROS), which can cause damage to proteins, lipids and DNA (Schützendübel & Polle, 2002). Therefore, ROS production and removal must be efficiently controlled. To minimize the damaging effects of ROS, the plant cells possess evolved non-enzymatic and enzymatic antioxidative defense mechanism. The latter mainly include superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Miller *et al.*, 2008). Early studies showed that lead can inhibited seedlings growth and decreased the biomass, as well as induced the changes of SOD, POD and CAT activity in some plant species (Verma & Dubey, 2003; Xiong *et al.*, 2006; Qureshi *et al.*, 2007; Gao *et al.*, 2009). These results were important to understand the possible relationship between the effects of lead toxicity and the changes of some enzyme activities involved in defense mechanism of plants.

Luffa cylindrica is an annual vegetable upland crop cultivated throughout the world and is distributed mainly in tropical to warm-temperate areas. Its fruit is used in the traditional Chinese medicine as an anthelmintic, stomachic and antipyretic phytomedicinal drug. It is used as a vegetable either prepared like squash or eaten raw like cucumbers (Yang *et al.*, 1999; Oboh & Aluyor, 2009). However little is known about the effects of heavy metals on growth and antioxidant responses in *L. cylindrica* plant. Hence the present study was aimed to understand the effects of lead toxicity on the growth and changes of SOD, POD, CAT and PAL activity of *in vitro* cultured *L. cylindrica* seedlings in order to better understand the defensive

To cite this paper: Jiang, N., X. Luo, J. Zeng, Z. Yang, L. Zheng and S. Wang, 2010. Lead toxicity induced growth and antioxidant responses in *Luffa cylindrica* seedlings. *Int. J. Agric. Biol.*, 12: 205–210

mechanisms of *L. cylindrica* under heavy metals stress.

MATERIALS AND METHODS

Luffa cylindrica seeds were obtained from a traditional Chinese medicine market, Chengdu, China. Seeds were selected and stored at 4°C in a plastic box with labeled (No. 20080910) until processing. The seeds were surface-sterilized by 70% (v/v) ethanol for 30 sec and 0.1% (m/v) mercuric hloride ($HgC1_2$) for 8 min, followed by four times with sterile distilled water. The seeds were soaked in sterile distilled water for 24 h at room temperature and embryos were surgically isolated from the seeds in a clean bench. The embryos were rinsed three times with sterilized, distilled water and until processing. Murashige and Skoog (MS) media (20 mL) containing 6 g/L agar powder and 30 g/L sucrose in 100 mL wide-neck bottles were separated into five lots. One lot was allowed to culture with MS medium as controls. Other four lots were supplemented with lead added as $Pb(NO_3)_2$ in concentration of 100, 200, 400 and 800 μM, respectively. The pH of the media was adjusted to 5.7±0.1 with 0.1 M HCl or 0.1 M NaOH and then the media were autoclaved for 20 min at 120±2°C. The embryos (3 embryos per bottle) were placed in the MS media for germination and growth at 25/20°C under a 16/8 h (light/dark) photoperiod with light provided by cool-fluorescent lighting in a growth chamber. Germination was recorded every second day. After growth for 7 days (the cotyledons emergence), the cotyledons, hypocotyls and radicles of seedling were separately harvested and weighted. They were either used immediately for analysis or stored in an -80°C freezer. Three sets of seedlings were analyzed for each lead concentration, with 15 embryos per set.

All procedures were carried out at 4°C. Approximately 0.2 g of plant material was homogenized in 2 mL ice-cold extraction buffer containing 50 mM Tris–HCl (pH 7.0), 0.5 mM EDTA and 1% w/w polyvinylpyrrolidone. The homogenate was centrifuged at 15294 × *g* for 10 min and the supernatant was used as the crude extract for the assay of enzyme activity. Protein content was quantified by the Lowry's method using bovine serum albumin as standard (Lowry *et al.*, 1951).

Superoxide dismutase (SOD, EC 1.15.1.1) activity, were assayed by measurement of its capacity of inhibiting the photochemical reduction of nitro-blue tetrazolium (NBT) in a 3 mL reaction mixture (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 10 mM methionine, 1.17 mM riboflavin, 56 mM NBT and 100 μL enzyme extract. The absorbance of solution was measured at 560 nm using TU-1901 UV-Vis spectrophotometer (Purkinje General Instrument Co. Ltd., Beijin, China). One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium to blue formazan by 50%.

Peroxidase (POD, E.C. 1.11.1.7) activity was measured in a 4 mL reaction mixture containing 3.7 mL 3% guaiacol (water solution), 0.1 mL 2% H_2O_2 and 0.2 mL diluted extract. The dilution ratio of the extract depended on the protein concentration of the extract. POD activity was measured following the change of absorbance at 470 nm due to guaiacol oxidation using TU-1901 UV-Vis spectrophotometer (Purkinje General Instrument Co. Ltd., Beijin, China). One unit of POD activity was defined as the change in absorbance per minute and per gram fresh weight (FW) of the tissue (Abeles & Biles, 1991).

Catalase (CAT, EC 1.11.1.6) activity was assayed by the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 1 min according to the Aebi method with some modifications (Aebi, 1984). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 μL protein extract in 3 mL reaction mixture. One unit of CAT activity was expressed as enzyme units per gram fresh weight (U/g FW).

POD isoenzymes were separated on the discontinuous polyacrylamide gels (stacking gel 4% & separating gel 10%) under the non-denaturing conditions. A vertical electrophoresis apparatus (Mini-Protein II system, Bio-Rad, USA) was used. Proteins were electrophoresed at 4°C and 80 V in the stacking gel followed by 120 V in the separating gel. Electrophoretic pattern of POD isoenzymes was obtained by staining the gels using benzidine (Ros Barcelo, 1987). The gels were rinsed in water and immersed in 0.03% H_2O_2, 0.2% (w/v) benzidine and 0.1% (v/v) acetic acid for 3-5 min at room temperature till the brown color.

Fresh tissue (0.2 g) were homogenized with chilled Tris-HCl (50 mM, pH 8.0), supplemented with 0.5 mM EDTA and 1% polyvinyl pyrrolidone. Other steps were the same as previously. PAL activity was measured by monitoring the reaction product trans-cinnamate at 290 nm (Hahlbrock & Ragg, 1975). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 20 mM l-Phe and 100 μL enzyme extract. PAL activity was calculated as the changes in optical density during 30 min and one enzyme unit was defined as the amount causing an increase of 0.01 in A290 per minute using TU-1901 UV-Vis spectrophotometer (Purkinje General Instrument Co. Ltd., Beijin, China).

Each result shown in this paper was the mean of at least three replicated treatments. Significance of differences between the treatments was statistically evaluated by standard deviation and Student's *t*-test methods. Data were tested at significant levels of $P < 0.05$ using one way analysis of variance (Kleinbaum *et al.*, 1998).

RESULTS

No significant changes in the fresh weights of seedlings and visible toxicity symptoms were observed at 100 μM lead concentrations treatment compared to the controls. However a significant growth reduction was observed at the higher lead concentrations and the fresh weight in the cotyledons, hypocotyls and radicles were reduced by 44.8%, 26.5% and 31.7% at the highest lead

Fig. 1: Effects of lead on the fresh weight of cotyledons, hypocotyls and radicles in *L. cylindrica* seedlings, Values are the means ± SD (*n*=3)

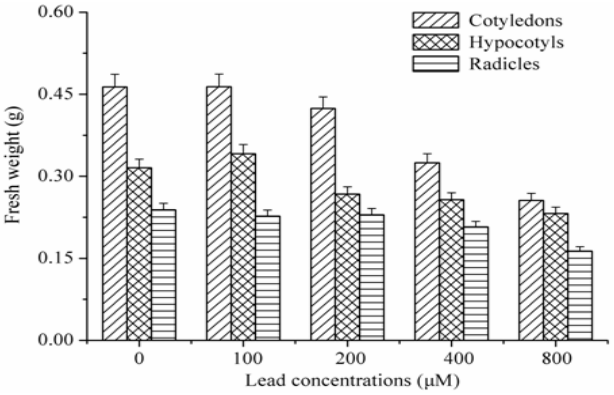
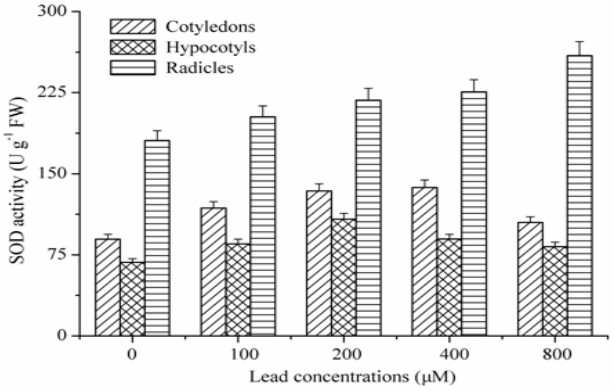


Fig. 2: Effects of lead on SOD activity in the cotyledons, hypocotyls and radicles of *L. cylindrica* seedlings, Values are the means ± SD (*n*=3)



concentration (800 μm) compared to the controls, respectively (Fig. 1).

Total SOD activity involved in scavenging ROS increased significantly in *L. cylindrica* seedlings under lead stress. In the cotyledons, the activity increased sharply, peaked at 400 μm lead concentration treatment and then decreased. Similarly, the activity in the hypocotyls and radicles was also significantly induced at all lead levels treatment, with the peaks at 200 and 800 μm, which increased 58.9% and 43.4% compared to the controls, respectively (Fig. 2).

The application of different Pb concentrations had significant effects on POD activity in *L. cylindrica* seedlings. In the cotyledons, the activity increased by 18.3%, 86.7%, 92.1% and 4.2% at lead concentrations of 100, 200, 400 and 800 μm compared to the control, respectively. In the hypocotyls and radicles, the peak activity increased by 65.3% and 25.2% at lead concentrations of 200 and 400 μm compared to the controls, respectively (Fig. 3). Results presented in Fig.4 showed significant variations in isoenzyme pattern of the cotyledons, hypocotyls and radicles under different lead treatments. There were four bands in the cotyledons and hypocotyls, five in the radicles under lead stress, respectively. The staining densities of isoenzymes (II, III & IV) in the cotyledons and hypocotyls, were affected by all lead treatments, while that of isoenzyme I showed no significant change compared to the control. In the radicles, the staining densities of isoenzymes (I, II & V) were significantly affected by different lead concentrations treatment, and those of isoenzymes (III & IV) appeared to decline in response to lead stress (Fig. 4).

CAT activity in the cotyledons, hypocotyls and radicles was significantly affected by different lead supply levels. In the cotyledons, the activity increased gradually with the increasing lead concentration up to 400 μm at which, the peak (132.2% increment) in the enzyme activity was observed. Similarly the peak in the hypocotyls and radicles occurred at the highest lead concentration (800 μm) and the activity showed increases of 65.2% and 146.9% compared to the controls, respectively (Fig. 5).

PAL activity in the cotyledons and hypocotyls showed highest value at the highest lead concentrations (800 μm) and the activity increased by 199.2% and 67.3% compared to the controls, respectively. In the radicles, the activity increased gradually with the increasing lead concentrations up to 200 μm and representing 73% increase compared to the control. However a further lead application led to decrease the activity (Fig. 6).

DISCUSSION

Lead is one of heavy metal widely used in modern industry that has been recognized as highly toxic and carcinogenic. It can affect growth and metabolism of plant to varying degrees depending on the concentration and tissue types of plant species (Sharma & Dubey, 2005). Previous studies have suggested that lead exerts its adverse actions by promoting or exacerbating oxidative damage to the cells and it has been shown that antioxidant defense enzymes play a key role in the protection against heavy metal toxicity (Thomas *et al.*, 2004; Reddy *et al*., 2005; Wang *et al.,* 2008). These findings are important to understand the behavior of those enzymes in the presence of highly toxic metals.

Growth inhibition is a common response to heavy metal stress and is also one of the most important agricultural indices of heavy metal tolerance. The effects of lead on seedlings growth seems to be different with regards to plant species, cultivars, organs and the metabolic processes (Sharma & Dubey, 2005). In the present study, application of higher lead concentrations treatments (above 200 μm) caused the decrease of the fresh weight in the cotyledons, hypocotyls and radicles. Growth inhibition had been well documented in *Elsholtzia argyi* (Islam *et al.,* 2002), *Brassica pekinensis* (Xiong *et al.*, 2006) and *Jatropha curcas* (Gao *et al.*, 2009) plants under lead stress conditions. With the support of these results, our finding showed that increased lead concentration can inhibit the

Fig. 3: Effects of lead on POD activity in the cotyledons, hypocotyls and radicles of *L. cylindrica* seedlings, Values are the means ± SD (*n*=3)

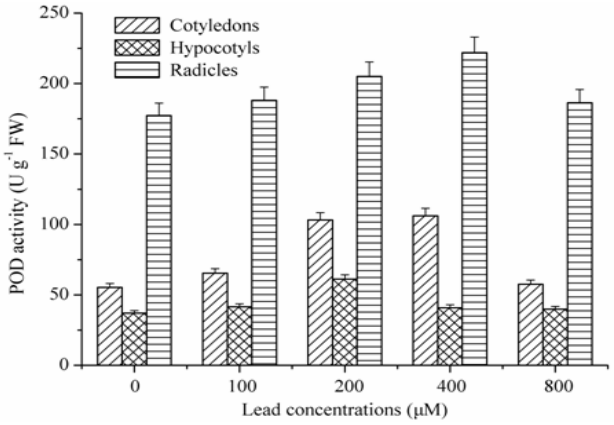
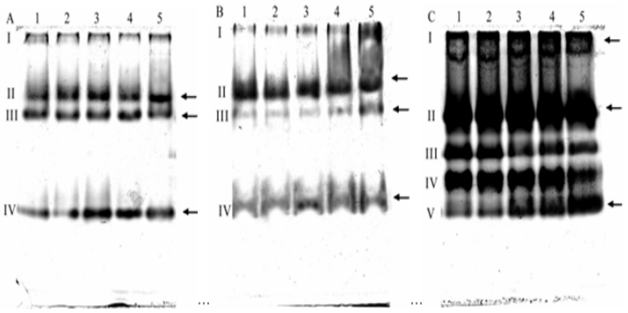


Fig. 4: Effects of different lead concentrations on POD activity in *L. cylindrica* seedlings revealed by native gel (10%) electrophoresis, A: patterns of POD isoenzymes in the cotyledons, B: patterns of POD isoenzymes in the hypocotyls, C: patterns of POD isoenzymes in the radicles, Lanes from 1 to 5 were 0, 100, 200, 400 and 800 μm, respectively, About 20 μL of each sample was loaded



normal growth and development of *L. cylindrica* seedling.

Up-regulation of SODs is essential for combating the oxidative stress and catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide. In plants environmental adversities often leads to the increased generation of ROS and consequently, production of SOD has been proposed to be important mechanism in plant stress tolerance (Alscher *et al.*, 2002). Our results showed SOD activity increased significantly in *L. cylindrica* seedlings under different lead concentration (Fig. 2). This increase had been reported in other plant species under lead stress, such as *Sesbania drummondii* (Thomas *et al.*, 2004), *Cassia angustifolia* (Qureshi *et al.*, 2007) and *J. curcas* (Gao *et al.*, 2009). Thus increased SOD activity is response to lead stresses appears to be due to the need for combating oxidative stress. SOD activity is of more relevance in heavy metal stress studies for the maintenance of the overall

Fig. 5: Effects of lead on CAT activity in the cotyledons, hypocotyls and radicles of *L. cylindrica* seedlings, Values are the means ± SD (*n*=3)

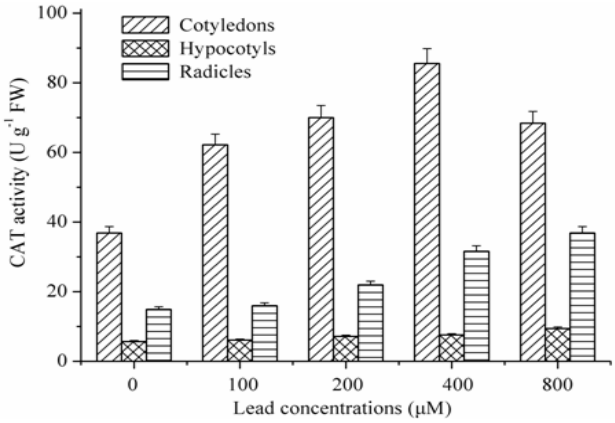
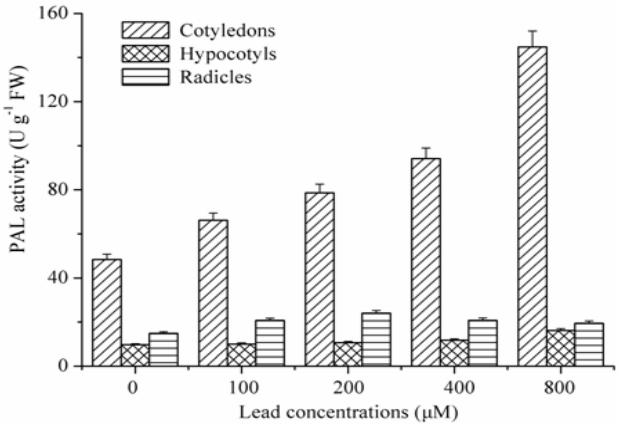


Fig. 6: Effects of lead on PAL activity in the cotyledons, hypocotyls and radicles of *L. cylindrica* seedlings, Values are the means ± SD (*n*=3)



defense system of a plant subjected to oxidative damage (Alscher *et al.*, 2002). Based on the above results, SOD is believed to be one of the most important factors of the plant biochemical defense against lead toxicity, which is actively involved in the self-regulation of plant metabolism.

For plant scientists, POD has often served as a parameter of metabolic activity during growth alterations. POD, SOD and CAT are redox metalloenzymes involved in cell defense against oxidative stress (Passardi *et al.*, 2005). Our results showed that an increase in the total POD activity are observed in *L. cylindrica* seedlings exposed to different lead concentrations. Similar results had been reported in rice (Verma & Dubey, 2003), *S. drummondii* (Thomas *et al.*, 2004) and *Vicia faba* (Wang *et al.*, 2008) exposed to lead toxicity. These findings showed that plants are able to overcome metal stress using an effective antioxidant defense mechanism in order to maintain the balance between ROS generation and their elimination. Thus increased POD activity might be associated with elevated ROS levels in *L. cylindrical* seedlings under lead stress. Plant POD is

encoded by multigenic families and is involved in several important physiological and development processes, resistance against biotic and abiotic stresses. Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants. The expression pattern of isoenzymes varies in the different tissues of healthy plants and is developmentally regulated and influenced by developmental and physiological conditions (Passardi *et al*., 2005). Our results showed the presence of four, four and five POD isoenzymes in the cotyledons, hypocotyls and radicles and they were differentially affected by lead stress (Fig. 4). In addition, the changes in the staining densities of these isoenzymes were consistent with the changes of POD activity as assayed in extract solutions (Fig. 3 & 4). There have been numerous reports with respect to the presence of several isoenzymes in *Lemna minor* (Garnczarska & Rataczak, 2000), *V. faba* (Wang *et al.*, 2008) and *J. curcas* (Gao *et al.*, 2009) plants under lead stress. These findings are consistent with its multiple physiological functions suggesting that different POD isoenzymes might be involved in distinct processes. Expression of POD isoenzymes is complicated since they are affected by different kinds of abiotic and biotic stress conditions (Passardi *et al*., 2005). Our results showed that the changes in the staining densities of POD isoenzymes are related to plant tissues and lead concentrations in *L. cylindrica* seedlings.

CAT can promote the dismutation of H_2O_2 into H_2O and O_2 and is a major ROS-scavenging enzyme in all aerobic organisms. CAT activity in plant cells has been correlated with development of increased tolerance to a variety of chemical compounds and physical stresses (Miller *et al.*, 2008). In our study, an increase of CAT activity in the cotyledons, hypocotyls and radicles exposed to lead toxicity was observed (Fig. 5). Increased CAT activity is generally regarded as a response to heavy metal stress due to the generation of ROS in plant cells (Schützendübel & Polle, 2002). Increased CAT activity under lead stress had also been reported in rice (Verma & Dubey, 2003), horsegram and bengalgram (Reddy *et al.*, 2005) as well as *V. faba* (Wang *et al.*, 2008). Therefore, increased CAT activity in *L. cylindrica* seedlings might be due to increased requirement of each organelle to combat the stress conditions. In addition CAT activity shows a similar trend with the changes of SOD and POD activity (Figs. 2, 3 & 5). These findings showed that SOD, POD and CAT activity are positively correlated to maintain the balance between the formation of ROS and their removal in *L. cylindrica* seedlings.

PAL converts L-phenylalanine to trans-cinnamic acid and is the rate-limiting enzyme of the phenylpropanoid pathway. Due to the defense related function of phenolic compounds, the changes in PAL activity under stress condition have been considered a part of defense mechanism (Dixon & Paiva, 1995). In the present study, there was also an increase in PAL activity in *L. cylindrica* seedlings with increasing lead concentrations (Fig. 6). Under heavy metal stress, earlier data have suggested that increased PAL activity is significantly relative to the accumulation of such phenolic compounds (Ali *et al.*, 2006; Kováčik & Bačkor, 2007). Thus increased PAL in *L. cylindrica* seedlings might modulate the resistance mechanism to lead stress by regulating the biosynthesis of phenolic compounds. The interaction between heavy metal and synthesis of phenolic compounds is complex, further research concerning the effects of heavy metal on phenolic compounds need to be done.

In conclusion, our findings showed that there is an imbalance between ROS and ROS scavenging enzymes, and although we can not discern whether this fact reflects a defense mechanism of *L. cylindrica* seedlings. Increased SOD, POD, CAT and PAL activity might play a role in the defense response of *L. cylindrica* seedlings exposed to lead toxicity. Thus, these findings may contribute to a better understanding of the antioxidant response mechanisms in *L. cylindrica* seedlings to heavy metal stress.

Acknowledgement: We gratefully acknowledge the contribution and enthusiasm of our coworkers and collaborators in the course of the studies in our laboratories.

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(Received 02 November 2009; Accepted 30 November 2009)