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Original Scientific Paper

Induction of peroxidase isoforms in the roots of two *Verbascum thapsus* L. populations is involved in adaptive responses to excess Zn^{2+} and Cu^{2+}

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ABSTRACT: To investigate metal specific responses of root class III peroxidases (POD, EC 1.11.1.7), two populations of *Verbascum thapsus* L. were exposed to excess Zn^{2+} or Cu^{2+} for three weeks in hydroponic culture. One population originating from an uncontaminated area (NMET) and one from an industrial disposal area for jarosite residues from zinc refining (MET) were chosen to test the capacity of *V. thapsus* to adapt to excess metal in the soil. Exposure to 60 μM Zn^{2+} led to increased levels of protein carbonyl groups only in the roots of NMET, which was accompanied by higher increase of POD activity and NADH-oxidase activity compared with MET plants. New anionic and cationic POD isoforms were induced in the roots of both populations in response to Zn^{2+} treatment, while IAA-oxidase activity decreased. On the other hand, root growth was more affected by Cu^{2+} than Zn^{2+} in both populations, which was correlated with increased auxin-oxidase (IAA-oxidase) activity. Cu^{2+} induced an increased activity of anionic POD isoforms in the roots of both populations, yet the ratio of NADH-oxidative to peroxidative POD activity remained higher in NMET than in MET plants. Overall results show differential effects of Zn^{2+} and Cu^{2+} on POD activity in the roots of *V. thapsus* L. In addition, higher tolerance to Zn^{2+} in MET plants than in NMET indicated that these plants have developed an adaptive mechanism to cope with Zn^{2+} excess.

Key words: auxin, copper, NADH, peroxidase, populations, *Verbascum thapsus*, zinc.

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INTRODUCTION

Class III peroxidases (POD, E.C.1.11.1.7) are ubiquitous enzymes with numerous functions in plant growth and development. Peroxidases are heme-containing enzymes and their catalytic function is determined by oxidation-reduction reactions of iron (Yamazaki & Yokota, 1973). The antioxidative function of peroxidases is based on scavenging of H_2O_2 using various hydrogen donors, preferably phenolics, resulting in conversion of H_2O_2 into water (Passardi *et al.* 2005; Kukavica 2005). Simultaneously, these reactions trigger polymerization reactions of phenoxyl radicals and cross-linking of cell wall components (Christensen *et al.* 1998). On the other hand, PODs may act as pro-oxidants in the oxidative cycle, by catalyzing the formation of superoxide anion ($O_2^{\bullet -}$) and subsequently H_2O_2, through reduction of molecular oxygen in the presence of strong reductants such as NAD(P)H, auxin and cysteine (Chen & Schopfer 1999; Schopfer *et al.* 2008; Liszkay *et al.* 2003). Moreover, ascorbate/NADH cooxidation systems function as H_2O_2 scavengers and regenerate phenolics contributing to cellular antioxidative protection (Hadži-Taškovic *et al.* 2008). Peroxidase-dependent formation of hydroxyl radicals ($OH^{\bullet}$) from $O_2^{\bullet -}$ and H_2O_2 has also been demonstrated in isolated cell walls of the roots of *Pisum sativum* (Kukavica *et al.* 2009). Cell wall-bound PODs mediate cell growth and cell wall differentiation through regulation of reactive

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oxygen species (ROS) and H_2O_2 levels in the cell wall and apoplast, but they are also involved in plant responses to various biotic and abiotic stresses (Passardi *et al.* 2005; Almagro *et al.* 2009). Distinguishing the specific functions of individual POD isoforms is difficult *in vivo*, although numerous studies have attempted to assign some functions to soluble, ionically and covalently cell wall-bound, anionic, cationic and neutral POD isoforms (Barcelo 1998; Quiroga *et al.* 2001; Minibayeva *et al.* 2009; Kukavica *et al.* 2009, 2012).

We have shown that auxin (indole-3-acetic acid, IAA) and chitosan (a widely-used elicitor) application induced covalently cell wall-bound PODs involved in formation of cell walls in non-elongating root tissue (Kukavica *et al.* 2012). In addition, apoplastic PODs were induced in *Pisum sativum* roots following IAA application (Kukavica *et al.* 2007; Vidović *et al.* 2010).

Excess zinc and copper in the environment, often a result of anthropogenic activities, may impose oxidative stress in plants (Broadley *et al.* 2007; Burkhead *et al.* 2009). Both Zn and Cu are essential micronutrients, cofactors of various enzymes (Marschner 2012). Zinc is involved in DNA and RNA replication and gene expression, protein synthesis and cell division and maintains membrane integrity (Marschner 2012). Copper participates in mitochondrial respiration, photosynthetic electron transfer, cell wall metabolism and hormone signalling (Marschner 2012). However, at supra-optimal concentrations Zn and Cu interfere with plant metabolism, leading to a decrease in biomass production and photosynthesis rate, shoot chlorosis and necrosis, unbalanced nutrient uptake and membrane disruption (Clemens 2001; Cuypers *et al.* 2001; Ebbs & Uchil 2008; Tewari *et al.* 2008; Zhang *et al.* 2008). In addition, mechanisms of Cu toxicity include direct interactions with proteins, generation of ROS and cation displacement at specific binding sites (Sharma & Dietz 2009; Morina 2010). Copper as a redox active metal efficiently promotes the Fenton-type reaction, generating very reactive $OH^{\bullet}$ radicals and disturbing redox homeostasis in plants (Sharma & Dietz 2009). On the other hand, Zn, although redox non-active, may also promote oxidative stress in plants (Cuypers *et al.* 2001; Broadley et al. 2007; Morina *et al.* 2010; Morina 2011).

The aim of our study was to determine the effects of Zn^{2+} and Cu^{2+}, differing in their redox activity, on POD functions and isoform profiles in the roots of *Verbascum thapsus* L. We have previously shown that high concentrations of Zn induced POD activity in the leaves of *V. thapsus* plants originating from a jarosite smelter contaminated with zinc (Morina *et al.* 2010; Morina 2011). Here we compared the responses of plants originating from two populations, one from an uncontaminated and one from a zinc-contaminated site, to excess Zn^{2+} and Cu^{2+} to reveal the involvement of PODs in metal tolerance.

MATERIAL AND METHODS

Seed collection and plant cultivation. Seeds from two *V. thapsus* populations were randomly collected at two locations, one unpolluted (NMET) and one metal-polluted area (MET). The NMET population was found in a ruderal meadow where the soil was degraded by erosion, on mountain Zlatibor (N 43° 42' E 19° 46'), while MET was an early colonizer species on a jarosite smelter from a zinc production process (MET1) in the Zorka Šabac Industry which has been in use since 2000 (N 44° 44' E 19° 43'). Seeds were germinated on moist sand, and after four weeks, seedlings were transferred to 0.1-strength Hoagland nutrient solution (0.5 mM KNO_3, 0.4 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 mM ferric ethylenediamine-di-(2-hydroxyphenylacetate) (FeEDDHA) (Duchefa Biochemie, Netherlands), 10 μM H_3BO_3, 2 μM $MnCl_2$, 0.2 μM $CuSO_4$, 0.2 μM $ZnSO_4$, and 0.1 μM Na_2MoO_4). Plants were grown under controlled conditions, at 25°C, 60% humidity and a 16/8 h day/night regime. Nutrient solutions were continuously aerated and replaced weekly. After four weeks, plants were exposed to metal-amended nutrient solutions with either 60 μM $ZnSO_4$ or 20 μM $CuSO_4$. Roots from eight plants per treatment were sampled after three weeks and their fresh biomass measured. Samples for enzymatic analysis were frozen and kept at -80°C until further analysis.

Extraction of peroxidases. For extraction of PODs, the root samples of NMET and MET were frozen in liquid nitrogen, powdered using a mortar and pestle and extracted in 100 mM sodium phosphate buffer (pH 6.5) with 0.05% (w/v) Triton X-100, 0.5 mM NaCl, 2 mM PMSF and 5% (w/v) insoluble polyvinylpyrrolidine (PVP). The homogenate was centrifuged at 10000 g for 10 min at 4°C, and the supernatant used for analysis.

Determination of POD activity and carbonyl content. Peroxidative activity of POD was measured as absorbance increase at 470 nm using 20 mM guaiacol ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$) as hydrogen donor in 100 mM potassium phosphate buffer (pH 6.5) with 1.3 mM H_2O_2 and an aliquot of the extract diluted 20 times. Oxidative activity of peroxidases was determined as absorbance decrease at 340 nm due to NADH oxidation ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$). The reaction mixture consisted of 50 mM phosphate buffer (pH 5.5) with 0.25 mM $MnCl_2$ and 0.2 mM *p*-coumaric acid, 0.2 mM NADH and root extract diluted 20 times according to Hadži-Tašković Šukalović *et al.* (2005).

The level of protein carbonylation was determined spectrophotometrically using 2,4-dinitrophenylhydrazine to form stable dinitrophenyl adducts according to Morina *et al.* (2010).

The protein contents in the samples were determined according to Bradford (1976). All spectrophotometrical measurements were performed in triplicate at 25°C using

a temperature-controlled spectrophotometer (Shimadzu, UV-160, Kyoto, Japan).

Electrophoresis. Isoelectrofocusing (IEF) was carried out in 7.5% polyacrylamide gel with 3% ampholyte on a pH gradient of 3–9. To detect peroxidative POD activity after electrophoresis, the gel was incubated with 10% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM sodium phosphate buffer (pH 6.5).

For detection of IAA-oxidase activity, three reagents were prepared according to Hoyle (1977), reagent A: 4 mg/ml Fast Blue salt (Sigma Aldrich) in ethanol, reagent B: 2 μM *p*-coumaric acid, 2 μM IAA and 2 μM H_2O_2 in ddH_2O and reagent C: 2 M acetate buffer (pH 4.2). Gels were incubated in a mixture of A, B and C reagents in 1:2:1 ratio.

Statistical analysis. The significance of differences between metal-treated and control plants was tested using Student's *t*-test ($p < 0.05$).

RESULTS

Effect of Zn^{2+} and Cu^{2+} on root growth. Although Cu^{2+} concentration in the nutrient solution was 3-fold lower than Zn^{2+}, it induced greater root growth inhibition, especially in NMET plants (Fig. 1). Excess zinc had no significant effect on root growth. On the other hand, Cu^{2+} significantly reduced root FW in NMET plants, to 70% compared with controls, while only a 10% decrease was observed in the root FW of MET plants (Fig. 1).

Level of carbonylated proteins. Accumulation of protein carbonyl groups is used as a marker of oxidative injury. Excess Zn^{2+} significantly increased the level of protein carbonyls in the roots of NMET plants, without any effect on the MET roots. However, Cu^{2+} slightly increased the level of protein carbonyls in the roots of plants from both populations (Fig. 2).

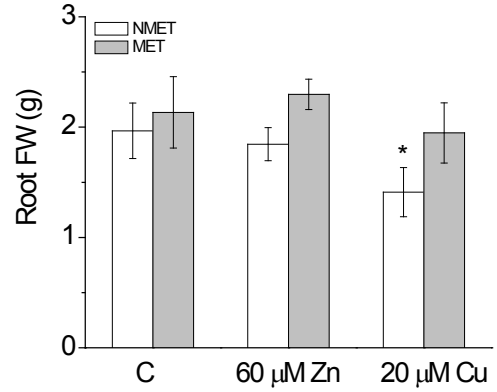

Fig. 1. Effect of 60 μM Zn^{2+} and 20 μM Cu^{2+} on fresh weight of roots from two *V. thapsus* populations, NMET and MET, after three weeks of treatment (n=4). * denotes significant difference compared with control plants ($p < 0.05$) C-controls.

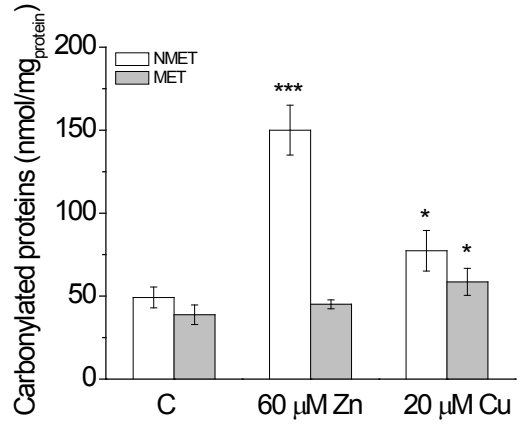

Fig. 2. Effect of 60 μM Zn^{2+} and 20 μM Cu^{2+} on the level of carbonylated proteins in root extracts of two *V. thapsus* populations, NMET and MET, after three weeks of treatment (n = 4). * denotes significant differences compared with control plants, $p < 0.05$, *** $p < 0.001$.

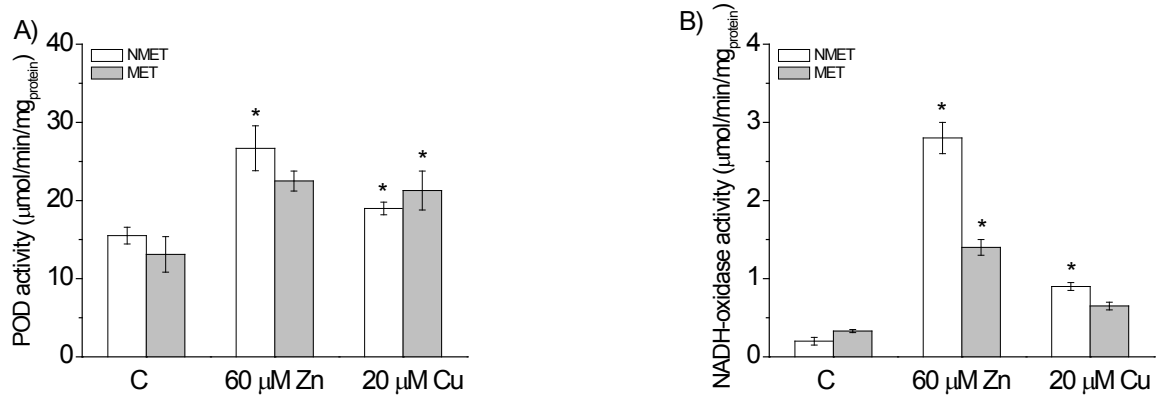

Fig. 3. Specific peroxidative activity of POD using guaiacol as electron donor (A) and NADH-oxidative activity of POD (B) in the root extracts of NMET and MET after Zn^{2+} and Cu^{2+} treatment (n = 3). * denotes significant differences compared with control plants ($p < 0.05$).

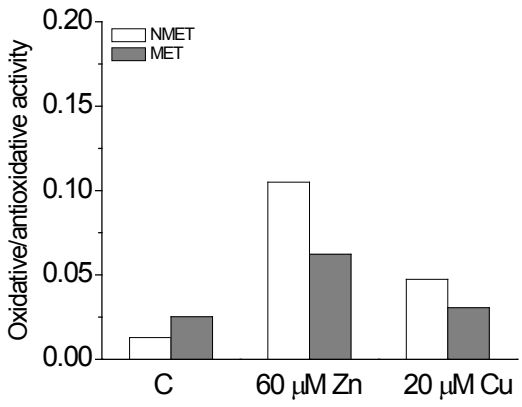

Fig. 4. Ratio of NADH-oxidative to peroxidative activity of POD in root extracts of NMET and MET after Zn^{2+} and Cu^{2+} treatment.

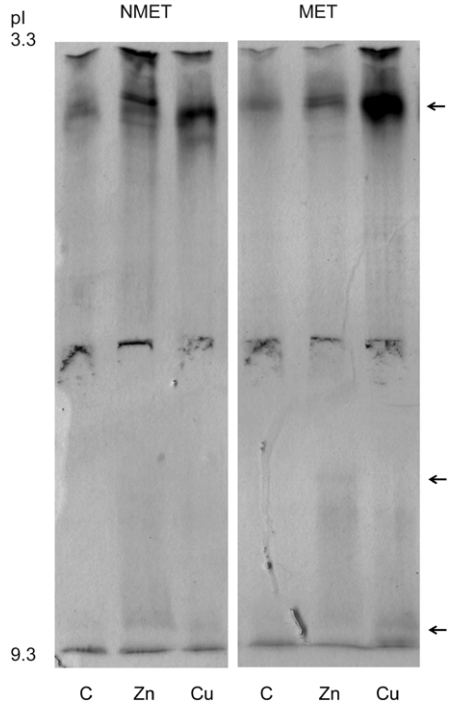

Fig. 5. Separation of POD isoforms in the root extracts of NMET and MET plants after 60 μM Zn^{2+} and 20 μM Cu^{2+} treatment on an IEF gel and stained with α-chloro-naphthol. C-controls. The same amount of proteins was applied in each lane.

Oxidative and peroxidative activities of POD and isoenzyme profiles. Specific POD activity in the roots was determined using guaiacol as electron donor. POD activity in Zn-treated NMET roots significantly increased, while in the roots of MET plants this effect was not so pronounced (Fig. 3A). On the other hand, Cu^{2+} slightly increased POD activity compared with control plants in the roots of both populations.

Oxidative activity of POD, determined as capacity for oxidation of NADH in the presence of H_2O_2, $MnCl_2$ and *p*-coumaric acid, increased in response to Zn^{2+} treatment about 15-fold and 3-fold compared with control roots in NMET and MET plants, respectively (Fig. 3B), while Cu induced an increase in NADH-oxidase activity only in the roots of NMET.

The ratio of oxidative to peroxidative activity of PODs is shown in Fig. 4. In untreated plants, this ratio was higher in the roots of MET than in NMET plants. However, both Zn^{2+} and Cu^{2+} increased the oxidative capacity of PODs for NADH-oxidation to a greater extent in NMET than in the roots of MET plants.

IEF analysis of control root extracts showed several anionic and cationic isoforms, with a similar pattern and activity in both NMET and MET plants (Fig. 5). Cu^{2+} and Zn^{2+} induced different isoforms in both populations. Zinc induced anionic (pI 3.6-4.6) and cationic POD isoforms (pI 8.8-9.3) in the roots of both NMET and MET. A higher increase of POD activity in response to Zn was observed in NMET than in MET roots. On the other hand, Cu increased the activities of anionic isoforms (pI 4.2-4.6) in the roots and this effect was more pronounced in MET than in NMET. In addition, a new cationic isoform in MET roots was induced by Cu (pI around 9).

Contrary to effects on NADH-oxidase activity, Cu^{2+} had a pronounced effect on IAA-oxidase isoforms in the roots of both NMET and MET (Fig. 6), observed as increased activity of anionic isoforms (pI 4.2-4.6). On the other hand, Zn^{2+} inhibited overall IAA-activity. In the roots of MET plants, the POD isoform (pI 4.2) was insensitive to Zn^{2+} treatment.

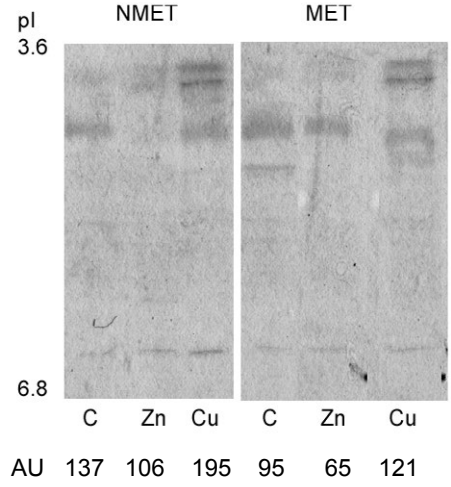

Fig. 6. Separation of POD isoforms in the root extracts of NMET and MET plants after 60 μM Zn^{2+} and 20 μM Cu^{2+} treatment on an IEF gel stained for IAA-oxidative activity. C-controls. IAA-oxidative activity of PODs determined as staining intensity is shown in arbitrary units measured using ImageJ program. The same amount of proteins was applied in each lane.

DISCUSSION

The plant root is in first direct contact with excess metal ions in the environment, and it rapidly responds to metal toxicity. One of the earliest symptoms of metal toxicity is overproduction of ROS and deterioration of antioxidative metabolism in the root. It has been shown that Cu is involved in Fenton reactions inducing the generation of $OH^{\bullet}$, and H_2O_2 (Halliwell 1982; Schutzendubel & Polle 2002; Sharma & Dietz 2009), while Zn may also induce oxidative damage, through the induction of lipoxygenase (Chaoui *et al.* 1997; Anwar *et al.* 2015) and stabilisation of phenoxyl radicals in the cell wall (Morina *et al.* 2010).

Exposure of *V. thapsus* plants to excess Zn^{2+} and Cu^{2+} resulted in oxidative injury, evidenced by increased levels of carbonylated proteins, as well as Cu-induced growth inhibition, especially in the roots of NMET plants (Figs. 1,2). A significantly higher content of carbonylated proteins and higher induction of both antioxidative and NADH-oxidative POD activities in the roots of NMET plants compared with MET plants indicated differential sensitivity to Zn^{2+} between the two populations. The antioxidative defence system in the roots of NMET plants following Zn exposure failed to maintain the cellular redox balance, contrary to MET plants (Fig. 2,4-5). These results indicate that MET plants originating from Zn-polluted jarosite smelter have acclimated to excess Zn in the soil. Local adaptations of plant species to excess metal levels in the soil have been noted (Antonovics *et al.* 1971).

The ability of MET plants to tolerate excess Zn^{2+} may be explained by the existence of efficient zinc tolerance mechanisms, such as chelation, vacuolar sequestration, cell-wall binding (Hall 2002, Broadley et al. 2007) and/or activity of antioxidative components other than PODs (Foyer & Noctor 2005). Moreover, the concentrations of Zn accumulated in the roots were similar in NMET and MET plants (Morina 2011).

In the roots of NMET plants, the 3-fold increased level of carbonylated proteins by Zn was accompanied by increased NADH-oxidizing activity (Fig. 3,4). The stimulating effect of Zn^{2+} on NADH-oxidase activity has been shown previously (Di Toppi *et al.* 2009), leading to formation of $O_2^{\bullet-}$ and H_2O_2 (Chen & Schopfer 1999). Class III PODs can oxidize various substrates in H_2O_2–scavenging peroxidative reactions (Passardi *et al.* 2005), but may also produce $O_2^{\bullet-}$ and $OH^{\bullet}$, and H_2O_2 in oxidative reactions, using NAD(P)H, auxin and ascorbate as reductants (Chen & Schopfer 1999; Liszkay *et al.* 2003; Schopfer *et al.* 2008). It has been shown that $O_2^{\bullet-}$ is transformed to H_2O_2 by superoxide dismutase, while PODs may further use H_2O_2 for the production of $OH^{\bullet}$ (Kukavica 2005; Kukavica *et al.* 2009). In this study, new POD isoforms were induced in response to Zn^{2+} in both populations (Fig. 5), while the ratio of NADH-oxidative/peroxidative activity of PODs increased (Fig. 4). We have previously shown that high concentrations of Zn^{2+} increased the activity of anionic POD isoforms in the roots of *V. thapsus*, in a short time period, without induction of new isoforms (Morina *et al.* 2008). New Zn-induced POD isoforms were reported by Chaoui and co-workers (1997) and Fang & Kao (2000). However, no induction of POD isoforms was observed in response to Zn^{2+} in bean (Cuypers *et al.* 2002) and maize (Vuletic *et al.* 2014). Overall, the response of PODs in Zn-treated plants has shown no specific patterns, depending on plant species, Zn concentration and duration of exposure (Fang & Kao 2000; Madhava Rao & Sresty 2000; Cuypers *et al.* 2002; Wojcik *et al.* 2006; Wang *et al.* 2009, Di Toppi *et al.* 2009; Vuletic et al. 2014).

Unlike Zn, which can be translocated from roots to shoots, Cu accumulation is restricted to the roots in most plant species (Marschner 2012; Krzesłowska 2011; Colzi *et al.* 2012). Excess Cu^{2+} is preferentially bound to the root cell wall, which is one of the protective mechanisms against its toxicity (Marschner 2012). Furthermore, in addition to H_2O_2 accumulation in the roots, excess Cu induced the accumulation of phenolics and lignification, thus limiting root growth (Kováčik & Klejdus 2008; Feigl *et al.* 2013). In our experiment, Cu^{2+} inhibited root growth of NMET plants to a greater extent than Zn^{2+}, and this was correlated with increased IAA oxidation (Fig. 1,6). Indole-3-acetic acid is a major auxin in plants, involved in growth regulation, but also in adaptation to stressful conditions (Ludwig-Müller 2011; Yuan *et al.* 2013). Increased IAA catabolism was observed in the roots of both populations upon Cu^{2+} treatment (Fig. 6). However, root growth was significantly reduced only in the roots of NMET. Induction of IAA-oxidase activity in response to metal treatments has been shown previously (Chaoui & El Ferjani 2005; Pető *et al.* 2011; Yuan *et al.* 2013).

Both antioxidative and NADH-oxidative activities of PODs in the presence of Cu^{2+} treatment increased in the roots of *V. thapsus* plants, and activities of PODs were more induced in MET than in NMET plants (Fig. 5). The lower ratio of oxidative to peroxidative activities of PODs in Cu^{2+} treatment compared to Zn^{2+} probably contributed to lower oxidative damage of proteins and efficient H_2O_2 scavenging in the POD/Phe/H_2O_2 cycle. Inhibition of oxidase activity of PODs was noted in the *Zea mays* roots treated with Cu (Hadži-Tašković Šukalović *et al.* 2010). A protective mechanism against Cu^{2+} toxicity included auxin degradation, and lignification observed as intensive root browning in both populations (not shown). In addition, MET plants were able to maintain root growth regardless of IAA-degradation.

CONCLUSION

Differences have been observed in the response of PODs to Zn^{2+} in the roots of metal-sensitive and metal-tolerant plants reflecting their origin. NMET showed higher POD induction in response to Zn^{2+} than MET, while MET plants had higher capacity for POD induction in Cu^{2+} treatment. Although there were no constitutive differences in the activity and isoform profiles between NMET and MET, the results implicate the importance of PODs in adaptive responses to excess Zn^{2+} and Cu^{2+}. MET plants, originating from zinc polluted jarosite smelter, have developed adaptive mechanisms including an efficient antioxidative network for regulation of ROS levels, probably due to selective pressure favouring more tolerant genotypes.

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Botanica SERBICA

REZIME

Indukovane izoforme peroksidaza u korenovima dve populacije divizme (*Verbascum thapsus* L.) uključene su u adaptivni odgovor na povišene koncentracije cinka i bakra

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Dve populacije divizme (*Verbascum thapsus* L.) izložene su povišenim koncentracijama Zn^{2+} ili Cu^{2+} hidroponično, tokom tri nedelje, kako bi se ispitao specifični odgovor peroksidaza klase III (POD, EC 1.11.1.7) na prisustvo metala. Prva populacije sa nezagađene lokacije (NMET), i druga populacija nađena na industrijskoj deponiji otpadnog mulja iz hidrometalurškog jarosit procesa proizvodnje cinka (MET), odabrane su kako bi se ispitala sposobnost divizme da se adaptira na povećane koncentracije metala u zemljištu. Tretman sa 60 μM Zn^{2+} doveo je do značajnije akumulacije karbonilovanih proteina i povećanja peroksidazne i NADH-oksidazne aktivnosti POD u korenovima NMET u odnosu na MET. Cink je indukovao nove anjonske i katjonske izoforme, i istovremeno inhibirao IAA-oksidaznu aktivnost u korenovima obe populacije. Suprotno tome, Cu^{2+} je inhibirao rast korenova u većoj meri u odnosu na Zn, što je korelisano sa indukcijom IAA-oksidazne aktivnosti. Bakar je uticao na povećanje peroksidazne aktivnosti anjonskih POD u korenovima obe populacije, ali je odnos NADH-oksidazne/peroksidazne aktivnosti POD bio veći u NMET. Rezultati ukazuju na različit efekat Zn^{2+} and Cu^{2+} na aktivnost POD u korenovima divizme. Veća otpornost biljaka iz populacije MET u odnosu na NMET, ukazuje na razvijen adaptivni mehanizam pri povišenim koncentracijama cinka.

Ključne reči: auksin, bakar, cink, NADH, peroksidaze, populacije, *Verbascum thapsus* L.