PEROXIDASE, PHENOLICS, AND ANTIOXIDATIVE CAPACITY OF COMMON MULLEIN (*VERBASCUM THAPSUS* L.) GROWN IN A ZINC EXCESS

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Abstract — Common mullein *(Verbascum thapsus* L.) is the dominant plant species at a disposal site polluted with metal from the hydrometallurgical jarosite zinc production process. Seeds collected at the site were germinated and plants were grown hydroponically under controlled conditions in a excess of Zn. Induction of total soluble POD activity in the root occurred at 1, 5, and 10 mM Zn, indicating Zn accumulation within the root. Accumulation of Zn in leaves was not accompanied by changes in POD activity, but resulted in gradual increase of total antioxidative capacity, which could be partly attributed to accumulation of soluble phenolics. The role of the phenolics/POD system in defense of *V. thapsus* against zinc is discussed.

Key words: Antioxidative capacity, peroxidase, phenolics, zink, *Verbascum thapsus* L., Serbia

INTRODUCTION

Long exposure of plants to soil contaminated with an excess of various metals may lead to the accumulation of metals in plant organs and organelles and to the induction of defense mechanisms. Plants possess several mechanisms by which they protect themselves from the deleterious effects of metals such as: exclusion, inactivation, and storage as complexes with various natural compounds called phytochelators in the vacuole (B r u n e et al., 1994; Z e n k, 1996). One of the aspects of metal toxicity in plants is a disruption of redox homeostasis due to increased production of reactive oxygen species (ROS) (H a l l i w e l l, 1982; L u n a et al., 1994; S c h u t z e n d u b e l and P o l l e, 2002), accompanied by a response in antioxidative metabolism (P r a s a d et al., 1999; R a o and S r e s t y, 2000; B o n n e t et al., 2000). Overloading of cells with metals and exhaustion of the plant's detoxification capacity leads to oxidative damage to cellular components such as DNA, proteins, and membrane lipids, causing cell death (C a s a n o et al., 1997; T a p p e l, 1973; S g h e r r i et al., 2003). The redox-active

metals copper and iron have the ability to induce oxidative stress in cells by promoting a Fenton-type of reaction (H a l l i w e l l and G u t t e r i d g e, 1986; N a v a r i – I z z o et al., 1998). However, metal ions unable to perform univalent oxido-reduction reactions, such as zinc, can also induce oxidative damage by yet unknown mechanisms (L u n a et al., 1994; P r a s a d et al., 1999; R a o and S r e s t y, 2000).

Plant cells possess powerful antioxidative systems composed of non-enzymatic scavengers of ROS such as ascorbate, glutathione, phenolics, and numerous antioxidant enzymes such as superoxide dismutase, peroxidase (POD), and catalase (A s a d a, 1992; N o c t o r and F o y e r, 1998). Class III POD with various physiological functions in plant cells can participate in many reactions, among which lignification, auxin metabolism, regulation of cell elongation, and hydrogen peroxide scavenging through the ascorbate/phenolic cycle have been widely studied (O t t e r and P o l l e, 1997; T a k a h a m a et al., 1999; S c h o p f e r et al., 2002). Results showing a positive correlation between POD activity and metal concentrations in plants suggest

a key role for POD in the cellular defense mechanism against metal toxicity (V a n   A s s c h e   and C l i j s t e r s , 1990; M a c f a r l a n e   and   B u r c h e t t , 2001; C u y p e r s et al., 2002; S g h e r r i et al., 2002). Phenolics, besides their function as electron donors to hydrogen peroxide in reactions catalyzed by Class III POD, have the ability to scavenge radical species directly (R i c e - E v a n s et al., 1997). In addition, some phenolics (o-diphenols and catechins) have the ability to bind metals and can thus be considered as an important class of phytochelators (S a k i h a m a et al., 2002). Even their metal complexes have been shown to be stable (Ya m a s a k i and G r a c e , 1998; S a k i h a m a et al., 2002), yet evidence on their role in the defense mechanism against metal toxicity is scarce.

Common mullein (*Verbascum thapsus* L.) is a biennial plant that usually grows on bare and disturbed soils. It was found to be the dominant plant species at a site contaminated by the hydro-metallurgical jarosite zinc production process in Šabac, Western Serbia. The ability of *V. thapsus* L. to accumulate heavy metals was observed previously (K f a y a t u l l a h et al., 2001; J o v a n o v i ć et al., 2007). At concentrations above 0.4 mg/g DW in the plant, zinc may inhibit growth and disrupt nutrient uptake (C h a o u i et al., 1997; Q u a r i t i et al., 1997); inhibit chlorophyll formation in young leaves (N a g et al., 1984, K a y a et al., 2000); and change the activities of antioxidative enzymes (V a n A s s c h e   and   C l i j s t e r s , 1986; O u z o u n i d o u et al., 1995; Q u a r i t i et al., 1997). The aim of our study was to investigate the antioxidative capacity of *V. thapsus* L. as part of its adaptive strategy in plants grown at extremely high Zn concentrations comparable with Zn concentrations observed in soil at the polluted site.

MATERIAL AND METHODS

Plant material and cultivation

Seeds of *Verbascum thapsus* L. obtained from populations grown at an industrial waste disposal area in Šabac, Western Serbia, were used for experiments under controlled conditions. Those plants accumulated high concentrations of several metals (J o v a n o v i ć et al., 2007). After sterilization in 15% H_2O_2 for 5 min, the seeds were germinated on an agar nutrient medium containing essential microelements (6.7 mM KCl, 23.5 mM $NaNO_3$, 7.3 mM KH_2PO_4, 2.24 mM $MgSO_4x7H_2O$, 0.36 mM $FeSO_4x7H_2O$) and 6% sucrose. After 2 weeks, plantlets were transferred to aerated pots containing modified half-strength Hoagland nutrient solution (HNS) (N i k o l i ć and R ö m h e l d , 2002) with iron supplied as 10 μM FeEDTA in a growth chamber with constant humidity of 65%, temperature of 25˚C, photoperiod with 16 h of light, and irradiance of 250 μE/m²s. After 4 weeks, plants were transferred to continuously aerated full HNS, which was renewed every 3 days. Plants were grown in this solution for the next 2 weeks before treatment.

Treatment with zinc and sampling

Eight-week-old plants were treated with different concentrations of Zn (1, 5, and 10 mM $ZnSO_4·7H_2O$). Samples of leaves and roots were collected on the 4th, 7th, and 10th day after the beginning of treatment.

Content of Zn in leaves

All plant samples were dried at 60˚C for 24 h and milled afterwards. The milled plant samples were digested in 1 M HNO_3, and contents of soluble metals in the leaves were determined by atomic absorption spectrometry (using a Varian Spectra 220 instrument).

Protein extraction

Leaf and root samples were powdered in a mortar containing liquid N_2 and extracted in 100 mM Na-phosphate buffer (pH 6.5) with 2 mM phenylmethylsulfonyl fluoride and 5% (w/v) polyvinylpyrrolidine. The homogenate was sonicated for 60 s and centrifuged at 10000g for 10 min at 4˚C. Protein content was measured according to B r a d f o r d (1976).

Enzyme analysis

Total POD (EC. 1.11.1.7) activity in soluble leaf and root fractions was measured as absorbance increase at 430 nm with pyrogallol (A_{430} ε=2.47 mM⁻¹cm⁻¹) as the hydrogen donor. The reaction mixture consisted of 30 mM pyrogallol, 1.3 mM H_2O_2, 100 mM

Na–phosphate buffer (pH 6.5), and an aliquot of the extract diluted 30 times. Absorbance was measured in a UV/VIS spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Isoelectrofocusing (IEF) was carried out in 7.5% polyacrylamide gel with 3% ampholite in a pH gradient from 3 to 9.3. To determine POD activity, the gel was incubated with 10% 4-chloro-a-naphthol and 3% H_2O_2 in 100 mM K-phosphate buffer (pH 6.5).

Determination of total antioxidative capacity (TAC)

Frozen and powdered samples of leaves and roots were extracted in methanol, and sonicated for 60 s. The homogenate was centrifuged at 10000g for 10 min at 4˚C. Total antioxidative capacity was estimated in the supernatant according to R e et al. (1998) and A r n a o et al. (1996). The reaction mixture contained 2 mM ABTS, 0.1 mM H_2O_2, and 0.25 nM HRP in 50 mM phosphate buffer (pH 7.5) and 100 µl of extract. Absorbance was measured at 730 nm. The ascorbic acid standard curve was used to determine the relative antioxidant capacity of samples.

Total phenolics content

The content of total phenolics was determined using Folin-Ciocalteu reagent and absorbance changes at 725 nm were recorded (S i n g l e t o n and R o s s i, 1965). The gallic acid standard curve was used for determination of phenolics content in extracts.

Statistical analysis

Data were analyzed with the Statistica 6 for Windows program. Repeated ANOVA measurements and the non-parametric Mann-Whitney test were applied after performing the Kolmogorov-Smirnof-Lilliefors test for normality. The level of significance was set at 0.05.

RESULTS

Two-month-old plants of *Verbascum thapsus* L. with four fully developed leaves were transferred to nutrient media supplied with different concentrations of $ZnSO_4$ (1, 5, and 10 mM). Those plants, which were grown hydroponically under controlled conditions, initially had 400 times lower Zn content in leaves and 90 times lower Zn content in roots (20 µg/g and
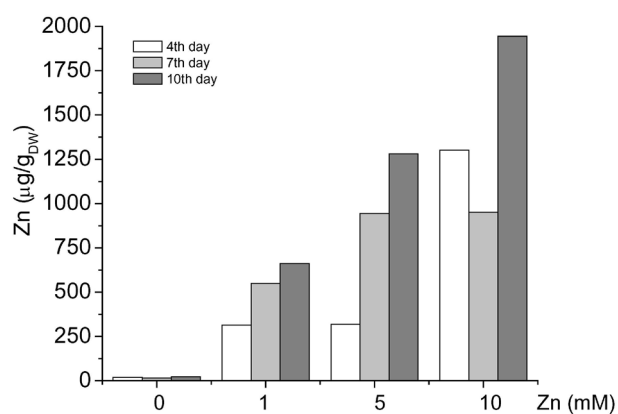

Fig. 1. Content of zinc in leaves of *Verbascum thapsus* L. after 4, 7, and 10 days of treatment with 1, 5, and 10 mM of ZnSO4 added to the nutrient solution.
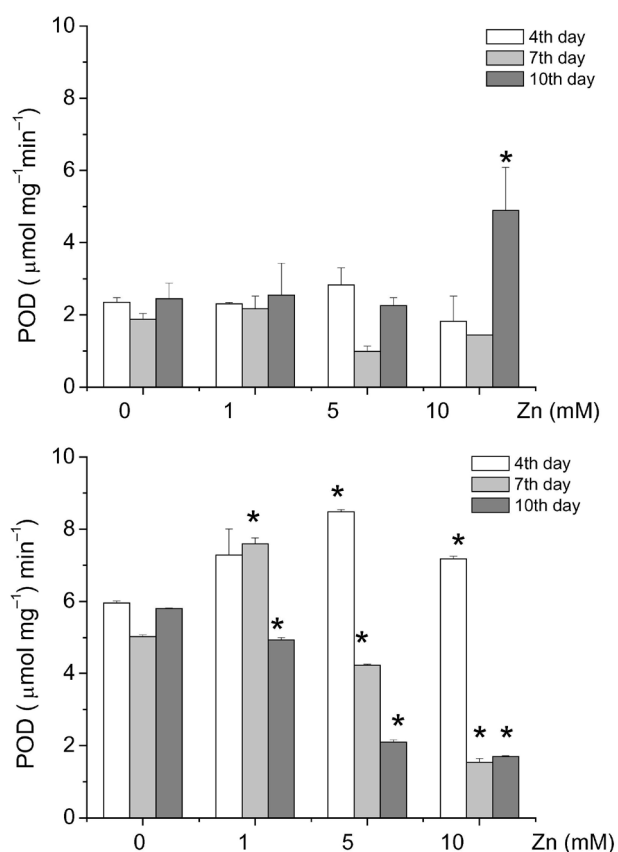

Fig. 2. Activity of soluble peroxidase in leaves (up) and roots (down) of *Verbascum thapsus* L. after 4, 7, and 10 days of the treatment with three concentrations of ZnSO4 (1, 5, and 10 mM). Pyrogallol was used as an electron donor, and absorbance increase was measured at 430 nm. Data are means of three or four replicates. The asterisk (*) indicates statistically significant differences in relation to the control (ANOVA, p≤ 0.05).
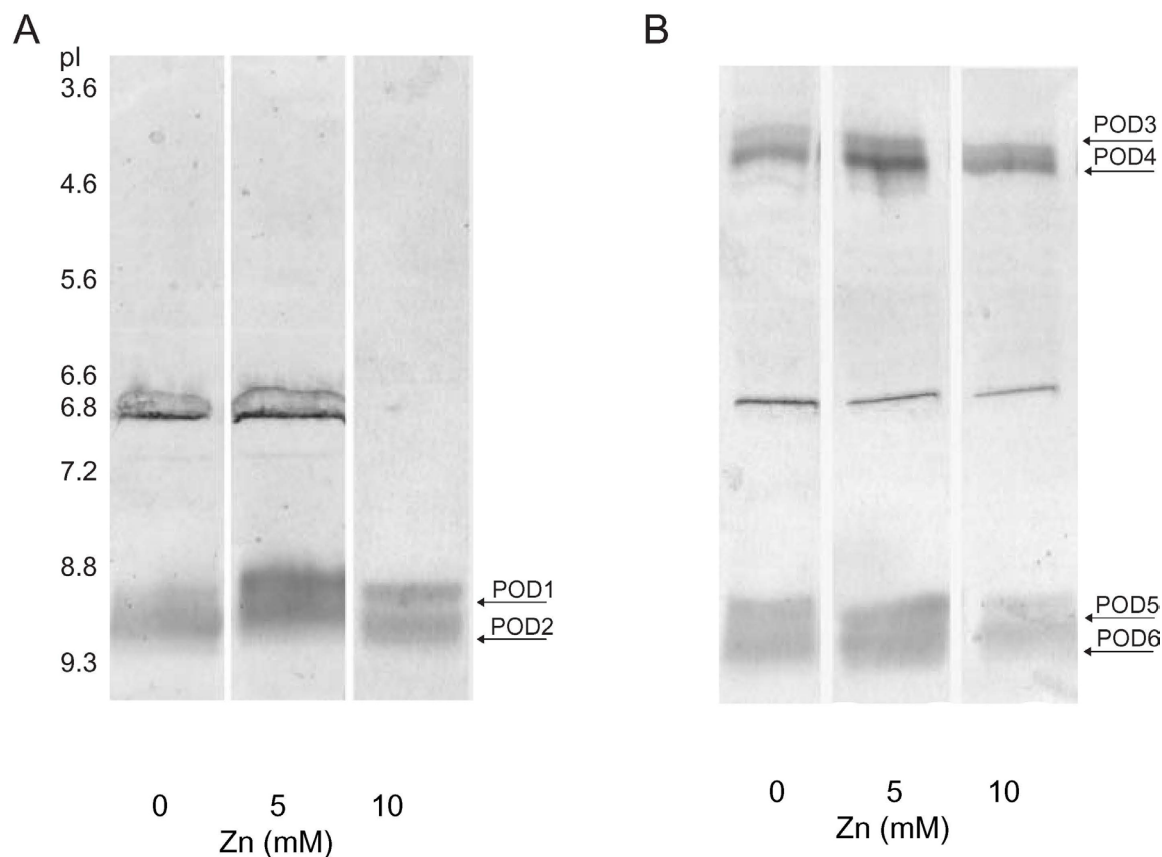
Fig. 3. Isoelectrofocusing pattern of soluble leaf (A) and root (B) peroxidase of *Verbascum thapsus* L. in control plants and after 4 days of treatment with 5 mM and 10 mM Zn. Arrows indicate different POD isoforms.

330 μg/g, respectively) when compared to 2-yr-old plants found at the industrial waste disposal site in Šabac (Jovanović et al., 2007). Leaves accumulated Zn progressively, with increasing concentration and time of exposure, reaching a 60 times higher concentration at 10 mM Zn compared to the control (Fig 1). For induction of POD activity in leaves, higher Zn concentrations and longer exposure time were required compared to roots (Fig. 2). Peroxidase activity in roots was induced by all Zn concentrations applied (1, 5, and 10 mM) at the beginning of the treatment, after which it decreased with time (Fig. 2 B). Isoelectrofocusing showed that higher total POD activity was not due to induction of new POD isoforms in either leaves or roots (Fig. 3). Two cationic isoforms, POD1 (pI 8.9) and POD2 (pI 9.1) were present in leaves (Fig. 3A). In roots, IEF analysis resolved two groups of POD isoforms: two anion-

ic isoforms, POD3 and POD4 (pI 3.6 and 3.8), and two cationic ones, POD5 and POD6 (pI 8.9 and 9.1), similar to those in leaves. Class III POD can oxidize a wide range of different substrates, among which phenolics are preferred. We measured changes in total content of soluble phenolics in leaves and roots during the experiment. Under all conditions, we found that leaves contained six times higher levels of phenolics compared to roots (Fig. 4). Increased content of phenolics in leaves was recorded on the 7th day from the beginning of treatment at 1, 5, and 10 mM Zn. Besides ascorbate, phenolics are the main component of total antioxidative capacity of plant methanol extracts, a parameter which can be defined as the ability of water-soluble low-molecular-weight antioxidants to scavenge free radicals. Total antioxidative capacity (TAC) in leaves was higher than in roots (Fig. 5). Decrease of phenolics
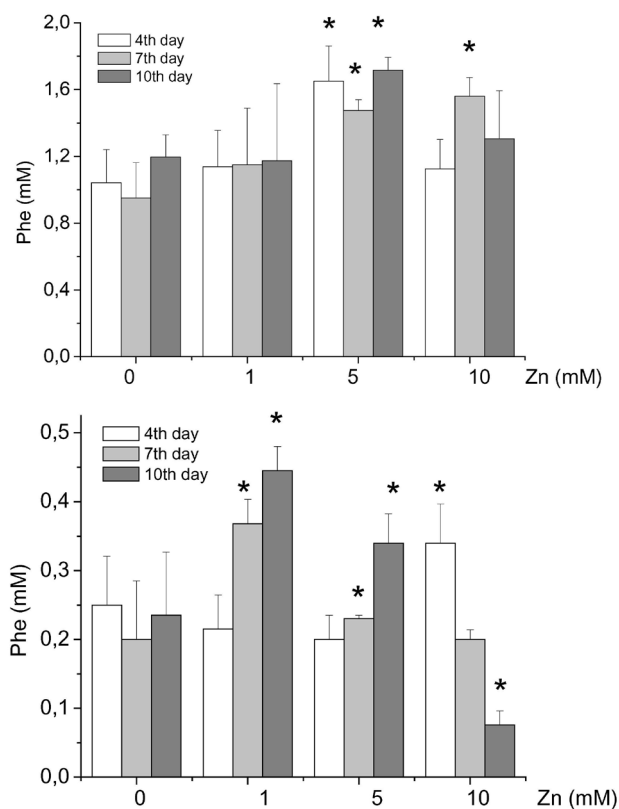
Fig. 4. Total phenolics content in leaves (left) and roots (right) of *Verbascum thapsus* L. after 4, 7, and 10 days of treatment with three concentrations of ZnSO4 (1, 5, and 10 mM). Content of total phenolics was determined using Folin-Ciocalteu reagent with the gallic acid curve as standard. Data are means of three or four replicates. The asterisk (*) indicates statistically significant differences in relation to the control (ANOVA, p≤ 0.05).
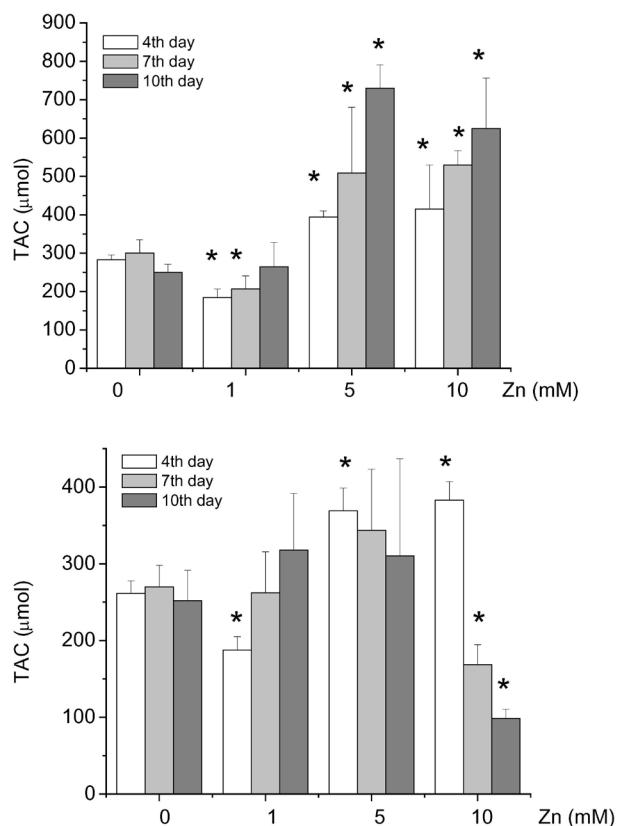
Fig. 5. Total antioxidative capacity (TAC) in leaves (left) and roots (right) of *Verbascum thapsus* L. after 4, 7, and 10 days of treatment with 1, 5, and 10 mM of ZnSO4.
The value of TAC was estimated with the ABTS test. Data are means of three or four replicates. The asterisk (*) indicates statistically significant differences in relation to the control (ANOVA, p≤ 0.05).

content in leaves at 10 mM Zn was not accompanied by a corresponding decrease of TAC (Figs. 4 A, 5 A). In roots, content of phenolics and total antioxidative capacity decreased with time. A linear correlation between phenolics content and TAC was found in roots (R^2=0.9911 for the 10th day) (Fig. 6). In leaves, on the other hand, increase of TAC was not paralleled by increase of phenolics content.

DISCUSSION

The optimal concentration of Zn in plants grown on unpolluted soil varies from 0.02 to 0.4 mg/g DW (B o w e n, 1979). Most plants show symptoms of Zn toxicity at leaf tissue concentrations of Zn above 0.2 mg/g DW (D a v i s and B e c k e t t, 1978; L o n g et

al., 2003). However, there are plants that when grown in soil containing high levels of heavy metals are capable of accumulating high metal concentrations in shoots. According to B a k e r and B r o o k s (1989), a plant is considered to be a zinc hyperaccumulator if concentrations of this metal reach 10000 mg/kg DW in shoots, which should be from 10 to 500 times higher than in plant shoots grown at an unpolluted site. We showed that *V. thapsus* L., with concentrations of 40000 mg Zn/kg DW of leaves in treated plants after 10 days of treatment, can be considered to be highly tolerant to zinc (Fig. 1). It has been shown that zinc is easily translocated from roots to shoots of the species *Sedum alfredii* and is stored in vacuoles (T i n g – Q u i a n g et al., 2006). C u p y e r s et
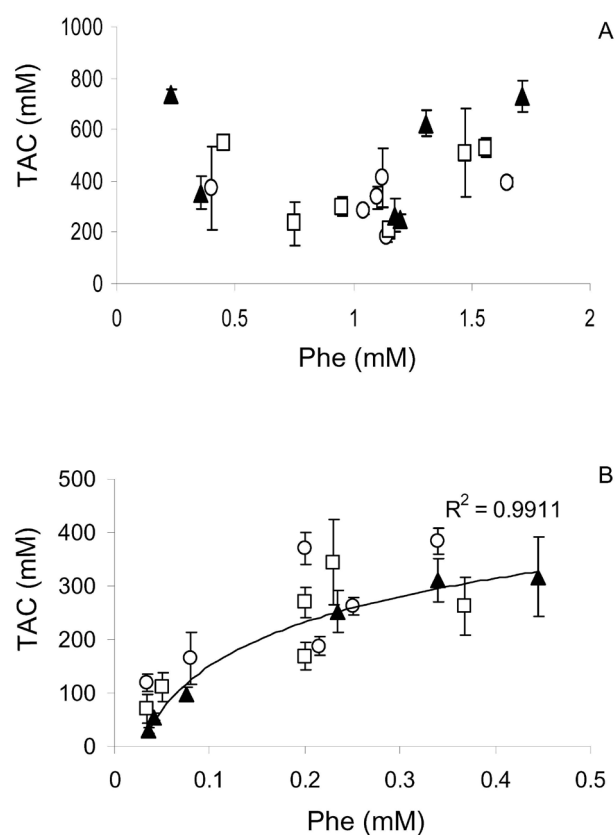
Fig. 6. Correlation between total phenolics content and total antioxidative capacity in leaves (A) and roots (B) of *Verbascum thapsus* L. induced by different Zn concentrations.

al. (2002) showed that application of Zn to seedlings of *Phaseolus vulgaris* resulted in immediate increase of zinc concentration in root tissue, which remained at the same level throughout the time of exposure. Bonnet et al. (2000) showed that Zn concentration in leaves increased with time of exposure and with Zn concentration in the culture medium. It has been shown that an excess of zinc may act as a generator of reactive oxygen species in plant tissue, causing oxidative injury (Kappus, 1985; Weckx and Clijsters, 1997; Prasad et al., 1999; Rao and Sresty, 2000), but also inducing changes in POD activity (Van Assche et al., 1986; Weckx and Clijsters, 1997; Hagemeyer, 1999; MacFarlane and Burchett, 2001; Cuypers et al., 2002). Our results confirmed that Zn had a significant effect on total POD activity in the roots and leaves of *V. thapsus* L. (Fig. 2). Compared to root

POD, leaf POD required higher Zn concentrations and longer exposure for induction of activity (induction of POD activity was obtained in leaves only at 10 mM Zn after 10 days). However, zinc did not induce changes in the POD isoform pattern in either roots or in leaves (Fig. 3). Similar results were obtained for *Phaseolus* leaves and roots (Cuypers et al., 2002). On the other hand, Fang and Kao (2000) resolved one new POD isoform in rice leaves induced by zinc treatment. Peroxidases are involved in the last step of lignification, a process that can be considered as a part of the defense mechanism against heavy metal toxicity (Degenhardt and Gimmler, 2000; Cuypers et al., 2002). These enzymes also act as an efficient H_2O_2-scavenging system in the presence of phenolics as electron donors in the apoplast and in plant vacuoles. Our results showed that increase of peroxidase activity in roots was accompanied by increase of total phenolics content (Figs. 2 B, 4 B), whereas in leaves induction of phenolics accumulation proceeded at lower Zn concentrations than induction of POD activity (Figs. 2 A, 4 A). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Morel et al., 1994; Rice–Evans et al., 1997); and metal chelators (Sakihama et al., 2002). The effect of heavy metal stress on phenolics metabolism in plants has been studied extensively (Pandolfini et al., 1992; Parry et al., 1994; Diaz et al., 2001; Sgherri et al., 2003). We observed that total antioxidative capacity in roots was in linear correlation with total phenolics content (Fig. 7 B). A linear relationship between total antioxidative capacity values and total phenolics contents has been shown previously for different plants (Ivanova et al., 2005). In leaves, however, decrease of total phenolics content was not accompanied by decrease of TAC values, indicating that another component has an important role as part of the total antioxidative capacity of *V. thapsus* (Figs. 6 A, 7 A). Cypers et al. (1999) proposed that ascorbate/glutathione may represent the main antioxidant mechanism in leaves.

CONCLUSION

Our results on Zn accumulation in leaves indicate that *V. thapsus* can be considered as a metal-tolerant

species. We showed that both peroxidase activity and phenolics content were prior to growth arrest and cell death in leaves and roots. Earlier induction of peroxidase in roots compared to leaves and the different ratio between total antioxidative capacity and phenolics content indicate that roots and leaves of *V. thapsus* L. have different antioxidative strategies as part of the defense mechanism against zinc toxicity.

Abbrevations: ABTS, 2,2'-azinobis(3-ethylbenzo-thiazo-line-6-sulfonate); DW, dry weight; HNS, Hoagland nutrient solution; HRP, horseradish peroxidase; IEF, isoelectrofocusing; Phe, phenolics; POD, peroxidase; ROS, reactive oxygen species; TAC, total antioxidative capacity.

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ПЕРОКСИДАЗЕ, ФЕНОЛНА ЈЕДИЊЕЊА И АНТИОКСИДАТИВНИ КАПАЦИТЕТ ДИВИЗМЕ (*VERBASCUM THAPSUS* L.), ИЗЛОЖЕНЕ ВИСОКИМ КОНЦЕНТРАЦИЈАМА ЦИНКА

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Доминантна врста на депонији јаловишта цинка, из металуршке индустрије, загађеној металима је дивизма (*Verbascum thapsus* L). Младе биљке наклијане из семена биљака са депоније су одгајане у хранљивом раствору са додатком цинка (1,5 i 10 mM) од осме недеље старости. Индукција пероксидазне активности у корену указује на акумулацију цинка у корену. Акумула-ција цинка у листовима није праћена променама у активности пероксидаза, већ постепеним порастом у укупном антиоксидативном капацитету, који се делимично може објаснити порастом нивоа солубилних фенолних једињења. Дискутован је значај фенолних једињења и пероксидаза за одбрамбени механизам изазван стресом вишка цинка код дивизме.