Journal of Hazardous Materials 215–216 (2012) 83–89

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Cadmium accumulation and strategies to avoid its toxicity in roots of the citrus rootstock Citrumelo

Griselda Podazza [a], Marta Arias [b], Fernando E. Prado [c,*]

[a] Instituto de Ecología, Fundación Miguel Lillo, Miguel Lillo 251, CP 4000, Tucumán, Argentina
[b] Cátedra de Anatomía Vegetal, Facultad de Ciencias Naturales e IML, Miguel Lillo 205, CP 4000, Tucumán, Argentina
[c] Cátedra de Fisiología Vegetal, Facultad de Ciencias Naturales e IML, Miguel Lillo 205, CP 4000, Tucumán, Argentina

ARTICLE INFO

Article history:
Received 13 December 2011
Received in revised form 4 February 2012
Accepted 13 February 2012
Available online 21 February 2012

Keywords:
Cadmium
Citrus rootstock
Histochemical localization
Oxidative stress

ABSTRACT

In order to assess implications of Cd-induced oxidative stress in roots of the citrus rootstock Citrumelo, seedlings were hydroponically exposed to two relatively realistic Cd concentrations during 7 days. Our results showed that increasing Cd concentrations in external solution were associated with higher Cd accumulations in roots. At 5 μM Cd the accumulation of Cd in roots was over 70-f higher than in aerial part (stem + leaves). Malondialdehyde (MDA), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and lipoxygenase activity (LOX) increased in Cd-exposed roots, suggesting a metal-induced oxidative stress. The Cd treatment enhanced the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and guaiacol-type peroxidase (G-POD), as well as the content of secondary metabolites *i.e.* soluble phenolics (SPs) and lignin. Histochemical analyses of roots showed that Cd, H_2O_2, ($O_2^{\bullet -}$), lignin and G-POD displayed a similar location pattern. Almost all analyzed parameters showed a similar dynamic tendency with increases under 5 μM Cd followed by decreases under 10 μM Cd, suggesting that a complex coordinated Cd-defensive mechanism is operating in Citrumelo roots exposed to environmental realistic Cd concentrations.

1. Introduction

Cadmium (Cd) is nowadays among the most widespread toxic heavy metals in the environment [1]. It causes many toxic effects on plants being one of the most harmful symptoms the generation of reactive oxygen species (ROS) under Cd-induced oxidative stress [2]. Oxidative stress has often been discussed as the primary effect of Cd exposure [3]. Contrarily to the ability of redox-active metals to produce ROS, Cd is a non-redox-active metal being unable to catalyze the ROS formation through Fenton and/or Haber–Weiss reactions [4]. Mechanisms by which the Cd induces the generation of ROS include inefficient operation of ROS quenching systems including the glutathione–ascorbate cycle, inhibition of antioxidant enzymes, disruption of the mitochondrial electron transport chain and/or metabolic disturbances of essential elements [3,5]. Cd also produces oxidative stress either *via* the induction of both LOX and plasma membrane-bound NADPH oxidase activities which cause the oxidation of polyunsaturated fatty acids (PUFAs), or by the peroxidase-mediated production of H_2O_2 [6]. Roots are the main interface to Cd exchange between the environment and the plant, so the root metal immobilization constitutes an important barrier to Cd entry [7]. The binding of Cd to functional groups of the cell wall (*e.g.* carboxyl, phenolyl, hydroxyl, amino acyl) forming stable complexes is considered as the pivotal reaction to block the Cd absorption [8]. Increases in peroxidase (POD) activity and lignin synthesis are important features observed during cell wall-Cd-binding [9]. Last synthesising steps in lignin polymerization are catalyzed by different POD isoenzymes that use hydrogen peroxide (H_2O_2) as co-substrate [10]. POD activity is also involved in the Cd-induced antioxidant defence mechanism [11]. It requires a concerted action of enzymatic and non-enzymatic antioxidant compounds [4]. Glutathione (GSH) and ascorbate (AS) are the major non-enzymatic antioxidants, and together with antioxidant enzymes *i.e.* SOD, CAT, PDO and APX (ascorbate peroxidase), may control the cellular levels of H_2O_2 and superoxide radical ($O_2^{\bullet -}$) [2].

Despite that responses of citrus rootstocks to several environmental stresses such as salinity and drought have been extensively studied [12,13]; scarce information can be found on plants' ability to cope with the Cd-induced oxidative stress. Most available studies have been only focused on the uptake and tissular distribution of Cd [13,14], without considering biochemical and physiological events underlying in the roots exposed to different Cd concentrations [15]. Thus, in this work we analyzed the changes of both oxidant and antioxidant markers occurring in the roots of Citrumelo seedlings

* Corresponding author. Tel.: +54 381 4239456; fax: +54 381 4330633.
E-mail addresses: prad@arnet.com.ar, fepra@csnat.unt.edu.ar, pra@tucbbs.com.ar (F.E. Prado).

0304-3894/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2012.02.031

exposed to different Cd concentrations. We hypothesized that combining biochemical and histochemical indications given by oxidant and antioxidant markers will allow us to identify and locate physiological processes characterizing the responses of roots to Cd and also to confirm its role as stress factor.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of Citrumelo (*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.), a well known commercial citrus rootstock, were used as plant material. Seeds were kindly provided by CITRUSVIL SA (Tucumán, Argentina). Seeds were soaked in distilled water at 45 °C overnight, and then surface-sterilized with 10% sodium hypochlorite for 15 min. Sterilized seeds were thoroughly washed with distilled water, sown on wet vermiculite in 15 cm × 20 cm × 5 cm plastic boxes and transferred to a germination chamber at 30 °C in darkness. To avoid water evapotranspiration the boxes were sealed with a plastic film. After 7-d incubation, seedlings of equal size were carefully selected and transplanted to 250-mL plastic pots filled with wet vermiculite (three seedlings per pot). Seedlings were allowed to grow for further 4 weeks in a growth chamber under 30/25 °C day/night temperature, 180 μmol m^{-2} s^{-1} photon flux density, 12-h photoperiod and 70% relative humidity. Weekly, each pot was supplied twice with 25 mL distilled water. After this time, seedlings with 2 leaves and equal root length and similar weight were transferred to 600-mL plastic pots (six seedlings per pot; 15 pots per treatment) filled with 500 mL Cd solution. Cd was supplied as $CdCl_2 \cdot 2½H_2O$ and the Cd solution was made with distilled water, but not with Hoagland nutrient solution to avoid the Cd chelation and/or Cd competition with Hoagland components. Cd concentrations were 0 (control), 5 and 10 μM. Treatment solutions were continuously aerated by bubbling filtered air. Seedling transpiration was compensated by replenishing the pots once with distilled water. Seven days after beginning the Cd treatment, uniformly sized roots were cut from 30 seedlings (two seedlings per pot) for each treatment and stored at −20 °C prior to chemical determinations. For microscopical analyses uniform roots from 40 seedlings (two to three seedlings per pot) for each treatment, were either collected and used without prior fixation for *in situ* observations of Cd accumulation and studied oxidant and antioxidant markers (25 roots), or fixed in FAA (formaldehyde/acetic acid/ethanol, 5:5:90) for lignin and suberin staining (15 roots).

2.2. Growth parameters

Five 7-d old seedlings from 5 pots for each treatment were collected, washed with distilled water and divided into roots, stems and leaves. Root and stem lengths were measured with a plastic ruler (accuracy ±0.5 mm). Samples were weighed to determine the fresh weight (FW), dried at 60 °C for 48 h and reweighed to dry weight (DW) determination.

2.3. Cadmium content

Samples of powdered roots, stems and leaves, digested in a concentrated $HNO_3/HClO_4$ (4:1, v/v) mixture, were used to determine the Cd content by atomic absorption spectrometry.

2.4. Determination of H_2O_2, MDA and SPs

H_2O_2 was determined according to Alexieva et al. [16]. Roots (0.5 g FW) were homogenized with 3 mL 1% trichloroacetic and centrifuged at 3000 × *g* for 5 min, and the supernatant (0.5 mL) was added with 5 mL 100 mM potassium phosphate buffer (pH 6.8) and 2 mL 1 M KI. The resulting mixture was incubated in darkness at room temperature for 30 min and the absorbance was measured at 390 nm. H_2O_2 concentration was calculated from a calibration curve made with pure H_2O_2. MDA was determined using thiobarbituric acid (TBA) [17]. Roots (0.5 g FW) were homogenized with 7.5 mL 80% ethanol and centrifuged at 3000 × *g* for 10 min, and the supernatant (1 mL) was added with 1 mL 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid. The resulting mixture was heated in boiling water for 25 min, and then quickly cooled on ice. After centrifugation at 3000 × *g* for 10 min, the absorbance was measured at 440, 532 and 600 nm. MDA concentration was calculated using the extinction coefficient 155 mM^{-1} cm^{-1} [18]. SPs were measured colorimetrically using the Folin–Ciocalteu reagent [19]. Roots (0.2 g FW) were homogenized with 3 mL 96% ethanol incubated in darkness at room temperature for 48 h and centrifuged a 3000 × *g* for 5 min. The resulting supernatant (0.1 mL) was added with 0.2 mL (1:1, v/v) diluted Folin–Ciocalteu reagent and 1.8 mL distilled water. After incubation at room temperature for 2 min, the reaction mixture was added with 0.8 mL 7.5% Na_2CO_3 and incubated at room temperature for 5 min. The absorbance was read at 760 nm. SPs concentration was determined using a calibration curve made with pure phenol.

2.5. Enzyme activities

LOX was extracted according to Baracat-Pereira et al. [20]. Roots (0.5 g FW) were homogenized in 50 mM sodium phosphate buffer (pH 6.5) and centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was used as enzyme extract. LOX activity was measured reading the increase of absorbance at 234 nm (formation of conjugated dienes) from added linoleic acid as substrate, and expressed as ΔA_{234} min^{-1} g^{-1} FW [21]. SOD and CAT were extracted by homogenizing roots (0.5 g FW) in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and centrifuging at 10,000 × *g* for 5 min at 4 °C. The resulting supernatant was used as enzyme extract. SOD activity was measured as the inhibition of nitroblue tetrazolium (NBT) photoreduction at 560 nm [22]. One SOD unit was defined as the enzyme activity that inhibited the photoreduction of NBT by 50%. CAT activity was determined by monitoring the consumption of H_2O_2 at 240 nm during 1 min [23]. One CAT unit was defined as the enzyme activity that decomposed 1 μmol of H_2O_2 per min. POD activity was extracted by homogenizing roots (0.5 g FW) in 10 mM sodium phosphate buffer (pH 6.0) containing 1 M KCl and centrifuging at 10,000 × *g* for 5 min at 4 °C, and the supernatant was used as enzyme extract. Homogenization in presence of high salt concentration allowed us to obtain both soluble and ionically cell wall-bound POD activity [24]. POD activity was assayed by monitoring the oxidation of guaiacol (G-POD) and syringaldazine (S-POD) at 470 and 530 nm during 1 min, and expressed as $\Delta A_{470\,or\,530}$ min^{-1} g^{-1} FW, respectively [25].

2.6. Microscopical analyses

Cd histolocalization was made based on the chelating ability of QAI [2-(8-quinolylazo)-4,5-(diphenyl)imidazole] to produce a rosy-purple precipitate after reacting with Cd [26]. Transversal freehand root sections directly were cut approximately 2-cm from the apex in order to reduce the Cd leaching. Root sections were washed several times with distilled water and then washed with 50 mM borate buffer (pH 10). Sections were sequentially incubated at room temperature for 15 min with each of two masking solutions: (a) 2-aminoethylamine 0.33 mM, (b) triethylenetetramine 0.66 mM. This treatment prevents staining effects of other metals such as Zn, Cr, Cu, and Pb [26]. Sections were placed during 3 h in QAI staining solution, prepared by dissolving 3 mg of QAI in 0.5 mL dimethyl sulfoxide containing 50 μL 0.5 N NaOH and diluted with

29 mL distilled water. Stained sections were washed with distilled water, transferred to slides and mounted in 50% (v/v) glycerol for microscopical observations. To detect the accumulation of H_2O_2, 2-cm root segments taken approximately 2-cm above the apex were washed with distilled water and stained by vacuum infiltration with a recently prepared 0.1% DAB (3,3′-diaminobencidine) solution (pH 3.8) for 5 min [27]. Staining solution was not buffered because the DAB was insoluble under pH above 4.0. Infiltrated segments were incubated in the same DAB solution at room temperature for 24 h in darkness and fixed in FAA for at least 1 h. For visualizing *in situ* the $O_2^{\bullet-}$ generation, roots segments were stained by vacuum infiltration with 0.35% NBT solution containing 10 mM buffer potassium phosphate (pH 7.0) and 0.01% sodium azide for 5 min. Infiltrated segments were placed under a 40 W fluorescent lamp at room temperature for 60 min, washed with distilled water and fixed in FAA [28]. Transversal freehand sections were cut from both H_2O_2 and $O_2^{\bullet-}$ fixed segments and put on slides for microscopical observations. The accumulation of H_2O_2 was visualized by a brown color, whereas a dark blue color indicated the generation of $O_2^{\bullet-}$. Histological staining to visualize the POD activity was developed using guaiacol as enzyme substrate [9]. Transversal freehand root sections directly were cut approximately 2-cm from the apex and incubated in 50 mM phosphate buffer (pH 6.0) at room temperature for 3 min. Incubated sections were transferred to slides and treated with 50 μL 1.2 mM guaiacol and 50 μL 5 mM H_2O_2 [29]. Brown staining occurred where G-POD activity was present. An enzyme control was incubated using the same tissues and conditions but without H_2O_2. For lignin and suberin visualization, transversal freehand sections were cut approximately 2-cm from the apex and fixed in FAA at room temperature overnight. Fixed sections were clarified in 10% sodium hypochlorite solution for 12 h, washed with distilled water and stained either with 1% (w/v) toluidine blue in 96% ethanol (lignin) or 0.3% (w/v) Sudan IV in water–ethanol (3:1, v/v) (suberin) [30]. Stained sections were washed with 70% ethanol and mounted in 50% (v/v) glycerol for microscopical observations.

Histochemical observations were performed using a binocular light microscope and photomicrographs were taken with a digital camera.

2.7. Statistics

All analyses were carried out in triplicate. Data presented are means ± SD of three independent experiments. Data were subjected to analysis of variance and differences were significant at $p < 0.05$.

3. Results

3.1. Growth parameters and Cd accumulation

Citrumelo seedlings exposed to different Cd concentrations during 7 days did not produce visual symptoms of metal toxicity. At 5 and 10 μM Cd, the root and leaf biomass based on DW decreased 15.5 and 15.8% (root) and 14.5 and 14.9% (leaf) when comparing with 0 μM Cd (control), whereas the stem biomass was not affected. Root and stem lengths were unchanged by Cd exposure. Tissular accumulation of Cd was increased by increasing Cd concentration in the treatment solution. Under two Cd concentrations, the metal accumulation ranked as follows: roots > leaves > stems. Obtained Cd accumulations were: 316.68 and 346.15; 4.07 and 24.34; 0.35 and 1.11 μg g^{-1} DW at 5 and 10 μM Cd solution, in roots, leaves and stems, respectively. Root accumulation under 5 μM Cd was ~72-f higher than in aerial part (stem + leaves). Attempts to detect Cd in Cd-untreated (control) seedlings failed (Table 1).

Table 1
Growth parameters and Cd accumulation in Citrumelo roots treated with 0, 5 and 10 μM Cd. Data are means of three independent experiments ± SD ($n = 5$).

Parameter	Cd		
	0 μM	5 μM	10 μM
Root FW (g)	1.106 ± 0.10	1.088 ± 0.08	1.095 ± 0.10
Root DW (g)	0.248 ± 0.01	0.212 ± 0.01*	0.211 ± 0.02*
Root length (cm)	13.12 ± 2.19	12.85 ± 1.80	12.35 ± 2.02
Cd (μg g^{-1} DW)	ND	316.68 ± 15.23*	346.15 ± 12.30*
Stem FW (g)	0.731 ± 0.60	0.787 ± 0.43	0.750 ± 0.47
Stem DW (g)	0.209 ± 0.06	0.219 ± 0.05	0.216 ± 0.06
Stem length (cm)	7.67 ± 0.93	7.36 ± 0.69	7.43 ± 0.72
Cd (μg g^{-1} DW)	ND	0.35 ± 0.02*	1.11 ± 0.10*
Leaf FW (g)[a]	1.093 ± 0.12	1.136 ± 0.20	1.125 ± 0.14
Leaf DW (g)[a]	0.291 ± 0.02	0.246 ± 0.01*	0.245 ± 0.01*
Cd (μg g^{-1} DW)	ND	4.07 ± 0.8*	24.34 ± 2.12*
Root/shoot (DW/DW)	1.18 ± 0.14	0.97 ± 0.11	0.98 ± 0.10

[a] Corresponds to the first pair of leaves.
* Values significantly different to the control ($p < 0.05$).

3.2. H_2O_2, MDA and SPs contents

As shown in Table 2, the content of H_2O_2, MDA and SPs significantly increased in Cd-treated roots. H_2O_2 was strongly enhanced under two Cd concentrations being the highest value observed at 5 μM Cd. At 5 and 10 μM Cd, the contents of H_2O_2 were 3.3- and 2.4-f higher than the Cd-untreated content. Roots exposed to both Cd concentrations resulted in a strong MDA accumulation with the highest value observed at 10 μM Cd. Although there were no significant differences between Cd concentrations, MDA contents were 2.3- and 2.0-f higher than the control content. SPs content also increased by Cd exposure, but in less extent than H_2O_2 and MDA. The highest increase (53.7%) was observed at 5 μM Cd, whereas at 10 μM Cd it was only 18.1%.

3.3. Enzyme activities

Under Cd exposure the LOX activity strongly increased reaching at 10 μM Cd 3.6-f higher activity than the control. At 5 μM Cd the enzyme activity was 2-f greater (Fig. 1). This result indicates that an enzymatic lipid peroxidation is strongly occurring in Cd-exposed Citrumelo roots. Fig. 2A and B shows G-POD and S-POD activities under two Cd concentrations. G-POD strongly increased under 5 μM Cd reaching ~11-f higher activity than the Cd-untreated activity, whereas at 10 μM Cd this increase was only 3-f greater (Fig. 2A). Contrarily, the S-POD activity significantly decreased by Cd treatments (34.5% and 24.4% at 5 and 10 μM Cd, respectively) (Fig. 2B). Interestingly, both highest G-POD and lowest S-POD activities were observed at 5 μM Cd. SOD and CAT activities were enhanced by Cd exposure, but their activity patterns were different. SOD activity under two Cd concentrations was increased by a similar percentage (33.7%) compared with the Cd-untreated activity (Fig. 2C). CAT activity strongly increased at 5 μM Cd reaching 8.4-f higher activity than the control, whereas at 10 μM Cd, practically, no enzyme activity was detected (Fig. 2D).

Table 2
Concentration of H_2O_2, MDA and SPs in Cd-exposed Citrumelo roots. Data are means of three separate experiments ± SD ($n = 3$).

Marker	Cd		
	0 μM	5 μM	10 μM
H_2O_2 (μmol g^{-1} FW)	0.44 ± 0.03	1.45 ± 0.20*	1.04 ± 0.10*
MDA (nmol g^{-1} FW)	7.78 ± 0.06	17.89 ± 1.81*	16.54 ± 2.00*
SPs (μmol ph.eq. g^{-1} FW)	1.49 ± 0.20	2.29 ± 0.31*	1.76 ± 0.19

* Values significantly different to the control ($p < 0.05$).

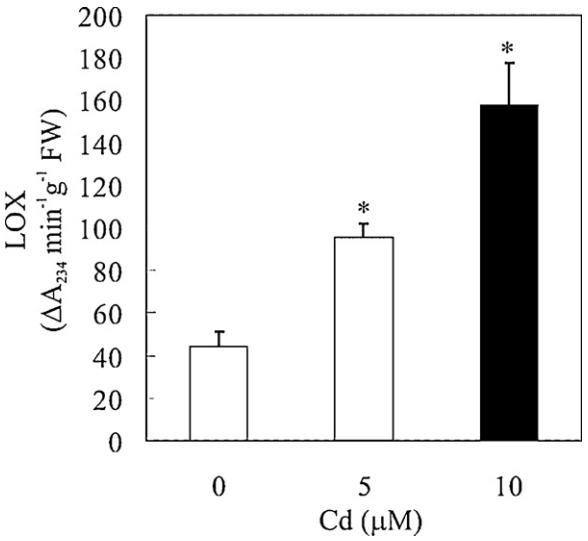

Fig. 1. LOX activity in Cd-treated Citrumelo roots. Data are means of three separate experiments ($n = 3$). Bars indicate standard deviation (SD). Asterisks in Cd-treated columns indicate values significantly different to the control ($p < 0.05$).

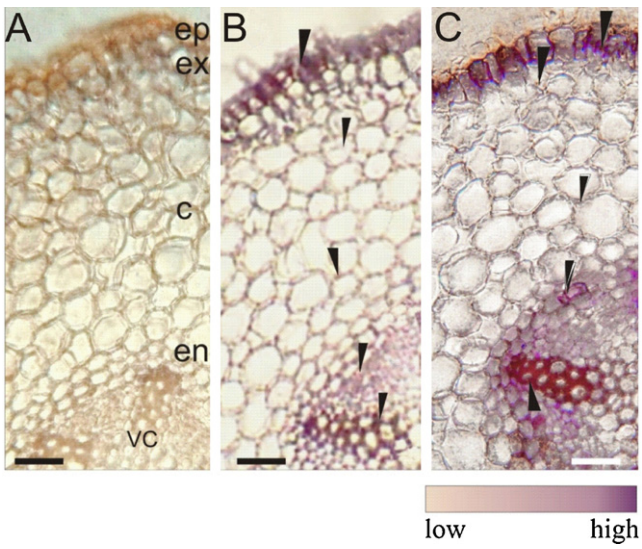

Fig. 3. Histochemical visualization of Cd in Citrumelo roots: (A) control; (B, C) 5 and 10 μM Cd. Arrows indicate major rosy-purple Cd-QAI deposits in exodermis and vascular cylinder, as well as small colored deposits in cortex and endodermis. c: cortex; en: endodermis; ep: epidermis; ex: exodermis; vc: vascular cylinder. Bar = 50 μm. Colored scale indicates the amount of Cd-QAI precipitate.

3.4. Histochemical localizations

As shown in Fig. 3B and C largest amounts of rosy-purple Cd-QAI precipitates were found in exodermis and vascular cylinder, whereas less amounts were observed in cortex and endodermis. Rosy-purple color was depending on the Cd concentration, but there were no significant differences in the distribution of colored precipitates between Cd concentrations. Rosy-purple precipitates were never observed in Cd-untreated roots (Fig. 3A). To visualize the Cd-induced ROS generation, root sections were histochemically

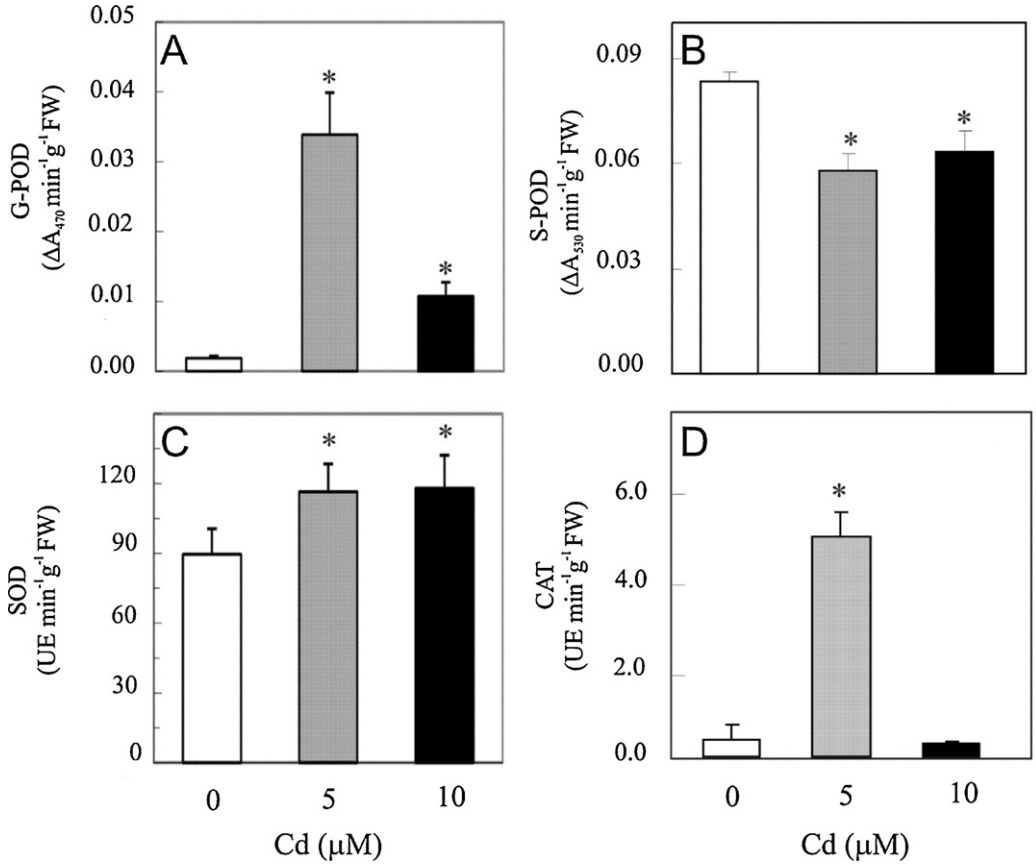

Fig. 2. Changes in G-POD (A), S-POD (B), SOD (C), and CAT (D) activities in Cd-treated Citrumelo roots. Data are means of three separate experiments ($n = 3$). Bars indicate standard deviation (SD). Asterisks in Cd-treated columns indicate values significantly different to the control ($p < 0.05$).

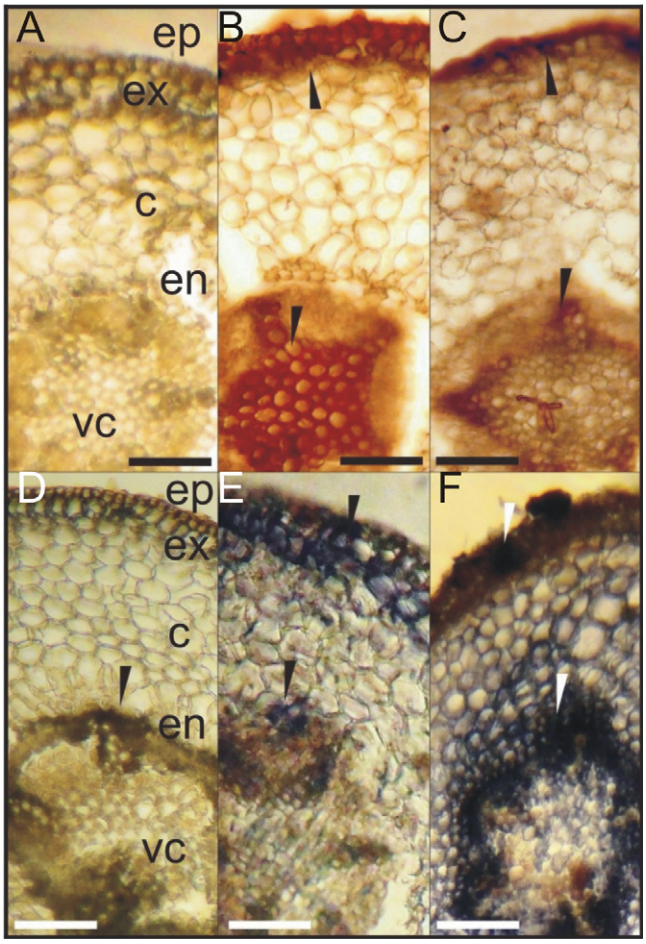

Fig. 4. Histochemical visualization of H_2O_2 (A–C) and radical $O_2^{\bullet-}$ (D–F) in Citrumelo roots exposed to 0, 5 and 10 μM Cd. White and black arrows indicate major and minor localizations of H_2O_2 and $O_2^{\bullet-}$, respectively. c: cortex; en: endodermis; ep: epidermis; ex: exodermis; vc: vascular cylinder. Bar = 50 μm.

stained for visualizing H_2O_2 and $O_2^{\bullet-}$. Roots exposed to two Cd concentrations significantly enhanced H_2O_2 production compared with Cd-untreated roots (Fig. 4A–C). Like to Cd, the accumulation of H_2O_2 was more abundant in vascular cylinder and exodermis than in endodermis and cortex (Fig. 4B and C). Agreeing with chemical measurements, the color intensity was higher at 5 μM Cd (Fig. 4B). The histolocalization of $O_2^{\bullet-}$ showed a similar distribution pattern than H_2O_2, but the difference in color intensity between Cd treatments was less pronounced (Fig. 4D–F). $O_2^{\bullet-}$ accumulation clearly was evident in the cortex at 10 μM Cd (Fig. 4F). G-POD was visualized in both Cd-exposed and control roots, but it was more intense in the former indicating an increased activity (Fig. 5A–C). Although G-POD was visualized in exodermis, cortex, and vascular cylinder, the highest color intensity was observed in exodermis and vascular cylinder under 5 μM Cd (Fig. 5B). Staining specificity of G-POD activity was confirmed by evidence that root sections incubated without H_2O_2 were not stained (data not shown). Histochemical detection of S-POD activity gave no reasonable color. Lignin deposition occurred in both Cd-treated and Cd-untreated root cell walls, but it was more intense in the former (Fig. 5D–F). Lignification was evident in exodermis and vascular cylinder being clearly higher under 5 μM Cd (Fig. 5E). Suberin deposition mainly occurred in exodermis and in minor extent in endodermis. It was slightly higher at 5 μM Cd, but practically, there were no visible differences between control and Cd-exposed roots (Fig. 5G–I).

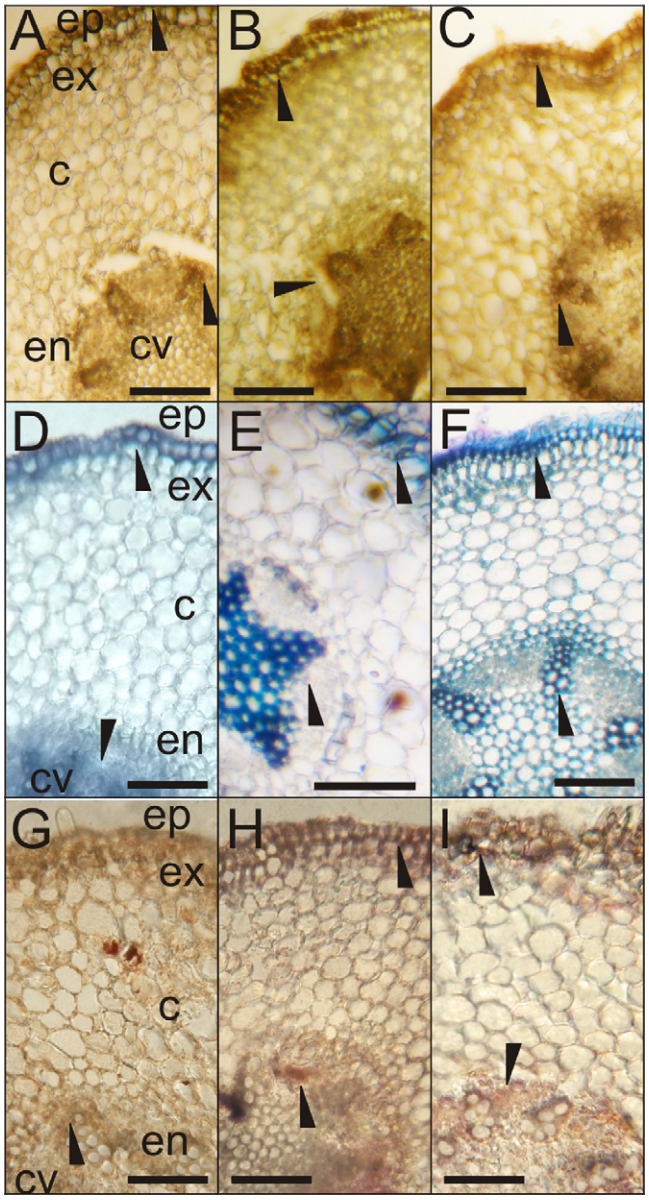

Fig. 5. Histochemical visualization of G-POD activity (A–C), lignin (D–F) and suberin (G–I) in Cd-treated Citrumelo roots. Arrows indicate major localization of G-POD, lignin and suberin in exodermis and vascular cylinder. c: cortex; en: endodermis; ep: epidermis; ex: exodermis; vc: vascular cylinder. Bar = 50 μm.

4. Discussion

Cd causes molecular damages directly itself or indirectly through the formation of ROS [31]. Our data showed a significant accumulation of H_2O_2, $O_2^{\bullet-}$ and MDA as well as a strong increase of the LOX activity in roots of Citrumelo seedlings exposed to different Cd concentrations (Fig. 1 and Table 2). These results indicate that in Cd-treated roots both ROS-dependent (non-enzymatic) and LOX-dependent (enzymatic) lipid peroxidation occurs. MDA accumulation, routinely used to estimate ROS-dependent lipid peroxidation, was less pronounced than LOX increase, so it is possible to accept that enzymatic lipid peroxidation is the predominant mechanism operating in Cd-exposed roots. This assumption could agree with previous findings indicating an earlier increase of the LOX activity in plant cells under heavy metal stress [32]. However, we do not measure the time-progress of LOX activity and MDA production and then we are unable to assume that this mechanism

is operating in Citrumelo roots. Cd affects several antioxidant enzymes, but contrasting results have been reported. Both stimulatory and detrimental effects were frequently observed [3,5]. In this study enhanced and declined CAT activity was observed in roots exposed to both low and high Cd concentrations (Fig. 2D). A decreased CAT activity produces a delay in H_2O_2 removal which enhances its accumulation and concomitantly produces a shift in the generation of ROS giving an increased free radical mediated lipid peroxidation [5]. Similar increase and decline in CAT activity was found in Cd- and Pb-stressed plants [33,34]. Our data also indicate an increase and decline of the guaiacol-type peroxidase activity (G-POD) at 5 and 10 μM Cd (Fig. 2A). Unlike our results, decreases of CAT activity are generally accompanied by increases in G-POD activity, which contributes to scavenging the excess of H_2O_2 [35]. Although we cannot explain the behavior of CAT and G-POD occurring in Citrumelo roots, it is possible that 5 μM of externally applied Cd represents an optimum threshold level for both oxidant and antioxidant processes. Supporting this assumption, maxima contents of H_2O_2, MDA and SPs as well as greater staining of H_2O_2, $O_2^{\bullet -}$ and lignin in histochemical analyses were also found at 5 μM Cd (Table 2, Figs. 4A–F and 5D–F). Based on these results we hypothesized that in Cd-exposed Citrumelo roots, antioxidant enzymes respond in a coordinated manner to combat the Cd-induced oxidative stress up to a moderate external Cd concentration (5 μM). However, LOX and SOD activities were not decreased by the high Cd concentration (Figs. 1 and 2C), suggesting that a disturbance in the coordination among antioxidant enzymes seems to be occurring. In these conditions the defense provided by antioxidant enzymes could become insufficient to cope up with the increased Cd load, leading to an oxidative stress. A similar hypothesis has been postulated to explain the distribution pattern of SOD and CAT activities in Cd-stressed *Ceratophyllum demersum* plants [36]. It has been assumed that root length is a more accurate indicator of heavy metal toxicity than the biomass production [37]; however, in our study there were no significant changes in the root length of Cd-treated seedlings when comparing with initial root length, suggesting that further analyses are needed to get a better knowledge of relationships between growth parameters and oxidative stress occurring in Cd-exposed Citrumelo roots.

It is known that SPs participate in ROS scavenging due to availability of hydroxyl groups in their molecules [38]. Soluble phenolics (SPs) pathway to ROS scavenging involves a G-POD-mediated electron donation to H_2O_2 detoxification [39]. Positive correlations between G-POD activity and SPs accumulation under heavy metal stress were found in many plants [31,38,40]. Agreeing with these findings, significant increases of both SPs and G-POD were observed in Cd-treated roots (Table 2 and Fig. 2A), suggesting that the SPs-dependent ROS scavenging pathway occurs in Citrumelo roots. Contrarily to powerful antioxidant activity, SPs can also act as prooxidant molecules *via* the phenoxyl radical ($POO^{\bullet}$) generation in both antioxidant reactions and lignin synthesis [10,11]. Under normal growth conditions these radicals have not a harmful prooxidant activity, because they are rapidly changed to non-radical products by polymerization reactions or to parent phenolics by enzymatic and non-enzymatic reductions [41]. However, under stressful conditions the heavy metals can act as spin stabilizers of $POO_s^{\bullet}$, giving a longer radicals' lifetime and consequently enhancing the SPs-prooxidant effects [39]. As prooxidant molecules, SPs can induce the lipid peroxidation of PUFAs [41]. According to our data, LOX, MDA and SPs contents increased in Cd-exposed roots, but the SPs accumulation was less pronounced; suggesting that ROS-induced- and LOX-catalyzed-lipid peroxidation are the main lipid oxidative pathways operating in Citrumelo roots. Therefore, we hypothesized that SPs mainly are channeled *via* G-POD activity toward the synthesis of lignin and other cell wall polymerized phenolic compounds which contribute to immobilize the Cd in roots. Cell wall lignification naturally occurs during the root development, but prematurely it also occurs in response to different biotic and abiotic stresses. Many POD isoenzymes catalyze lignin-related oxidations being G-POD and S-POD the best characterized POD activities involved in lignin synthesis [42]. In our study G-POD activity increased significantly under Cd exposure whereas S-POD activity decreased compared with Cd-untreated roots (Fig. 2A and B). This fact suggests that G-POD rather than S-POD is the main POD isoenzyme involved in Citrumelo root lignification. Our results disagrees with previous reports indicating that S-POD is the main POD activity involved in lignin synthesis due to only cell walls that are undergoing this activity are able to oxidize syringaldazine [43]. Conclusive evidence on the involvement of G-POD activity in the lignification occurring in Cd-treated Citrumelo roots was provided by us, *via* the direct correlation observed between measured G-POD activity and H_2O_2 accumulation with *in situ* histolocalization of lignin deposition, G-POD activity and H_2O_2 production in these roots (Figs. 2A, 4A–C, 5A–F and Table 2). Contrarily, the S-POD activity did not show any correlation with both lignin synthesis and H_2O_2 accumulation. Supporting the postulated protective mechanism:

SPs synthesis → lignin deposition → Cd immobilization

we found in Citrumelo roots under 5 μM Cd a metal concentrations over 70-f higher than the observed in aerial part (stem + leaves) (Table 1). Although the histochemical localization of Cd and lignin were coincident under both Cd concentrations, attempts to histochemical detection of SPs failed. Direct correlations between POD activity and SPs content under Cd stress were also found in *Matricaria chamomilla* roots [38], *Crotalaria juncea* leaves [40], and *Brassica juncea* shoots [44]. Despite the role that exodermis and endodermis suberization plays to avoid the entry of heavy metals into the plant [45], in our study practically no visible differences in suberin deposition between Cd-treated and Cd-untreated roots were observed (Fig. 5G–I)

5. Conclusion

Enhanced activities of antioxidant enzymes including G-POD activity associated to lignin synthesis, as well as increased contents of both SPs and H_2O_2 observed in this study suggest that in Citrumelo roots exposed to environmental realistic Cd concentrations an efficient ROS scavenging system to mitigate the oxidative stress and an active mechanism to retain the Cd in roots avoiding its transport to aerial parts, are occurring. Histochemical detection of Cd, lignin, H_2O_2, $O_2^{\bullet -}$ and G-POD also contribute to support this conclusion.

Acknowledgements

This work was supported by grant 26/G437 from Consejo de Investigaciones de la Universidad Nacional de Tucumán. FEP is a researcher of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

[1] L. Sanità di Toppi, R. Gabbrielli, Response to cadmium in higher plants, Environ. Exp. Bot. 41 (1999) 105–130.
[2] B. Halliwell, Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life, Plant Physiol. 141 (2006) 312–322.
[3] M.C. Romero-Puertas, M. Rodriguez-Serrano, F.J. Corpas, M. Gómez, L.A. del Rio, L.M. Sandalio, Cadmium-induced subcellular accumulation of O_2-, and H_2O_2 in pea leaves, Plant Cell Environ. 27 (2004) 1122–1134.
[4] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930.

[5] P. Kumar, R.K. Tewari, P.R. Sharma, Cadmium enhances generation of hydrogen peroxide and amplifies activities of catalase, peroxidases and superoxide dismutase in maize, J. Agron. Crop Sci. 194 (2008) 72–80.
[6] F. Zhang, H. Zhang, G. Wang, L. Xu, Z. Shen, Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes, J. Hazard. Mater. 168 (2009) 76–84.
[7] Y.X. Chen, Y.F. He, Y.M. Luo, Y.L. Yu, Q. Lin, M.H. Wong, Physiological mechanism of plant roots exposed to cadmium, Chemosphere 50 (2003) 789–793.
[8] M. Krzesłowska, The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy, Acta Physiol. Plant. 33 (2011) 35–51.
[9] Y.J. Yang, L.M. Cheng, Z.H. Liu, Rapid effect of cadmium on lignin biosynthesis in soybean roots, Plant Sci. 172 (2007) 632–639.
[10] A.M. Boudet, Lignins and lignification: selected issues, Plant Physiol. Biochem. 38 (2000) 81–96.
[11] A. Chaoui, E. El Ferjani, Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings, C. R. Biol. 328 (2005) 23–31.
[12] V. Arbona, V. Flors, J. Jacas, P. García-Agustín, A. Gómez-Cadenas, Enzymatic, non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive citrus rootstock, to different levels of salinity, Plant Cell Physiol. 44 (2003) 388–394.
[13] A. Gómez-Cadenas, F.R. Tadeo, M. Talon, E. Primo-Millo, Leaf abscission induced by ethylene in water stressed intact seedlings of Cleopatra mandarin requires previous abscisic acid accumulation in roots, Plant Physiol. 112 (1996) 401–408.
[14] M.F. López-Climent, V. Arbona, R.M. Pérez-Clemente, A. Gómez-Cadenas, Effects of cadmium on gas exchange and phytohormone contents in citrus, Biol. Plant. 55 (2011) 187–190.
[15] G. Podazza, M. Rosa, J.A. González, M. Hilal, F.E. Prado, Cadmium induces changes in sucrose partitioning, invertase activities and membrane functionality in roots of Rangpur lime (*Citrus limonia* L. Osbeck), Plant Biol. 8 (2006) 706–714.
[16] V. Alexieva, I. Sergiev, S. Mapelli, E. Karanov, The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat, Plant Cell Environ. 24 (2001) 1337–1344.
[17] D.M. Hodges, J.M. DeLong, C.F. Forney, R.K. Prange, Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds, Planta 207 (1999) 604–611.
[18] D. Du, W.J. Bramlage, Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts, J. Agric. Food Chem. 40 (1992) 1566–1570.
[19] T. Swain, W.E. Hillis, The phenols constituents of *Prunus domestica*. I. The quantitative analysis of phenolics constituents, J. Sci. Food Agric. 10 (1959) 63–68.
[20] M.C. Baracat-Pereira, M. Goreti de Almeida-Oliveira, E. Gonçalves de Barros, M. Alves-Moreira, M. Matos-Santero, Biochemical properties of soybean leaf lipoxygenases: presence of soluble and membrane-bound forms, Plant Physiol. Biochem. 39 (2001) 91–98.
[21] U. Szymanowska, A. Jakubczyk, B. Baraniak, A. Kur, Characterisation of lipoxygenase from pea seeds (*Pisum sativum* var. Telephone L.), Food Chem. 116 (2009) 906–910.
[22] C. Beauchamp, I. Fridovich, Superoxide dismutase: improved assays and an assay applicable to acrylamide gels, Anal. Biochem. 44 (1971) 276–287.
[23] H. Aebi, Catalase in vitro, Methods Enzimol. 105 (1984) 121–126.
[24] G. Peyrano, E. Taleisnik, M. Quiroga, S.M. Forchetti, H. Tigier, Salinity effects on hydraulic conductance, lignin content and peroxidase activity in tomato roots, Plant Physiol. Biochem. 35 (1997) 387–393.
[25] A. Polle, T. Otter, F. Seifert, Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L), Plant Physiol. 106 (1994) 53–60.
[26] Y. Sumi, T. Suzuki, Recent advances in the histochemical staining of heavy metals by means of chelating agents, with special reference to cadmium, Microsc. Res. Tech. 56 (2002) 332–340.
[27] H. Thordal-Christensen, Z. Ziguo, W. Yangdou, D.B. Collinae, Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction, Plant J. 11 (1997) 1187–1194.
[28] R. Hückelhoven, K.H. Kogel, Tissue-specific superoxide generation at interaction sites in resistant and susceptible near-isogenic barley lines attacked by the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*), Mol. Plant Microbe Interact. 11 (1998) 292–300.
[29] S. García-Lara, J.T. Arnason, D. Díaz-Pontones, E. Gonzalez, D.J. Bergvinson, Soluble peroxidase activity in maize endosperm associated with maize weevil resistance, Crop Sci. 47 (2007) 1125–1130.
[30] A. D'Ambrogio de Argüeso, Manual de técnicas en histología vegetal, Hemisferio Sur, Buenos Aires, 1986.
[31] A. Schützendübel, P. Schwanz, T. Teichmann, K. Gross, R. Langenfeld-Heyser, D.L. Godbold, A. Polle, Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots Pine roots, Plant Physiol. 127 (2001) 887–898.
[32] E. Skórzyńska-Polit, Lipid peroxidation in plants cells, its physiological role and changes under heavy metal stress, Acta Soc. Bot. Pol. 76 (2007) 49–54.
[33] N. Nikolić, D. Kojić, A. Pilipović, S. Pajević, B. Krstić, M. Borišev, S. Orlović, Responses of hybrid poplar to cadmium stress: photosynthetic characteristics, cadmium and proline accumulation, and antioxidant enzyme activity, Acta Biol. Cracov. Bot. 50 (2008) 95–103.
[34] S. Verma, R.S. Dubey, Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants, Plant Sci. 164 (2003) 645–655.
[35] U.H. Cho, N.H. Seo, Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation, Plant Sci. 168 (2005) 113–120.
[36] S. Mishra, S. Srivastava, R.D. Tripathi, S. Dwivedi, M.K. Shukla, Response of antioxidant enzymes in coontail (*Ceratophyllum demersum* L.) plants under cadmium stress, Environ. Toxicol. 23 (2008) 294–301.
[37] D.L. Godbold, C. Kettner, Use of root elongation studies to determine aluminium and lead toxicity in *Picea abies* seedlings, J. Plant Physiol. 138 (1991) 231–235.
[38] J. Kováčik, B. Klejdus, Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots, Plant Cell Rep. 27 (2008) 605–615.
[39] S.C. Grace, H. Yamasaki, W.A. Pryor, Spin stabilizing approach to radical characterization of phenylpropanoid antioxidants: an ESR study of chlorogenic acid oxidation in the horseradish peroxidase, tyrosinase, and ferrylmyoglobin protein radical systems, in: G.G. Gross, R.W. Hemingway, T. Yoshida (Eds.), Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology, Plenum Press, New York, 1999, pp. 435–450.
[40] S. Uraguchi, I. Watanabe, A. Yoshitomi, M. Kiyono, K. Kuno, Characteristics of cadmium accumulation and tolerance in novel Cd-accumulationg crops, *Avena strigosa* and *Crotalaria juncea*, J. Exp. Bot. 57 (2006) 2955–2965.
[41] Y. Sakihama, M.F. Cohen, S.C. Grace, H. Yamasaki, Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants, Toxicology 177 (2002) 67–80.
[42] A.R. Barceló, L.V. Gómez Ros, C. Gabaldón, M. López-Serrano, F. Pomar, J.S. Carrión, M.A. Pedreño, Basic peroxidases: the gateway for lignin evolution? Phytochem. Rev. 3 (2004) 61–78.
[43] N.G. Lewis, E. Yamamoto, Lignin: occurrence, biogenesis and biodegradation, Annu. Rev. Plant Physiol. Plant Mol. Biol. 41 (1990) 455–496.
[44] B. Irtelli, F. Navari-Izzo, Influence of sodium nitrilotriacetate (NTA) and citric acid on phenolic and organic acids in *Brassica juncea* grown in excess of cadmium, Chemosphere 65 (2006) 1348–1354.
[45] L. Schreiber, K. Hartmann, M. Skrabs, J. Zeier, Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls, J. Exp. Bot. 50 (1999) 1267–1280.