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Early interconnectivity between metabolic and defense events against oxidative stress induced by cadmium in roots of four citrus rootstocks

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Abstract. The effect of cadmium on roots of four citrus rootstocks was studied to assess the relationships between oxidative stress, carbohydrates, phenolics and antioxidant responses. Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) genotypes were exposed to 0, 5 and 10 µM Cd over 7 days, after which Cd accumulation was markedly higher in roots compared with stems and leaves. Malondialdehyde (MDA) and lipoxygenase (LOX) activity increased in Cd-treated SC and RL roots, suggesting that a lipid peroxidation is the main driver of plasma membrane damage. In contrast, in TC and VL genotypes, LOX-mediated lipid peroxidation does not appear to play a key role in Cd-induced lipid peroxidation, but H_2O_2 accumulation seems to be responsible of less plasma membrane damage. Catalase (CAT), superoxide dismutase (SOD) and guaiacol and syringaldazine peroxidases (G-POD and S-POD respectively) were differentially affected by Cd. Lipid profile and ATPase-dependant proton extrusion indicated higher disfunctionalities of root plasma membrane in SC and RL genotypes than in TC and VL genotypes. Differences in carbohydrates and phenolic compounds were also observed. Histochemical analysis of G-POD activity and lignin and suberin deposition revealed differences among genotypes. A model to explain the relationships among carbohydrates, soluble phenolics, lipid peroxidation and H_2O_2 accumulation in Cd-exposed roots was proposed.

Additional keywords: cadmium, defence, genotype, metabolism, oxidative stress, root.

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Introduction

The increased use of fertile lands for human activities other than agricultural practices has pushed crop cultivation to less productive lands and even polluted areas. In these conditions an increased application of fertilisers to soils is required to improve the productivity of plants. Phosphate mineral is the most used fertiliser worldwide, but contains cadmium (Cd) as a contaminant at levels varying from trace amounts to as much as 300 mg kg^{-1} on a total DW basis (Alloway and Steinnes 1999). It represents the major source of Cd input into agricultural lands (Irfan *et al*. 2013). Cd is a non-essential metal for plants and animals: it is one of the most widespread pollutants and is the most ubiquitous heavy metal in the environment (Kabata-Pendias 2011). Phytotoxic effects of Cd are related to disturbances of basic physiological processes such as the inhibition of root growth and decrease in both photosynthesis and biomass accumulation (Benavides *et al*. 2005). It also affects the normal balance between generation and scavenging of reactive oxygen species (ROS), and causes oxidative stress (Tran and Popova 2013). Cd-induced ROS accumulation is driven by both non-enzymatic and enzymatic mechanisms (Tamás *et al*. 2010). The main members of the ROS family include free radicals such as $O_2^{\bullet-}$ (superoxide) and •OH (hydroxyl), as well as non-radicals like $^1O_2$ (singlet oxygen) and H_2O_2 (hydrogen peroxide) (Sharma *et al*. 2012). Major H_2O_2-generating enzymes induced by Cd include, among others, plasma membrane-bound NADPH oxidase (NOX), xanthine oxidase (XOD), polyamine oxidase (PAO) and apoplastic peroxidase (POD) (Cheeseman 2007). Lipid peroxidation catalysed by the lipoxygenase enzyme (LOX) is of particular concern in plants exposed to Cd ions (Skórzyńska-Polit and Krupa 2006). LOX are ubiquitous enzymes in both animal and plant organisms. In plants, they play important roles in biotic and abiotic stress defence mechanisms and also in different morphological processes and the jasmonate signalling pathway (Liavonchanka and Feussner 2006). Many studies have reported a close connection between increased LOX activity and lipid peroxidation induced by different heavy metals such as Cd, Hg,

Zn, Cu, Pb and Cr (Skórzyńska-Polit and Krupa 2006; Zhou *et al*. 2008; Prado *et al*. 2010). In plants exposed to transitions metals, both non-enzymatic and LOX-dependant lipid peroxidation may be initiated in parallel, which results in a severe lipid peroxidation and increased level of lipid peroxides. Although LOX activity is increased first in plants exposed to Cd (non-transition metal), non-enzymatic lipid peroxidation begins as a result. However, transition metals produce a stronger stimulation of non-enzymatic lipid peroxidation than that induced by Cd (Gallego *et al*. 1996). Furthermore, lipid peroxidation also depends on the amount and kind of metal, individual part of plant, plant age and plant species or genotype (Skórzyńska-Polit 2007).

To alleviate Cd-induced oxidative stress, plants have a set of enzymatic and non-enzymatic antioxidant molecules (Benavides *et al*. 2005). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (AsP), glutathione peroxidase (GP), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate (DHAR) reductase, as well as reduced glutathione (GSH), ascorbic acid (AsA), α-tocopherol, metallothioneins (MTs) and phytochelatins (PCs), participate actively in the scavenging of ROS of plant cells (Mohamed *et al*. 2012). Since Cd is absorbed easily by roots, plants have evolved different strategies to avoid its toxicity. Some species use an avoidance strategy to retain most Cd on roots (Rascio *et al*. 2008), whereas others use internal mechanisms to cope Cd toxicity (Zhang *et al*. 2010). The cell wall is the major environment–plant exchange interface in roots and constitutes a barrier to Cd entry. Binding Cd to functional groups of the cell wall such as carboxyl, phenolyl, hydroxyl and amino acyl leads to the formation of stable complexes, which play a pivotal role to block the entry of Cd in root cells (Parrotta *et al*. 2015). In this regard, increases in both POD activity and lignin synthesis have been reported as common responses of Cd-exposed roots (Elobeid *et al*. 2012; Podazza *et al*. 2012; Rui *et al*. 2016). Inter- and intra-genotype variability in anatomical and physiological responses against Cd toxicity has been frequently found (Gaudet *et al*. 2011; Gill *et al*. 2011); however, little is known about the biochemical mechanisms underlying such variability in woody plants.

Since commercial citrus plants are scion–rootstock combinations, selection of suitable rootstocks becomes a key strategy to improve the performance of scion cultivars under unfavourable conditions (Ribeiro *et al*. 2014). Responses of citrus rootstocks to some environmental stresses such as salinity, drought, flooding, low temperature and boron, were studied extensively; however, heavy metal effects have not been similarly analysed (Arbona *et al*. 2008; Balal *et al*. 2012; Chen *et al*. 2012; Merlin *et al*. 2012; Liao *et al*. 2015). Studies on the effects of Cd have been focussed on Cd-induced changes of non-enzymatic and enzymatic antioxidants, but the relationships between Cd-induced oxidative stress and primary (carbohydrates) and secondary (phenolic compounds) metabolisms has not been analysed. As far as we know, studies related to this subject on Cd-exposed citrus rootstocks have been made only in our laboratory (Podazza *et al*. 2012), thus, specifics relating to inter-genotype cross-talk between oxidative stress and primary and secondary metabolisms remains unreported. We hypothesised that variability in responses of citrus rootstocks to Cd stress is a key factor for better understanding the performance of scion–rootstock cultivars growing in soils treated with mineral phosphate fertilisers. The aim of this study was to evaluate inter-genotype relationships among H_2O_2 accumulation, lipid peroxidation, antioxidant responses, soluble carbohydrates and soluble phenolics occurring in four citrus rootstocks exposed to different Cd concentrations. For this, internal accumulation of H_2O_2, MDA, soluble phenolics (SP), soluble sugars (sucrose, glucose and fructose), and lipids (phospholipids, sterols and glycolipids), as well as LOX, CAT, SOD, G-POD, and S-POD activities and plasma membrane proton extrusion were measured in roots of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) seedlings. Cd accumulation in roots, stems and leaves was determined. Histochemical analyses were performed to detect localised G-POD activity, lignin and suberin deposition in roots of both Cd-untreated (control) and Cd-treated seedlings.

Materials and methods

Plant material

Seedlings of Swingle citrumelo (*Citrus paradise* Macf. × *Poncirus trifoliata* (L.) Raf.), Rangpur lime (*Citrus* × *limonia* Osbeck), Troyer citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.), and Volkamer lemon (*Citrus volkameriana* V. Ten. & Pasq.) genotypes were used as plant material. Seeds were provided by CITRUSVIL SA (Tucumán, Argentina). Before sowing, seeds were soaked overnight in distilled water at 45°C, and then surface-sterilised with 10% sodium hypochlorite for 15 min. Sterilised seeds were thoroughly washed with distilled water, sown on wet vermiculite in plastic boxes (15 × 20 × 5 cm) and transferred to a germination chamber at 30°C under darkness. To avoid water loss by evapotranspiration, plastic boxes were sealed with plastic film. After 7 days, uniform seedlings were carefully selected, planted in 250 mL plastic pots filled with wet vermiculite (three seedlings per pot), and transferred to growth chamber under 180 µmol m^{-2} s^{-1} photon flux density, 30/25°C day/night temperature, 12 h photoperiod and 70% RH. Pots were supplied with 1/4 strength Hoagland's nutrient solution (25 mL per pot) each week. After 4 weeks, seedlings with two leaves and similar root length were washed thoroughly with distilled water to eliminate residual vermiculite and transferred to 600 mL plastic pots (six seedlings per pot, 15 pots per treatment) containing 500 mL of different Cd solutions prepared in distilled water. Next, pots were transferred to a growth chamber for 7 days under similar conditions. We choose a 7 day treatment period based on a previous study conducted in our laboratory, which showed that citrus seedlings are able to grow and stay healthy, without visible chlorosis symptoms, in distilled water without nutrient supply for at least 9 days (G Podazza, unpubl. data). Cd solutions were prepared from a 500 µM stock solution. Hoagland's nutrient solution was not used to prepare treatment solutions to avoid competition between Cd and cations of the Hoagland's solution either by cell wall binding sites and/or plasma membrane ion transporters (Sterckeman *et al*. 2011). The use of nutrient solution could also result in algal growth that can interfere with root Cd uptake.

Selected Cd concentrations were 5 and 10 µM, which are equivalent to 0.62 and 1.24 mg Cd kg^{-1} soil DW (Kabata-Pendias 2011). These Cd concentrations are found frequently in soils added with phosphate mineral fertiliser or sewage sludge (Alloway and Steinnes 1999). Treatment solutions were aerated every day by bubbling filtered air for 6 h and were changed every 2 days to avoid Cd depletion during the experiment. Distilled water was used as a control solution (0 µM Cd). At the end of Cd treatment, seedlings were harvested and used for both biochemical and histochemical studies. For enzymatic analyses, plant material was stored at –20°C.

Biomass accumulation and Cd determination

After harvest, seedlings with similar height (30 per treatment) were washed with deionised water, blotted dry onto absorbent paper, and sectioned into roots, stems and leaves. Sectioned organs were weighed to obtain the FW and dried in a hot air oven at 60°C for 48 h to obtain the DW. Biomass accumulation was expressed as g DW. After that, dry samples were ground to fine powder in an electrical mill. Powdered samples (roots, stems and leaves) were digested in concentrated $HNO_3 : HClO_4$ (4 : 1, v/v) mixture, following a modified USEPA 3051 protocol (USEPA 1994). Cd content was determined by atomic absorption spectrophotometry (Perkin-Elmer 373 Spectrophotometer) and expressed as µg g^{-1} DW. Data presented are the mean values of triplicates. The ratio between Cd accumulated in root and Cd accumulated in shoot, ($Cd_{root} : Cd_{shoot}$), was calculated as follows:

$$Cd_{root}/Cd_{shoot} = R/(S + L), \quad (1)$$

where R is root Cd content × root DW, S is stem Cd content × stem DW and L is leaf Cd content × leaf DW.

Enzyme activities

SOD, CAT and LOX activities were extracted and assayed as described by Prado *et al*. (2010). SOD and CAT activities were expressed as UE min^{-1} g^{-1} FW whereas LOX activity was expressed as ΔA_{234} min^{-1} g^{-1} FW. G-POD and S-POD activities were extracted and assayed according to work by Peyrano *et al*. (1997). Enzyme activities were expressed as ΔA_{470} min^{-1} g^{-1} FW (G-POD) and ΔA_{530} min^{-1} g^{-1} FW (S-POD) respectively.

Quantification of H_2O_2, MDA and SP

H_2O_2, MDA and SP were extracted and determined according to work by Prado *et al*. (2010). Concentrations were expressed as µmol g^{-1} FW (H_2O_2), nmol g^{-1} FW (MDA), and µmol phenol equivalents g^{-1} FW (SP) respectively.

Determination of phospholipids (PL), sterols (ST) and glycolipids (GL)

Root lipids were extracted with methanol : chloroform : H_2O, (50 : 50 : 10), mixture and determined as described by Zenoff *et al*. (1994). Lipid concentrations were expressed as µmol Pi g^{-1} FW (PL), µmol stigmasterol g^{-1} FW (ST) and µmol glucose g^{-1} FW (GL).

Proton extrusion

Proton extrusion from Cd-untreated and Cd-treated roots was measured as described by Zenoff *et al*. (1994) and expressed as ΔpH% by assuming that maximum ΔpH (100%) correspond to Cd-untreated (control) roots. To assess the involvement of plasma membrane H^+ ATPase activity in proton extrusion, ΔpH of control roots was also measured in presence of Na_3VO_4, a specific inhibitor of the plasma membrane H^+ ATPase activity.

Quantification of soluble sugars

Sucrose, glucose and fructose were extracted and determined as described by Prado *et al*. (2000). Sugar concentrations were expressed as µmol g^{-1} FW.

Histochemical localisation of G-POD, lignin and suberin

For histochemical analyses, roots (10 per treatment) were washed thoroughly with distilled water and blotted dry onto absorbent paper. Next, transversal freehand sections were cut ~2-cm from root apex from both control and Cd-treated seedlings. Visualisation of G-POD activity, lignin and suberin was performed according to Podazza *et al*. (2012). Microscopic observations were performed using a binocular light microscope and photomicrographs were taken with a digital camera (Olympus BX51 Microscope and DP70 Digital Camera Systems).

Statistics

For chemical and enzymatic determinations, at least three replicates were analysed and two independent experiments were performed. Results were analysed by one-way ANOVA using the Sigma Stat program for Windows, ver. 3.5 (Systat Software). Significant differences in numerical results between treatments were tested using the Tukey's multiple comparison tests at $P < 0.05$. Data in tables and figures are presented as mean values ± s.e.

Results

Biomass and Cd accumulation in seedling organs

Table 1 shows both biomass accumulation and Cd content in roots, stems and leaves of SC, RL, TC and VL seedlings exposed to different Cd concentrations. Root, stem and leaf DW were quickly affected by increasing Cd concentrations in almost all genotypes, but whereas root DW decreased significantly in RL genotype (21.1%), in VL genotype a significant increase (30.8%) occurred. Cd accumulation increased under increasing Cd concentrations in all studied genotypes. Accumulation of Cd was markedly higher in roots compared with stems and leaves. There were no significant inter-genotype differences in root Cd content, but in stem and leaf contents significant differences were observed. Under low Cd exposure, metal content in roots was between 40- (TC) and 124-fold (SC) higher than in stems, whereas under high Cd concentration was between 45- (TC) and 122-fold (SC) higher. For all assayed genotypes the pattern of Cd accumulation was: roots > stems > leaves. At the whole-plant level, metal accumulation was higher in VL and TC genotypes under 5 µM Cd, but there were no significant difference at 10 µM Cd. Higher and lower values of the $Cd_{root} : Cd_{shoot}$ ratio occurred

Table 1. Biomass accumulation, Cd content and tolerance index (Ti) in roots, stems and leaves of SC, RL, TC, and VL seedlings exposed to 0, 5 and 10 μM Cd during 7 days

Root Cd accumulation to shoot (stem + leaves) Cd accumulation ratio (Cd_{root} : Cd_{shoot}) is also showed. Different lowercase letters indicate significant differences inside each genotype for each evaluated parameter. Different uppercase letters indicate significant differences among genotypes for each Cd concentration and each evaluated parameter. Values are means ± s.e. ($n = 6$)

Concentration	SC			RL			TC			VL		
	Biomass[A] (g DW)	Cd (μg g^{-1} DW)	Ti	Biomass[A] (g DW)	Cd (μg g^{-1} DW)	Ti	Biomass[A] (g DW)	Cd (μg g^{-1} DW)	Ti	Biomass[A] (g DW)	Cd (μg g^{-1} DW)	Ti
Root												
0 μM	0.16 ± 0.02aB	–	–	0.19 ± 0.02aA	–	–	0.19 ± 0.03aA	–	–	0.16 ± 0.01bC	–	–
5 μM	0.13 ± 0.02bB	632.2 ± 57bB	0.81 ± 0.04bB	0.15 ± 0.01bB	582.1 ± 65bB	0.79 ± 0.06bB	0.22 ± 0.03aA	730.1 ± 75bA	1.16 ± 0.12aA	0.18 ± 0.01bA	766.8 ± 100bA	1.13 ± 0.12aA
10 μM	0.15 ± 0.02aB	1332.3 ± 118aA	0.93 ± 0.07bB	0.16 ± 0.01bB	1320.3 ± 165aA	0.84 ± 0.06bB	0.22 ± 0.04aA	1213.8 ± 146aA	1.16 ± 0.14aA	0.20 ± 0.02aA	1316.7 ± 187aA	1.23 ± 0.12aA
Stem												
0 μM	0.20 ± 0.03aA	–	–	0.15 ± 0.02bB	–	–	0.19 ± 0.03aA	–	–	0.19 ± 0.02aA	–	–
5 μM	0.23 ± 0.04aA	5.1 ± 0.8dD	1.15 ± 0.10aA	0.20 ± 0.02aA	7.3 ± 1.1dC	1.25 ± 0.11aA	0.22 ± 0.05aA	18.3 ± 2.1dA	1.16 ± 0.10aA	0.20 ± 0.02aA	12.7 ± 0.9dB	1.05 ± 0.10aA
10 μM	0.23 ± 0.04aA	10.9 ± 0.6cC	1.15 ± 0.09aA	0.19 ± 0.01aA	18.8 ± 1.4cB	1.19 ± 0.10aA	0.22 ± 0.04aA	26.7 ± 1.9cA	1.11 ± 0.09aA	0.18 ± 0.02aA	26.4 ± 2.1cA	1.00 ± 0.12aA
Leaf												
0 μM	0.22 ± 0.04aB	–	–	0.20 ± 0.03aB	–	–	0.28 ± 0.06aA	–	–	0.18 ± 0.01aC	–	–
5 μM	0.24 ± 0.06aB	1.1 ± 0.1fB	1.09 ± 0.08aA	0.23 ± 0.02aB	1.2 ± 0.1fB	1.15 ± 0.13aA	0.27 ± 0.06aA	1.5 ± 0.2fA	0.96 ± 0.10aA	0.19 ± 0.02aC	1.8 ± 0.2fA	1.05 ± 0.10aA
10 μM	0.23 ± 0.05aB	3.0 ± 0.1eB	1.04 ± 0.10aA	0.20 ± 0.02aB	3.6 ± 0.3eB	1.00 ± 0.09aA	0.27 ± 0.04aA	3.7 ± 0.4eA	0.96 ± 0.09aA	0.18 ± 0.02aC	4.4 ± 0.3eA	1.00 ± 0.11aA
$Cd_{root} : Cd_{shoot}$												
5 μM		57.2 ± 4.9aA			47.5 ± 3.4aB			36.2 ± 3.7aC			45.2 ± 3.1aB	
10 μM		62.5 ± 6.1aA			49.3 ± 5.1aB			38.8 ± 3.9aC			47.5 ± 4.4aB	

[A]Corresponds to a single plant.

in SC and TC genotypes compared with VL and RL genotypes respectively. There were no significant differences between VL and RL genotypes. Attempts to detect Cd in control seedlings failed in all genotypes. The tolerance index (Ti), which was calculated as the ratio between DW of plant growth in Cd solution and DW of plant grown in control solution, ranged between 0.79 and 1.25 in roots, stems and leaves. Ti was significantly higher ($P < 0.05$) in roots of TC and VL genotypes compared with roots of SC and RL genotypes, whereas in stems and leaves there were no significant differences among genotypes. For each genotype there was no significant difference in Ti values between the two Cd concentrations (Table 1).

Effect of Cd on LOX activity, and MDA and H_2O_2 accumulation

LOX activity was differentially affected by Cd treatment: in SC and RL roots LOX activity increased markedly under increasing Cd concentrations. Maximum increases of 3.5-fold (SC) and 78.8% (RL) occurred at 10 µM Cd. In contrast, in TC and VL roots it decreased by 61.4% in the former (5 µM Cd) and 68.7% in the latter (10 µM Cd) (Fig. 1*a*). Cd treatment also induced changes in the concentration of MDA. At 10 µM Cd, MDA increased significantly in SC (2.4-fold) and RL (58.9%) roots. In contrast, in both TC and VL roots MDA content was not affected by Cd treatment, but decreased significantly (73.3%) in TC roots exposed to 10 µM Cd (Fig. 1*b*). H_2O_2 concentration increased in Cd-treated genotypes, being significantly higher in VL and TC roots (Fig. 1*c*). Maximum increases were observed in VL (10.5-fold) and TC (2.6-fold) genotypes under 10 µM Cd. In SC and RL genotypes, highest increases (71.2% and 28.7%) occurred at 5 µM Cd.

Effect of Cd on antioxidant enzymes of seedling roots

Except G-POD activity, SOD, CAT and S-POD activities were differently affected by Cd in all studied genotypes (Fig. 2). Enzyme activities were either increased or decreased under increasing Cd concentrations when compared with Cd-untreated controls. SOD activity decreased by 40.6% in TC roots exposed to 5 µM Cd, but displayed a non-significant increase at 10 µM Cd. By contrast in SC, RL and VL roots, SOD activity increased markedly at 5 and 10 µM Cd respectively. Maximum increases 5.1-fold (VL) and 2.9-fold (RL) were observed at 10 µM Cd (Fig. 2*a*). CAT activity increased significantly in both SC (12.5-fold) and VL (9.7-fold) roots exposed to 5 µM Cd but sharply decreased at 10 µM Cd, reaching even much lower values than that control values. In an opposite trend, in TC and RL roots CAT activity showed a Cd-dependant increment, reaching maximum values of 83.3% (TC) and 165.9% (RL) under 10 µM Cd (Fig. 2*b*). G-POD showed a similar activity pattern in all rootstocks with significant increases occurring at 5 µM Cd. Maximum increases were 11.6-fold (VL) and 8.7-fold (RL and TC) respectively. At 10 µM Cd, G-POD activity decreased in all genotypes (Fig. 2*c*). Excepting in TC roots, S-POD activity decreased in Cd-treated SC, RL and VL roots. Maximum reductions were 58.3% (VL) and 22.9% (RL), respectively. Lowest values of S-POD activity were observed at both 5 µM (SC, VL) and 10 µM (RL) Cd respectively. Maximum

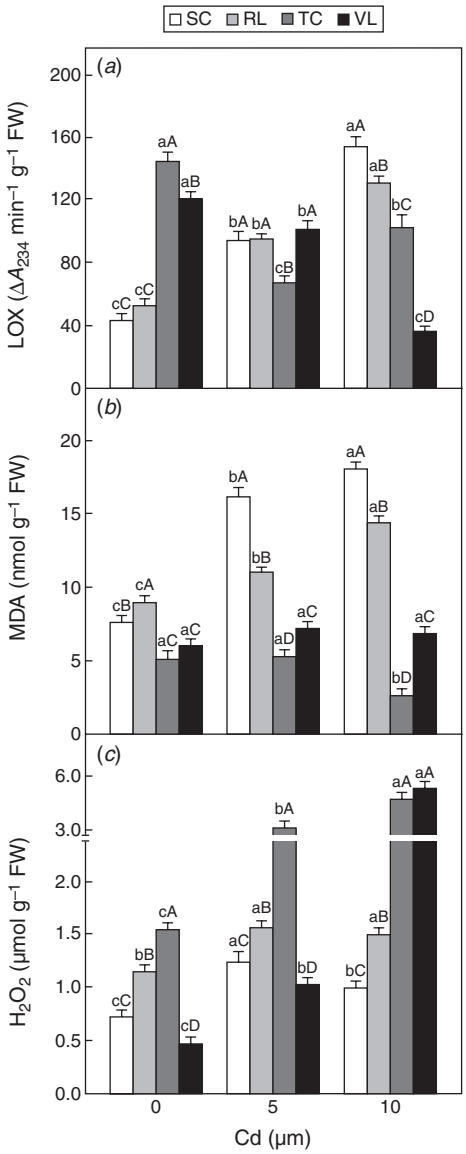

Fig. 1. Effect of Cd on lipoxygenase (LOX) activity, and H_2O_2 and malondialdehyde (MDA) concentrations of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) roots. Bars indicate means ± s.e. ($n = 6$). Different lowercase letters on bars indicate significant differences for each measured parameter and for each genotype. Different uppercase letters on bars indicate significant differences among genotypes, for each Cd concentration and each measured parameter.

increase of S-POD activity (23.4%) occurred in TC roots under 10 µM Cd (Fig. 2*d*).

Effect of Cd on root lipids

Phospholipids (PL), sterols (ST) and glycolipids (GL) were differentially affected by Cd in all genotypes (Table 2). PL showed a Cd-dependant reduction in both SC and RL roots. Maximum reductions (31.5% and 27.6%) were observed at 10 µM Cd. In contrast, there was no significant change in PL concentrations in TC and VL roots. ST decreased in SC and RL roots, but was more pronounced in the first one. Maximum

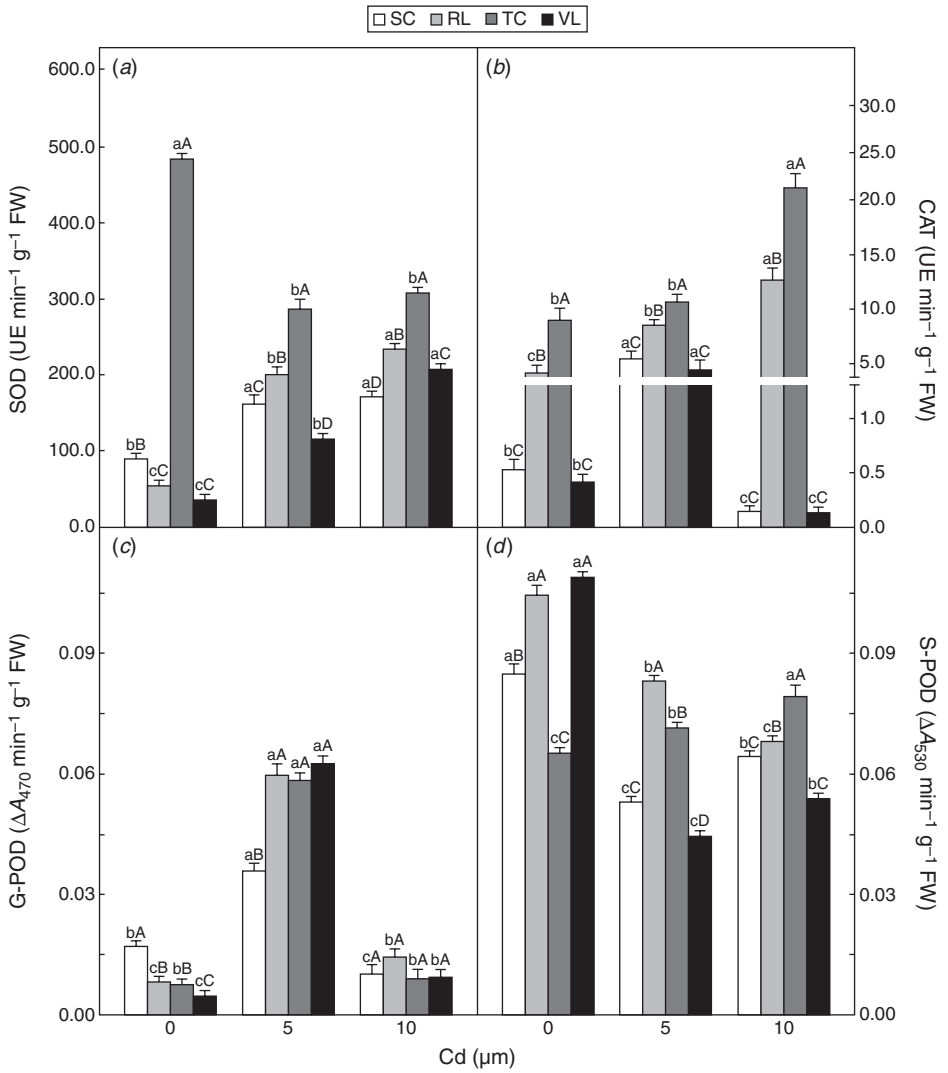

Fig. 2. Effect of Cd on superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD) and syringaldazine peroxidase (S-POD) activities in Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) roots. Bars indicate means ± s.e. (n = 6). Different lowercase letters on bars indicate significant differences for each enzyme activity and for each genotype. Different uppercase letters on bars indicate significant differences among genotypes, for each Cd concentration and each enzyme activity.

reductions (45.1 and 24.6%) occurred at highest Cd concentration. Further, in TC and VL genotypes, ST content was increased by 16.5 and 61.0% at 5 and 10 μM Cd respectively. GL content decreased in Cd-exposed roots of SC (14.4%) and RL (25.0%) genotypes under 10 μM Cd, whereas no significant changes occurred in TC roots. In VL roots GL was increased by 22.8% at 10 μM Cd. Total lipids (PL + ST + GL) were decreased by Cd treatment in SC and RL genotypes whereas in VL one was increased by 25.0%. There was no significant change of total lipids in TC roots. In Cd-exposed roots the ST/PL ratio decreased by 21.1% in SC genotype, but in TC and VL genotypes increased by 20.9 and 28.6% respectively. No significant changes were observed in RL roots. The GL : PL ratio increased by 15.3% in the SC genotype, but in the rest of genotypes there were no significant changes.

Effect of Cd on root proton extrusion

Root proton extrusion decreased at 10 μM Cd in all genotypes, but was higher in SC and RL roots. Maximum reductions were 52.4% (RL), 50.3% (SC), 37.9% (VL) and 37.1% (TC). Proton extrusions of control roots were strongly decreased by Na_3VO_4, but there were no significant differences with Cd-induced decreases (Fig. 3*a–d*). At 5 μM Cd less decreases of proton extrusion occurred (data not shown).

Effect of Cd on root soluble phenolics (SP)

SP content was increased in Cd-exposed roots of all genotypes, being higher at 5 μM Cd concentration. Maximum increases were 155.2% (VL), 90.6% (TC), 42.9% (SC) and 23.3% (RL).

Table 2. Phospholipids (PL), sterols (ST), glycolipids (GL) and total lipids (PL+ST+GL), and ST : PL and GL : PL ratios in roots of SC, RL, TC and VL rootstocks exposed to 0, 5 and 10 µM Cd during 7 days

Different lowercase letters indicate significant differences for each genotype and for each lipid type. Different uppercase letters indicate significant differences among genotypes, for each Cd concentration and each lipid type. Values are means ± s.e. ($n = 6$)

	SC			RL			TC			VL		
	Cd (0 µM)	Cd (5 µM)	Cd (10 µM)	Cd (0 µM)	Cd (5 µM)	Cd (10 µM)	Cd (0 µM)	Cd (5 µM)	Cd (10 µM)	Cd (0 µM)	Cd (5 µM)	Cd (10 µM)
Phospholipids (µmol Pi g^{-1} FW)	1.08 ± 0.09aA	0.88 ± 0.07bA	0.74 ± 0.04cA	1.05 ± 0.11aA	0.82 ± 0.08bA	0.76 ± 0.06bA	0.58 ± 0.04aB	0.56 ± 0.07aB	0.50 ± 0.04aB	0.61 ± 0.06aB	0.69 ± 0.05aB	0.64 ± 0.06aB
Sterols (µmol stigmasterol g^{-1} FW)	2.35 ± 0.20aA	1.51 ± 0.15bA	1.29 ± 0.17bB	1.75 ± 0.12aB	1.43 ± 0.11bA	1.32 ± 0.17bA	1.33 ± 0.12bC	1.55 ± 0.20aA	1.28 ± 0.10bC	1.00 ± 0.10bD	1.35 ± 0.13aB	1.61 ± 0.12aB
Glycolipids (µmol glucose g^{-1} FW)	15.22 ± 1.43aA	13.43 ± 1.23aA	13.02 ± 1.43aA	14.12 ± 1.40aA	11.76 ± 1.20bB	10.59 ± 1.05bB	10.96 ± 1.21aB	10.29 ± 1.09aB	10.82 ± 1.00aB	10.54 ± 1.10aB	11.80 ± 1.00bB	12.94 ± 1.30aA
Total lipids	18.65 ± 1.72aA	15.82 ± 1.43bA	15.05 ± 1.50bA	16.92 ± 1.65aA	14.01 ± 1.22bB	12.67 ± 1.22bB	12.87 ± 1.16aB	12.40 ± 1.15aC	12.60 ± 1.12aB	12.15 ± 1.23cB	13.84 ± 1.43bB	15.19 ± 1.33aA
ST : PL	2.18 ± 0.18aB	1.72 ± 0.16bC	1.74 ± 0.14bC	1.67 ± 0.14aD	1.74 ± 0.18aC	1.74 ± 0.17aC	2.29 ± 0.23aB	2.77 ± 0.25aA	2.56 ± 0.21aA	1.96 ± 0.20aB	1.96 ± 0.19aB	2.52 ± 0.20aB
GL : PL	14.09 ± 1.32bB	15.26 ± 1.53bB	17.59 ± 1.78aA	13.44 ± 1.45aB	14.34 ± 1.42aB	13.93 ± 1.32aB	18.89 ± 2.11aA	18.37 ± 1.76aA	21.64 ± 2.10aA	17.29 ± 1.80aA	17.10 ± 1.71aA	20.22 ± 2.00aA

At 10 µM Cd concentration, increases were 73.9, 57.4, 30.3 and 15.7% respectively (Fig. 4).

Effect of Cd on soluble sugar accumulation

Exogenous Cd caused changes in sucrose, glucose and fructose concentrations in SC, TC, RL and VL roots. Sugars were differentially affected by Cd treatment, and showed different distribution patterns among genotypes (Fig. 5). The sucrose pattern revealed that in SC, TC, RL and VL roots a Cd-dependant sucrose reduction occurred, being more pronounced in TC and VL genotypes. Maximum reductions occurring at 10 µM Cd were 61% (TC), 52.4% (VL), 45.2% (RL) and 27.5% (SC) (Fig. 5*a*). The fructose pattern was similar in all genotypes with higher reductions occurring under 5 µM Cd. Maximum decreases were 46.5% (VL), 44.1% (TC), 20.8% (RL) and 12.9% (SC) respectively (Fig. 5*b*). Glucose accumulation did not show a similar pattern in all genotypes. In SC and RL roots glucose displayed Cd-dependant increases with maximum values of 69.3% (RL) and 57.4% (SC) occurring at 10 µM Cd. In contrast, in TC and VL genotypes glucose contents decreased under low Cd concentration, but was greater than control values at 10 µM Cd. Maximum decreases occurring at 5 µM Cd were 39.1% (TC) and 37.7% (VL) respectively (Fig. 5*c*).

Histochemical localisation of lignin, suberin and G-POD activity

G-POD activity and lignin and suberin depositions were observed in both control and Cd-treated roots, but staining, in general, was more intense in the latter (Fig. 6*a–x*). At 5 µM Cd colour intensity was higher in TC and VL roots compared with SC and RL ones. Under 10 µM Cd fewer differences among genotypes were observed. All attempts to detect S-POD activity failed.

Discussion

Biomass and Cd accumulation

Many woody plant species retain large amounts of Cd in roots without transporting it to aerial parts (Gogorcena *et al*. 2011). A high accumulation of Cd has been observed in roots of Cd-exposed Cleopatra mandarin and Carrizo citrange genotypes (López-Climent *et al*. 2014). Data of the present work show a markedly higher content of Cd in roots of all studied citrus rootstocks than in stem and leaf contents (Table 1). At 5 µM Cd, higher Cd accumulation occurs in roots, stems and leaves of TC and VL genotypes than in SC and RL ones. Since the $Cd_{root} : Cd_{shoot}$ ratio did not show a similar trend among genotypes, it may be assumed that there exists a complex heterogeneity in the mechanism that regulates the transport of Cd towards aerial part in Cd-treated roots. Further, the difference in the ratio may also indicate a metal-induced disruption of the root-to-shoot transport mechanism, as well as reflect a genotypic difference in metal translocation strength. Indeed, since at a 5 µM Cd concentration both DW and Ti changes were similar to Cd accumulation, it can be assumed either that a higher threshold of Cd toxicity or a more efficient detoxification mechanism, or even both processes, could be operating in TC and VL genotypes regarding to SC and RL ones. However, there were no significant genotypic differences in root Cd

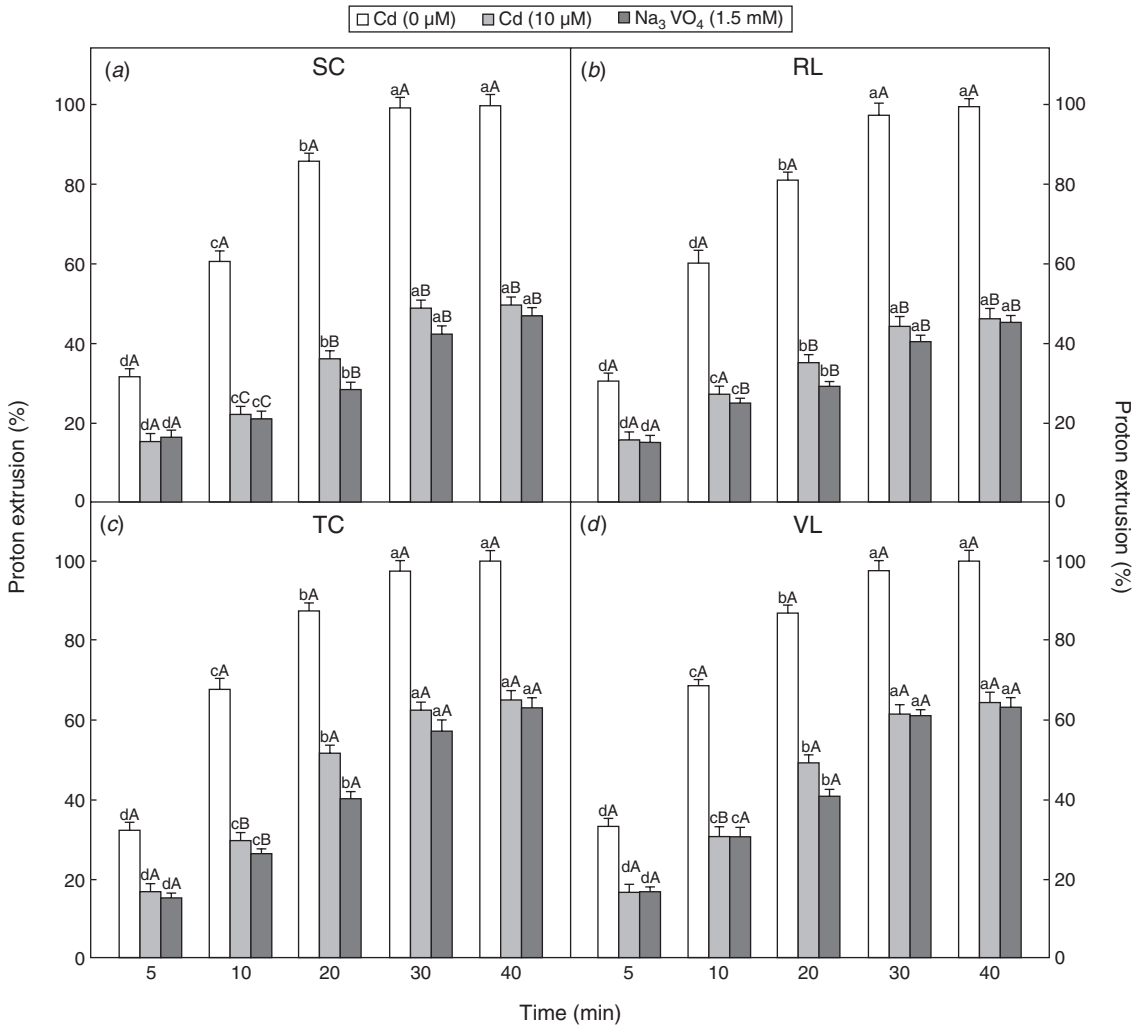

Fig. 3. Effect of Cd and Na_3VO_4 on the temporal evolution of proton extrusion from roots of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) seedlings. Bars indicate means ± s.e. ($n = 6$). Different lowercase letters on bars indicate significant differences for each measurement condition and for each genotype. Different uppercase letters on bars indicate significant differences among genotypes, for each Cd concentration and each measurement condition.

accumulation under the highest Cd concentration. Although this fact may be related to saturation of Cd-binding sites onto cell wall (Nocito *et al*. 2011), other unknown processes could also be occurring. In this regard, both accumulation and translocation of metals in plants are often not clearly identifiable entities because they depend upon species/genotype, metal concentration and metal speciation, and also may be the results of complex interactions of metal ion with other essential or non-essential ions and with several metabolic events (internal factors), and even with the environment (external factors) (Ernst 2006). It has also been suggested that root-to-shoot Cd transport is a genetically regulated process, rather than one dependent on external factors (Obata and Omebayashi 1997). Additional experiments are being planned to clarify this subject and will be reported elsewhere.

Lipid peroxidation and LOX activity

Cd, like the transition metals, is able to induce lipid peroxidation of polyunsaturated fatty acids (PUFAs) (Skórzyńska-Polit and Krupa 2006). Heavy metal-induced lipid peroxidation in plants mainly occurs by enzymatic (LOX-dependant) or non-enzymatic (H_2O_2-dependant) mechanisms, or by both (Sharma *et al*. 2012). LOX activity and H_2O_2 content in roots of citrus rootstocks were affected differently by Cd treatment. In SC and RL genotypes LOX activity increased strongly whereas H_2O_2 increased less markedly (Fig. 1*a*, *c*). These results could indicate that both enzymatic and non-enzymatic lipid peroxidation took place in SC and RL genotypes, but a LOX-mediated oxidative event would be primarily responsible of plasma membrane damage. In agreement with this assumption is that the accumulation of MDA, an indicator of lipid peroxidation, strongly increased in these genotypes. In contrast, in TC and VL Cd-treated roots, H_2O_2 increased markedly whereas both MDA and LOX activity decreased significantly, indicating that in these genotypes LOX-mediated lipid peroxidation is not a key event, being accumulated H_2O_2 responsible of Cd-induced oxidative stress. Also supporting this assumption, it has been demonstrated that

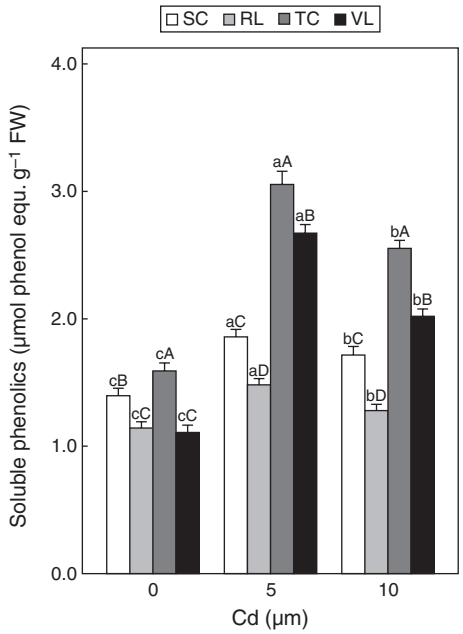

Fig. 4. Effect of Cd on soluble phenolic contents of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) roots. Bars indicate means ± s.e. (n = 6). Different lowercase letters on bars indicate significant differences for each genotype. Different uppercase letters on bars indicate significant differences among genotypes for each Cd concentration.

the increase of LOX activity is involved in Cd-induced oxidative stress responses but not in harmful lipid peroxidation in barley roots (Liptáková *et al*. 2013). Furthermore, in Cd-treated leaves of *Phaseolus coccineus*, LOX activity was similar to Cd-untreated plants, whereas non-enzymatic lipid peroxidation was significantly increased (Skórzyńska-Polit and Krupa 2006). Moreover, lipid peroxidation also depends on plant species or genotype. Because we did not measure the time progress of LOX activity and both H_2O_2 and MDA accumulation, we cannot fully confirm our hypothesis.

H_2O_2 and SP accumulation

High levels of both non-radical (e.g. H_2O_2, $^1O_2$) and radical (e.g. $O_2^{\bullet-}$, •OH) oxygen species are commonly associated to cellular oxidative damage (Sharma *et al*. 2012). According with biomass yield and H_2O_2 accumulation found in TC and VL roots, this trait seems not to be occurring in these genotypes. This could indicate that in TC and VL roots efficient mechanisms exist to remove the excess of H_2O_2. In this way, excess of H_2O_2 could be accumulated and detoxified inside a safe cell compartment such as the vacuole (Yamasaki *et al*. 1997), or channelled to H_2O_2-dependant synthesis of phenolic compounds (Kováčik and Klejdus 2008). Agreeing with this last suggestion is the result that the highest levels of SP were observed in TC and VL roots (Fig. 4). In metal stressed plants, SP consume H_2O_2 in both POD-dependant vacuolar ROS scavenging and POD-dependant synthesis of cell wall protective compounds, i.e. lignin and suberin (Michalak 2006). Increased POD activity and SP accumulation were found in all studied genotypes, but were

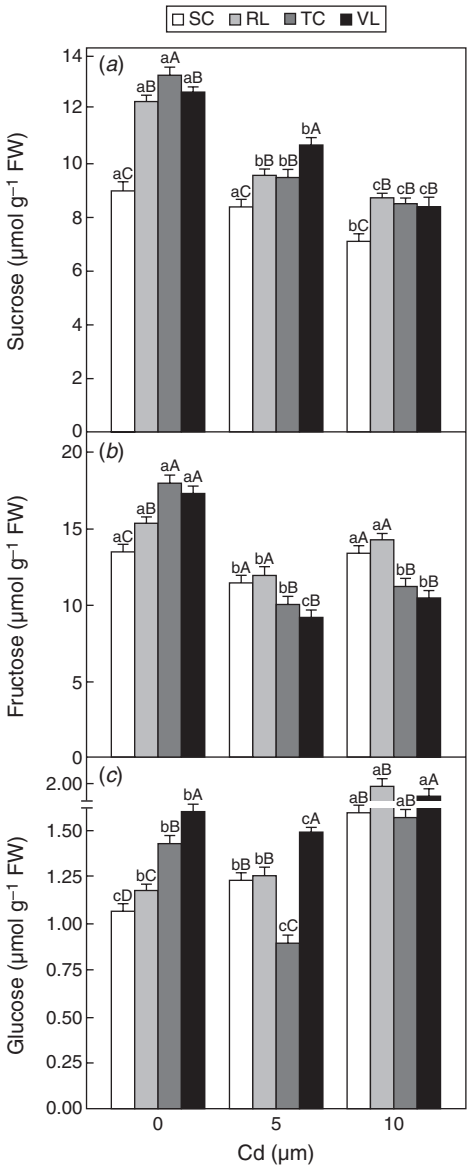

Fig. 5. Effect of Cd on sucrose, fructose and glucose concentrations of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) roots. Bars indicate means ± s.e. (n = 6). Different lowercase letters on bars indicate significant differences for each genotype and for each sugar. Different uppercase letters on bars indicate significant differences between genotypes and for each sugar.

higher in TC and VL roots. Additionally, histochemical observations of Cd-treated roots also showed higher G-POD activity and lignin and suberin deposition in TC and VL genotypes than SC and RL genotypes (Fig. 6). In agreement with our results, in a recent study conducted with two varieties of *Vicia sativa*, a differential increase of both G-POD activity and lignin deposition in roots of Cd-exposed plants has been demonstrated (Rui *et al*. 2016). Although both G-POD and S-POD activities have been related to protective compounds synthesis (Prado *et al*. 2011), our results indicate that G-POD is the main activity involved in lignin and suberin accumulation

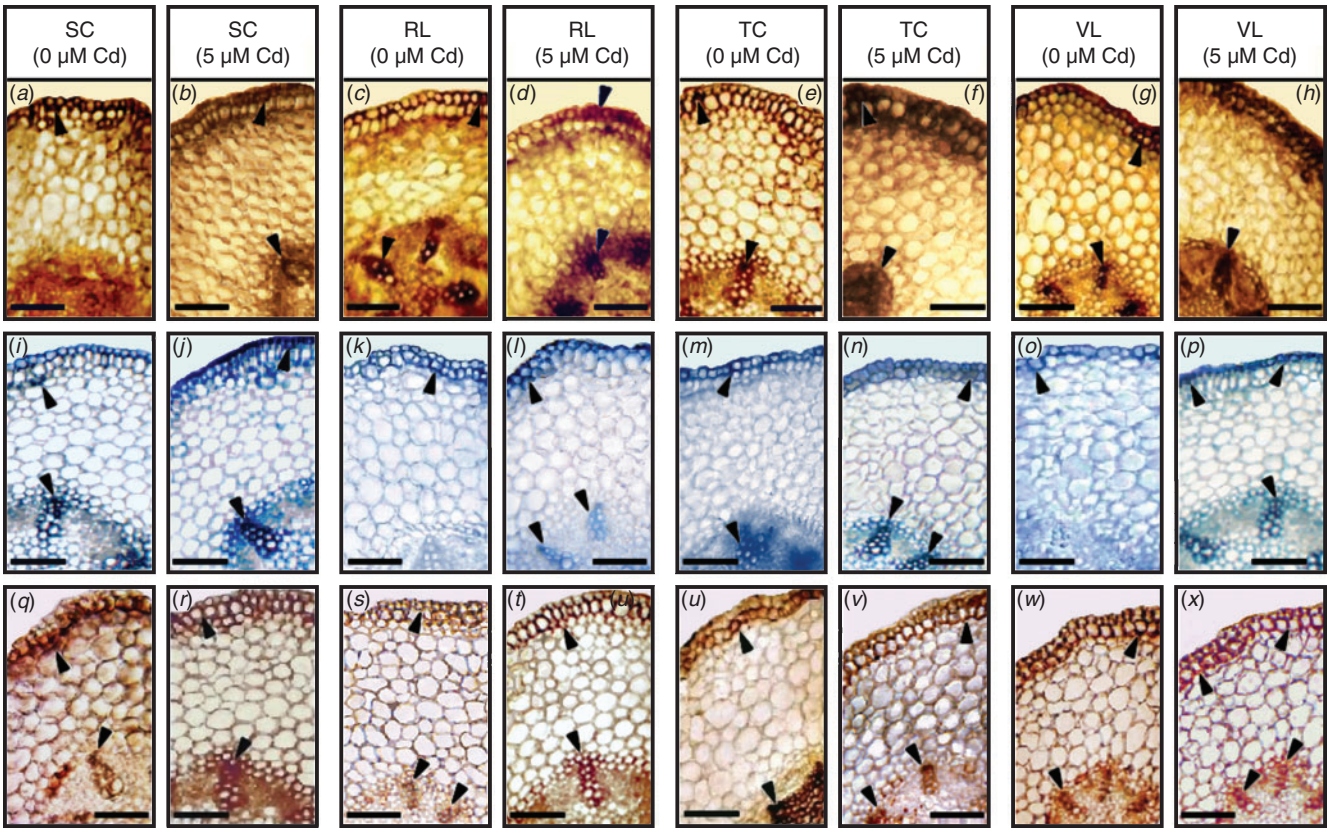

Fig. 6. Histochemical visualisation of guaiacol peroxidase (G-POD) activity (*a–h*), lignin (*i–p*), and suberin (*q–x*) in Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) roots exposed to 0 (control) and 5 µM Cd during 7 days. Arrowheads indicate enzyme activity, lignin and suberin deposition in both exodermis and vascular cylinder. Scale bar = 50 µm.

in citrus rootstock roots. However, higher levels of H_2O_2 in TC and VL roots than in SC and RL roots can be faster channelled through another H_2O_2-dependant signal pathway to orchestrate an early Cd-stress response. This assumption could be supported considering the hypothesis proposed by Petrov and Van Breusegem (2012). According to these authors a low level of H_2O_2 creates a signal for mild stress, whereas a higher amount of H_2O_2 produces a message for more severe stress. Based on this assumption, roots of TC and VL could respond faster to Cd-induced oxidative stress and then will be able to cope with oxidative stress better than SC and RL roots.

Regarding detoxification of Cd-induced radical oxygen species, both SOD and CAT activities responded differently to increasing Cd concentrations among studied genotypes, indicating that in Cd-exposed roots of citrus rootstocks can be acting through different antioxidant mechanisms. Further research is needed to gain new insights in this topic.

Lipids and plasma membrane proton extrusion

Decreases of PL in root cell membranes dramatically affect both fluidity and activity of intrinsic membrane enzymes such as H^+ ATPase and H^+ PPase (Fodor *et al*. 1995). Total lipids and PL in SC and RL roots were significantly reduced by Cd treatment, wheras in TC and VL roots total lipids were increased, but in PL there were no significant changes. According to Elloumi *et al*. (2014), decreases of both total lipids and PL are related to less plasma membrane stability against Cd toxicity. Thus, root plasma membranes of TC and VL genotypes would be less affected by Cd toxicity. In contrast though, ST modulates the physical state of lipid bilayer by restricting the motion of fatty acid chains, and also regulating the fluidity of cell membranes (Hartmann 1998). Moreover, ST becomes crucial for structural and functional properties of plant membranes (Roche *et al*. 2008). Further increasing values of ST : PL ratio have been related to stress tolerance (Wu *et al*. 1998). Data of this study show that ST and ST : PL ratio increased in Cd-treated roots of TC and VL genotypes. Together, data related to lipid content seem indicate a higher tolerance of plasma membrane against cadmium toxicity in TC and VL roots compared with SC and RL roots. Plasma membrane fluidity is determined by both unsaturated and polyunsaturated fatty acids, which, in turn, are highly dependent of LOX activity. Since LOX activity decreased in Cd-treated TC and VL roots, higher plasma membrane fluidity is expected to occur in these genotypes. Supporting this assumption, proton extrusion, which is frequently used as an indicator of membrane fluidity, showed less decreases in TC and VL roots in absence and presence of Na_3VO_4 (specific inhibitor of the plasma membrane H^+ ATPase activity). Additionally, proton extrusion of Cd-untreated roots did not show significant inter-genotypical differences. Hence, plasma membrane functionality seems to be less affected by Cd treatment in TC and VL roots.

However, both H^+ ATPase and H^+ PPase activities involved in plasma membrane proton extrusion were not measured in this work, so caution should be taken before accepting this assumption.

Soluble sugars

Under heavy metal stress a high demand for sucrose occurs to support the respiratory metabolism and synthesis of stress-related metabolites (e.g. phenolic compounds, amino acids, organic acids, glutathione, phytochelatins, metallothioneins and stress-related proteins) (Prado *et al*. 2011). In this regard, all genotypes showed significant reductions in sucrose under increasing Cd concentrations, indicating a high metabolic demand to cope Cd toxicity. However, decreased levels of sucrose can also reflect a Cd-induced photosynthate flux reduction from leaves. Although in this study did not include measurement of gas-exchange parameters, in a previous study performed on Carrizo citrange and Cleopatra mandarin rootstocks exposed to 30 and 150 µM Cd during 85 days, López-Climent *et al*. (2014) demonstrated only a slight decrease in CO_2 assimilation in Cd-treated plants. Hence, higher decreases occurring in sucrose contents of TC and VL roots (Fig. 5*a*) could be related to higher release of fructose to increase the synthesis of phenolic compounds. Fructose, through its phosphorylated derivative fructose-6-phosphate (F-6-P) produces erythrose-4-phosphate (E-4-P), enters into shikimate and phenylpropanoid pathways to produce phenolic compounds (Mustafa and Verpoorte 2007). Supporting this assumption in Cd-treated roots of TC and VL genotypes, a strong decrease of free fructose and strong increase of SP were observed. By contrast, in SC and RL genotypes, a smaller decrease of fructose and less synthesis of SP were observed (Figs 4, 5*b*). The decreases observed in glucose content at the highest Cd concentration in all genotypes probably reflect a generalised metabolic reduction induced by metal toxicity. Beyond their metabolic functions, soluble sugars (mainly sucrose and glucose) also act as signal molecules, playing a central role in both regulation and fine tuning of source–sink relationships and heavy metal stress responses (Bolouri-Moghaddam *et al*. 2010).

Figure 7 summarises a possible model of interactions among oxidative stress, H_2O_2 accumulation, lipid peroxidation, soluble carbohydrates and soluble phenolic compounds occurring in root cells of citrus rootstocks exposed to Cd: in both SC and RL roots, Cd enters cells and triggers oxidative stress with increased accumulation of ionic ROS through increased LOX activity (high lipid peroxidation) and also, but to a lesser extent, intrinsic H_2O_2 accumulation produced by Cd-induced both apoplastic and plasma membrane-bound H_2O_2-generating enzymes (POD, NOX, XOD, PAO) (Cheeseman 2007). High lipid peroxidation concomitantly drives a plasma membrane damage, which, in turn, is accompanied by a possible dysfunction of the plasma membrane H^+ ATPase activity (lower proton extrusion). Furthermore, the lower consumption of fructose occurring in Cd-exposed SC and RL roots compared with TC and VL ones can also drive a decrease in synthesis of both soluble and polymerized-derived phenolic compounds (lower lignin and suberin deposition on cell walls). In contrast, in cells of TC and VL roots, accumulated Cd does not trigger

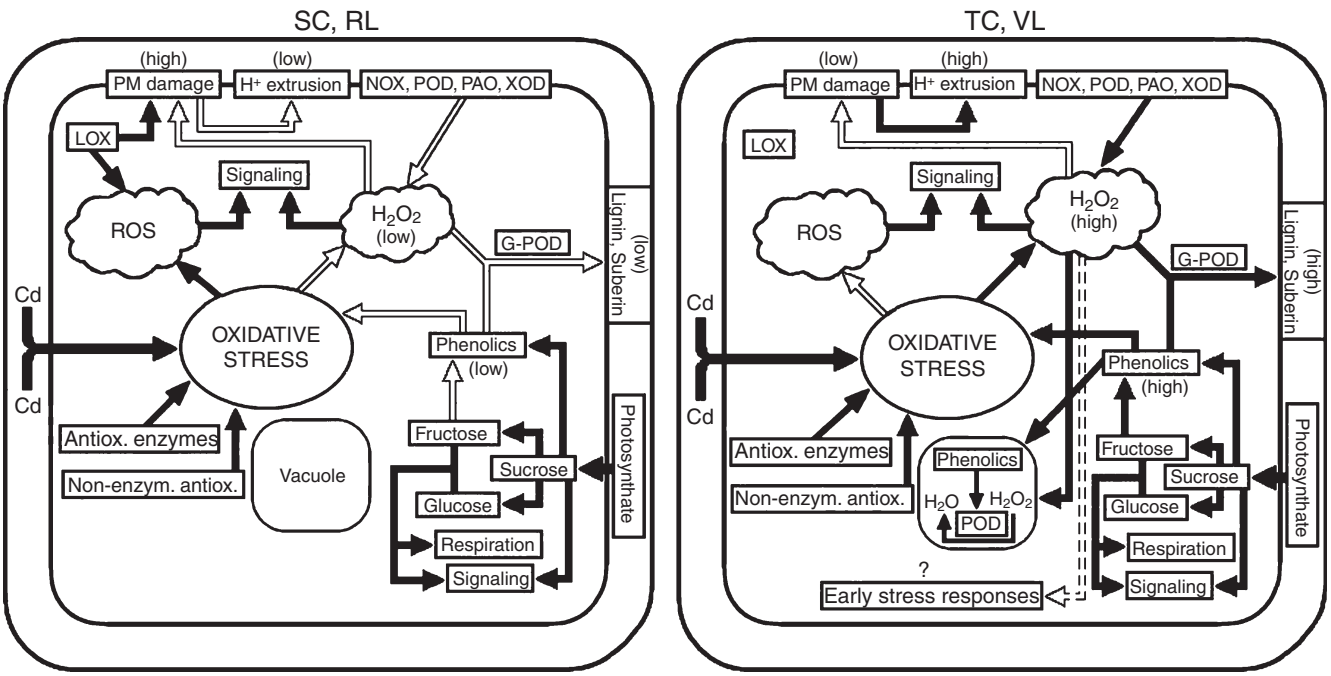

Fig. 7. Hypothetical scheme of major metabolic and defence events involving soluble carbohydrates, soluble phenolics, H_2O_2 and antioxidant enzymes that occurs in Cd-exposed roots of Swingle citrumelo (SC), Rangpur lime (RL), Troyer citrange (TC) and Volkamer lemon (VL) seedlings. Abbreviations: PM, plasma membrane; LOX, lipoxygenase; NOX, plasma membrane NADPH oxidase; POD, apoplastic and vacuolar peroxidase; PAO, polyamine oxidase; XOD, xanthine oxidase; G-POD, guaiacol peroxidase. In this figure ——— denotes high contribution; ═══ denotes low contribution; = = = = denotes probable effect.

LOX-induced lipid peroxidation, and then less severe plasma membrane damage and less H^+ ATPase dysfunction occurs (higher proton extrusion). However, Cd-toxicity triggers higher intrinsic H_2O_2 accumulation (non-ionic ROS), possibly, via enhanced activities of H_2O_2-generating enzymes. Furthermore, increased H_2O_2 would trigger a higher synthesis of secondary defence compounds through G-POD-catalysed reactions that use fructose-derived phenolics as substrates (higher SP accumulation and increased lignin and suberin deposition on cell walls). Additionally, increased synthesis of SP in TC and VL roots can also contribute to eliminate the excess of H_2O_2 inside the vacuole through a vacuolar POD-catalysed reaction (Yamasaki *et al*. 1997). Beyond these H_2O_2-dependant reactions, H_2O_2 in TC and VL root cells could also act as secondary signal molecule to trigger other early stress responses to counteract metal toxicity. Together, these traits would allow to TC and VL genotypes get greater fitness to tolerate Cd-induced stress.

Conclusions

Overall results of this study indicate that different metabolic events linked to Cd tolerance occur in SC, RL, TC and VL roots. Moreover, this study also represents a good approach to understand how low Cd concentrations in agricultural soils could affect the performance of citrus rootstocks.

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