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Response of the pea roots defense systems to the two-element combinations of metals (Cu, Zn, Cd, Pb)*

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The presence of the single metals (Cd, Pb, Cu, Zn) induces ROS (reactive oxygen species) production and causes oxidative stress in plants. While applied in two-element combinations, trace metals impact organisms in a more complex way. To assess the resultant effect we treated the pea grown hydroponically with the trace metals in variants: CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd in concentrations of 25 μM for each metal ion. Abiotic stress inhibited root elongation growth, decreased biomass production, induced changes in root colour and morphology. It changed rate of ROS production, malondialdehyde content, increased activity and altered gene expression of defence enzymes (superoxide dysmutase, catalase, ascorbate peroxidase, glutathione reductase, γ-glutamylcysteine synthetase).

Key words: antioxidants, antioxidative enzymes, heavy metals, oxidative stress

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INTRODUCTION

Trace metals are naturally present in soil, though human activity altered their concentration and bioavailability. Metal ions binding to cell wall and macromolecules inside cell cause multiple indirect and direct effects on plants and microorganisms, leading to the decrease of microbial activity, soil fertility and crop yields.

Advancing pollution prompts biologists to understand the mechanisms of plant resistance (Qureshi *et al*., 2007, Khatun *et al*., 2008). Intensive studies on metal-induced abiotic stress offer evidences for cross-talk in complex network of stress signaling. Metals lead to the generation of reactive oxygen species (ROS): superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). ROS are generated in different compartments of the plant cells, such as: cell wall (peroxidases and polyamine oxidases), cytoplasm, peroxisomes (xanthine oxidase), mitochondria and chloroplasts (Vianello *et al*., 2007; Malecka *et al*., 2009). At high concentrations ROS damage cell components: proteins, lipids and nucleic acids, but also function as effectors and regulators of the programmed cell death.

Growth in a heavy-metal rich environment depends on the ability of plant to synthesize metal chelating molecules, activate antioxidant mechanisms and alter gene expression. Over 150 genes in plants encode enzymes involved in ROS production and processing (Mittler *et al*., 2004). In antioxidant defense, ROS are counteracted by enzymatic and non-enzymatic elements e.g.: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR), glutathione (Ahmad *et al*., 2008). Transgenic plants overexpressing enzymes of Haliwell-Asada cycle provide insights into the oxidative stress tolerance mechanism and increased tolerance to abiotic stress. Several recent studies have been aimed at enhancing ROS protection with constitutive overexpression of antioxidant defense enzymes (Lee *et al*., 2007).

Most of the available studies focus on impact of the single trace elements on plants, whereas plants must overcome simultaneous influence of various metal ions in soil. Therefore, in our study we applied two-element combinations of metals to assess the resultant effect on pea seedlings.

MATERIALS AND METHODS

Plant material. Pea seedlings (*Pisum sativum* L., cv. Bohun) were grown hydroponically on the Hoagland medium for 72 h with 16/8 h day/night photoperiod, at RT and light intensity of 82 μmol m^2 s^{-1}. Next medium was diluted (100×) and metals were applied in the following combinations: CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd at concentration 25 M of each. We used $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, $ZnSO_4$ solutions. Roots were cut after 0, 24, 48, 72 and 96 h of incubation. Metals adsorbed to root surface were cleansed with 10 mM of $CaCl_2$ and 10 mM EDTA.

Stress factors determination. Index of tolerance (IT) was calculated according to Wilkins (1957). Malondialdehyde (MDA) content was determined by reaction with thiobarbituric acid (Heath and Packer 1968). The material was homogenized in 1:5 ratio with 5% trichloroacetic acid (TCA). MDA concentration was estimated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm, using an absorbance coefficient of extinction 156 $mM^{-1}cm^{-1}$.

Reactive oxygen species analysis. Superoxide anion content was determined according to Doke (1983) at 580 nm. 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 tetra-

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Abbreviations: APOX, ascorbate peroxidase; CAT, catalase; ECS, γ-glutamylcysteine synthetase; IT, Index of Tolerance; H_2O_2, hydrogen peroxide; GR, glutathione reductase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; $O_2^{-\bullet}$, superoxide anion

Table 1. Index of tolerance (IT) [%] for the plants exposed to metals in the following combinations: CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd (25 μM of each metal).
Mean values and S.D. were calculated from the three independent experiments.

	CuPb	CuCd	CuZn	PbCd	ZnPb	ZnCd
24 h	82%±4.1	86%±4.4	81%±3.9	97%±4.6	99%±4.9	84%±4.2
48 h	72%±2.9	78%±3.1	66%±2.0	70%±3.0	81%±3.8	78%±3.3
72 h	67% ±2.7	65%±2.1	58%±1.6	77%±3.1	77%±3.2	75%±3.2
96 h	62%±1.9	69% ±2.9	60%±1.6	72%±3.0	86%±4.3	68%±2.8

zolium) 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 85°C for 10–15 min, cooled in the ice for 5 min and the absorbance was measured at 580 nm against the control.

Hydrogen peroxide content was determined according to Patterson *et al.* (1984). 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, 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).

Determination of enzyme activities. Total soluble protein contents were determined according to Bradford (1976), using the Bio-Rad assay kit with bovine serum albumin as a calibration standard. Activity of SOD was assayed according to Beauchamp and Fridovich (1971), with slight modification. The activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT. 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. Activity of CAT was determined according to Aebi (1983) at 240 nm. The activity of CAT was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min 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} cm^{-1} for H_2O_2. Activity of APOX was acc. to Nakano and Asada (1981). The method relies on the monitoring the rate of ascorbate oxidation at 290 nm (extinction coefficient of 2.9 mM^{-1} cm^{-1}) for 3 min. A reaction mixture consisted of 25–50 μL supernatant, 50 mM phosphate buffer (pH 7.0); 20 μM H_2O_2; 0.2 mM ascorbate; 0.2 mM EDTA. GR (EC 1.6.4.2) activity was measured acc. to Foyer and Halliwell (1976). Assay mixture consisted of 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 0.5 mM GSSG, 0.2 mM NADPH and 0.5 mM EDTA and enzyme extract. The reaction was monitored by the following of the decrease in absorbance at 340 nm. γ-ECS activity was determined according to Orlowski and Meister (1971). Gamma glutamylcysteine synthetase was determined in the mixture reaction containing sodium L-glutamate (10 mM), L-a-amino-butyrate (10 mM), MgCl, (20 mM), Na,ATP (5 mM), Na,EDTA (2 mM), Tris/HCl buffer (100 mM); pH 8.21, and bovine serum albumin (10 pg) in a final volume of 0.5 ml. The reaction was initiated by adding an enzyme.

Enzyme activities were undetectable in the absence of extract or any of the substrates.

Gene expression analysis. Total RNA was isolated with TRIzol reagent and tested spectrophotometrically for the purity at 260 and 280 nm. RNA was reverse-transcribed with oligo (dT) primers using RevertAid Reverse Transcriptase Kit (Thermo Science) after DNA denaturation with DNase I (Thermo Science). Primers were designed with the Primer3 program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). *P. sativum* beta-tubulin 1 (Tub1) was used as the internal control. Primer pairs sequences of are as follows (forward/reverse, gene accession number): gtgattgcttgcagggtttt/cagaatacggaagcaaatgtca, X54844.1 (TUB1), gctatttgccactggtagg/tgcaatagcaatacctga, X98274.1 (cytosolic GR), gcatatcattggagccaggt/ggaaaccaatccccagaaat, AF128455.1 (γ-ECS), ggagcaagtttggttccatt/aaggttattcggccagattg, U30841.1 (MnSOD), gaacaatggtgaaggctgtg/gtgaccacctttccaagat M63003.1 (Cu,Zn-SOD). PCRs were performed with 29–35 cycles of 95°C 30 s; 53, 50, 55, 53, 53°C 30 s; 72°C 30 s, respectively, using 1:100 diluted cDNA template and REDAllegro*Taq* DNA Polymerase (Novazym). PCR products were separated by the electrophoresis on a 1% agarose gel with the ethidium bromide in a TBE buffer and visualized under the UV light. CP Atlas 2.0 and MS Office Excel were used for densitometric analysis of relative gene expression.

RESULTS

The appearance and shape of the pea roots treated with the trace metals were significantly changed, especially after treatment with CuPb, PbCd and ZnCd. We observed inhibition of root elongation growth, a decrease in dry and fresh weight, the root sliming and changes in the root colour from a creamy white to the dark brown, which was probably caused by an intense suberification or an overproduction of the phenol substances. Index of Tolerance (IT) for the pea roots indicates that after 96 h of a treatment the pea exhibits highest sensitivity to the CuZn combination (IT 58%) as well as to the CuPb combination (IT 62%) (Table 1).

We observed a high concentration of the superoxide anion (Fig. 1) in CuCd and CuPb variants: after 24 h of exposition to CuPb level of $O_2^{-\bullet}$ increased by about eight-fold, while in CuCd plants level of $O_2^{-\bullet}$ peaked after 72 h. After 96 hours we observed decrease in $O_2^{-\bullet}$ concentration, probably because of the action of a defense system. After 24 hours H_2O_2 level (Fig. 1) peaked in plants treated with CuPb to twofold higher than control. Day later concentrations of H_2O_2 in all variants except ZnCd were two- to threefold higher than in control. Over time H_2O_2 level was gradually returning

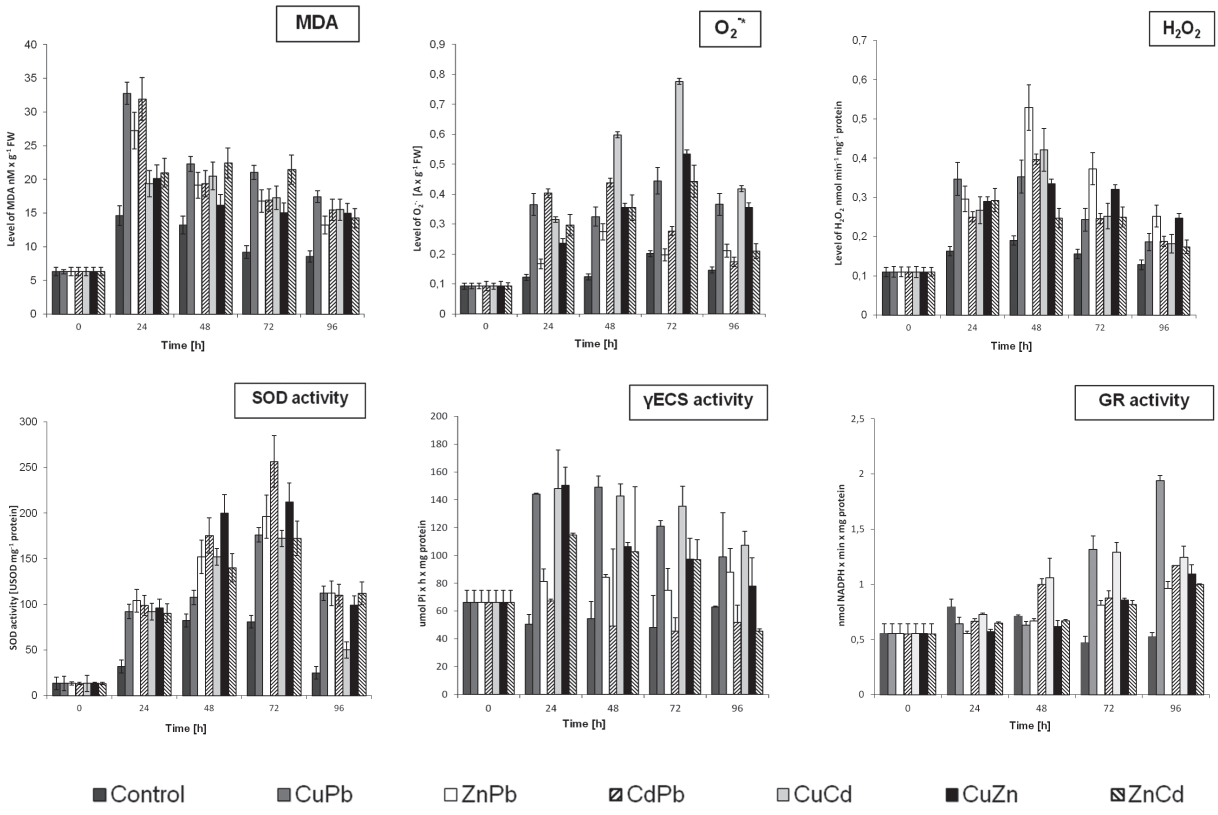
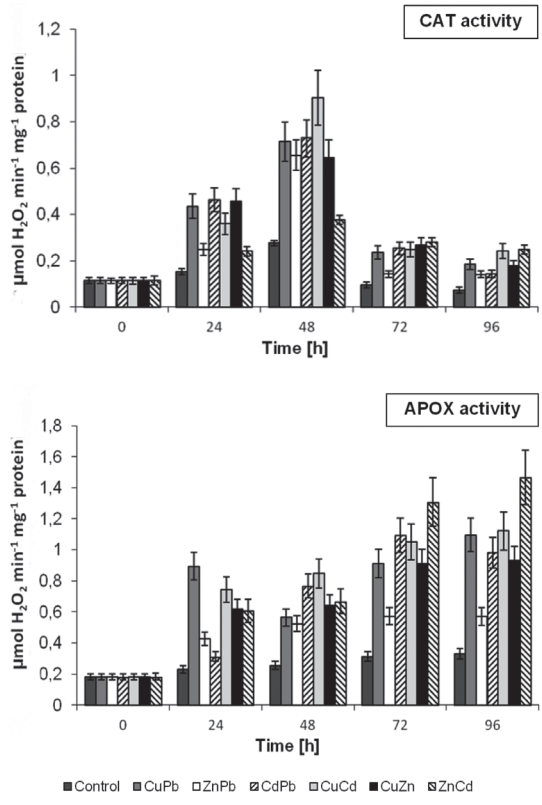


Figure 1. Level of MDA (nmol g^{-1} FW) , $O_2^{\cdot-}$ (A580 g^{-1} FW), H_2O_2 (nmol g^{-1} FW) and activity of SOD (U SOD mg^{-1} protein), γ-ECS (µmol Pi × h × mg protein), GR (nMol NADPH × min × mg protein) in roots of *P. sativum* treated for 96 h with CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd (25 µM each metal).
Mean values and S.D. were calculated from the three independent experiments.



to base line, though it was always higher in the treated plants than in the control ones.

We assessed MDA level to examine nonenzymatic lipid peroxidation in the membranes (Fig. 1). After 24 hours MDA level peaked for all combinations and was over twofold higher in CuPb, PbCd and ZnPb than in others. From 48- to 96-hours of incubation MDA level was relatively lower, but always higher (for around 1,5-fold) in plants exposed to abiotic stress than in control ones.

SOD activity in roots treated with metals was gradually increasing and peaked after 72 hours (Fig. 1). Highest activity of SOD was observed in PbCd and CuZn combinations: 260% and 330% higher compared to the control. In other variants (CuPb, CuCd, ZnPb, ZnCd) we noted twofold increase in SOD activity compared to control. CAT activity (Fig. 2) was highest —- four times higher than in control — in plants treated for 48 hours with CuPb, CuCd and PbCd. Subsequently, CAT activity decreased in plants treated with heavy metals to about two-threefold level compared to control. APOX activity (Fig. 2) was highest after 4 days of incubation in all variants exposed to abiotic stress. We observed five- and seven times higher APOX activity in all variants except for ZnPb, in which enzyme activity was only two times higher than in control.

Figure 2. Activity of CAT (µmol min^{-1} mg^{-1} protein) and APOX (µmol min^{-1} mg^{-1} protein in roots of *P. sativum* treated for 96 h with CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd (25 µM each metal).
Mean values and S.D. were calculated from the three independent experiments.

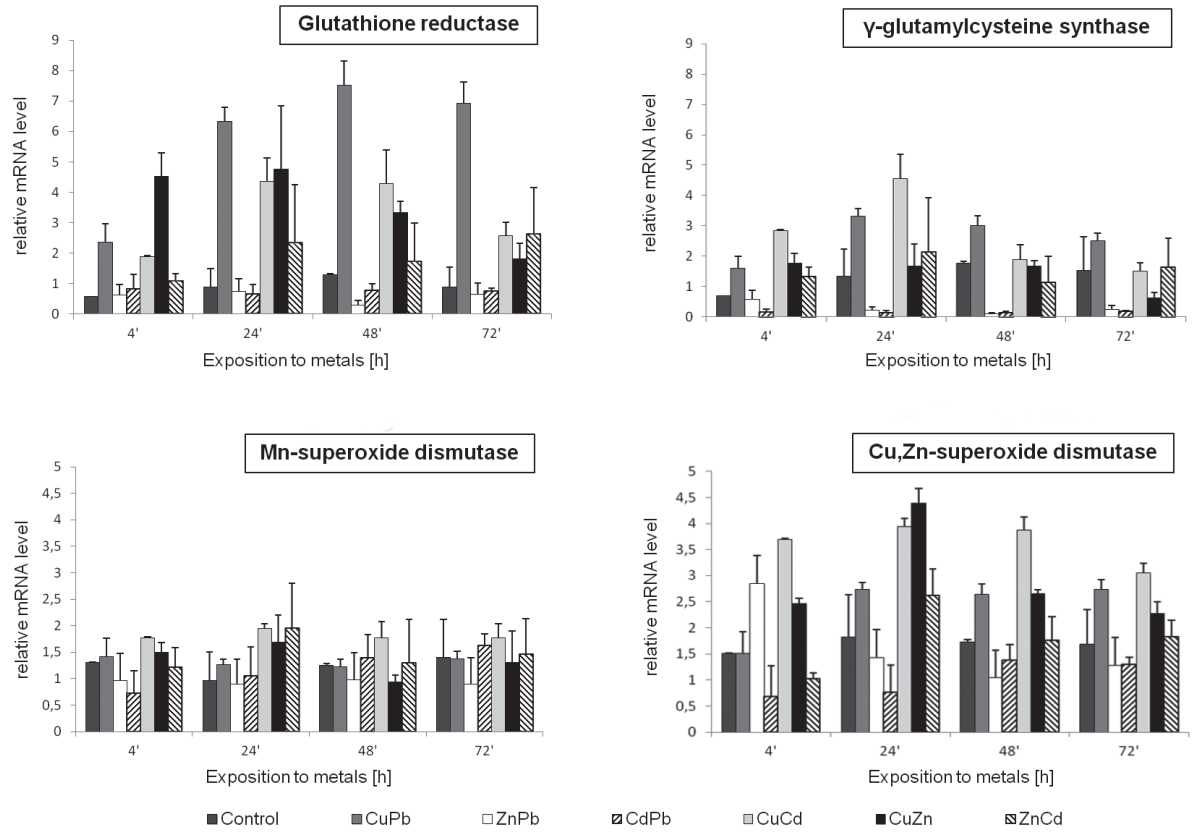


Figure 3. Changes in mRNA levels of genes encoding GR, γ-ECS, MnSOD, CuZnSOD (RT-PCR) in roots of *P. sativum* treated for 96 h with CuPb, CuCd, CuZn, PbCd, ZnPb, ZnCd (25 μM each metal).
Mean values and S.D. were calculated from the three independent experiments.

We observed changes in activity of γ-glutamylcysteine syntethase (ECS) and glutatione reductase (GR) (Fig. 1). ECS activity increased after 24 h of treatment in all combinations, in CuZn, CuCd, CuPb, ZnCd activity was for about three times higher. Later on ECS activity was decreasing, though in CuCd, CuPb and ZnPb combinations stayed for 70–80% higher than in control. Glutathione reductase activity after 24 hours remained similar to control, first changes appeared after 48 h in CuCd and CdPb combinations (73% and 64% increase). After 72 h GR activity was increased in all tested variants, with highest level after CuCd and CuPb treatment. After 96 hours GR activity was increased in plants treated with CuPb (three times higher than in control).

We studied changes in expression of genes encoding four enzymes: GR, γ-ECS, Mn-SOD and Cu,Zn-SOD (Fig. 3). mRNA level for genes encoding GR and γ-ECS was highest after 24–48 hours of treatment with CuCd, CuPb and CuZn. Increase in transcript level was observed also for *CuZnSOD*, especially for plants exposed for 24 hours to CuCd and CuZn.

DISCUSSION

Although studies on trace elements' influence on plants are numerous, the simultaneous impact of various metal ions and their cross talk remains poorly understood. In our previous study we determined the generation of ROS and an activation of the antioxidative systems in *Pisum sativum* treated with individual heavy metals, such as: Pb, Cu, Cd and Zn. We noticed that the least toxic trace element for pea plants was zinc, which caused in plants a noticeable but low increase in activity of SOD, CAT and GR with relatively low level of ROS. The most toxic for plants were Cu and Cd ions: they caused rapid generation of ROS in pea cells (Malecka *et al.* 2012). In this paper we want to show the influence of the two-element combinations of trace metals in the pea plants.

Of the metals tested in our study, two are essential (Cu and Zn) and two are non-essential (Pb and Cd) to plants, though each element is toxic in excess. Plants had highest IT values (highest tolerance) to ZnPb (72% after 96 hours) and lowest to CuZn (60%). We suggest such explanation: plants actively use metal transporters to uptake essential metals like Cu and Zn; non-essential metals lack in dedicated mechanisms of transport. Additionally, lead shows lower mobility than the other metals (Kumchai *et al.* 2013).

Trace elements cause generation of ROS, which induce the antioxidant response. ROS scavenging mechanisms decrease oxidative damage and increase resistance to metals (Gill & Tuteja, 2010). In our experiment every combination of metals lead to oxidative stress in pea roots. Cu and Zn ions induced highest ROS production especially in CuPb, CuZn, CuCd and ZnPb combinations. The increase in ROS level under the influence of heavy metals was shown by many authors (e.g. Malecka *et al.*, 2009; 2012; Lehotai *et al.*, 2011). According to Gill and Tuteja (2010) and Dalvi and Bhalerao (2013) ROS influence the expression of many genes, which validate their role as the controllers of a genetic stress-response program.

Stress-induced ROS accumulation is counteracted by an antioxidant system, including: SOD, CAT, APOX and

GR. Highest activities of SOD and CAT were observed in plants treated for 24–48 hours with CuCd, CuZn and CuPb — all of combinations with Cu. In our earlier experiment we showed that also individual ions (Pb, Cu, Cd and Zn) cause changes in enzyme activity in the pea seedlings (Malecka *et al.* 2012). SOD is perceived as a key player in plant stress tolerance and defense against $O_2^{-\bullet}$ (Gill & Tuteja, 2010; Sharma *et al.*, 2012), whereas CAT is crucial in H_2O_2 scavenging. Increased SOD activity is correlated with the plant tolerance against the environmental stress. The plants overexpressing different SOD isomers are often more resistant to the abiotic stress (Sharma *et al.*, 2012). Tobacco overexpressing *CuZnSOD* was tolerant to multiple stresses (Badawi *et al.*, 2004), while overexpression of MnSOD in *Arabidopsis* increased its salt tolerance (Wang *et al.*, 2004). Combined expression of *CuZnSOD* and *APOX* in *Festuca arundinacea* led to increased tolerance to mosaic virus, hydrogen peroxide, Cu, Cd and As (Lee *et al.*, 2007). In our study, molecular analysis showed an increase in *CuZnSOD* mRNA level in plants treated with CuCd, CuPb and CuZn — same combinations that induced SOD activity. Similarly, these combinations stimulated also CAT activity, an enzyme performing disproportionation of H_2O_2. Gabara and others (2003) proposed catalase as key regulator of H_2O_2 level involved in cell signaling network. Decreased activity or inactivation of catalase can flood cells with hydrogen peroxide and induce apoptosis. Hsu and Kao (2007) reported that rice seedlings pretreated with H_2O_2 showed increased CAT activity and higher tolerance to cadmium. Similarly, tobacco plants overexpressing CAT from *B. juncea* had enhanced tolerance to cadmium. Although catalase has a fast turnover rate, it has a lower affinity to H_2O_2 than APOX, one of the most widely distributed antioxidant enzymes in the plant cells (Sharma *et al.*, 2012). In our study, extended exposure to metals affected APOX activity, stimulating it to a gradual growth complementing decreasing activity of CAT over time. Many researchers reported enhanced activity of APOX in response to the abiotic stresses such as drought, salinity, chilling, metal toxicity, and UV irradiation (Sharma *et al.*, 2012). Overexpression of a pea cytosolic APOX-gene in tomato plants stimulated resistance to cold and salt stress (Wang *et al.*, 2005), while in tobacco and in *Arabidopsis* additional copies of *tApx* gene increased tolerance to the oxidative stress (Yabuta *et al.*, 2002). Combination ZnPb affected the pea seedlings in an unlikely manner causing low levels of superoxide anion, high levels of hydrogen peroxide and low activity of both CAT and APOX. Radic *et al.* (2010) showed that zinc can inhibit CAT synthesis or change the assembly of enzyme subunits, which might explain our results. Hyperaccumulator *Brassica juncea* responds to zinc with increased CAT activity, but pea and Indian mustard differ in their tolerance to a metal (Prasad *et al.*, 1999).

Plants exposed to the metals exhibited a decreased glutathione level. The major changes were observed during first 24 hours of an exposition. GSH reduction was due to its use as an antioxidant, heavy metal ligand and substrate in the phytochelatin biosynthesis. In further exposure was observed GSSG accumulation and the consequent decline in the GSH/GSSG ratio (data not included). Glutathione is synthesized in two ATP-dependent steps catalyzed by: (1) γ-glutamylcysteine synthetase (ECS), (2) glutathione synthetase. It acts as an antioxidant, regenerates other antioxidants, detoxifies metal ions by binding them to its thiol (-SH) group and serves as a substrate in phytochelatin biosynthesis (Anjum *et al.*, 2012). NADPH dependant glutathione reductase (GR) regenerates reduced GSH and responds to different stresses (Kornyeyev *et al.* 2003; Logan *et al.* 2003; Yannarelli 2007; Mhamdi *et al.*, 2010). In our experiment, decline in GSH level during first 24 hours induced expression of *ECS* gene, especially in CuCd, CuPb and CuZn combinations. High inducibility of *ECS* expression leads to higher GSH levels and stress resistance: overexpression of *ECS* induced resistance to herbicides in poplar and tolerance to Cd, Zn, Pb in Indian mustard (Gullner *et al.*, 2001; Noctor *et al.*, 2013; Zhu *et al.*, 1999, Reisinger *et al.*, 2008). We observed that glutathione reductase showed a delayed response: induction of *GR* expression and enzyme activity took place after 48–72 hours (mainly in CuCd, CuPb and CuZn combinations). This may suggest that GR responds not only to GSH level (like ECS), but to the changes in the GSH/GSSG ratio. Also other authors proposed that GSSG accumulation and the resulting change in glutathione redox status are involved in the network controlling the gene expression (Yadav *et al.*, 2010; Noctor *et al.*, 2013).

In conclusion, heavy metals in combinations: CuPb, CuCd, CuZn, PbCd, PbZn and ZnCd induced an oxidative stress in the pea roots. We found differences between plants treated with different combinations of heavy metals, regarding the generation of ROS, MDA and activation of the antioxidative and detoxicative systems. Combinations with copper (CuZn, CuPb, CuCd) stimulated highest response of the pea defense mechanisms, causing an increase in activity of SOD, CAT, APOX and GR enzymes, as well as increased expression of *GR*, *γ-ECS*, *CuZnSOD* genes. Copper toxicity observed in the two-element combinations of metals was not diminished by the presence of other metals, as compared with the influence of an individual copper treatment in our previous study.

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