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# Changes in non Protein Thiols, some Antioxidant Enzymes Activity and Ultrastructural Alteration in Radish Plant (*Raphanus sativus* L.) Grown under Lead Toxicity

Hossam Saad EL-BELTAGI[1)], Amal A. MOHAMED[2)]

[1)] *Cairo University, Faculty of Agriculture, Department of Biochemistry, Gamma Street, Giza, Cairo, Egypt; lbltg@yahoo.com*

[2)] *National Research Center, Plant Biochemistry Deptment, El Behouth Street, Dokki, Cairo-Egypt; amin_amal@yahoo.com*

**Abstract**

Forty days old radish plants (*Raphanus sativus* L.) were exposed to different regimes of lead stress as $Pb(NO_3)_2$ at the following concentrations 0, 25, 50, 100, 150, 250 and 500 ppm. The possible generation of oxidative stress, antioxidant metabolism and changes in the chloroplast and cell membrane ultrastructure were investigated. Greater loss of the photosynthetic pigments (Chl. a, Chl. b and total carotenoids) were observed especially under 500 ppm lead (Pb). The accumulation of lead in roots and leaves of plant were measured and the results showed that lead accumulation increased with increasing of the metal treatment concentration. An increasing trend was observed in levels of ascorbate and decreasing trend in glutathione. Also, the antioxidant enzymes, viz., guaiacol peroxidase (GPX) ascorbate peroxidase (APX), catalase (CAT) and glutathione S-transferase (GST) showed significant variation with the increase in lead stress compared to control (untreated) plants. The rapid inducibility of some of these enzymes is useful early and sensitive indicators of heavy metal toxicity. Native polyacrylamide gel electrophoresis revealed an increase in the isoenzymes profile of CAT in both leaves and roots. While POD isoenzymes bands prominently increased in leaves and slightly decreased in roots at the higher Pb concentration in the growth media. The ultrastructural studies at selected concentrations; 100 and 500 ppm of Pb showed distortion of the structure and cell membranes in roots. Therefore, the changes in the levels of some antioxidants may play an important role against oxidative injury.

***Keywords:*** antioxidative enzymes, chlorophyll, isoenzymes, transmission electron microscopy

## Introduction

The continuous increase of heavy metal ions in the environment is imposing serious problems in agricultural yield and increases human health threats through accumulation in the food chain. Lead (Pb) is a highly toxic metal which can be potentially harmful to plants, animals and humans at elevated levels. Therefore, accumulation of Pb in plants poses a potentially serious health risk to humans through contamination of the food chain. It exerts direct and indirect effects on nutrient uptake, seedling growth, and photosynthetic processes of plants and causes inhibition of enzyme activities, water imbalance, and alterations in membrane permeability (Moustakas *et al.,* 1994; Sharma and Dubey, 2005). Pb can cause change in chloroplast ultrastructure in plants, thereby reducing the growth (Poskuta *et al.,* 1987; Moustakas *et al*., 1994). In plants, reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($^{\cdot}OH$) are produced continuously as byproducts of various metabolic pathways that are localized in different cellular compartments (Reddy *et al.,* 2005). However, under stressful conditions, their formation might increase to excess of antioxidant scavenging capacity, thus creating oxidative stress by reaction and damage to all biomolecules (Halliwell and Gutteridge, 1999). Many heavy metals, including Pb, are known to induce over production of ROS and consequently enhance lipid peroxidation, decrease the saturated fatty acids and increase the unsaturated fatty acid contents of membranes of several plant species (Malecka *et al*., 2001). To control the level of ROS and to protect the cells, plants possess low molecular weight antioxidants compounds (ascorbic acid (AsA), reduced glutathione (GSH), carotenoids, tocopherols) and antioxidant enzymes such as peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) which remove, neutralize and scavenge the ROS (Gratão *et al.,* 2005; Mishra *et al.,* 2009). Peroxidases are heme containing proteins that utilize $H_2O_2$ in the oxidation of various organic and inorganic substrates (Asada, 1994). Peroxidases utilizing guaiacol as electron donor *in vitro* are guaiacol peroxidases and participate in developmental processes, lignification, ethylene biosynthesis, defense, wound healing (Asada, 1992). Catalases are involved in scavenging $H_2O_2$ generated during the photo-respiration and β-oxidation of fatty acids (Morita *et al.,* 1994). 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 meta-

bolic and environmental signals (Alscher, 1997). Glutathione S-transferases (GST) are a family of multifunctional detoxification enzymes that are mainly cytosolic and catalyse the conjugation of a wide variety of xenobiotics to glutathione (Chasseaud, 1979). However, the effect of heavy metals on plant GSTs is rather poorly studied. Regulation of the level of antioxidative enzymes gives plants an additional protective ability against oxidative stress (Foyer, 1994). As Pb is one of the most abundant heavy metal pollutants in both aquatic as well as terrestrial environments, the present study was undertaken to examine the uptake Pb in radish seedlings, to determine Pb-induced possible induction of the oxidative stress, likely alterations in behavior of the enzymes of antioxidant defense, and related ultrastructural of radish plants.

**Materials and methods**

*Plant material and stress conditions*

The healthy, homogenous seeds of radish (*Raphanus Sativus* L.*)* were subjected to surface sterilization with 0.1% sodium hypochlorite solution for 10 min and then rinsed with double distilled water. After 24 h imbibitions of seeds in water, seedlings were raised in sand cultures in plastic pots saturated with either Hoagland nutrient solution (Hogland and Arnon, 1950), which served as control or nutrient solutions supplemented with $Pb(NO_3)_2$ to achieve concentrations of 0, 25, 50, 100, 150, 250 and 500 ppm $Pb^{2+}$ which served as treatment solutions. Pots were maintained at field saturation capacity and received control and respective treatment solutions when needed to saturate the sand and the plants were watered as needed. Pots were kept for growth of seedlings at 28±1°C under 80% relative humidity and 12 h photoperiod with 40-50 $\mu mol^{-2}\ s^{-1}$ irradiance. All treatments were tested in four replications. For further study, the plant samples were collected at 40 day from pot culture and the biochemical parameter were analyzed.

*Determination of Pb content*

To determine the amount of absorbed lead in the seedling, fresh root/shoot samples were surface sterilized with 1 M HCl and then with 1 mM $Na_2EDTA$ to resolve excess surface bound Pb and then dried in oven at 70°C for 3-4 days. Dried samples were ground to a fine powder in a mortar and pestle and digested using the $HNO_3/HClO_4$ digestion method. Digested samples were dissolved in deionized distilled water and lead content was estimated using atomic absorption spectrometer (Unicam Sp 1900 model). Pb content of roots and shot was calculated in mg $g\ DW^{-1}$, where DW stands for dry weight.

*Pigments contents*

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids were extracted and estimated according to the method of Lichtenthaler (1987). About 100 mg of leaves from each Pb treatment was cut into tiny segments and kept in 10 ml of chilled 80% acetone in a capped glass tube. After 48 h extraction in dark at 4°C, the leaf segments were well-extracted for residual pigments. The contents of Chl *a*, Chl *b* and carotenoids were measured at 666, 653 and 470 nm, respectively. Pigment contents were calculated in mg $g\ FW^{-1}$. Where FW stands for fresh weight.

*Glutathione (GSH) determination*

Glutathione level was determined with Ellman's reagent as described by Tukendorf and Rauser (1990). The plant material (1 g of fresh weight) was homogenized with mortar and pestle in 5 ml of cold extraction buffer containing: Tris–HCl (0.1 M, pH 8.0), 10 mM $MgCl_2$, 3 mM Na-EDTA. The homogenate was centrifuged for 30 min at 15 000 g in a refrigerated centrifuge at 4°C. The reaction mixture contained 1 ml of the supernatant, 2 ml 0.4 M Tris–HCl buffer (pH 8.9), 50 µL 10mM 5,5′-ditiobis(2-nitrobenzoic acid) (DTNB).

All samples were incubated for 1 h at 37°C. The absorbance was read at 412 nm after 2 min by spectrophotometer ($\sum 412=13.6\ mM^{-1}\ cm^{-1}$).

*Ascorbic acid (AsA) determination*

One gram of fresh leaf sample was ground in 2.0 ml of 2% (w/v) metaphosphoric acid using mortar and pestle then, centrifuged at 13000 g for 20 min at 4°C. The ascorbate content was determined in a reaction mixture consisting of 0.2 ml of supernatant, 0.5 ml of 150 mM phosphate buffer (pH 7.4, containing 5mM EDTA) and 0.2 ml of deionized water. Colour was developed by the addition of 2, 4-dinitrophenolindophenol. The absorbance of red color was measured at 520 nm according to Omaye *et al.* (1979). The concentration of ascorbic acid in the extracts was calculated using ascorbic acid as standard.

*Preparation of crude enzyme extracts*

The method described by Vitória *et al.* (2001) was used to prepare the crude enzymes extracts. In this method, roots and leaves tissues were homogenized in a chilled pastel and mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinyl pyrrolidone. The homogenates were centrifuged at 10.000 g for 30 min and then the supernatants were kept stored in separate aliquots at -20°C until analysis.

*Protein determination*

Soluble protein was estimated by using the Coomassie Brillent Blue G-250 reagent according to the method of Bradford (1976) with bovine serum albumin as standard.

*Antioxidant enzyme activities*

Guaiacol peroxidase (GPX) activity was assayed according to the method of Hemeda and Klein (1990). A 100 ml of reaction mixture contained 10 ml of 1% guaia-

col (v/v), 10 ml of 0.3% $H_2O_2$ and 80 ml of 50mM phosphate buffer (pH 6.6). Enzyme extract (75 µl) was added to reaction mixture in a final volume of 3 ml. The increase in absorbance due to oxidation of guaiacol (extinction coefficient 26.6 $mM^{-1}$ $cm^{-1}$) was monitored at 470 nm. Enzyme activity was expressed as units $min^{-1}$ $mg^{-1}$ protein.

The activity of APX was measured by estimating the rate of ascorbate oxidation (extinction coefficient 2.8 $mM^{-1}$ $cm^{-1}$). The 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM $H_2O_2$, 0.5 mM sodium ascorbate, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The change in absorbance was monitored at 290 nm (Nakano and Asada, 1981) and enzyme activity was expressed as units $min^{-1}$ $mg^{-1}$ protein.

For measurement of the catalase (CAT) activity the method of Aebi (1974) was used. The 3 ml reaction mixture comprised of 50 mM sodium phosphate buffer (pH 7.0), 20 mM $H_2O_2$ and a suitable aliquot of enzyme. Decrease in the absorbance was taken at 240 nm (molar extinction coefficient of $H_2O_2$ was 0.04 $mM^{-1}$ $cm^{-1}$). Enzyme activity was expressed as units $min^{-1}$ $mg^{-1}$ protein.

Glutathione S-transferase (GST) was determined by the method of Habig and Jacoby (1981). The reaction mixture consisted 100 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM 1-chloro 2,4-dinitrobenzene (CDNB), 1 mM reduced glutathione and enzyme extract. The enzyme activity was measured at 340 nm. The activity of the enzyme was calculated using the extinction coefficient of the conjugate 9.6 $mM^{-1}$ $cm^{-1}$ and expressed as units $min^{-1}$ $mg^{-1}$ protein.

*Native gel electrophoresis and isoenzyme staining*

PAGE for peroxidase POD and catalase CAT isoenzymes assay was performed with 7% (w/v) polyacrylamide gel as described by Laemmli (1970). POD isoenzymes were detected by the Ros Barcelo method (Ros Barcelo, 1987). The gels were rinsed in water and the gel was stained in a solution containing 0.06% (v/v) $H_2O_2$, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till the brown colour. CAT isoenzymes were detected by the Woodbury method (Woodbury *et al.,* 1971). Gels were incubated in 0.01% $H_2O_2$ for 10 min and developed in a 2% (m/v) $FeCl_3$ and 2% $K_3Fe(CN)_6$ (m/v) solution for 10 min until the colourless bands were appeared.

*Transmission electron microscopy*

For transmission electron microscopy (TEM) studies, small sections of treated roots (100, 500 ppm) with $Pb(NO_3)_2$, 1-3 mm in length, were fixed in 4% glutaraldehyde (v/v) in 0.2 M sodium phosphate buffer (pH 7.2) for 6-8 h and post fixed in 1% $OsO_4$ (Osmium oxide) for 1 h, then in 0.2 M phosphate-buffered saline (PBS) (pH 7.2) for 1-2 h as suggested by Makela *et al.* (2000). Dehydration was done in a graded ethanol series (50, 60, 70, 80, 90, 95 and 100%) followed by acetone; then samples were infiltrated and embedded in the Spurr's resin. Ultra thin sections (80 nm) were prepared and mounted on copper grids for viewing in the transmission electron microscope (10-Zeiss West Germany) at an accelerating voltage of 80.0 kV.

*Statistical analyses*

Statistical analysis was done using SPSS (version 10) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. $P \leq 0.05$ were considered significant. Values reported here are means of three replicates.

**Results and discussion**

*Lead accumulation*

The concentration of Pb was determined in leaves and roots of radish plants and illustrated in (Fig. 1). When

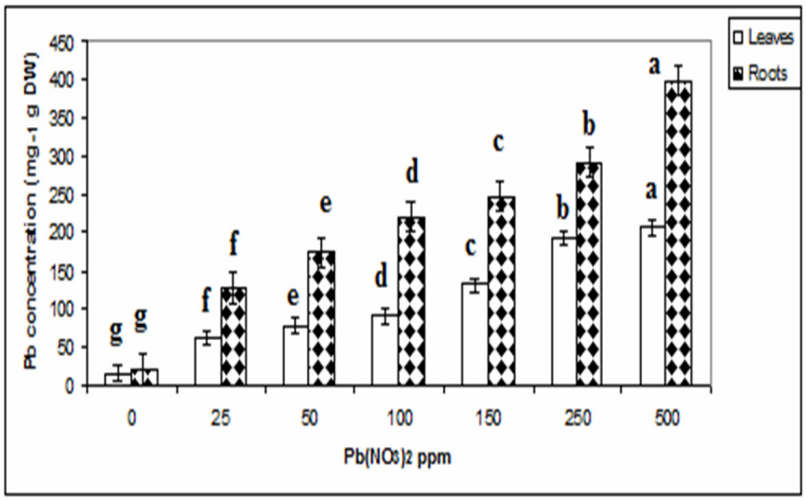


Fig. 1. Pb accumulation ($mg^{-1}$ g DW) in leaves and roots of radish plants (*Raphanus sativus* L.) at harvest time (40 days of growth). All the values are means ± SD. ANOVA significant at $p \leq 0.05$

seedlings grown under Pb treatments (from 25 to 500 ppm), Pb contents recorded 62.3, 76.3, 90.3, 131, 193 and 207 ppm with radish leaves against 128, 174, 220, 248, 292 and 398 mg $g^{-1}$ d. wt, in roots with regard to treatments of 25, 50, 100, 150, 250 and 500 ppm, respectively, compared to the corresponding control (14.3 and 20.3 mg $g^{-1}$ d. wt). Most lead was found accumulating in roots and less was transported to stems and leaves. Panich-Pat *et al.* (2004) studied the removal of lead from contaminated soils by *Typha angustifolia* and found the ability to accumulate high concentration of lead in roots. These results corroborate with our earlier findings with the heavy metal Cd which was also found to accumulate in maize seedlings to a greater extent in roots than in leaves and that the uptake of Cd was against concentration gradient (Mohamed, 2002).

*Chlorophylls and carotenoids content*

Data of chlorophylls and carotenoids are presented in Tab. 1. All lead concentrations in the nutrient solution caused a significant reduction of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids content. The smallest reduction of chlorophylls and carotenoids were recorded when plants grown under control treatment, while the highest reduction were found when radish plants treated with 500 ppm Pb. Chlorophyll content is often measured in plants in order to assess the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic productivity (Parekh *et al*., 1990). Researchers have reported decreased chlorophyll in several different plant species under the impact of heavy metals and this may be the most important cause of inhibition. Decreased chlorophyll content associated with heavy metal stress may be the result of inhibition of the enzymes responsible for chlorophyll biosynthesis. Reduction in the chlorophyll content might be attributed to the inhibition of δ-aminolevulinic acid dehydratase (ALAD) caused by Pb uptake (Prasad and Prasad, 1987), to impaired uptake of essential elements, such as Mn and Fe, to damaged photosynthetic apparatus, or due to chlorophyll degradation by increased chlorophyllase activity (Sharma *et al.,* 2005).

Tab. 1. Chlorophylls and carotenoids concentration (mg $g^{-1}$ FW) in leaves of lead (Pb) treated radish plants

| Pb Treatments ppm | Chlorophyll a | Chlorophyll b | Total Chlorophyll (a+b) | Carotenoids |
|---|---|---|---|---|
| 0 | 0.793±0.033$^{a}$ | 0.454±0.029$^{a}$ | 1.247±0.008$^{a}$ | 0.464±0.009$^{a}$ |
| 25 | 0.737±0.016$^{b}$ | 0.372±0.012$^{b}$ | 1.109±0.018$^{b}$ | 0.449±0.005$^{ab}$ |
| 50 | 0.675±0.021$^{c}$ | 0.321±0.005$^{c}$ | 0.996±0.019$^{c}$ | 0.429±0.006$^{b}$ |
| 100 | 0.617±0.009$^{d}$ | 0.290±0.009$^{d}$ | 0.907±0.019$^{d}$ | 0.370±0.032$^{c}$ |
| 150 | 0.553±0.006$^{e}$ | 0.268±0.003$^{de}$ | 0.821±0.019$^{e}$ | 0.319±0.005$^{d}$ |
| 250 | 0.524±0.009$^{f}$ | 0.241±0.009$^{e}$ | 0.750±0.008$^{f}$ | 0.298±0.005$^{e}$ |
| 500 | 0.419±0.049$^{g}$ | 0.186±0.025$^{f}$ | 0.605±0.057$^{g}$ | 0.238±0.006$^{f}$ |
| LSD 5 % | 0.048 | 0.0283 | 0.0443 | 0.0254 |

Values are expressed as the means ± SD of three independent assays. Values with different letters in the same column were significantly different ($p \leq 0.05$)

*Total glutathione (GSH) and total ascorbate (AsA)*

A gradual decrease in glutathione value after Pb treatments (Tab. 2) in leaves and roots was observed. Compared to the corresponding control, GSH content was significantly decreased in leaves and roots of radish by Pb treatments. These results clearly indicate that lead toxicity induced oxidative damage. Glutathione (GSH) is the main non-protein thiol of the cell and it is one of the main soluble antioxidant compounds in plants. The reduction level of GSH might be due to its utilization as a reducing substrate in the synthesis of ascorbate (Dixit *et al.,* 2001). GSH is consumed and degraded in order to protect cellular membranes from lipid peroxidation. It has been reported that the activity of the glutathione (GSH)-(ASA) cycle is responsible for an increase in tolerance of plants to oxidative stress (Hegedus *et al.,* 2001; Larsson *et al.,* 1998).

Furthermore, the marked increase of ascorbate (AsA) by Pb treatments in both leaves and roots of radish plants were illustrated in Tab. 2. The ascorbate was increased in leaves by (118%) and roots by (119%) of radish at 500 ppm of Pb compared with the control. The AsA-dependent formation of hydroxyl radicals (·OH) involves in the cleavage of cell wall components also plays an active roll

Tab. 2. Total glutathione (GSH) and total ascorbate (AsA) content in leaves and roots of lead treated radish plants

| Pb treatments (ppm) | Total glutathione (GSH) content µmol $g^{-1}$ FW | | | | Total ascorbate (AsA) content µmol $g^{-1}$ FW | | | |
|---|---|---|---|---|---|---|---|---|
| | Leaves | % | Roots | % | Leaves | % | Roots | % |
| 0 | 109±1.15$^{a}$ | 100 | 60.6±2.55$^{a}$ | 100 | 15.1±0.08$^{g}$ | 100 | 4.79±0.09$^{f}$ | 100 |
| 25 | 98.5±3.73$^{b}$ | 90 | 48.3±1.23$^{b}$ | 80 | 15.6±0.06$^{f}$ | 103 | 4.95±0.05$^{e}$ | 103 |
| 50 | 84.6±5.49$^{c}$ | 77 | 45.1±0.54$^{c}$ | 74 | 16.1±0.09$^{e}$ | 107 | 5.05±0.05$^{c}$ | 105 |
| 100 | 75.8±1.74$^{d}$ | 69 | 42.1±0.83$^{d}$ | 70 | 16.5±0.16$^{d}$ | 109 | 5.24±0.04$^{d}$ | 109 |
| 150 | 69.7±1.71$^{e}$ | 64 | 39.3±0.61$^{e}$ | 65 | 16.8±0.10$^{c}$ | 111 | 5.37±0.21$^{c}$ | 112 |
| 250 | 62.3±0.97$^{f}$ | 57 | 36.0±1.99$^{f}$ | 59 | 17.2±0.03$^{b}$ | 114 | 5.54±0.02$^{b}$ | 116 |
| 500 | 46.7±2.41$^{g}$ | 43 | 25.0±0.79$^{g}$ | 41 | 17.8±0.21$^{a}$ | 118 | 5.70±0.1$^{a}$ | 119 |
| LSD | 5.046 | | 2.469 | | 0.205 | | 0.106 | |

Values are expressed as the means ± SD of three independent assays. Values with different letters in the same column were significantly different ($p \leq 0.05$)

in cell extension (Piquery *et al.,* 2002). A high level of cellular oxidants promotes synthesis of ascorbate (Foyer *et al.,* 1997). So the enhancement of total ascorbate in leaves and roots of radish under Pb treatments may be due to its active participation in detoxification of oxygen species/ free radicals directly (non-enzymatic), as well as through certain enzymes (Asada and Takahashi, 1987). The same results have been reported by Cho and Park (2000) in tomato plants grown under cadmium stress. The reason for this is because the entrance of GSH in pathway for phytochelatin synthesis and therefore the substrate for ascorbate reduction reduced.

*Antioxidant enzymes activity*

Changes in antioxidant enzymes are shown in Tab. 3. Under Pb treatment the activity of GPX showed an initial increase with increase in metal concentration in leaves and roots, respectively in comparison with untreated-Pb plants. In case of APX, a marked increases was noticed in leaves (2.1, 3, 3.4, 5.1, 4 and 3.5 times) and roots (1.3, 1.8, 2.2, 2.7, 2.62 and 2.63 times) of radish plants under Pb treatments (25, 50, 100, 150, 250 and 500 ppm) respectively, in comparison with control plants. A comparison of the catalase activity in radish treated Pb and control (untreated) indicated an increase in enzyme activity following increasing Pb concentrations in the nutrient media. CAT activity in leaves did not exhibit significant variation in both 0.0 and 25 pb treatments. A progressive induction of CAT activity was noticed after Pb treatments at 25, 50, 100, 150, 250 and 500 ppm, in both leaves (7.36, 12.5, 31.9, 59.2, 65.6 and 42.7) followed by roots (10.4, 16.1, 27.7, 42.7, 46.8 and 30) compared with control. A decrease was noticed at 500 ppm in both leaves and roots.

The activity of GST increased gradually in leaves and roots under both control as well as Pb treatments. Induction of GST activity in Pb treatments was found in leaves (1.7, 1.8, 2.5, 4, 4.5 and 6.6 times) followed by obviously increment of GST activity in the roots (1.3, 2.2, 2.4, 3.6, 5.3 and 8.4 times) at Pb-treated plants against control. It was suggested that the elevated GPX activity could be the consequence of either ionic microenvironment or the tissue specific gene expression in the leave and root (Blinda *et al.,* 1996). The role of peroxidases as stress enzymes in plants has been widely accepted and it used as a potential biomarker for sublethal metal toxicity in examined plant species (Radotic *et al.,* 2000). Furthermore, GPX participating in the lignin biosynthesis might build up a physical barrier against poisoning heavy metals (Attila *et al.,* 2001). In addition, our results agreement with many researchers who reported that, Pb enhances GPX activity in many plants such as *Phaseolus vulgaris and Salix acmophylla* (Wouter *et al.,* 2002; Ali *et al.,* 2003). Therefore, an increase in GPX activity prevents plant from toxic effects of $H_2O_2$. Generally, the hyper activity of antioxidant enzyme GPX in leaves and roots of radish might be consequence of strategy adapted by plant for its survival under stress by metals like Pb (Ali *et al.,* 2003). Under sublethal salinity, metal deficiency and toxicity conditions, level of peroxidase activity has been used as potential biomarker to evaluate the intensity of stress (El-Beltagi *et al.,* 2008). The induction of APX specific activity in leaves and roots of radish plants exposed to different Pb levels suggested its role in the detoxification of $H_2O_2$, as has been reported by Weckx and Clijsters (1996). Similar induction in ascorbate peroxidase was reported in response to salt stress (El-Beltagi *et al.,* 2008), chilling (Fadzillah *et al.,* 1996), drought (Mittler and Zilinskas, 1994), Cu toxicity (Weckx and Clijsters, 1996), Fe deficiency (Salama *et al.,* 2009) and UV-B radiation (Hideg *et al.,* 1997). The principal harmful oxygen species-scavenging enzyme in plants is catalase, which decomposes hydrogen peroxide and thus maintains the redox balance during oxidative stress (Bowler *et al.,* 1992). The reasons for the increase in catalase activity after Pb treatments may be due to the scavenging role of CAT to $H_2O_2$, which could be quenched by the induction of specific enzymes like catalase (Elstner *et al.,* 1988). An increase in CAT activity was also observed by Vitória *et al.* (2001), El-Beltagi *et al.* (2008) and Salama *et al.* (2009) for *Raphanus sativus* and Flax Cultivars. CAT is only present in peroxisomes, but it is indispensable for ROS detoxification during stress, when high levels of ROS are produced (Gupta *et al.,* 2009). The induction of GST activity may be due to its role in the conjugation between the glutathione (GSH) and xenobiotic substrates (CDNB) to produce

Tab. 3. Specific activities of antioxidant enzymes in leaves and roots of lead treated radish plants

| Pb treatments (ppm) | CAT unit $min^{-1}$ $mg^{-1}$ protein | | GST unit $min^{-1}$ $mg^{-1}$ protein | | GPX unit $min^{-1}$ $mg^{-1}$ protein | | APX unit $min^{-1}$ $mg^{-1}$ protein | |
|---|---|---|---|---|---|---|---|---|
| | Leaves | Roots | Leaves | Roots | Leaves | Roots | Leaves | Roots |
| 0 | 5.7±0.8$^{f}$ | 3.7±0.6$^{f}$ | 0.03±0.0008$^{f}$ | 0.025±0.001$^{e}$ | 0.1±0.002$^{e}$ | 3.1±0.25$^{g}$ | 0.8±0.09$^{f}$ | 1.42±0.12$^{e}$ |
| 25 | 7.4±2.7$^{f}$ | 10±1.3$^{e}$ | 0.05±0.0005$^{e}$ | 0.032±0.003$^{e}$ | 1.8±0.129$^{d}$ | 7.6±0.25$^{f}$ | 1.8±0.07$^{e}$ | 1.84±0.09$^{b}$ |
| 50 | 13±1.7$^{e}$ | 16±1.5$^{d}$ | 0.05±0.0005$^{e}$ | 0.06±0.002$^{d}$ | 1.9±0.002$^{d}$ | 9.3±0.37$^{e}$ | 2.5±0.05$^{d}$ | 2.59±0.18$^{c}$ |
| 100 | 32±1.2$^{d}$ | 28±1.4$^{c}$ | 0.07±0.0047$^{d}$ | 0.06±0.002$^{d}$ | 2.0± 0.052$^{d}$ | 16±0.22$^{d}$ | 2.8±0.05$^{c}$ | 3.12±0.14$^{b}$ |
| 150 | 59±1.4$^{b}$ | 43±1.9$^{b}$ | 0.12±0.0007$^{c}$ | 0.09±0.003$^{c}$ | 3.4± 0.099$^{c}$ | 25±1.12$^{c}$ | 4.2±0.06$^{a}$ | 3.89±0.27$^{a}$ |
| 250 | 66±1.2$^{a}$ | 47±2.2$^{a}$ | 0.13±0.0056$^{b}$ | 0.13±0.006$^{b}$ | 4.0±0.099$^{b}$ | 29± 1.46$^{b}$ | 3.1±0.06$^{b}$ | 3.73±0.26$^{a}$ |
| 500 | 43±1.8$^{c}$ | 30 ±1.0$^{c}$ | 0.19±0.0035$^{a}$ | 0.21±0.007$^{a}$ | 4.4±0.109$^{a}$ | 38± 0.57$^{a}$ | 2.9±0.14$^{c}$ | 3.74±0.29$^{a}$ |
| LSD | 2.4 | 2.6 | 0.01 | 0.007 | 0.15 | 1.313 | 0.15 | 0.36 |

Values are expressed as the means ± SD of three independent assays. Values with different letters in the same column were significantly different ($P \leq 0.05$)

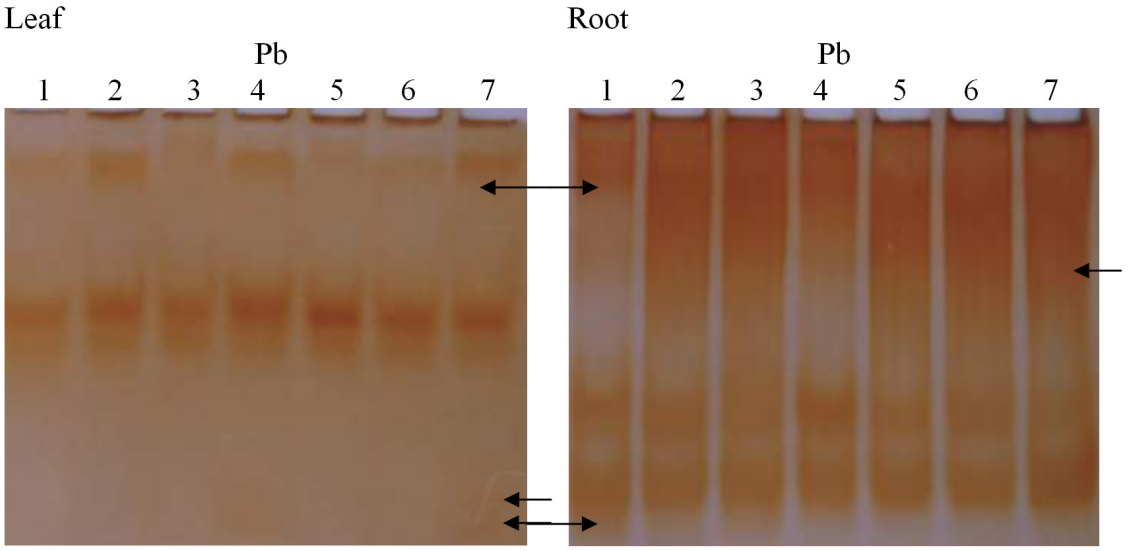


Fig. 2. Peroxidase (POD) isoenzyme patterns of radish tissues (leaves and roots) in plants grown in Pb containing media. Lanes: 1: Pb 0.0 ppm; 2: Pb 25 ppm; 3: Pb 50 ppm; 4: Pb 100 ppm; 5: Pb 150 ppm; 6: 250 ppm; 7: Pb 500 ppm

GSTC in roots and leaves of the Pb-treated plants (Dixit *et al.,* 2001). GST is known to be responsive to biotic and abiotic stresses. This enzyme has not been characterized with respect to its antioxidative roles in plants (Dixit *et al.,* 2001). The glutathione S-transferase is considered to serve in the intracellular detoxification of mutagens, carcinogens and other noxious chemical substances in animal cells. In plant cells also GST may have a similar role. In the present study, elevated activity of GST in both leaves and roots of radish under lead stress may be attributed that it could catalyze the conjugation of lead ions to glutathione (GSH) or directly act as binding protein to accommodate lead ions (Reddy *et al.,* 2005). Higher increase in the activity of GST under lead stress conditions may detoxifies the lead ions at high degree in horsegram further support its tolerant nature.

*Antioxidant Isoenzymes*

The induction of new isoenzymes and the change in the isoenzyme profile is considered to play an important role in the cellular defense against oxidative stress. Pb treatments induced different changes in POD isoenzyme patterns. The activity of POD increased in extracts of Pb-treated leaves, and this rise coincided with Pb concentration. Two additional bands could also be distinguished at the end of the gel in samples of 100 and 500 ppm Pb treated leaves (Fig. 2). These results agreed with that of Rucinska *et al.* (1999). These fastest migrating isoenzymes of POD was almost undetectable in the control and low treatments of Pb, but was clearly visible in 100 and 500 ppm Pb treatments. In spite of the fact that leaves and roots POD was tested in crude extract, without considering its distribution within the cell, it is possible that the Pb induced new iso-POD which might associated with

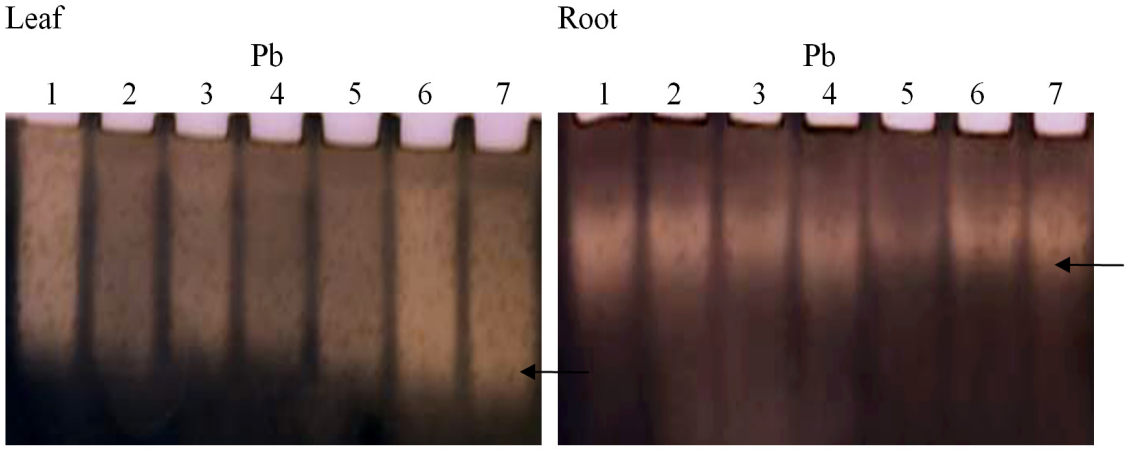


Fig. 3. Catalase (CAT) isoenzyme patterns of radish tissues (leaves and roots) in plants grown in Pb containing media. Lanes: 1: Pb 0.0 ppm; 2: Pb 25 ppm; 3: Pb 50 ppm; 4: Pb 100 ppm; 5: Pb 150 ppm; 6: 250 ppm; 7: Pb 500 ppm

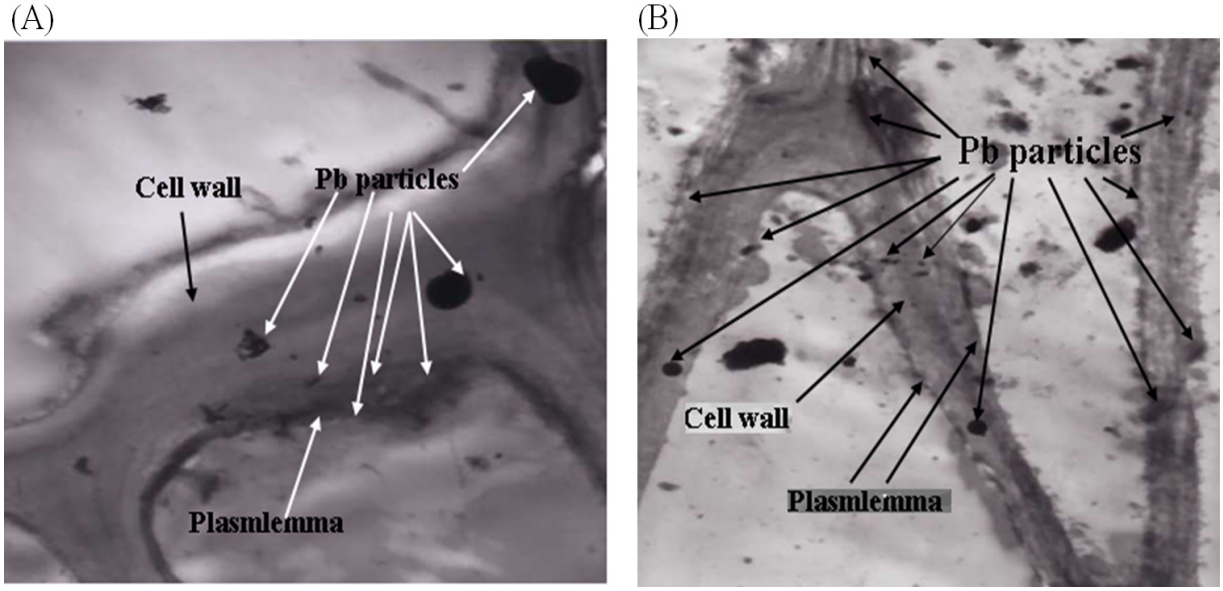


Fig. 4. Ultrastructure of the Pb100 ppm treatment showed several large and small Pb-particles closely associated with the cell wall and with the plasmalemma accommodating them in each case

cell wall lignification. This function of POD has proposed by Lamport (1986). Iso-POD analysis appears to be particularly useful for the evaluation of phytotoxicity of metal polluted soils (Van Assche and Clijsters, 1990). Our results are in agreement with (El-Beltagi *et al.,* 2008; Salama *et al.,* 2009) who found some changes in POD isoenzyme profile under salt and iron stress respectively.

CAT isoenzymes in both leaves and roots subjected to Pb stress are presented in Fig. 3. Only one band with different density was observed in both leaves and roots in Pb treated radish plants. In leaves, a clear increase in the density of this band was verified following Pb treatments. The recover in the CAT activity in roots samples after the exposure Pb high doses may be due to the great formation of ROS, as a result of de novo protein synthesis (Pereira *et al*., 2002).

*Ultrastructure electron microscopy*

Within sections treated with 100 and 500 ppm Pb (Fig. 4 and 5) several large lead particles can be seen closely associated with the cell wall, with the plasmalemma accommodating them in each case.

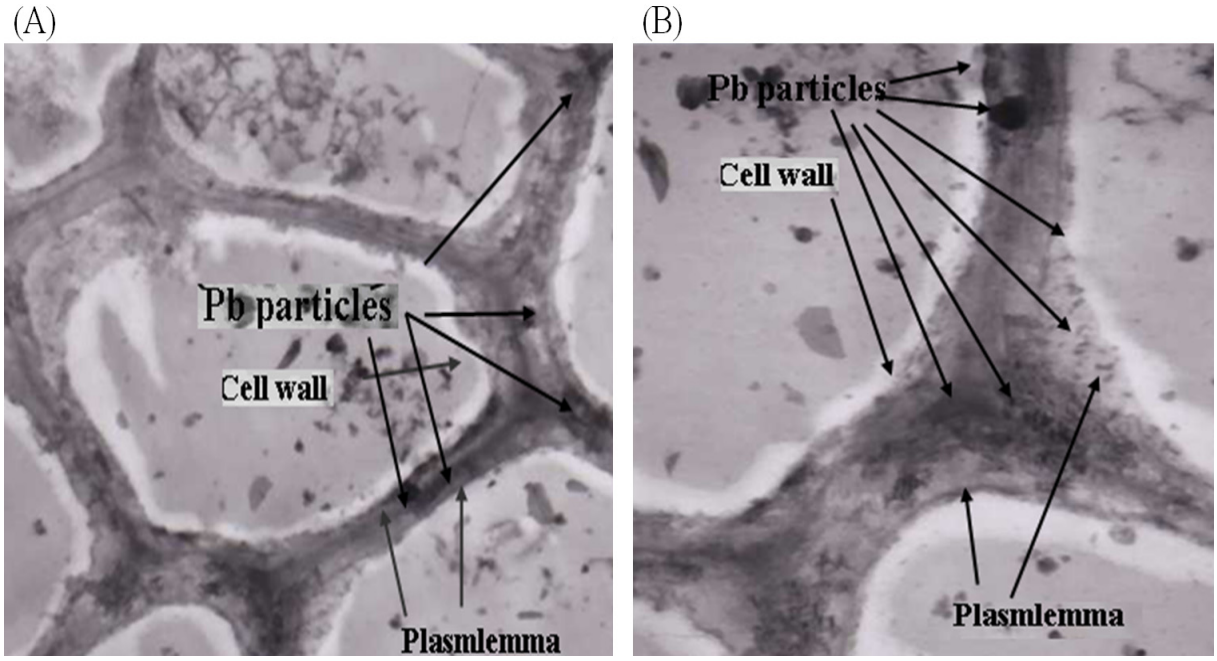


Fig. 5. (a) Ultrastructure of the Pb500 ppm treatment showed several large and small Pb-particles closely associated with the cell wall and with the plasmalemma accommodating them in each case. (b) Close-up view of Pb particles in the intercellular space associated with these cells and little Pb was found in the cell wall itself

In close-up view of the same sections of the two treatments (Fig. 4a, b and 5a, b) it is possible to distinguish the plasmalemma that traverses the cell wall at the point at which the Pb particle is located. The Pb particle itself has an irregular outline and is completely electron opaque. Heavy deposits of Pb were found in the intercellular space associated with these cells and little Pb was found in the cell wall itself, a phenomenon that was observed on several occasions. In ultra-thin sections of radish roots, after 40 days of lead treatment, Pb supplied was never found in vacuoles but often found embedded in or adjacent to the cell wall, in many cases near plamodesmata. Pb particles, particularly the large ones, always had sharp angular edges with a very grainy, crystalline appearance (Jarvis and Leung, 2002). Malone *et al.* (1974) described a situation where the Pb deposits in the spaces between the cell wall and plasmalemma were caused by Pb-containing dictyosome vesicles migrating to the cell periphery, focusing with the plasmalemma and discharging the Pb contents into the extracellular space. Also, the exist of heavy metals like Cu and Pb in cell wall of root cells may be due to the ability of cell wall proteins to bind with heavy metals. Our observations are agreement with Jarvis and Leung (2002) and many others who observed heavy depositions of Pb in intracellular spaces, material adhering to the cell wall and the middle lamellae of roots, while no Pb was observed in the upper plant parts by using transimation electron microscopy (Wouter *et al*., 2002).

## Conclusions

In summary, Pb toxicity negatively affected the plant growth and enzymes activity, which consequently reduce the plant productivity. Also, these assays appear to be simple, sensitive and correlated well with heavy metals stress, such assays, would be a good biochemical tools to predict heavy metals pollution.

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