American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Biochemistry and Molecular Biology

ISSN 2150-4210
DOI: 10.3923/ajbmb.2016.84.94

Research Article
Effect of Lead Stress on the Hydrolytic Enzyme Activities and Free Radical Formation in Radish (*Raphanus sativus* L.) Plant

[1]Hossam S. El-Beltagi, [2]Amal A. Mohamed, [1]Abdel-Kader M. Abdel-Samad and [1]Mohamed M. Rashed

[1]Department of Biochemistry, Faculty of Agriculture, Cairo University, P.O. Box 12613, Gamma Street, Giza, Cairo, Egypt
[2]Department of Plant Biochemistry, National Research Center, Cairo, Egypt

Abstract

Background and Objective: The effect of lead on several metabolic reactions in the radish plant has been investigated. The present study was conducted to evaluate the potential role of enzymes activity, their isoenzyme profile pattern and free radical formation as biomarkers of Pb pollution in the radish (*Raphanus sativus* L.) plant. **Materials and Methods:** Varied concentrations of $Pb(NO_3)_2$ ranging from 25-500 ppm in the growth media were used. The plant samples were collected after 40 days growth period. Lead and micronutrients concentration, the profile of electron spins resonance determination and hydrolytic enzymes activities were analyzed. **Results:** Lead concentrations in roots and leaves increased with increasing Pb concentration in the tested media; however the most accumulation were observed in leaves. Generally, the concentration of micronutrients such as Fe, Zn, Mn and Cu declined in leaves compared to the roots. Results of the profile of Electron Spin Resonance (ESR) determination showed a decrease in unstable free radical level in the roots, followed by a significant increase with increasing Pb concentrations. The Pb induced changes in some enzymes activity and its isoenzyme profiles such as acid phosphatase (AP), esterase (EST) and polyphenol oxidase (PPO) in leaves and roots of radish plant. Results of isoenzymes suggested that the staining intensities of isoform patterns were consistent with the changes of the activities assayed in solutions. **Conclusion:** These results suggested that (*Raphanus sativus* L.) seedlings may have a better protection against oxidative stress by increasing antioxidant enzymes activity exposed to Pb toxicity.

Key words: Lead stress, radish, electron spin resonance, acid phosphatase, esterase, polyphenol oxidase

Received: June 26, 2016 Accepted: August 12, 2016 Published: September 15, 2016

Citation: Hossam S. El-Beltagi, Amal A. Mohamed, Abdel-Kader M. Abdel-Samad and Mohamed M. Rashed, 2016. Effect of lead stress on the hydrolytic enzyme activities and free radical formation in radish (*Raphanus sativus* L.) plant. Am. J. Biochem. Mol. Biol., 6: 84-94.

Corresponding Author: Hossam S. El-Beltagi, Department of Biochemistry, Faculty of Agriculture, Cairo University, P.O. Box 12613, Gamma Street, Giza, Cairo, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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[1]. The Pb can cause changes in chloroplast ultra structure in plants, thereby reducing their growth[2]. Furthermore, deleterious effects of heavy metal stress in plants may be coupled to other physiological processes via the stimulation of some enzymatic activities that limit cell growth and consequently, accelerate tissue ageing. Several hydrolytic enzymes including glucosidases, esterases, acid and alkaline phosphatases were pro-posed to be involved in plant morphogenesis, especially in cell wall metabolism that is necessary for its turnover and plasticity[3]. 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[4,5]. However, under stressful conditions, their formation might increase to exceed the antioxidant scavenging capacity, thus creating oxidative stress by reaction and damage to all biomolecules[6,7]. Many heavy metals, including Pb are known to induce overproduction 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[8]. In addition, ROS are highly reactive to membrane lipids, protein and DNA[9,10]. They are believed to be the major contributing factors to stress injuries and to cause rapid cellular damage[11,12]. Acid Phosphatases (AP) form a group of enzymes catalyzing hydrolysis of a variety of phosphate esters in acidic environments. The AP is believed to increase orthophosphate (Pi) availability under phosphorous deficient conditions[13]. Salt, drought and osmotic stresses have also been reported to increase AP activity[14-16]. In addition, the activity of acid phosphatase (AP) increased in leaves and roots of tomato and radish plants grown at high zinc and cadmium concentration in the growth media[17]. Esterases, a group of hydrolases, catalyze the formation or cleavage of ester bonds of water soluble substrates. Generally, these enzymes have a broad spectrum of substrates and act on a variety of natural and xenobiotic compounds[18]. Polyphenol oxidase (PPO) has been the subject of several reviews because enzymatic browning in fruits and vegetables provokes unpleasant sensory qualities and losses in nutrient quality[19]. Stress-tolerant plants usually contain high constitutive levels of protective metabolites, while the more sensitive ones show their induction under different kind of stress. Isoenzymes are an easily obtained class of molecular markers, co-dominant, reliable and can provide valuable information about abiotic stress tolerance[20]. Esterase isoenzymes have been used as a genetic marker in barley crop, identification of esterase loci was reported to encode monomeric isoenzymes[21]. Free radicals and hydrogen peroxides formation were reported to cause the membrane damage, which is often related to lipid peroxidation[22]. The ESR techniques have been implicated to detect the formation of free radicals in plants during nutrient stress[23]. The ESR method can also be utilized for estimating the type and content of more stable free radicals that accumulated in cells as markers of oxidative stress due to O_2 enrichment[24]. As Pb is one of the most abundant heavy metal pollutants in both aquatic and terrestrial environments, the present study was conducted to evaluate the potential role of enzymes activity, their isoenzyme profile pattern and free radical formation as biomarkers of Pb pollution in the radish (*Raphanus sativus* L.) plant.

MATERIALS AND METHODS

Plant material and stress conditions: 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 imbibition of seeds in water, seeds were grown in sand cultures in plastic pots saturated with Hoagland nutrient solution[25], which served as control or nutrient solutions supplemented with $Pb(NO_3)_2$ to achieve different concentrations of 0, 25, 50, 100, 150, 250 and 500 ppm Pb^{2+} which served as treatment solutions. The incubation temperature was set at 28°C with a 12 h photoperiod with 40-50 μmol^{-2} sec^{-1} irradiance. The experiment was arranged in a completely randomized design with three replicates, each replicate with about 10 radish seeds. The plant samples were collected after 40 days growth period and the following biochemical parameters were analyzed.

Pb accumulation and micronutrient contents: Lead and micronutrient content (Zn, Fe, Mn and Cu) were determined in the fine powdered dry matter of plants (leaves and roots).

The samples were washed and then mineralized with perchloric acid. Metal concentrations (ppm) were estimated by atomic absorption spectrometry (Unicam Sp 1900 model) according to the method of Lu[26].

Spectra of electron spin resonance (ESR): Representative sample composed of 10 roots from each treatment were frozen in liquid N_2 and were then ground in a mortar. The powder material (500 mg) was packed into ESR flat quartz cuvette. The ESR spectra were recorded at room temperature using ESR spectrometer ELEXSYS E500 (Bruker-Germany). The experimental condition was as follows: Field mod. amplitude 0.001, field mod. frequency (Hz) 100000, microwave frequency (Hz) 9.8145e+09, microwave power (W) 0.0202637, receiver grain 40, receiver time constant (S) 0.00512. The standardization of "g" value was carried out using 1,1 Diphenyl-2-picryl-hydrazyl (DPPH) as a standard. The areas under ESR curve for each sample were calculated. The area under control sample was represented as 100% and was used to plot the relative radicals versus cadmium concentration[27].

Preparation of crude enzyme extracts: The method described by Vitoria *et al.*[28] was applied to prepare the crude enzyme extracts. In this method, 0.5 g 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 10000×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[29] with bovine serum albumin as standard.

Enzyme specific activity: Acid phosphatase (AP) specific activity was determined spectrophotochemically according to Yoneyama *et al.*[30]. The assay mixture contained sodium acetate buffer 50 mM, pH 5.5, 3 mM pNPP and 20 µL enzyme extract. After 10 min at 37°C, the reaction was stopped by adding 1 mL of 0.2 M NaOH. The increasing in absorbance at 410 nm was measured.

Esterase (EST) specific activity was assayed spectrophotochemically according to Brahimi-Horn *et al.*[31]. The reaction mixture contained sodium phosphate buffer 66 mM pH 7.5, ρ-nitrophenyl acetate (100 µM) and 20 µL enzyme extract. The change in absorbance was followed for 5 min with 30 sec intervals at 405 nm.

Polyphenol oxidase (PPO) specific activity was assayed by using photochemical method as described by Coseteng and Lee[32]. The reaction mixture contained 50 mM potassium phosphate buffer pH 6.2 and 250 mM catechol and 50 µL enzyme extract. The increasing in absorbance at 420 nm was measured.

Native gel electrophoresis and isoenzyme staining: Isoenzymes were separated using native-polyacrylamide gel electrophoresis (PAGE) technique with 6% PAA in tris-borate buffer 0.125 M, pH 8.9 on slab gel according to the method of Stegmann *et al.*[33].

PPO isoenzyme staining: Immediately after stopping the electrophoretic run, the slabs were dipped and agitated in 15 mM catechol in 0.1-0.2 M phosphate buffer (pH 5) and 0.05 M p-phenylenediamine until bands showing PPO activity were evident (30-40 min). The gels were placed in deionized water overnight and transferred to 50% methanol for storage[34].

AP isoenzyme staining: The staining solution was prepared according to Wendel and Weeden[35]. The Na-a naphthyl acid phosphate 100 mg, $MgCl_2$ 100 mg and 100 mg of fast garent GBG salt were dissolved in 100 mL of 50 mM Na-acetate buffer, pH 5.0. Gels were incubated in dark at room temperature until black bands appeared.

EST isoenzyme staining: After electrophoresis, the gels were removed from the glass plates and incubated for 30-40 min at 37°C in 100 mL 0.2 M sodium phosphate buffer (pH 6.5) containing 50 mg fast blue RR salt with the addition of 2 mL 1% α-naphtyl acetate in acetone at 50%[33].

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

RESULTS AND DISCUSSION

Pb accumulation and micronutrient contents: Lead is one of the 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[1]. Regarding to the data of Pb accumulation, which

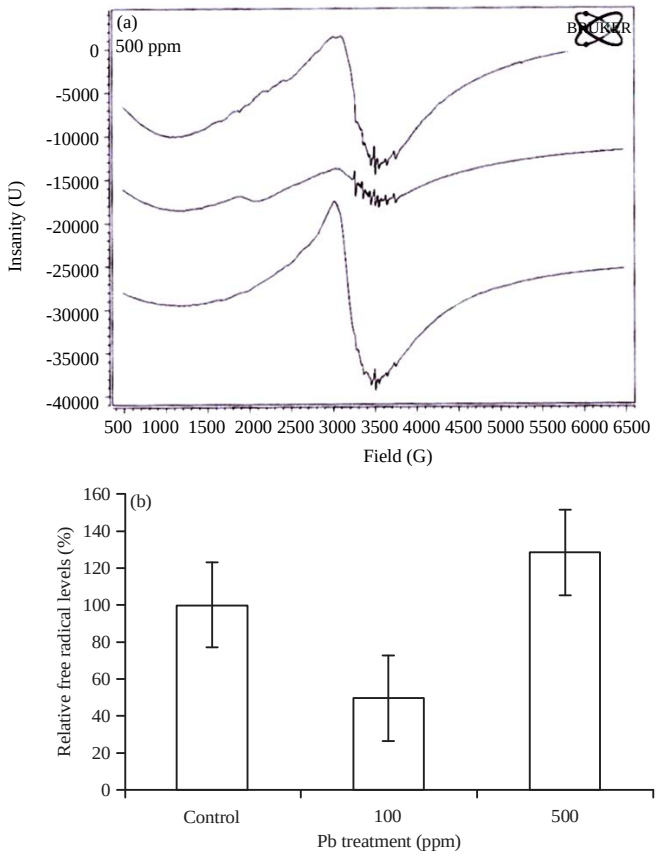

Fig. 1(a-b): (a) Signals of free radical formation in Pb-treated radish roots 0, 100, 500 ppm and (b) Percent of free radical level in Pb-treated radish roots in relation the control

represented in Table 1, when radish seedlings were grown under increasing concentrations of lead in the growth medium, during a 40 days growth period increasing lead levels caused a continuous increase in the content of lead. The absorbed lead was localized to a greater extent in roots than in shoots. The Pb concentration was ranged between 20.3 and 207 ppm in leaves and 20.3-398 ppm in roots. It means a progressive accumulation of Pb in roots, which translocated to leaves. Lead increase was paralleled by a decline in the concentration of Fe, Zn, Mn and Cu was decreased by increasing Pb concentration in the growth media. The lowest concentration of micronutrients was found at 500 ppm Pb in both leaves and roots. Nevertheless this increase is not toxic to the plant and may be render to continuous addition of nutrient solution but its translocation from the roots to the leaves differ from one element to other. These results are in harmony with previous studies, who found an accumulation of lead in plants with increasing Pb concentration in the growth media in *Sesbania exaltata* and *Jatropha curcas* plants, respectively[36-39].

Effect of Pb on stable free radical formation in radish roots: Electron Spin Resonance (ESR) spectra of radish untreated and treated roots grow under Pb treatments (100 and 500 ppm) were measured using X-band ESR spectrometer at room temperature and the results are represented in Fig. 1a and b. The ESR lines recorded with g-value 2.003. Under control condition, the spectrum area was lower than the high treated sample with Pb (g = 105, considered as 100%). While, the treatment of Pb (100 ppm) was lower caused lower g value than control sample (g = 52.3 considered 49.8%) this combined with the appearance of six characteristic fine peaks of Mn. For this reason, the free radical peaks did not appear clearly in those two treatments. Heavy metals can influence the physical and chemical processes in plants by directly inducing ROS production (Fenton reaction), by blocking functional groups of proteins and glutathione and by displacing essential metals like zinc or selenium from proteins and zinc-finger motifs of transcription factors[40]. One possible mechanism by which excess heavy metals may damage plant tissues is the stimulation of free radical production, by

Table 1: Lead accumulation and its effect on micronutrient contents (ppm) in leaves and roots of radish plants under Pb stress after 40 days growth

Pb treatment	Pb		Fe		Zn		Mn		Cu	
(ppm)	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
0 (Control)	20.3±0.6^{g}	20.3±0.3^{g}	817±40^{a}	948±23^{a}	36.3±1.2^{a}	18±1.5^{c}	327±15^{a}	302±7.1^{a}	39±1.5^{a}	77±4.6^{a}
25	62.3±1.5^{f}	128±8.0^{f}	536±65^{b}	768±25^{b}	33.6±2.9^{a}	35±1.3^{a}	237±5.8^{b}	244±5.5^{b}	32±1.0^{b}	71±2.6^{b}
50	76.3±5.3^{e}	174±11^{e}	339±7.1^{c}	648±13^{c}	32.6±1.5^{a}	34±1.9^{a}	190±10^{c}	224±6.0^{c}	26±0.6^{c}	63±2.5^{c}
100	90.3±2.9^{d}	220±14^{d}	344±56^{c}	375±13^{d}	22.0±4.6^{b}	25±0.7^{b}	166±5.1^{d}	189±10^{d}	21±1.5^{d}	54±3.5^{d}
150	131±8.1^{c}	248±14^{c}	240±43^{d}	396±16^{d}	21.3±4.0^{bc}	19±1.8^{c}	146±5.3^{e}	156±6.0^{e}	18±0.6^{e}	45±3.1^{e}
250	193±3.8^{b}	292±12^{b}	197±46^{d}	300±9.0^{e}	17.0±3.0^{bc}	15±1.1^{d}	131±2.1^{f}	135±4.5^{f}	14±1.0^{f}	36±1.5^{f}
500	207±2.1^{a}	398±16^{a}	171±19^{d}	229±18^{f}	16.0±3.0^{c}	13±0.6^{d}	102±7.0^{g}	113±6.7^{g}	11±1.2^{g}	27±1.7^{g}
LSD	7.32	20.6	76.6	30.7	5.32	2.37	14.17	11.85	1.91	5.18

Mean values are presented ±SE (n = 3). Values with different letters in the vertical column differ significantly at p<0.05

Table 2: Enzymes activity (U mg^{-1} protein) in leaves and roots of Pb treated radish plants after 40 days growth period

Pb treatments	Acid phosphatase		Esterase		Polyphenol oxidase	
(ppm)	Leaves	Roots	Leaves	Roots	Leaves	Roots
0 (Control)	3.96±0.07^{b}	4.16±0.15^{e}	97.64±3.2^{d}	108.6±1.8^{e}	3.35±0.45^{f}	5.21±0.08^{g}
25	3.29±0.05^{d}	3.87±0.08^{f}	155.6±4.4^{b}	139.7±2.8^{d}	8.50±0.51^{e}	14.9±1.96^{f}
50	3.26±0.07^{d}	3.55±0.08^{g}	175.1±2.9^{a}	159.2±1.6^{c}	9.72±0.5^{de}	18.7±0.66^{e}
100	3.04±0.01^{d}	4.50±0.05^{d}	123.5±1.9^{c}	192.0±1.7^{b}	10.6±1.80^{d}	22.8±1.25^{d}
150	3. 50±0.10^{c}	5.25±0.12^{c}	118.7±2.3^{c}	254.0±4.0^{a}	19.1±0.23^{c}	34.6±0.96^{c}
250	4.11±0.16^{b}	6.25±0.18^{b}	91.91±2.4^{e}	194.3±2.4^{b}	21.6±0.74^{b}	38.8±1.68^{b}
500	4.47±0.08^{a}	7.95±0.21^{a}	62.22±4.5^{f}	154.7±3.6^{c}	26.1±0.92^{a}	52.4±1.32^{a}
LSD 5 (%)	0.183	0.237	5.663	4.727	1.247	2.226

Mean values are presented ±SE (n = 3). Values with different letters in the vertical column differ significantly at p<0.05

imposing oxidative stress[41]. Reactive Oxygen Species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are formed in metabolic processes occurring in plants, but when they are created on non-controlled levels they can lead to disturbances and injury in cell metabolism or even to cell death[42,43]. The higher Pb concentration in the soil environment results in an enhanced Pb uptake by roots and leaves. In particular, the level of antioxidant enzyme is a good indicator for the impacts of pollutants like heavy metals. The heavy metal damage is an important factor in many pathological and toxicological processes[44]. The presence of toxic metal in the cell led to formation of free radical species, which cause severe damage to different cell organelles and biomolecules[45,46]. On contrary in the highest treatment of Pb (500 ppm) the spectrum area was higher than control sample (g = 134.9, considered 128.5%). The intensity which related to free radical formation may be correspond to the stable free radicals came from the reaction of superoxide free radical with one or more of the constituents of the root cells such as DNA, lipids and proteins. It was found that two g-values were consistent with quinone radicals, although the exact origin of these radicals was not determined. Atherton *et al.*[47] suggested that precursors of these radicals might be either quinone involved in electron transport pathways, simple phenolic secondary metabolites or more complex polyphenols. The present results are in agreement with Rucinsk *et al.*[48] who found that Pb treatment in lupine roots induced ESR signal for paramagnetic radical, which derived from quinine.

Enzyme specific activities: A significant inhibition in acid phosphatase (AP) activity was observed in seedlings of both leaves and roots of radish with low Pb treatments (Table 2). The results appeared that with Pb treatments (25, 50, 100 and 150 ppm) a clearly reduction in AP activity in leaves (17, 17.7, 23 and 12%), respectively, in comparison with control treatments. Additionally, the concentration (25 and 50 ppm) of Pb caused also reduction of acid phosphatase activity in roots (7 and 15%), respectively, compared with control plants. Under (250 and 500 ppm) Pb treatment about 4 and 13% increase in AP activity was observed in leaves of radish plants as compared with control. Furthermore, AP activity significantly sharply increased at (8, 26.2, 50.2 and 91%) in roots with the highest levels of Pb (100, 150, 250 and 500 ppm) as compared with control. The change of enzyme activity, however, cannot support the presumption that AP is directly involved in Pb detoxification, although it might help the plant to resist the nutrient inadequacy that often accompanies heavy metal stress[49]. In addition, the increases in AP activity with Pb treatments might be due to the decline of phosphate (Pi) level in the cell of Pi starvation and that intracellular and extracellular AP are integral components of plants response to Pi deficiency[50]. Abiotic environmental

stress such as salinity, water stress and heavy metals lead to an alteration of gene expression in plants, which in turn leads to alteration in its metabolism and development[51]. However, over expression of AP, accelerating the degradative processes in cellular metabolism, are non-specific or exclusive responses to heavy metal toxicity[52]. Thus, some investigators have suggested that AP activity increases under the stress of high levels of heavy metals and is one possible process of detoxification and resistance[53].

Esterase (EST) specific activity show different pattern in leaves and roots of radish plant under Pb treatments as presented in Table 2. The results of Pb treatments (25, 50, 100, 150 ppm) appeared that a significant increase of EST activity (59.4, 79.3, 26.5 and 21.6%) in leaves compared to control sample. While in high dose of Pb (250 and 500 ppm) showed a noticed inhibition in EST activity (6 and 36.3%), respectively, in comparison with control sample. Furthermore, Pb treatments in root, showed a variable significant induction of EST activity (28.6, 46.6, 76.8, 134, 80 and 42.4%) as compared to control of radish plants. These results are in agreement with Amal *et al.*[54] who found the trend of EST activity in leaves and roots of radish under Cd stress. The increase of EST activity in leaves and roots of radish plants in low doses of Pb treatments may be due to its role in detoxification mechanisms for heavy metals resistance in the medium or due to the de novo protein synthesis under conditions of metal stress[55]. In addition, the increased activity of enzyme after treatment of plants with heavy metals can be interpreted as an indication of damage to membranes, which leads to liberation of enzymes from cell structures[56]. While, the reduction in enzyme activity in the high level of Pb may be due to the interaction of both metals with functional sulphydryl (SH)-groups of the enzyme[57].

Polyphenol oxidase (PPO) is very important enzyme for plant respiration. The PPO is a terminal oxidase which can directly pass electrons to O_2 when the intermediate products of plant respiration are oxidized. It could catalyze the oxidization of such a group of compounds as phenol to quinone. The PPO has some relationship with the synthesis of cell compounds containing phenol groups such as lignin[58]. The activity of polyphenol oxidase enzyme was increased by increasing Pb treatments in the growth media and reached its maximum activity at 500 ppm as shown in Table 2. The results showed that a marked significant increase in PPO specific activity with Pb treatments in roots about 10 fold (5.21 and 52.4) and followed by leaves about 7.6 fold (3.35-26.1) (U mg^{-1} protein) compared to control. The PPO specific activity were enhanced by increasing Pb concentration and reached its maximum increase with 500 ppm even in roots 52.4 and leaves 26.1 as noticed in Table 2. It was suggested that activity of PPO enhanced under stress conditions caused by the presence of toxic heavy metals is responsible for binding and detoxification of heavy metals in *Nymphet* epidermal glands. In *Arabidopsis thaliana* the responses to Pb toxicity and to bacterial infection are similar and include an increase in peroxidase activity with the accumulation of polyphenol deposits[59]. Increases in phenol concentration in response to heavy metal stress have also been noted in several plants such as tobacco, maize and pine[60-62]. Saffar[63] reported that in *Arabidopsis thaliana* PPO activity might be the result from prolonged heavy metal stress.

Isoenzymes detection: Isoenzyme profiles of acid phosphatase (AP) in leaves and roots at 40 day-old grown under control and Pb treated radish plants are shown in Fig. 2. It is evident from the zymogram that, an obvious decrease in leaves AP band under Pb treatments (25, 50, 100 and 150 ppm) were observed, while at highest Pb treatments (250 and 500 ppm) the intensity and mobility of this band was enhanced comparing with control band. Moreover, the results of roots treated with Pb appeared that, a marked increase in the intensity of the basic three-isoenzyme bands of acid phosphatase with Pb treatments. In addition, two new bands were appeared at high treatments of Pb (100, 150, 250 and 500 ppm) with increasing in their intensity compared with control sample. According to Mukherji and Gupta[64], the increased level of enzyme activity can be attributed to an accelerated synthesis of protein. The stimulation of enzyme by metal ions is assumed to be an indirect effect. However, this stimulation is known to be caused by a metal induced de novo synthesis of the enzyme protein[65]. Generally, increased the activity of hydrolytic enzymes (ribonuclease, deoxyribonuclease and acid phosphatase) after treatment of plants with heavy metals reflect a general senescence response induced by heavy metal ions[66]. These results in agreement with many researchers who reported that heavy metals modified the pattern of AP isoenzymes in many plants such as *Glycine max*, *Zea mays* and lupin[67,68].

Esterase (EST) isoenzyme polymorphism was photographed and presented in Fig. 3. Zones Est1 and Est2 at leaves of radish showed a decrease in their intensity at high doses of Pb. While zones Est3 and Est4 were represent appearance of new isoenzyme bands and disappearance of others at Pb exposed sample compared with unexposed ones. Moreover, no changes were seen at the pattern zones of Est1 and Est2 in root exposed to Pb compared with control. Otherwise, zones Est3 and Est4 showed different changes under Pb stress. New isoenzyme bands appeared at strong intensity and others at weak intensity in the exposed samples

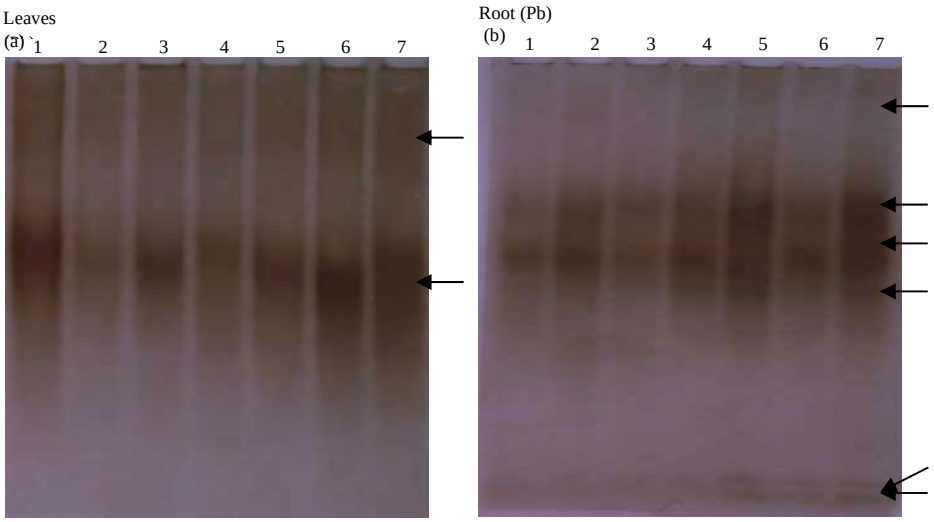

Fig. 2(a-b): Acid phosphatase isoenzyme patterns of radish tissues (a) Leaves and (b) Roots at harvest time (40 days growth) in plants grown in Pb containing media, lanes: 1: Pb 0.0 ppm, 2: Pb 25 ppm, 3: Pb 50 ppm, 4: Pb 100 ppm, 5: Pb 150 ppm, 6: Pb 250 ppm, 7: Pb 500 ppm

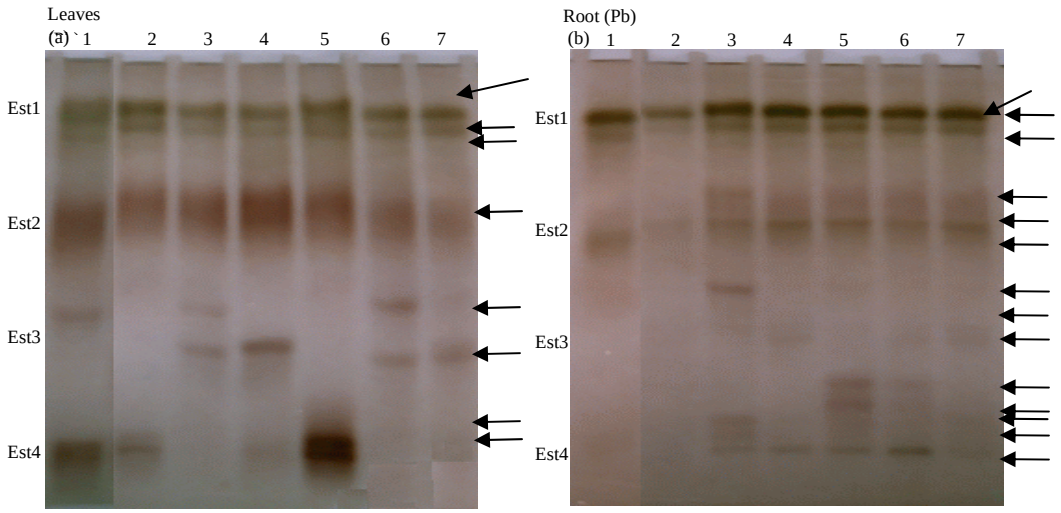

Fig. 3(a-b): Esterase isoenzyme patterns of radish tissues (a) Leaves and (b) Roots at harvest time (40 days growth) in plants grown in Pb containing media, lanes: 1: Pb 0.0 ppm, 2: Pb 25 ppm, 3: Pb 50 ppm, 4: Pb 100 ppm, 5: Pb 150 ppm, 6: 250 ppm, 7: Pb 500 ppm

compared with unexposed sample. Therefore, the appearance of new bands or disappearance of others at zones Est3 and Est4 in the metal exposed radish samples could be due to adaptation of the leaves and roots to the metal exposed environment[69]. In addition, the reduction of iso-esterase bands intensity or the disappearance of the fast bands may be due to heavy metals acts at the nucleic acid level in which transcriptional, post-transcriptional, translational or other inhibition processes may be involved. Evidence has been found of inhibition of RNA synthesis and the inhibition of DNA polymerase I and II activity has been confirmed[69]. The isoenzyme patterns of EST are particularly affected by metals. Finally, these results are agreement with previous studies who noted that, Treatment of *Triticum aestivum*, *Silene vulgaris* and *Raphanus sativus* with heavy metals (Zn, Cu, Pb, Cd and Ni) concentrations of verifying toxicity resulted in

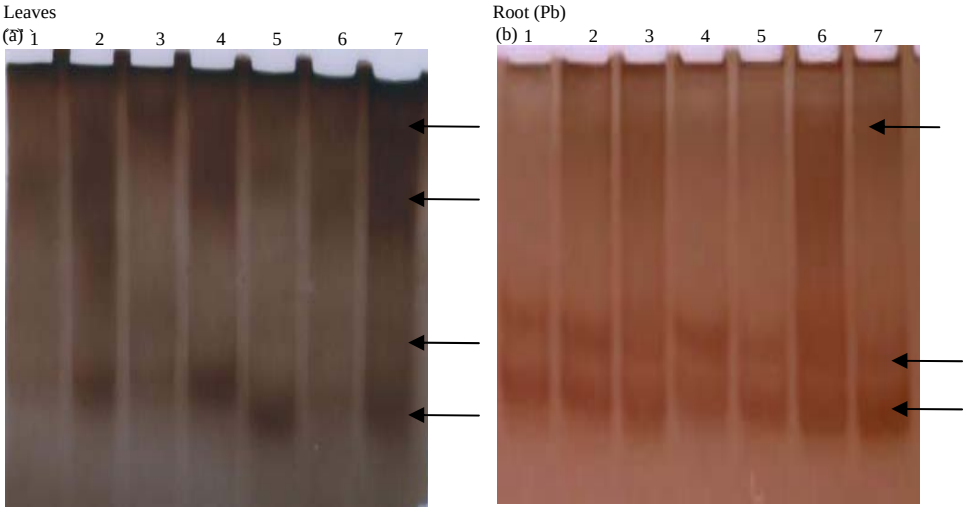

Fig. 4(a-b): Polyphenol oxidase isoenzyme patterns of radish tissues (a) Leaves and (b) Roots at harvest time (40 days growth) in plants grown in Pb containing media, lanes: 1: Pb 0.0 ppm, 2: Pb 25 ppm, 3: Pb 50 ppm, 4: Pb 100 ppm, 5: Pb 150 ppm, 6: Pb 250 ppm, 7: Pb 500 ppm

differenced in the number of iso-esterase bands and in quantitative changes of band activity[54,70].

The results of PPO profile in Pb-treated radish plants appeared that, a marked induction at the intensity of two isoenzyme bands of PPO with Pb treatments (Fig. 4). The fastest migrating isoenzyme of PPO was almost undetectable in the control, but was clearly visible in Pb-treated leaves. The mobility of this fast band was increased at the high treatments of Pb (150, 250 and 500 ppm) compared with control. In addition, the root extracts used for PPO staining following native PAGE showed a dramatic increase at the intensity of three PPO isoenzyme bands under Pb treatments. However, the induction of PPO activity may be due to its role in phenolic compound synthesis which playing an important role in detoxification of heavy metals in plants[71]. Detoxification of heavy metals in plants was achieved by the formation different complexes and amino acids and phenolic compounds have all been implicated in the chelation and precipitation of heavy metals[72].

CONCLUSION

Based on the present study, it can be concluded that Pb causes oxidative stress as evidenced by significant increase in enzyme activity. Generally, Fe, Zn, Mn and Cu declined in leaves compared to the roots. Moreover, the data demonstrated that Pb induced changes in some enzymes activity such as acid phosphatase (AP), esterase (EST) and polyphenol oxidase (PPO) in leaves and roots of radish plant.

The Pb stress also induced several changes in AP, EST and PPO isoenzyme profiles. It remains to be seen whether these increases may be due to induced gene transcriptional and de novo synthesis of protein or are due to posttranslational modification of existing protein. Finally, the results of Electron Spin Resonance (ESR) showed a clear disorder in the Pb treated plant. The detection of such parameters is useful, early and sensitive indicators for prediction of heavy metal toxicity in plant.

SIGNIFICANCE STATEMENT

- Lead concentrations in roots and leaves increased with increasing Pb concentration
- Micronutrients decline in leaves compared with increasing Pb concentration
- The ESR show decrease in free radical in roots, followed by increase with increasing Pb
- The Pb induces changes in enzymes activity leaves and roots of radish plant
- Results of isoenzymes were consistent with changes of enzyme activities assayed

ACKNOWLEDGMENTS

Authors would like to thank the management of the Faculty of Agriculture, Cairo University and National Research Center for ongoing cooperation to support

research for providing funds and facilities necessary to achieve the desired goals of research.

REFERENCES

1. Sharma, P. and R.S. Dubey, 2005. Lead toxicity in plants. Braz. J. Plant Physiol., 17: 35-52.
2. Poskuta, J.W., E. Parys and E. Ramanowska, 1987. The effects of lead on the gaseous exchange and photosynthetic carbon metabolism of pea seedlings. Acta Soc. Bot. Pol., 56: 127-137.
3. Balen, B., M. Krsnik-Rasol and V. Simeon-Rudolf, 2003. Isoenzymes of peroxidase and esterase related to morphogenesis in *Mammillaria gracillis* Pfeiff. tissue culture. J. Plant Physiol., 160: 1401-1406.
4. Reddy, A.M., S.G. Kumar, G. Jyothsnakumari, S. Thimmanaik and C. Sudhakar, 2005. Lead induced changes in antioxidant metabolism of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.). Chemosphere, 60: 97-104.
5. El-Beltagi, H.S. and H.I. Mohamed, 2013. Reactive oxygen species, lipid peroxidation and antioxidative defense mechanism. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 41: 44-57.
6. El-Beltagi, H.S. and H.I. Mohamed, 2013. Alleviation of cadmium toxicity in *Pisum sativum* L. seedlings by calcium chloride. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 41: 157-168.
7. Mohamed, A.A., A. Castagna, A. Ranieri and L.S. di Toppi, 2012. Cadmium tolerance in *Brassica juncea* roots and shoots is affected by antioxidant status and phytochelatin biosynthesis. Plant Physiol. Biochem., 57: 15-22.
8. Malecka, A., W. Jarmuszkiewicz and B. Tomaszewska, 2001. Antioxidative defense to lead stress in subcellular compartments of pea root cells. Acta Bioquimica Polonica, 48: 687-698.
9. Afify, A.M.R. and H.S. El-Beltagi, 2011. Effect of the insecticide cyanophos on liver function in adult male rats. Fresenius Environ. Bull., 20: 1084-1088.
10. El-Beltagi, H.S., Z.A. Salama and D.M. El-Hariri, 2008. Some biochemical markers for evaluation of flax cultivars under salt stress conditions. J. Nat. Fibers, 5: 316-330.
11. El-Beltagi, H.S. and A.A. Mohamed, 2010. Changes in non protein thiols, some antioxidant enzymes activity and ultrastructural alteration in radish plant (*Raphanus sativus* L.) grown under lead toxicity. Not. Bot. Hort. Agrobot. Cluj, 38: 76-85.
12. El-Beltagi, H.S., O.K. Ahmed and W. El-Desouky, 2011. Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of Rosemary (*Rosmarinus officinalis* L.) callus culture. Radiat. Phys. Chem., 80: 968-976.
13. Vance, C.P., C. Uhde-Stone and D.L. Allan, 2003. Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. New Phytol., 157: 423-447.
14. Szabo-Nagy, A., G. Galiba and L. Erdei, 1992. Induction of soluble phosphatases under ionic and non-ionic osmotic stresses in wheat. J. Plant Physiol., 140: 629-633.
15. Sharma, A.D. and R. Kaur, 2007. Drought-induced changes in acid phosphatase activities in wheat in relationship with phosphorus. Emir. J. Food Agric., 19: 31-38.
16. El-Beltagi, H.S., H.I. Mohamed, A.M.A. Mohamed, L.M. Zaki and A.M. Mogazy, 2013. Physiological and biochemical effects of γ-irradiation on cowpea plants (*Vigna sinensis*) under salt stress. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 41: 104-114.
17. Kaya, C., 2002. Effect of supplementary phosphorus on acid phosphatase enzyme activity and membrane permeability of zinc-toxic tomato plants. J. Plant Nutr., 25: 599-611.
18. Cummins, I., M. Burnet and R. Edwards, 2001. Biochemical characterisation of esterases active in hydrolysing xenobiotics in wheat and competing weeds. Physiol. Planta., 113: 477-485.
19. Matheis, G., 1987. Polyphenol oxidase and enzymatic browning of potatoes (*Solanum tuberosum*) I. Properties of potato polyphenol oxidase. Chem Mikrobiol. Technol. Lebensm, 11: 5-12.
20. Mohamed, A.A., M.A. Matter and M.M. Saker, 2010. Effect of salt stress on some defense mechanisms of transgenic and wild potato clones (*Solanum tuberosum* L.) grown *in vitro*. Nat. Sci., 8: 181-193.
21. Kahler, A.L. and R.W. Allard, 1970. Genetics of isozyme variants in Barley. I. Esterases.. Crop Sci., 10: 444-448.
22. Hendry, G.A.F., A.J. Baker and C.F. Ewart, 1992. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus* L. Acta Botanica Neerlandica, 41: 271-281.
23. Mohamed, A.A. and A.A. Aly, 2004. Iron deficiency stimulated some enzymes activity, lipid peroxidation and free radicals production in Borage officinalis induced *in vitro*. Int. J. Agric. Biol., 6: 179-184.
24. Goodman, B.A., D.B. McPhail and D.J. Linehan, 1986. Oxygen-induced free radical in wheat roots. Free Radical Res. Commun., 2: 173-178.
25. Hoagland, D.R. and D.I. Arnon, 1950. The water culture method for growing plant without soil. Calif. Agric. Expt. Stat. Circ., 347: 1-32.
26. Lu, R.K., 1999. Analytical Methods for Soils and Agricultural Chemistry. China Agricultural Science and Technology Press, Beijing.
27. Cakmak, I. and H. Marschner, 1988. Zinc-dependent changes in ESR signals, NADPH oxidase and plasma membrane permeability in cotton roots. Physiol. Plant., 73: 182-186.

28. Vitoria, A.P., A.P.M. Rodriguez, M. Cunha, P.J. Lea and R.A. Azevedo, 2003. Structural changes in radish seedlings exposed to cadmium. Biol. Planta., 47: 561-568.
29. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
30. Yoneyama, T., M. Shiozawa, M. Nakamura, T. Suzuki and Y. Sagane *et al*., 2004. Characterization of a novel acid phosphatase from embryonic axes of kidney bean exhibiting vanadate-dependent chloroperoxidase activity. J. Biol. Chem., 279: 37477-37484.
31. Brahimi-Horn, M.C., M.L. Guglielmino, L. Elling and L.G. Sparrow, 1990. The esterase profile of a lipase from *Candida cylindracea*. Biochimica et Biophysica Acta (BBA)-Lipids Lipid Metabol., 1042: 51-54.
32. Coseteng, M.Y. and C.Y. Lee, 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. J. Food Sci., 52: 985-989.
33. Stegmann, H., W. Purgermeister, A. Shah, H. Franckssen and E. Krogerrecklenfo, 1987. Gel electrophoresis between glass plate in polyacrylamide or other gel. Manual of Institute for Biochemistry, pp: 31-37.
34. Benjamin, N.D. and M.W. Montgomery, 1973. Polyphenol oxidase of royal Ann cherries: Purification and characterization. J. Food Sci., 38: 799-806. J. Food Sci., 38: 799-806.
35. Wendel, J.F. and N.F. Weeden, 1989. Visualization and Interpretation of Plant Isozymes. In: Isozymes in Plant Biology, Soltis, D.E. and P.S. Soltis (Eds.). Champer and Hall, London, pp: 5-45.
36. McComb, J., S. Hentz, G.S. Miller and M. Begonia, 2012. Effects of lead on plant growth, lead accumulation and phytochelatin contents of hydroponically-grown *Sesbania exaltata*. World Environ., 2: 38-43.
37. Chinmayee, M.D., M.S. Anu, B. Mahesh, A.M. Sheeba, I. Mini and T.S. Swapna, 2014. A comparative study of heavy metal accumulation and antioxidant responses in *Jatropha curcas* L. IOSR J. Environ. Sci. Toxicol. Food Technol., 8: 58-67.
38. Nareshkumar, A., G.V. Nagamallaiah, M. Pandurangaiah, K. Kiranmai and V. Amaranathareddy *et al*., 2015. Pb-stress induced oxidative stress caused alterations in antioxidant efficacy in two groundnut (*Arachis hypogaea* L.) cultivars. Agric. Sci., 6: 1283-1297.
39. Zafari, S., M. Sharifi, N.A. Chashmi and L.A.J. Mur, 2016. Modulation of Pb-induced stress in *Prosopis* shoots through an interconnected network of signaling molecules, phenolic compounds and amino acids. Plant Physiol. Biochem., 99: 11-20.
40. Malecka, A., M. Derba-Maceluch, K. Kaczorowska, A. Piechalak and B. Tomaszewska, 2009. Reactive oxygen species production and antioxidative defense system in pea root tissues treated with lead ions: Mitochondrial and peroxisomal level. Acta Physiol. Plant., 31: 1065-1075.
41. Foyer, C.H., H. Lopez-Delgado, J.F. Dat and I.M. Scot, 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol. Plant., 100: 241-254.
42. Salin, M.L., 1988. Toxic oxygen species and protective systems of the chloroplast. Physiol. Plant., 72: 681-689.
43. Scandalios, J.G., 1993. Oxygen stress and superoxide dismutases. Plant Physiol., 101: 7-12.
44. Ates, B., I. Orun, Z.S. Talas, G. Durmaz and I. Yilmaz, 2008. Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) exposed to Pb^{2+} and Cu^{2+}. Fish Physiol. Biochem., 34: 53-59.
45. Radotic, K., T. Ducic and D. Mutavdzic, 2000. Changes in peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium. Environ. Exp. Bot., 44: 105-113.
46. Shah, K., R.G. Kumar, S. Verma and R.S. Dubey, 2001. Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. Plant Sci., 161: 1135-1144.
47. Atherton, N.M., G.A.F. Hendry, K. Mobius, M. Rohrer and J.T. Torring, 1993. A free radical ubiquitously associated with senescence in plants: Evidence for a quinone. Free Radic. Res. Commun., 19: 297-301.
48. Rucinsk, R., S. Waplak and E.A. Gwozdz, 1999. Free radical formation and activity of antioxidant enzymes in lupin roots exposed to lead. Plant Physiol. Biochem., 37: 187-194.
49. Zheng, W., Y. Fei and Y. Huang, 2009. Soluble protein and acid phosphatase exuded by ectomycorrhizal fungi and seedlings in response to excessive Cu and Cd. J. Environ. Sci., 21: 1667-1672.
50. Duff, S.M., W.C. Plaxton and D.D. Lefebvre, 1993. Phosphate-starvation response in plant cells: De novo synthesis and degradation of acid phosphatases. Proc. Natl. Acad. Sci. USA., 88: 9538-9542.
51. Low, R., B. Rockel, M. Kirsch, R. Ratajczak and S. Hortensteiner *et al*., 1996. Early salt stress effects on the differential expression of vacuolar H^+-ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. Plant Physiol., 110: 259-265.
52. Pandey, N., G.C. Pathak, A.K. Singh and C.P. Sharma, 2002. Enzymic changes in response to zinc nutrition. J. Plant Physiol., 159: 1151-1153.
53. Tsekova, K. and D. Galabova, 2003. Phosphatase production and activity in copper (II) accumulating *Rhizopus delemar*. Enzyme Microb. Technol., 33: 926-931.
54. Mohamed, A.A., H.S. El-Beltagi and M.M. Rashed, 2009. Cadmium stress induced change in some hydrolytic enzymes, free radical formation and ultrastructural disorders in radish plant. Electron. J. Environ. Agric. Food Chem., 8: 967-983.

55. Keshav Raj, K., J. Deepak, W. Ravindra, K. Sandhya and R. Derek, 2001. Pyrethroid resistance and mechanisms of resistance in field strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol., 94: 253-263.
56. Foy, C.D., R.L. Chaney and M.C. White, 1978. The physiology of metal toxicity in plants. Ann. Rev. Plant Physiol., 29: 511-566.
57. Prassad, D.D.K. and A.R.K. Prassad, 1987. Altered δ-aminolevulinic acid metabolism by lead and mercury in germinating seedlings of Bajra (*Pennisetum typhoideum*). J. Plant Physiol., 127: 241-249.
58. Chen, Y.X., Y.F. He, Y.M. Luo, Y.L. Yu, Q. Lin and M.H. Wong, 2003. Physiological mechanism of plant roots exposed to cadmium. Chemosphere, 50: 789-793.
59. Lummerzheim, M., M. Sandroni, C. Castresana, D.D. Oliveira, M.V. Montagu, D. Roby and B. Timmerman, 1995. Comparative microscopic and enzymatic characterization of the leaf necrosis induced in *Arabidopsis thaliana* by lead nitrate and by *Xanthomonas campestris* pv. *Campestris* after foliar spray. Plant Cell Environ., 18: 499-509.
60. Edreva, A. and E. Apostolova, 1989. Manganese toxicity in tobacco. A biochemical investigation. Agrochimica, 33: 441-451.
61. Baccouch, S., A. Chaoui and E. El Ferjani, 1998. Nickel toxicity: Effects on growth and metabolism of maize. J. Plant Nutr., 21: 577-588.
62. Giertych, M.J., P. Karolewski and L.O. de Temmerman, 1999. Foliage age and pollution alter content of phenolic compounds and chemical elements in Pinus nigra needles. Water Air Soil Pollut., 110: 363-377.
63. Saffar, A., M.B. Bagherieh Najjar and M. Mianabadi, 2009. Activity of antioxidant enzymes in response to cadmium in *Arabidopsis thaliana*. J. Biol. Sci., 9: 44-50.
64. Mukherji, S. and B.D. Gupta, 1972. Characterization of copper texicity in lettuce seedlings. Physiol. Plant., 27: 126-129.
65. Pereira, G.J.G., S.M.G. Molina, P.J. Lea and R.A. Azevedo, 2002. Activity of antioxidant enzymes in response to cadmium in *Crotalaria juncea*. Plant Soil, 239: 123-132.
66. Lee, K.C., B.A. Cunningham, G.M. Paulsen, G.H. Liang and R.B. Moore, 1976. Effects of cadmium on respiration rate and activities of several enzymes in soybean seedlings. Physiol. Plant., 36: 4-6.
67. Maier, R., 1978. Studies on the effect of lead in the acid phosphatase in *Zea mays* L. Zeitschrift Fuer Pflanzenphysiologie, 87: 347-354.
68. De Varennes, A. and I. Carvalho, 1993. Trace elements and isoenzyme activities in white lupin. Optim. Plant Nutr., 53: 471-476.
69. Mukherjee, S., S. Mukherjee, P. Bhattacharyya and A.K. Duttagupta, 2004. Heavy metal levels and esterase variations between metal-exposed and unexposed duckweed *Lemna minor*: Field and laboratory studies. Environ. Int., 30: 811-814.
70. Karataglis, S., L. Symeonidis and M. Moustakas, 1988. Effect of toxic metals on the multiple forms of esterases of *Triticum aestivum* cv. Vergina. J. Agron. Crop Sci., 160: 106-112.
71. Ruiz, J.M., P.C. Garcia, R.M. Rivero and J. Romero, 1999. Response of phenolic metabolism to the application of carbendazim plus boron in tobacco. Physiol. Plant., 106: 151-157.
72. Neuman, D. and U. zur Nieden, 2001. Silicon and heavy metal tolerance of higher plants. Phytochemistry, 56: 685-692.