Pol. J. Environ. Stud. Vol. 21, No. 6 (2012), 1721-1730

Original Research

Antioxidative Defense System in *Pisum sativum* Roots Exposed to Heavy Metals (Pb, Cu, Cd, Zn)

Arleta Malecka[1]*, Aneta Piechalak[1], Anna Mensinger[1], Anetta Hanć[2], Danuta Barałkiewicz[2], Barbara Tomaszewska[1]

[1]Department of Biochemistry, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Collegium Biologicum, Umultowska 89, 61-614 Poznań, Poland.
[2]Department of Trace Element Analysis by Spectroscopy Method, Faculty of Chemistry, Adam Mickiewicz University, Grunwaldzka 6, 60-780 Poznań, Poland

Received: 23 January 2012
Accepted: 15 May 2012

Abstract

Heavy metals (Cd, Pb, Cu, Zn) absorbed by roots brought about oxidative stress conditions through ROS production ($O_2^{-\cdot}$, H_2O_2) for pea plants cultivated hydroponically for 96 h on a Hoagland medium with the addition of 50 µM: $CdCl_2$, $Pb(NO_3)_2$, $CuSO_4$, and $ZnSO_4$. We shows, using laser ablation ICP MS, that Cd, Cu, Pb, and Zn elements are located along a cross-section through the roots of pea plants. We observed increasing activities of antioxidative enzymes (SOD, CAT, GR) in oxidative stress conditions. We have shown changes in redox state (GSH/GSSG) in pea root grown with Pb, Cu, Cd, and Zn.

Keywords: antioxidants, antioxidative enzymes, heavy metals, oxidative stress

Introduction

Heavy metals derived from various anthropogenic sources (industrial effluents and wastes, urban runoff, sewage treatment plants, boating activities, agricultural fungicide runoff, domestic garbage dumps, and mining operations) have progressively affected the environment and ecosystems [1]. Of major concern with respect to plant exposure (as well as the human food-chain) are the metalloids: arsenic (As), selenium (Se), and the metals cadmium (Cd), mercury (Hg), and lead (Pb) [2]. Heavy metals can influence the physical and chemical processes in living organisms by directly inducing reactive oxygen species (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 from zinc-finger motifs of transcription factors [3-5].

*e-mail: arletam@amu.edu.pl

Cadmium is one of the most toxic heavy metals in plants due to its high solubility in water and phytotoxicity [2, 6]. Cd toxicity causes leaf rolls, chlorosis, and reduced growth of plants [7, 8], and a reduction of several physiological processes including photosynthesis, respiration, and transpiration. Cadmium is known to affect cellular processes such as: membrane damage and disruption of membrane electron transport, interaction with nucleic acids, and reduction of mitotic activity [6, 8, 9].

Lead (Pb) exists in many forms in natural sources throughout the world. According to the U.S. Environmental Protection Agency (EPA), Pb is the most common heavy metal contaminant in the environment [10]. Lead concentration over 30 $\mu g \cdot g^{-1}$ dry biomass is toxic to most plant species. Pb toxicity leads to inhibition of seed germination root and shoot growth, disturbed mineral nutrition, and inhibition of photosynthetic activity [11, 12]. The effect of Pb depends on concentration, type, and properties of soil and plant species [12].

Zinc is toxic at concentrations above between 100 and 300 μg·g^{-1}. Visible Zn deficiency symptoms range from the early appearance of old leaves or slight yellowing of younger leaves to the formation of yellow chlorotic or even necrotic areas on leaves. Critical Zn deficiency concentrations in leaves are given as 15 to 20 μg·g^{-1} dry biomass. Severely Zn-deficient plants appear stunted and exhibit reduced elongation and tip growth. Zn also has a central role in the cytoplasm, primarily in the process of translation and as a cofactor of a number of tRNA synthetases [13]. After Marschner [14] in most crops, the typical leaf Zn concentration required for adequate growth approximates 15-20 mg Zn·kg^{-1} DW (15-20 μg·g^{-1}). Toxicity symptoms usually become visible at $Zn_{leaf} > 300$ mg Zn·kg^{-1} leaf DW, although some crops show toxicity symptoms at $Zn_{leaf} < 100$ mg Zn·kg^{-1} DW [14, 15].

Copper is an essential trace element for all higher plants, and has several roles in metabolic processes in plants. This metal takes part in numerous redox reactions in biological systems and there is the metal component in Cu oxidases. Molecularly, little is known about the effects of and responses to Cu deficiency in plants. The concentration of Cu in vegetative plant parts is between 1 and 5 μg·g^{-1} dry biomass. Cu levels above which toxicity symptoms can be observed in plant tissues are in the range of 20 to 30 μg·g^{-1} dry biomass [13, 16].

Increasing contamination of the environment with heavy metals has resulted in a growing interest of biologists in understanding the mechanisms of plant resistance to this kind of stress factor [11, 16-18]. One of the effects of heavy metals is increased generation of ROS, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), which results in oxidative stress. ROS are generated in different compartments of plant cells, including cell wall (peroxidases and polyamine oxidases), cytoplasm, peroxisomes (xanthine oxidase), mitochondria, and chloroplast [5, 19]. At high concentrations ROS damage major cell components: proteins, lipids, and nucleic acids. ROS can function as important effectors and regulators of plant PCD (programmed cell death). Plant cells can tolerate ROS by endogenous protective mechanisms involving antioxidant molecules (ascorbate, cysteine, glutathione, phytochelatins, and α-tocopherol) and enzymes (superoxide dismutases SOD; catalase CAT; glutathione reductase GR), imbalance between production and quenching of ROS leads to plant damage breaking down the defense system of cells [20].

Thus, the grown of some organisms in heavy-metal polluted environments depends on their ability to synthesize different heavy-metal chelating molecules and activate efficient antioxidant mechanisms. In this study we examined the changes in $O_2^{\bullet-}$ and H_2O_2 generation together with changes in activity of some antioxidative enzymes in pea roots treated with cadmium, lead, cooper, and zinc ions during 4 days. Simultaneously, the root cells state redox GSH/GSSG was also determined.

Materials and Methods

Plant Material

Pea seedlings (*Pisum sativum* L., cv. Bohun) were grown hydroponically on the Hoagland medium for 72 h in a growth room with 16/8 h photoperiod, day/night at room temperature and light intensity of 82 μmol·m^{2}·s^{-1}. Then, the medium was changed into 100 x-diluted Hoagland medium and the $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, $ZnSO_4$ solution at a concentration of 50 μM was applied. The roots were cut off after 0, 24, 48, 72, and 96 h of cultivation. Heavy metals accumulated on the surface of roots were rinsed with 10 mM of $CaCl_2$ and 10 mM EDTA.

Index of Tolerance

The index of tolerance (IT) was calculated according to Wilkins [21]:

$$\mathrm{IT} = \frac{\text{average length of roots in tested solution}}{\text{average length of roots in control}} \times 100\%$$

Laser Ablation

Laser Ablation system CETAC 500 (model LSX-500, CETAC Technologies, USA) coupled to a quadruple ICP-MS (model Elan 9000 DRC II, Perkin-Elmer Sciex, Canada) was use to evaluate Pb, Cd, P, K, Ca, Mg, and Cu distribution immediately in thick (about 3 mm) segments of root plants. The root tissues were ablated along the predefined line across the sample surface. In order to minimize the variability that occurs in the ablating process, we used an internal standard. In the present work, to compensate for signal fluctuation the ^{13}C isotope was used as a natural internal standard for mapping selective elements [22]. A standard reference material (SRM NIST 610 – a quartz plate) with known Pb, Cd, P, K, Ca, Mg, and Cu concentration was used to optimize the LA ICP MS method.

Superoxide Anion Determination

Superoxide anion content was determined according to Doke [23]. The pea roots (0.5 g) were placed in the test tubes and filled with 7 mL of mixture containing 50 mM phosphate buffer (pH 7.8), 0.05 % NBT (nitro blue tetrazolium), and 10 mM of NaN_3. Next, the test tubes were incubated in the dark for 5 min, and then 2 mL of the solution were taken from the tubes, heated at 85ºC for 10-15 min, cooled in ice for 5 min, and the absorbance was measured at 580 nm against the control.

Hydrogen Peroxide Determination

Hydrogen peroxide content was determined using the method described by Becana et al. [24]. The decrease of absorbance was measured at 508 nm. The reaction mixture

contained 50 mM phosphate buffer (pH 8.4), reagent containing 0.6 mM 4-(-2 pyridylazo) resorcinol, and 0.6 mM potassium-titanium oxalate in (1:1). The corresponding concentration of H_2O_2 was determined against the standard curve of H_2O_2 (0.5-25 μM).

In vivo Detection of Superoxide Anion and Hydrogen Peroxide

After 24-hour cultivation with Pb, Cu, Cd, and Zn ions at 50 μM concentrations, pea roots were submerged for 12 h in 100 μM of $CaCl_2$ containing 20 μM of dihydroethidium (DHE), adopting the method of Yamamoto et al., [25] and in 4 μM dichlorodihydrofluorescein diacetate (DCFH-DA) in 5 mM dimethyl sulfoxide (DMSO), using the method modified according to Afzal et al., [26]. After rinsing with 100 μM of $CaCl_2$ or 50 mM phosphate buffer (pH 7.4) the roots were observed with a confocal microscope (Zeiss LSM 510, Axioverd 200 M, Jena, Germany) equipped with a filter set No. 10, excitation 450-490 nm, emission 520 nm or more.

Determination of Antioxidative Enzyme Activities

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT, adopting the method of Beauchamp and Fridovich [27]. The reaction mixture contained 13 μM riboflavin, 13 mM methionine, 63 μM NBT, and 50 mM potassium phosphate buffer (pH 7.8). Absorbance at 560 nm was then measured. One unit of SOD activity has been defined as the amount of enzyme, which causes a 50% decrease of the inhibition of NBT reduction. The activity of CAT was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi [28] in 50 mM phosphate buffer (pH 7.0) containing 5 mM H_2O_2 and enzyme extract. CAT activity was determined using the extinction coefficient of 36 $mM^{-1}\cdot cm^{-1}$ for H_2O_2. GR was assayed according to Yannarelli [29]. Assay mixture in 1 ml contained 100 mM-phosphate buffer, pH 7.8, 2 mM EDTA, 0.2 mM NADPH, and 0.5 mM GSSG. The GR activity was determined by monitoring the oxidation of NADPH at 340 nm. One unit of glutathione reductase was defined as the amount of enzyme-catalyzing oxidation of 1 μmol of NADPH/min.

GSH and GSSG levels

Total levels of thiols were measured spectrophotometrically by monitoring the reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, after the method of Griffith [30]. For the measurements of total glutathione (GSH + GSSG), 150 μL neutralized extract were added to 1.2 ml of 0.3 mM NADPH, 150 μl of 6 mM DTNB and 1 unit of glutathione reductase (GR). For oxidized glutathione (GSSG) determination, 150 μL of neutralized root extract were incubated with 2 μL of 2-vinylpyridine for 1h at 25ºC, and then added to 1.2 mL of 0.3 mM NADPH, 100 μL of 6 mM DTNB, and 1 unit of GR. The concentration of GSH was calculated as the difference between total glutathione and GSSG.

Table 1. Tolerance Index for *Pisum sativum* roots grown in Hoagland medium in the presence of 50 μmol : Pb, Cu, Cd, and Zn for 96 hours. Mean values and SD were calculated from three independent experiments.

	IT [%] 48 [h]	IT [%] 72 [h]	IT [%] 96 [h]
Pb [25 mM]	80.2±6.1	75.2± 4.9	71.1± 4.2
Cu [25 mM]	76.3±5.8	69.1±4.1	61.9±3.7
Cd [25 mM]	80.8±7.2	71.7±4.5	62.4±3.9
Zn [25 mM]	107.7±9.8	101.3±8.9	99.5±7.7

Protein Quantification

Total soluble protein contents were determined according to the method of Bradford [31] using the Bio-Rad assay kit with bovine serum albumin as a calibration standard.

Results

Pisum sativum seedlings were grown hydroponically in a medium supplemented with Pb, Cu, Cd, and Zn ions. The results shown in Table 1 demonstrate a varying tolerance of the seedlings to a 50 μM concentration of the metal ions. In comparison to the control plants grown in a medium without heavy metals, the appearance of the roots and their shape were significantly changed by the presence of the trace elements, especially when treated with Cu and Cd. The color of the roots changed gradually under the influence of heavy metal ions, from creamy white to dark brown, which probably was caused by an intense suberification or an overproduction of phenol substances. Differences in IT values between the metal treatments indicate that pea roots exhibit the highest sensitivity to Cu (IT=61.9%) and Cd (IT=62.4%) ions, and the highest resistance to Zn (IT=99.5%) (Table 1).

The LA-ICP-MS analysis showed that lead and cadmium were mostly localized in the epidermis, exodermis, and endodermis of the treated pea roots (Fig. 1). The regular level of Cu ions was observed in the tissues of the control roots. In treated roots the copper ions were located mainly in the epidermis and cortex. Already after 24 h of treatment with Cu ions a 100-fold increase of metal signal intensity was observed when compared to the control plants. In pea seedlings cultivated with 0.1 mM $ZnSO_4$, the LA analysis showed an equal dislocation of zinc ions in the root tissues. A 15-20 times rise of Zn- signal intensity was noted in comparison to the control plants. Cadmium accumulation in roots of the the control plants reached 0.17±0.06 $mg\cdot kg^{-1}$ DW. Linear increases of heavy metals accumulation in the treated plants were observed during exposition to Cu, Zn, Cd, and Pb (Fig.1). Accumulation analyses showed that zinc ions were the most absorbed and cadmium ions the

least. After 96 h of cultivation, the metal ion concentration in roots of the treated plants were: Cu – 20.9 mg·kg^{-1} (d.m.), Zn – 5,725.68 mg·kg^{-1} (d.m.), Cd -2.75 mg·kg^{-1} (d.m.), and Pb – 20.98 mg·kg^{-1} (d.m.).

A fast generation of superoxide anion was observed in the extracts of roots cultivated on the Hoagland medium with addition of 50 μM Pb, Cu, Cd, and Zn ions (Fig. 2). The level of $O_2^{-\cdot}$ reached the maximum after 24 h and increased twelvefold with Cu, tenfold with Cd, sevenfold with Pb, and fivefold with Zn. After 48-96 h of cultivation, we noted a gradual decrease in the $O_2^{-\cdot}$ level, which appeared most probably due to activation of the enzymatic defense system (SOD, CAT, GR).

The results of spectrophotometric studies on the level of $O_2^{-\cdot}$ in the pea roots were confirmed by the studies applying confocal microscopy (Fig. 3). The most intensive fluorescence was observed in the pea roots, treated for 24 h with 50 μM Cu and Cd, while in the pea roots treated with 50 μM Pb and Zn for 24 h the fluorescent signal was slightly lower. We found the $O_2^{-\cdot}$ mostly in the apex and epidermis of roots treated with heavy metals.

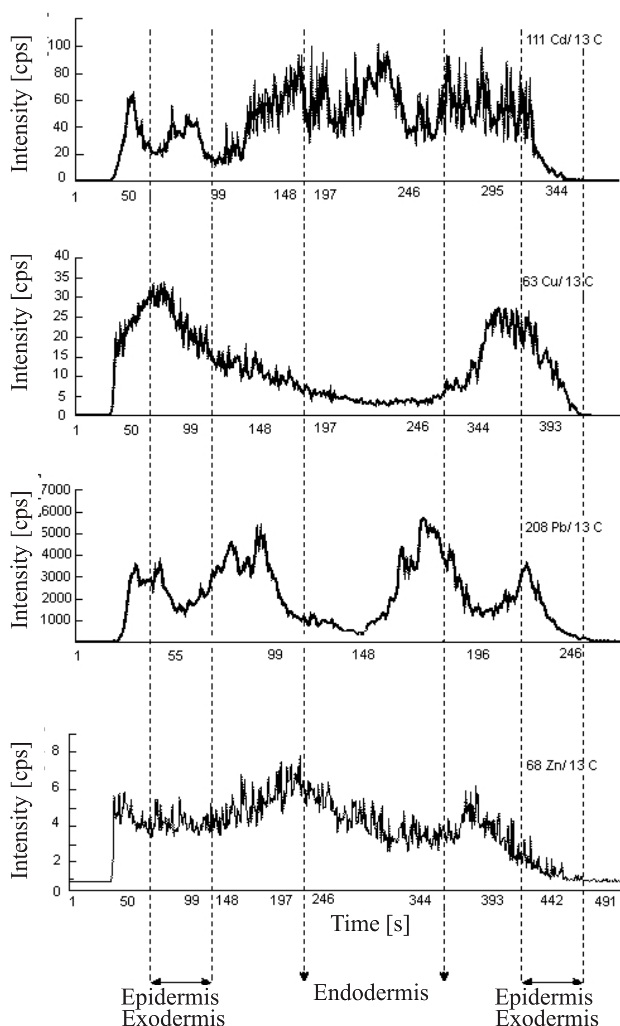

Fig. 1. Laser ablation ICP MS spectra showing the Cd, Cu, Pb, and Zn element locations along a cross-section through pea plant roots.

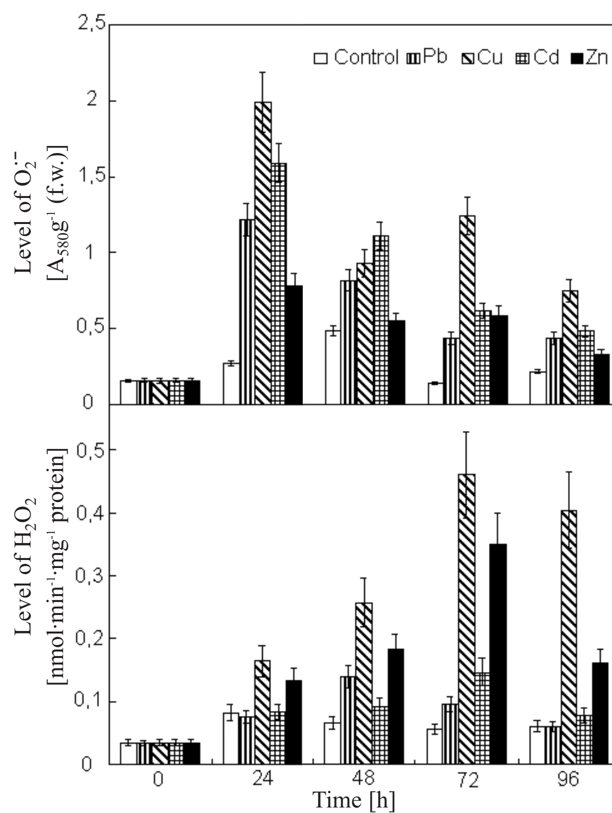

Fig. 2. Levels of $O_2^{-\cdot}$ (A_{580} g^{-1} FW) and H_2O_2 (nmol·g^{-1} FW) in roots of *Pisum sativum* grown hydroponically in Hoagland medium: control plants and in the presence of 50 μmol: Pb, Cu, Cd, and Zn for 96 hours. Mean values and SD were calculated from three independent experiments.

Simultaneously, we defined the changes in the hydrogen peroxide concentration in pea root tissues (Fig. 2). We observed a fast increase in hydrogen peroxide in the plants treated with four different heavy metal ions for 72 hours. The level of H_2O_2 in the roots reached the maximum and increased eightfold after 72 h of cultivation with Cu, sixfold with Zn, twofold with Pb, and threefold with Cd ions. In roots of the control plants, H_2O_2 was generated throughout the cultivation at the same low level. Similarly, as in the case of studies on the level of $O_2^{-\cdot}$, the H_2O_2 level was also confirmed by the studies applying confocal microscopy (Fig. 3). The most intensive fluorescence was observed in the pea roots, treated for 24 h with 50 μM Cd and Cu. The signal in the pea roots, treated with 50 μM Pb and Zn for 24h, was slightly lower.

The activities of SOD, CAT, and GR were always higher in the plants treated with heavy metals in comparison to the control plants (Fig. 4). In the extract of pea roots treated with 50 μM of lead nitrate, the SOD activity was at maximum after 48 h of cultivation, reaching 150% of the control value. In the roots exposed to 50 μM copper, the SOD activity increased about 100% during the first 24 h, and it was throughout the cultivation at the same level. After three days of cultivation with Cd, the SOD activities were the highest – 2.5-fold higher than in the control plants.

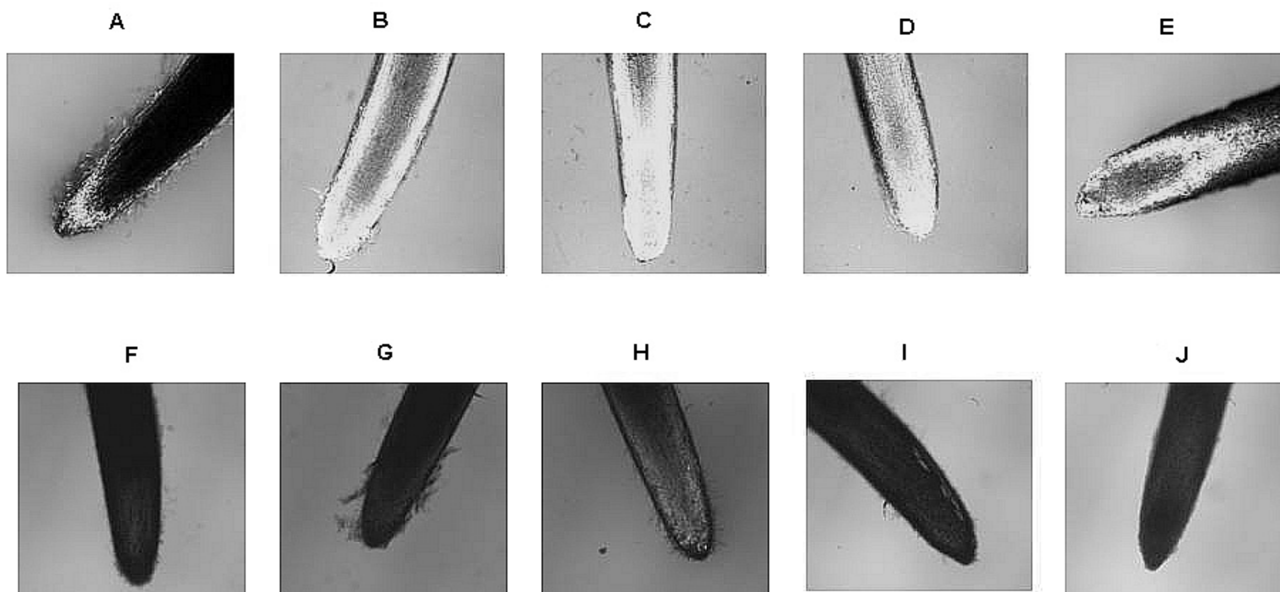

Fig. 3. Lead induced $O_2^{-\cdot}$ and H_2O_2 production in pea roots. Fluorescent images of pea roots grown in Hoagland medium in the presence of 50 μmol: Pb, Cu, Cd, and Zn for 24 hours: (A) control roots stained with DHE for 12 h; roots treated with heavy metals and stained with DHE for 12 h: (B) $Pb(NO_3)_2$ (C) $CuSO_4$ (D) $CdCl_2$ (E) $ZnSO_4$; (F) control roots stained with DCFH-DA for 4 h; roots treated with heavy metal and stained with DCFH-DA for 4 h:(G) $Pb(NO_3)_2$ (H) $CuSO_4$ (I) $CdCl_2$ (J) $ZnSO_4$. The bar indicates 1 μm.

The activity of SOD in the pea plants treated with Zn increased gradually during 72 h of the cultivation time, and it was the highest after 72 and 96 hours in comparison with all the experiments.

The smallest changes of CAT activity were observed in the pea roots treated with lead (Fig. 4). After 24 h of cultivation the CAT activity was 1.5-fold higher than in the control plants. The highest CAT acivity was observed in the plants exposed for 24-48 hours to Cu and Zn ions. Simultaneously, we could observe a low level of H_2O_2. On the next day, a gradual decrease of CAT activity was measured.

In the plant roots cultivated with Zn ions, the level of GR activity was slightly higher, only about 37% higher than in the control plants (Fig. 4). Cd caused a dramatic increase (2.3 times) of GR activity during the first 24 hours of treatment. In the roots of plants treated with Cu or Pb ions a progressive increase of GR activity was determined. After 96 hours the Cu ions induced 2,4 times increase of the enzymatic activity. Among all applied heavy metals, Pb was the strongest inductor of root GR activity. After 96 h cultivation with lead, GR activity was over 3 times higher than in roots of the control plants.

During exposure to heavy metals we observed a different response of pea plants. Only in the roots exposed to Cu ions did an increase of the reduced glutathione level take place when compared to the control plants. In these plants we observed also the highest increase of GSSG and the most significant changes in the GSH/GSSG ratio. During the first 24 hours after metal ion application, the GSSG level increased almost 5 times when compared to the control, while in the other variants about 4 times. The rise of GSH amount was also registered after 24 hours in the roots

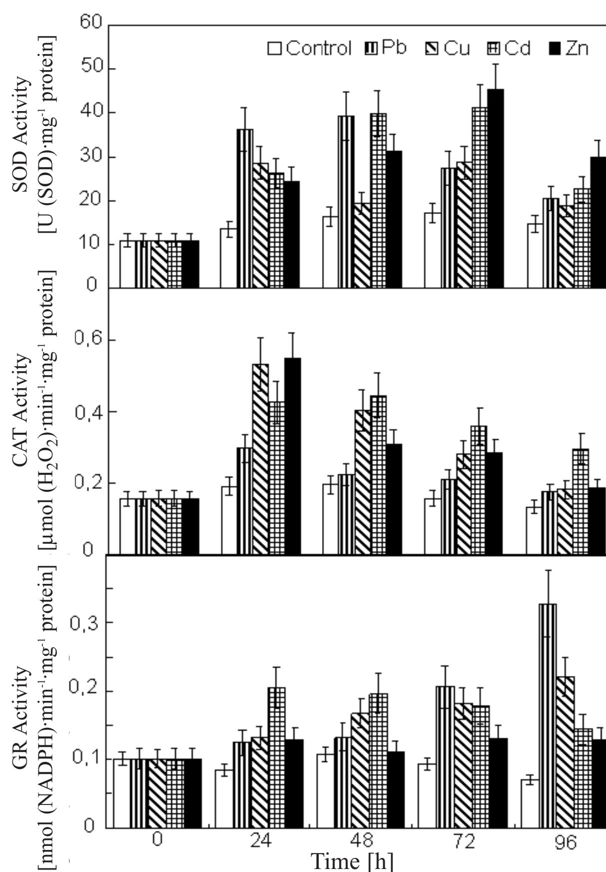

Fig. 4. Activity of: SOD (U SOD mg^{-1} protein), CAT ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein), and GR ($nmol \cdot NADPH \cdot min^{-1} \cdot mg^{-1}$ protein) in pea roots grown hydroponically in Hoagland medium: control plants and in the presence of of 50 μmol: Pb, Cu, Cd, and Zn for 96 hours. Mean values and SD were calculated from three independent experiments.

exposed to Zn^{2+}. But during a further 3 days growth of the GSH level was slightly lower than in the control. The amount of GSSG in these plants rose in the first hours, but afterward was measured at the control level. In the pea plants treated with Pb and Cd ions the quantity of GSH decreased, while the GSSG level increased about 4 times when compared to the control after 24 h of exposure. After 4 days of cultivation with lead and cadmium the amount of GSH was slightly lower, while the GSSG level was 2 times higher than in the control plants.

The level of total glutathione in the pea roots treated with four different heavy metals decreased during the first 24 h of cultivation (Table 2), probably due to the synthesis of phytochelatins, which are the main detoxification system in plants. At the same time, an increased level of oxidized GSSG was observed, which proves the occurrence of the oxidative stress conditions in the pea roots cultivated in the presence of Pb, Cu, Cd, and Zn. After 24 h of pea's exposure to heavy metals, a simultaneous decrease of GSSG level was observed in the pea plants.

An excellent indicator of the redox state in plant cells is a GSH/GSSG ratio (Table 2). A high amount of oxidized glutathione in the roots of the treated pea plants proves cellular oxidative stress, especially during the 24-48 h of heavy metals presence. The highest decrease in GSH/GSSG ratio was observed in plants treated with Cu ions during 4-day cultivation.

Discussion

Copper and zinc ions are essential for the normal growth and development of all organisms, as they serve as a cofactor for many physiological processes. However, they can be toxic at excessive levels. Lead and cadmium are nonessential elements and are toxic for plants, even at small levels. Only Zn did not inhibit the growth of plants, and in the first 48 h of cultivation this metal caused an increase in root length. Therefore, the highest IT values were found in plants exposed to Zn, achieving 108% after 24 h of cultivation.

According to Cakmak [32], Zn performs various important roles such as protecting cells from the damaging reactions caused by ROS. Palazzo and coauthors [33] observed grass growth on soil, rich in Zn, and found that the roots of these plants were longer and more heavy in comparison to plants grown in soil poor in this metal. For remaining heavy metals (Pb, Cu, Cd) the IT decreased to about 60% during the cultivation time. Other authors have shown a negative influence of Pb [10], Cu [34], and Cd [35, 36], especially on the growth and morphology of the treated plants. The reports of Wierzbicka [37] and Liu [10] indicated that heavy metals, like lead ions, may cause a water deficit by disturbing the water balance, which caused a poorer growth and development of the treatment metal-exposed plants. Inhibition of growth and reduction of biomass belong to the main responses of higher plants to heavy metal toxicity. The decline in biomass production could be explained, in part, due to the inhibition of both cell division and cell elonga-

Table 2. Level of total glutathione (GSH + GSSG) ($nmol \cdot SH \cdot g^{-1}$ FW) and the changes of the redox state (GSH/GSSG) in roots of *Pisum sativum* grown hydroponically in Hoagland medium (control plants and in the presence of 50 µmol): Pb, Cu, Cd, and Zn for 96 hours. Mean values and SD were calculated from three independent experiments.

	24 h			48 h			72 h			96 h		
	GSH+GSSG	GSSG	GSH/GSSG	GSH+GSSG	GSSG	GSH/GSSG	GSH+GSSG	GSSG	GSH/GSSG	GSH+GSSG	GSSG	GSH/GSSG
Control	2469±211	25±4	98±9	2031±198	49±5	40±4	1906±172	26±4	73±7	2119±209	24±4	86±8
Pb [50 mM]	1813±173	103±9	17±2	1938±166	94±8	20±3	1906±187	69±6	27±4	1863±182	59±6	30±4
Cu [50 mM]	2463±224	119±14	20±4	2438±213	132±16	17±2	2313±212	97±9	23±3	2250±215	72±6	30±4
Cd 50 [mM]	2000 ±189	92±8	21 ±3	1756±156	89±8	19±2	1844±175	56±5	32±4	1888±189	36±5	51±5
Zn [50 mM]	2219±219	108±11	20 ±3	2063±187	81±7	24±4	1906±187	41±5	46±5	1925±198	28±4	69±7

tion by heavy metals. Siddiqui et al. [1] and Zhang et al. [8] found that inhibition of root growth of *Pisum sativum* and *Vicia faba* treated with Cd was a result of the reduced mitotic activity of apical meristem and disturbed cell elongation in the extension root region. In addition, other visual effects of Cd toxicity, such as root browning and reduction, or disappearance of lateral roots, were observed by other authors [1, 38]. Khatun and coauthors [16] observed a net decrease in the leaf fresh and dry weight, shoot length, and pigment concentrations in *Withania somnifera* exposed to Cd. Likely, the inhibition of pea root and shoot growth, caused by 50 μM Cu, was observed elsewhere [35].

The present study confirms the occurrence of oxidative stress, which is reflected by an increased synthesis of ROS in the root cells of *Pisum sativum* affected by Pb, Cu, Cd, and Zn.

Superoxide anion is one of the major ROS, which can represent the intracellular degree of oxidative stress in organisms under biotic and abiotic stresses. Hydrogen peroxide is another important ROS, which also damages the plasma membrane lipids and other biomolecules when over-prodeced [39].

We found that in the pea root cells exposed to Pb, Cu, Cd, and Zn ions, there was an increased level of both $O_2^{-\cdot}$ and H_2O_2. The highest level of ROS was observed in plants treated with Cu and Zn that have prooxidative specifity, and when exposed to Cd, which is very phytotoxic. An increase of $O_2^{-\cdot}$ level was also shown by other authors [40] using confocal microscopy in lupine roots, treated with 1500 μM $Pb(NO_3)_2$. Ortega-Villasante et al. [41] showed an increase in ROS level in *Medicago sativa* roots, grown in the presence of 30 μM Cd and Hg. Wang et al. [6] have compared the level of H_2O_2 in metal accumulators (*Thlaspi caerulescens* and *Brassica juncea*) and non-accumulator plants (*Nicotiana tabacum*) exposed to cadmium stress. They have ascertained that the level of H_2O_2 in tobacco increased by 68% on the fourth day after 200 μM $CdCl_2$ addition, while the increases of H_2O_2 in *T. caerulescens* and Indian mustard were at about 25%, respectively, in comparison to the control. These results were in correlation with increases in the activity of antioxidative enzymes, such as SOD and CAT. An induced generation of H_2O_2 and $O_2^{-\cdot}$ was observed in the leaves in *Sedum alfredii* and authors supposed that the processes, such as NADPH-oxidase regulation, the changes in cytosolic Ca^{2+}, and protein dephosphorylation might be involved in Zn-induced ROS production [42].

According to Mittler et al. [43], plants with enhanced activities of the antioxidant enzymes have been shown to be tolerant to oxidative stress.

Along with an increase of ROS production, we could observe a definite increase in the activity of the antioxidative enzymes (SOD, CAT, and GR) in the root cells of *Pisum sativum*, exposed to four different heavy metals.

In plants treated by Pb and Cu ions after 24 hours we observed a rapid increase in SOD activity, which converts superoxide $O_2^{-\cdot}$ into H_2O_2, whereas Cd and Zn treatment resulted in a gradual increase of SOD with its maximum after 72 hours. The activity of CAT was the highest in plants after 24-48 hours of exposure to Cu and Cd ions. Pb affected the CAT activity only very slightly. In the roots of plants cultivated with all studied metals we detected an increase of the GR activity due to an enhanced ROS generation and GSH oxidation. Only in plants treated with Cd during further cultivation did we observe an inhibition and decrease of the GR activity. These results are confirmed by many authors [16, 44, 45], who reported a significant decrease of GR activity in response to heavy metals. Glutathione reductase contains a highly conserved disulphide bridge between Cys76 and Cys81 [46], which may undergo cleavage by heavy metals and decreases the level of GR activity. It is possible that this high concentration of Cd interacts with the cysteine thiol groups, disturbing the activity or even turnover of this enzyme.

Many authors have shown that heavy metals induced an increase of antioxidative enzyme activities. An increase in SOD, CAT, APX, and GR activities has been reported in Hg-treated *Medicago sativa* [47], SOD, APX, POD, and GR in Pb-exposed maize leaves [12], SOD, CAT POD, APX, and GPX in Cd-treated *Vicia faba* [8], and in tomato seedlings [48].

Other authors have shown that higher concentrations and a type of heavy metals or longer treatment bring about inhibition of the cellular metabolism and ROS production, which results in a decrease of enzyme activity at a higher intensity of oxidative stress.

A toxic effect of 100-200 μM of Cd was observed in *Lycopersicon esculentum*, but low concentrations of Cd (under 100 μM) lead to a generation of ROS and stimulation of the antioxidative defense system [49]. According to Mourato et al. [50] copper at high concentrations becomes phytotoxic, affecting plant development due to direct or indirect interference with numerous physiological processes. Verma and Dubey [51] showed that a high concentration of Pb (1 mM) inhibited CAT activity, since they observed a decrease in CAT activity in shoots. According to these reports, higher concentrations of lead or longer duration of the treatment bring about inhibition of the cell metabolism and H_2O_2 production, which result in a decrease of CAT activity at more intense lead stress. The hypothesis has been proven correct in our studies, carried out on pea cultivated with 1 mM of $Pb(NO_3)_2$, in which (with the use of electron microscopy) we showed the processes leading to a degradation of cellular membranes and organelles [20]. Jin et al. [42] showed that antioxidant activities change, depending on the ecotype of plant species. In the non- hyperaccumulating ecotype of *Sedum alfredii*, antioxidant enzyme activities, such as SOD, GPX, APX, and DHAR in both roots and leaves increased at 250 μM Zn. However, all enzymes studied in leaves of hyperaccumulating ecotype *S. alfredii* were at the peak when treated with 500 μM Zn.

SOD is the first frontline of defense mechanisms against ROS [50, 52, 53]. An increase of SOD activity in response to abiotic stress factors, including the following metals: Pb, Cu, Al, Cd, Mn, Fe, and Zn, occurs due to the

de-novo synthesis of enzymatic protein. It was shown that FeSOD overexpression in tobacco plants increased resistance to stress [54], whereas CAT is considered to be a key enzyme removing the toxic hydrogen peroxide. This, together with the increase of SOD activity, is thus indicative for the early activation of an antioxidative defense mechanism [50]. According to Gechev and coauthors [55], CAT plays a key role in tolerance to stress, since it may be a cellular "sink" of the produced H_2O_2. Gabara and others [56] postulated that CAT may be involved in the cell signaling process via controlling the H_2O_2 level. Inactivation of this enzyme brings about hydrogen peroxide accumulation and a change in the activity of the enzyme may induce oxidative damage, leading to PCD. It has been reported that when CAT activity is reduced, the activities of other ROS- scavenging enzymes increases as a compensatory mechanism [50].

According to Mazen [57] one of the possible mechanisms of heavy metals tolerance is the synthesis and accumulation of amino acids, whose contribution might be important to metal accommodation in the earlier stages of exposure of plants to metals before other mechanisms start to work.

In conclusion, Pb, Cu, Cd, and Zn ions induced an oxidative stress in the treated pea roots. We found big differences between plants treated with four heavy metals, regarding the generation of ROS and activation of antioxidative systems, caused by a varying accumulation of heavy metals and their translocation from the roots to the shoot. The least toxic trace element for pea plants is Zn. In plants treated with Zn we observed a conspicuous increase activity of SOD, CAT, and GR against relatively low levels of ROS. The GSH/GSSG ratio indicates a comparatively low oxidative stress in plant cells. Plants exposed to Zn ions showed the highest IT value. Cu and Cd were the most toxic for plants, became they caused large generation of ROS in the pea cells. In these plants we observed about 50% smaller GSH/GSSG ratio in comparison to the control plants and a high level of GSSG, suggesting a large oxidative stress. In the *Pisum sativum* roots exposed to four heavy metals, we observed the highest accumulation of Zn and Cu. Our results proved that Cu is the fastest trace element translocated from roots to shoots, at about 50% during a 4-day cultivation. The remaining metals were translocated up to: Zn – 27%, Cd – 15%, and Pb – 5% (data not shown). According to Wierzbicka et al. [58], Zn is less toxic for *Allium cepa* L. than lead and much less toxic than cadmium, because an excess of trace elements has always accompanied plants on soils of various composition, so they might have evolved effective and widespread defense mechanisms. Wierzbicka et al. [58] showed different distributions of the Cd and Pb in cells using electron microscopy. Both of these metals enter the plant cells, but the concentration of Pb in the form of insoluble deposits was higher than with Cd. Lead caused rapid and effective destruction. Other authors [59] have shown the reduction of toxic effects and bioaccumulation of one heavy metal in plant tissues in the presence of another one. In our study plants were grown hydroponically in Hoagland solution, which contained microelements such as Zn. This heavy metal may influence Cd translocation in *Pisum sativum*.

Thus, the response of pea plants to Pb, Cu, Cd, and Zn ions seems to depend on many factors: the presence of other heavy metals in the environment, mineral content, plant species, condition, etc.

Acknowledgements

This work was supported by NCN grant No. 3811/B/P01/2010/38.

References

1. SIDDIQUI S., MEGHVANSI M.K., WANI M.A., JABEE F. Evaluating cadmium toxicity in the root meristem of *Pisum sativum* L. Acta Physiol. Plant **31**, 531, **2009**.
2. CLEMENS S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochemie **88**, 1707, **2006**.
3. HARTWING A. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxidative Redox Signal **3**, 625, **2001**.
4. BARALKIEWICZ D., KOZKA M., KACHLICKI P., PIECHALAK A., TOMASZEWSKA B. Analysis of oxidized and reduced phytochelatins in pea and lupin plants using HPLC/MS. Int. Environ. Chem. **88**, 979, **2008**.
5. MALECKA A., PIECHALAK A., TOMASZEWSKA B. ROS production and antioxidative defense system in pea root cells treated with lead ions. Part 1. The whole roots level. Acta Physiol. Plant. **31**, 1053, **2009**.
6. WANG Z., ZHANG Y., HUANG Z., HUANG L. Antioxidative response of metal-accumulator and non-accumulator plants under cadmium stress. Plant Soil **310**, 137, **2008**.
7. PANDA S.K., PATRA K.P. Effect of salicylic acid potentiates cadmium-induced oxidative damage in *Oryza sativa* L. leaves. Acta Physiol. Plant. **29**, 567, **2007**.
8. ZHANG S., ZHANG H., QIN R., JIANG W., LIU D. Cadmium induction of lipid peroxidation and effects on root tip cells and antioxidant enzyme activities in *Vicia faba* L. Ecotoxicology **18**, 814, **2009**.
9. MOUSSA H.R., EL-GAMAL S.M. Effect of salicylic acid pretreatment on cadmium toxicity in wheat. Biol. Plant. **54**, 315, **2010**.
10. LIU D., ZOU J., MEN Q., ZOU J., JIANG W. Uptake and accumulation and oxidative stress in garlic (*Allium sativum* L.) under lead phytotoxicity. Ecotoxicology **18**, 134, **2009**.
11. QURESHI M.I., QADIR S., ZOLLA L. Proteomics-based dissection of stress-responsive pathways in plants. J. Plant Physiol. **164**, 1239, **2007**.
12. EKMECI Y., TANYOLAC D., AYHAN B. A crop tolerating oxidative stress induced by excess lead: maize. Acta Physiol. Plant. **31**, 319, **2009**.
13. KRAMER U., CLEMENS S. Functions and homeostasis of zinc, copper and nickel in plants. Topics in Current Genet., Springer: Heidelberg, **14**, 215, **2005**.
14. MARSCHNER H. Mineral nutrition of higher plants. Academic Press: San Diego, pp 3-651, **1995**.

15. CHANEY R.L. Zinc phytotoxicity. In: Robson A.D. (Ed.):. Zinc in soils and plants. Kluwer Academic Press, Dordrecht, pp 135-50, **1993**.
16. KHATUN S., ALI M.B., HAHN E.J., PEAK K.Y. Copper toxicity in *Withania somnifera*: Growth and antioxidant enzymes responses of *in vitro* grown plants. Environ. Exp. Bot. **64**, 279, **2008**.
17. PIECHALAK A., TOMASZEWSKA B., BARAŁKIEWICZ D., MAŁECKA A. Accumulation and detoxification of lead ions in legumes. Phytochemistry **60**, 153, **2002**.
18. LEE S.H., ASHAN N., LEE K.W., KIM D.H., LEE D.G., KWAK S.S., KWON S.Y., KIMD T.H., LEE B.H. Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J. Plant Physiol. **164**, 1626, **2007**.
19. VIANELLO A., ZANCANI M., PERESSON C., PETRUSSA E., CASOLO V., KRAJNAKOVA J., PATUI S., BRAIDOT E., MARCI F. Plant mitochondrial pathway leading to programmed cell death. Physiol. Plant **129**, 242, **2007**.
20. MALECKA A., PIECHALAK A., MORKUNAS I., TOMASZEWSKA B. Accumulation of lead in root cells of *Pisum sativum*. Acta Physiol. Plant. **30**, 629, **2008**.
21. WILLKINS D.A. A technique for the measurement of lead tolerance in plants. Nature **180**, 37, **1957**.
22. HANC A., BARAŁKIEWICZ D., PIECHALAK A., TOMASZEWSKA B., WAGNER B., BULSKA E. An analysis of long-distance root-to-leaf transport of lead in *Pisum sativum* plants by laser ablation-ICP-MS. Int. J. Environ. Anal. Chem. **89**, 651, **2009**.
23. DOKE N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Mol. Plant Pathol. **23**, 345, **1983**.
24. BECANA M., APARICIO-TEJO P., IRIGOYEN J.J., SNACHEZ-DIAZ M. Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. Plant Physiol. **82**, 1169, **1986**.
25. YAMAMOTO Y., KOBAYASHI Y., DEVI R.S., RIKIISHI S., MATSUMOTO H. Aluminium toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol. **128**, 63, **2002**.
26. AFZAL M., MATSUGO S., SASAI M., XU B., AOYAMA K., TAKEUCHI T. Method to overcome photoreaction, a serious drawback to the use of dichlorofluorescin in evaluation of reactive oxygen species. Biochem. Biophys. Res. Commun. **304**, 619, **2003**.
27. BEAUCHAMP C., FRIDOVICH I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44**, 276, **1971**.
28. AEBI H.E. Catalase *in vitro*, Methods in Enzymol. **105**, 121, **1984**.
29. YANNARELI G.G., FERNANDEZ-ALVAREZ A.J., SANTA-CRUZ D.M., TOMARO M.L. Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. Phytochemistry **68**, 505, **2007**.
30. GRIFFITH O.W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. **106**, 207, **1980**.
31. BRADFORD M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- dye binding. Anal. Biochem. **72**, 248, **1976**.
32. CAKMAK I. Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytologist **146**, 185, **2000**.
33. PALAZZO A.J., CARY T.J., HARDY S.E., LEE C.R. Root growth and metal uptake in four grasses on zinc-contaminated soils. J. Environ. Quality **32**, 834, **2003**.
34. CHEN L.M., LIN C.C., KAO C.H. Copper toxicity in rice seedlings: Changes in antioxidative enzyme activities, H2O2 level, and cell wall peroxidase activity in roots. Bot. Bull. Academia Sinica. **41**, 99, **2000**.
35. METWALLY A., SAFRONOVA V.I., BELIMOV A.A., DIETZ K.J. Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. J. Exp. Bot. **56**, 167, **2005**.
36. DEVI R., MUNJRAL N., GUPTA A.K., KAUR N. Cadmium induced changes in carbohydrate status and enzymes of carbohydrate metabolism, glycolysis and pentose phosphate pathway in pea. Environ. Exp. Bot. **61**, 167, **2007**.
37. WIERZBICKA M. How lead loses its toxicity to plants. Acta Societatis Botanicorum Poloniae **64**, 81, **1995**.
38. WOJCIK M., TUKENDORF A. Cd-tolerance of maize, rye and wheat seedlings. Acta Physiol. Plant. **21**, 99, **1999**.
39. WU G.L., CUI J., TAO L., YANG H. Fluoroxypyr triggers oxidative damage by producing superoxide and hydrogen peroxide in rice (*Oryza sativa*). Ecotoxicology **19**, 124, **2010**.
40. KOPYRA M., GWOZDZ E.A. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. Plant Physiol. Biochem. **41**, 1011, **2003**.
41. ORTEGA-VILLASANTE C., RELLAN-ALVAREZ R., DEL CAMPO F.F., CARPENA-RUIZ R.O., HERNANDEZ L.E. Cellular damage induced by cadmium and mercury in *Medicago sativa*. J. Exp. Bot. **56**, 2239, **2005**.
42. JIN X.F., YANG X.E., ISLAM E., LIU D., MAHMOOD Q., LI H., LI J. Ultrastructural changes zinc hyperaccumulation and its relation with antioxidants in two ecotypes of *Sedum alfredii* Hance. Plant Physiol. Biochem. **46**, 997, **2008**.
43. MITTLER R. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Sci. **7**, 405, **2002**.
44. SMEETS K., RUYTINX J., SEMANE B., VAN BELLEGHEM F., REMANS T., VAN SANDEN S., VANGRONSVELD J., CUYPERS A. Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. Environ. Exp. Bot. **63**, 1, **2008**.
45. NOUAIRI I., AMMAR W.B., YOUSSEF N.B., MILED D.D.B., GHORBAL M.H., ZARROUK M. Antioxidant defense system in leaves of Indian mustard (*Brassica juncea*) and rape (*Brassica napus*) under cadmium stress. Acta Physiol. Plant. **31**, 237, **2009**.
46. LEE H., JO J., SON D. Molecular cloning and characterization of the gene encoding glutathione reductase in *Brassica campestris*. Biochem. Biophys. Acta. **1395**, 309, **1998**.
47. ZHOU Z.S., WANG S.J., YANG Z.M. Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere **17**, 1500, **2008**.
48. AMMAR W.B., NOUAIRI I., ZARROUK M., GHORBEL M.H., JEMAL F. Antioxidative response to cadmium in roots and leaves of tomato plants. Biol. Plant. **52**, 727, **2008**.
49. HANA S., RACHID R., IBISSEN S., HOURIA B., MOHAMMED-REDA D. Induction of anti-oxidative enzymes by cadmium stress in tomato (*Lycopersicon esculentum*). Afr. J Plant Sci. **2**, 072, **2008**.
50. MOURATO M.P., MARTINS L.L., CAMPOS-ANDRADA M.P. Physiological responses of *Lupinus luteus* to different copper concentrations. Biol. Plant. **53**, 105, **2009**.

51. VERMA S., DUBEY R.S. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. **164**, 645, **2003**.
52. ZROBEK-SOKOLNIK A., GORSKA K, GORECKI R.J. The activity of antioxidant enzymes suspension cultured tobacco cells treated with heavy metals. Pol. J. Natur. Sci. **22**, 704, **2007**.
53. MISHRA P.K., PRAKASH V. Antioxidant modulation in response to zinc induced oxidative stress at different pH in *Glycine max* L. Cv. Merrill. Amer-Eurasian J. Agr. Environ. Sci. **6**, 485, **2009**.
54. CAMP W.V., CAPIAU K., MONTAGU M.V., INZE D., SLOOTEN L. Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplast. Plant Physiol. **112**, 1703, **1996**.
55. GECHEV T., GADJEV I., BREUSEGEM F.V., INZE D., DUKINDJIEV S., TONEVA V., MINKOV I. Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell. Mol. Life Sciences **59**, 708, **2002**.
56. GABARA B., SKLODOWSKA M., WYRWICKA A., GLINSKA S., GAPINSKA M.B. Changes in the ultrastructure of chloroplasts and mitochondria and antioxidant enzyme activity in *Lycopersicon esculentum* Mill. Leaves sprayed with acid rain. Plant Sci. **164**, 507, **2003**.
57. MAZEN A.M.A. Accumulation of four metals in tissues of *Corchorus olitorius* and possible mechanisms of their tolerance. Biol. Plant. **48**, 267, **2004**.
58. WIERZBICKA M., PRZEDPELSKA E., RUZIK R., QUERDANE L., POLEC-PAWLIK K., JAROSZ M., SZPUNAR J., SZAKIEL A. Comparison of the toxicity and distribution of cadmium and lead in plant cells. Protoplasma **231**, 99, **2007**.
59. GREJTOVSKY A., MARKUSOVA K., ELIASOVA A., SAFARIK P.J. The response of chamomile (*Matricaria Chamomilla* L.) pants to soil zinc supply. Plant Soil Environ. **52**, 1, **2006**.