Cadmium Induces Changes in Sucrose Partitioning, Invertase Activities, and Membrane Functionality in Roots of Rangpur Lime (*Citrus limonia* L. Osbeck)

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Abstract: **Cadmium (Cd) uptake effects on sucrose content, invertase activities, and plasma membrane functionality were investigated in Rangpur lime roots (*Citrus limonia* L. Osbeck). Cadmium accumulation was significant in roots but not in shoots and leaves. Cadmium produced significant reduction in roots DW and increment in WC. Leaves and shoots did not show significant differences on both parameters. Sucrose content was higher in control roots than in Cd-exposed ones. Apoplastic sucrose content was much higher in Cd-exposed roots than in control ones. Cd-exposed roots showed a significant decrease in both cell wall-bound and cytoplasmic (neutral) invertase activities; while the vacuolar isoform did not show any change. Alterations in lipid composition and membrane fluidity of Cd-exposed roots were also observed. In Cd-exposed roots phospholipid and glycolipid contents decreased about 50%, while sterols content was reduced about 22%. Proton extrusion was inhibited by Cd. Lipid peroxidation and proton extrusion inhibition were also detected by histochemical analysis. This work's findings demonstrate that Cd affects sucrose partitioning and invertase activities in apoplastic and symplastic regions in Rangpur lime roots as well as the plasma membrane functionality and H^+-ATPase activity.**

Key words: **Cd, *Citrus limonia*, invertases, membrane, roots, sucrose.**

Introduction

Cadmium is one of the major environmental contaminants known to accumulate in plants (Prassad, 1995; Das et al., 1997; Sanità di Toppi and Gabbrielli, 1999; Perfus-Barbeoch et al., 2002). It is a non-essential heavy metal pollutant found in soil, air, and water, mainly from various agricultural, mining, and industrial activities (Schützendübel et al., 2001). It has been considered an extremely dangerous contaminant due to its high toxicity and greater water solubility (Hart and Scaife, 1977).

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Cadmium is naturally present in soil and sediments at concentrations generally more than 1 μg g^{-1} of soil dry weight (Peterson and Alloway, 1979). Soil Cd concentration increases with time, since the addition of Cd is greater than the removal by leakage and plant harvesting (Greger and Johansson, 1992). It is rapidly taken up by plants and easily transported to the shoot; however, the amount that reaches the shoot is usually lower than the amount left in the roots because of formation of Cd-binding proteins and binding of Cd to cell walls in the roots (Greger and Lindberg, 1986; Robinson and Jackson, 1986; Greger and Johansson, 1992).

Studies of Cd toxicity have been developed on several plant physiological processes (Das et al., 1997; Sanità di Toppi and Gabbrielli, 1999). It has been shown that Cd damages the photosynthetic apparatus, decreases carbon assimilation and chlorophyll content, inhibits stomatal opening, and can generate oxidative stress (Stochs and Bagchi, 1995; Hsu and Kao, 2003; Yeh et al., 2004). Cadmium also depresses water uptake, and plants affected by metal toxicity have a low water content (Barceló et al., 1986). Additionally, Cd decreases water transport to leaves by reducing the number and radius of vessels and tracheids and by partial blockage of these with cellular debris and gums (Barceló et al., 1988).

Also, it is interesting to note that plant species vary widely in tolerance to Cd excess in the growth medium (Hertstein and Jager, 1986). In several species, these differences are genetically controlled (Das et al., 1997). However, the exact physiological mechanisms of Cd tolerance are still debated; these mechanisms may vary with plant species and varieties and may be controlled by different genes through different biochemical pathways. On the other hand, Cd-tolerant plants showing diminished accumulation of the heavy metal in shoots have been found to accumulate higher amount in roots as compared to non-tolerant plants of the same species (Baker, 1984).

Although inhibition of root elongation is considered to be the first evident effect of Cd toxicity (Breckle, 1991), the mechanisms involved in such toxicity are still not completely understood. Cadmium induced alterations in plasma membrane functionality by inducing changes in lipid composition and membrane fluidity and by affecting the enzymatic activities associated with it, such as the H^+-ATPase (Fodor et al., 1995; Ouariti et al., 1997; Sandalio et al., 2001). In addition, it has been demonstrated that Cd induces an increase in lipid perox-

idation in *Phaseolus vulgaris, Phaseolus aureus,* and *Helianthus annuus* (Somashekaraiah et al., 1992; Shaw, 1995; Gallego et al., 1996). In contrast, it is known that Cd decreases carbon assimilation by inhibiting photosynthesis (Greger and Lindberg, 1986; Greger and Bertell, 1992; Moya et al., 1993). Presently, information about Cd effects on sucrose content and cellular partitioning is scarce and unclear, especially in tree species. Thus, the aim of this study was to examine Cd effects on sucrose partitioning, invertase activities, and membrane functionality in Rangpur lime roots.

Materials and Methods

Seeds of lemon rootstock Rangpur lime (*Citrus limonia* L. Osbeck) were kindly provided by CITRUSVIL SA (Tucumán, Argentina). Seeds were germinated in plastic boxes containing moist vermiculite as substrate, in the dark at 30 °C. Boxes were sealed with plastic film to avoid water evaporation. Seven days after seeding, the boxes were transferred to a growth chamber at 30/25 °C day/night temperatures, 70% relative humidity, photosynthetic photon flux density (PPFD) of 190 µmol m^{-2} s^{-1} provided by white fluorescent tubes (Philips TLD 36 W/83, The Netherlands), and a 12-h photoperiod. After 30 days, seedlings with two leaves and equal root length were selected and transferred to 350-ml pots. Each pot contained 6 seedlings and 300 ml of continuously aerated solution of 0, 20, and 40 µM $CdCl_2$. These Cd concentrations are equivalent to 2.48 and 4.96 µg g^{-1} soil DW and correspond to the critical level of Cd soil toxicity (3 – 8 µg g^{-1} soil DW) according to Kabata-Pendias and Pendias (2000). After 7 days under above-growth conditions, plants were harvested and used for analysis.

Fresh weight (FW), dry weight (DW) after 48 h at 60 °C, and water content (WC) were measured in roots, shoots, and leaves of lemon rootstock.

Determination of sucrose

Sucrose was extracted from whole roots following the method of Prado et al. (2000): 0.5 g of tissue was homogenised with 2 ml of 80% ethanol using a cold pestle and mortar. After heating the homogenate in a water bath at 75 °C for 10 min, the insoluble residue was removed by centrifugation at 5000 × g for 10 min. The precipitate was re-homogenised with 2 ml of 80% ethanol and centrifuged as described above. The supernatants were pooled and dried under a stream of hot air and the residue was resuspended in 1 ml of distilled water and desalted using an ion-exchange (Amberlite MB3) column. To obtain the symplastic sucrose, roots (0.5 g) were cut into 5-mm long sections and rinsed in distilled water 7 – 8 times to eliminate the apoplastic sucrose. After this time, the remaining sucrose was extracted using the procedure indicated above.

Sucrose was determined by the method of Roe and Papadopoulos (1954) after eliminating free fructose from the extract (Cardini et al., 1955).

Cell wall-bound invertase

Cell wall-bound invertase was extracted from whole roots as follows (Vattuone et al., 1981): 1 g of tissue was homogenised with 2 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 5 µM $MnSO_4$ and 1 mM 2-mercaptoethanol using a cold pestle and mortar. The homogenate was squeezed through 2 layers of cheesecloth and centrifuged at 270 × g for 10 min. The pellet was resuspended in 2 ml of 10 mM sodium acetate buffer (pH 5.5) containing 5 µM $MnSO_4$ and 1 mM 2-mercaptoethanol (buffer A), frozen, and thawed 3 times to break cells and homogenised with a cold pestle and mortar. This final step enhances the fluidity of preparation. The suspension was centrifuged at 270 × g for 10 min and the pellet resuspended in 2 ml of buffer A. This procedure was repeated 3 times and the resulting suspension constitutes the cell wall preparation. To solubilise cell wall-bound invertase, the cell wall preparation was treated successively with the following solutions:

a) 0.2 M sodium phosphate buffer (pH 7.5) containing 1 M NaCl and 1 mM 2-mercaptoethanol.
b) 0.2 M sodium phosphate: sodium citrate buffer (pH 8.5) containing 1 mM 2-mercaptoethanol.
c) 0.2 M sodium phosphate: sodium citrate buffer (pH 8.5) containing 1 M NaCl, 30 mM EDTA, and 1 mM 2-mercaptoethanol.
d) 0.2 M sodium borate buffer (pH 8.5) containing 1 mM 2-mercaptoethanol.
e) Tween 20, 4% solution.

The cell wall suspension was centrifuged at 3000 × g for 10 min and resuspended for 30 min in the corresponding solution. Then, the suspension was centrifuged at 3000 × g for 10 min and the pellet washed twice and resuspended in 1.5 ml of buffer A. All steps were carried out at 4 °C.

Soluble (neutral and vacuolar) invertases

Soluble invertases were extracted from whole roots according to Rosa et al. (2004) using the following procedure: 1 g of tissue was homogenised with 2 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 5 µM $MnSO_4$ and 1 mM 2-mercaptoethanol using a cold pestle and mortar. The homogenate was squeezed through 2 layers of cheesecloth and centrifuged at 12 000 × g for 15 min and the pellet discarded. The resulting supernatant was dialysed against a 10-mM sodium acetate buffer (pH 5.5) containing 1 mM 2-mercaptoethanol and was then used for enzymatic activity determinations. Because the extraction buffer did not contain either chelating agent, high concentrations of salt or detergent, the probability of extracting the cell wall-bound invertase activity is very low. All steps were carried out at 4 °C.

Invertase activity

Cell wall-bound and soluble (neutral and vacuolar) invertase activities were assayed by determining the reducing sugars released, as described by Prado et al. (1982).

Extraction and quantitation of lipids in the root

Lipids were extracted from whole roots as follows (Zenoff et al., 1994): 2 g of tissue were homogenised with 10 ml of methanol – chloroform – water (50 : 50 : 10) using a cold pestle and mortar. The lower chloroformic phase was concentrated to dryness in vacuum and then the residual total lipids were dissolved in chloroform to a known volume (1 ml).

Total phospholipids were determined by the Ames method (Ames, 1966), total sterols by the Lieberman-Bouchard method (Lynch et al., 1963) and total glycolipids by the phenol sulfuric acid method (Roughan and Batt, 1967).

Determination of root membrane integrity

To determine membrane integrity, 40 root sections (1 cm long) were rinsed in distilled water, placed in sealed tubes containing 15 ml of distilled water, and incubated for 12 h at room temperature. After this time, the water conductivity ($\mu S\ cm^{-1}$) was measured using a conductimeter (Metrohm, Switzerland). Immediately, tubes were boiled in a water bath for 30 min and then the water conductivity was again measured.

Prior to each measurement, room temperature was recorded. Conductivity was calculated according to the following equation:

$$K_{(25\,^{\circ}C)}(\mu S\ cm^{-1}) = \frac{\text{Conductivity}\ (\mu S) \cdot C\ (cm^{-1})}{1 + 0{,}0191\ (\text{temperature}\,^{\circ}C - 25\,^{\circ}C)}$$

K: conductivity at 25 °C; C: constant of cell sensor (0.81).

Membrane integrity was expressed as the difference ($\Delta_{\text{conductivity}}$) between conductivity values after and prior to heating.

Histochemical staining of root for lipid peroxidation

To detect lipid peroxidation, intact roots were stained with Schiff's reagent for 20 min according to Pompella et al. (1987). Stained roots were photographed with a digital camera (Olympus D580, Japan).

Extrusion of protons from intact roots

To detect proton extrusion, 10 plants with intact roots were transferred to 50 ml of 1 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM $CaCl_2$ and 50 mM KCl (Chen et al., 1990). Recording of pH decrease was made over 30 min using a glass combination pH-sensitive electrode coupled to a pH meter (Hanna Instrument, Germany). The high potassium concentration used in the external medium was associated with a significant increase in the extent of acidification in Cd-unexposed and Cd-exposed roots. This stimulation permitted detection of more significant differences in ΔpH.

Visualisation of proton extrusion

To visualise proton extrusion, intact roots were embedded in a 0.7% agar containing 0.5 mM $CaSO_4$ and the pH indicator bromcresol purple (0.005%) for 20 min. The pH was adjusted to pH 6.0 and the acidification capacity was visualised by colour change around roots (Schmidt et al., 2003).

Plasma membrane H^+-ATPase activity

To confirm the presence of plasma membrane H^+-ATPase activity, 10 plants with intact roots were transferred to 50 ml of 1.5 mM Na_3VO_4, a specific inhibitor of plasma membrane H^+-ATPase (Krieger and Tashjia, 1983), for 20 h. After this time, roots were rinsed in distilled water and proton extrusion recorded for 30 min.

Cadmium analysis

Cadmium contents in roots, shoots, and leaves from 20 µM $CdCl_2$-exposed Rangpur lime seedlings were analysed by digestion of dried plant material in concentrated $HNO_3 : HClO_4$ (4 : 1 v/v). Metal ion concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer 373 Spectrophotometer, England).

Statistical analysis

The significance of the differences in numerical results from different treatments was tested using Tukey's test. Differences were accepted as significant if $p \leq 0.05$. Values are given as means ± SD.

Results

Cd effect on seedlings growth

Cadmium produced a significant growth inhibition of Rangpur lime seedling roots, measured as DW; however, shoots and leaves did not show significant alterations (Fig. **1**). The effect of higher Cd concentrations, between 50 and 80 µM, was also analysed, but seedlings were severely damaged (tip root and shoot apex necrosis and leaf chlorosis) (data not shown). For this reason, the study was focused on 20 and 40 µM $CdCl_2$. Under these concentrations, no visible symptoms of metal toxicity were observed. The decrease in root growth at 20 µM $CdCl_2$ was accompanied by WC increment. However, shoot and leaf WC did not show significant differences as compared with the control (Fig. **2**). In the presence of 40 µM $CdCl_2$, DW and WC showed a similar pattern (data not shown).

Sucrose content and invertase activities

Sucrose content was lower in 20 µM Cd-exposed roots than in control roots (Fig. **3**). To analyse sucrose influx into roots, we determined the apoplastic and symplastic sucrose content in Cd-exposed and Cd-unexposed Rangpur lime roots. Results showed that apoplastic sucrose content in Cd-unexposed roots was very low in relation to symplastic content, while in Cd-exposed roots a strong increment (12-fold) in the sucrose apoplastic content was observed (Fig. **3**). Cell wall-bound and soluble (neutral) invertase activities were lower (– 60% and – 80%) in 20 µM Cd-exposed roots than in control roots; however, vacuolar invertase activity did not change in either Cd-unexposed or Cd-exposed roots (Fig. **4**). In the presence of 40 µM $CdCl_2$, sucrose and invertase activities showed similar trends (data not shown).

Cd effect on lipid content

The effects of 20 µM cadmium on lipid composition of Rangpur lime roots are shown in Fig. **5**. Lipid content varied markedly between Cd-exposed and Cd-unexposed roots. In the former, total glycolipid and phospholipid contents decreased 50%, while sterol content only decreased 22%. The phospholipid/sterol ratio, an indicator of membrane fluidity, decreased from 0.99 in Cd-unexposed roots to 0.66 in Cd-exposed roots. In the presence of 40 µM $CdCl_2$ similar results were obtained (data not shown).

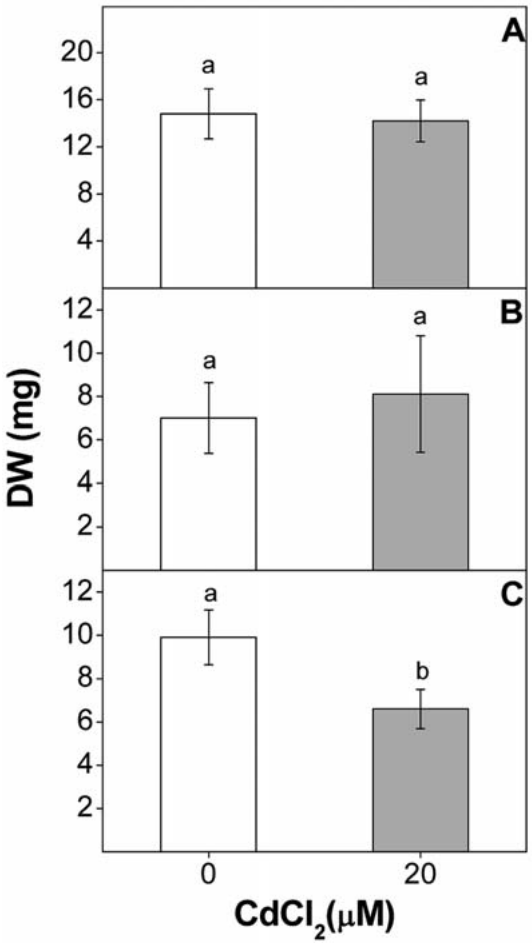

Fig. 1 Cadmium effect on Rangpur lime DW. (**A**) Leaf; (**B**) shoot; (**C**) root. Values are the mean ± SD (n = 40). Different letters indicate significant differences between means ($p < 0.05$) by the Tukey test.

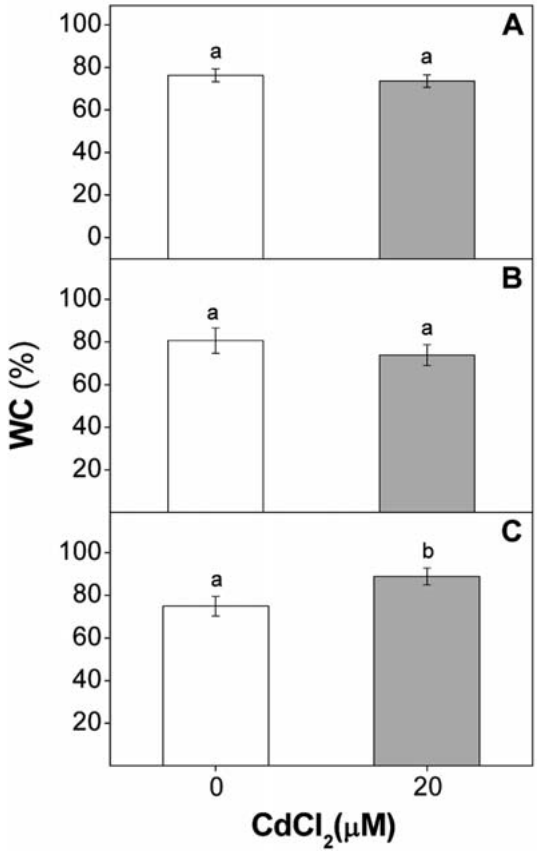

Fig. 2 Cadmium effect on Rangpur lime WC. (**A**) Leaf; (**B**) shoot; (**C**) root. Values are the mean ± SD (n = 40). Different letters indicate significant differences between means ($p < 0.05$) by the Tukey test.

Membrane integrity

Membrane integrity decreases, measured as $\Delta_{conductivity}$, were nearly 34% and 90% at 20 µM and 40 µM $CdCl_2$, respectively (Fig. **6**). These values represent a severe alteration (injury) in the transport/integrity ratio. Because membrane injury is frequently associated with lipid peroxidation, we investigated whether the decreases in $\Delta_{conductivity}$ observed in root membranes after Cd exposure were accompanied by an increase in lipid peroxidation. Intact roots were stained with Schiff's reagent for histochemical aldehyde detection (Fig. **7**). Roots exposed to 40 µM $CdCl_2$ showed a significant production of aldehyde (rose-coloured) as compared with Cd-unexposed roots.

Proton extrusion from intact roots

Cadmium treatment reduced proton extrusion by Rangpur lime roots at pH 7 (Fig. **8**). In Cd-unexposed roots, proton extrusion increased up to a maximum value (30 min) and then remained constant. However, in the presence of 20 and 40 µM $CdCl_2$ the maximum levels reached were 34% and 50% lower than the control. Na_3VO_4 addition to the measuring medium similarly decreased proton extrusion to 40 µM $CdCl_2$ (Fig. **8**). Proton extrusion visualisation from roots with or without Cd and in the presence of Na_3VO_4 is shown in Figs. **9a–d**. In Cd-exposed and Na_3VO_4-treated roots no change in medium colour was observed (Figs. **9b–d**). However, in Cd-unexposed seedlings roots, medium acidification was observed as a slight yellow colouration around roots; this effect was especially noted in the elongation zone (Fig. **9a**).

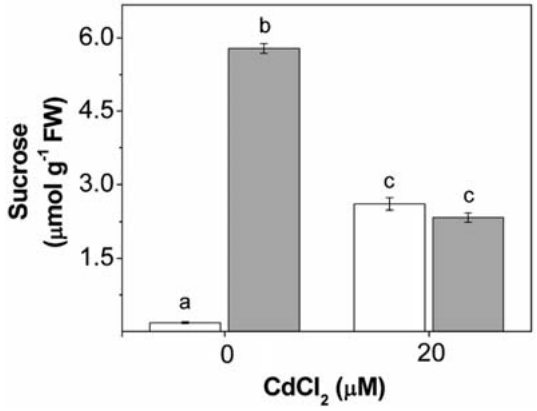

Fig. 3 Cadmium effect on Rangpur lime root sucrose content. Apoplastic (open bar) and symplastic (solid bar) sucrose. Each value is the mean ± SD of four separate measurements.

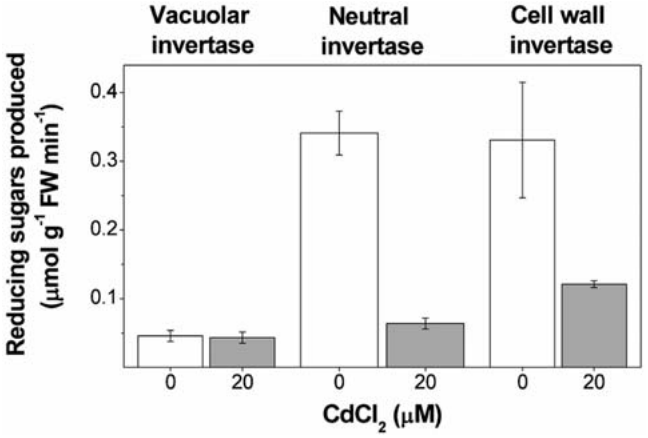

Fig. 4 Cadmium effect on Rangpur lime root activities of vacuolar, neutral, and cell wall-bound invertases. Each value is the mean ± SD of three separate measurements.

Cadmium content

Rangpur lime seedlings exposed to 20 μM $CdCl_2$ accumulated substantial amounts of the metal ion in the roots. Shoots accumulated cadmium at a lower, although significant extent; while in leaves, cadmium accumulation was not significant (Fig. **10**).

Discussion

We examined growth and other cellular events in relation to Cd toxicity in lemon rootstock roots, Rangpur lime *(Citrus limonia)*. Cadmium produced a significant reduction in root growth expressed as DW. Shoots and leaves did not show significant alterations. Root growth inhibition due to Cd supplied to soil or nutrient solution has been reported in several species (Bernal and McGrath, 1994; Ouariti et al., 1997; Wójcik and Tukendorf, 1999; Schützendübel et al., 2001; Liu et al., 2003; Yeh et al., 2004). However, Moya et al. (1993) and Arduini et al. (1994) reported increases in root biomass of Cd-exposed rice and pine plants. Parameters such as FW, DW, DW/FW ratio and shoot and root length have been used as heavy metal toxicity indicators (Baker and Walker, 1989; Moya et al., 1993). According to Moya et al. (1993), Arduini et al. (1994), Wójcik and Tukendorf (1999), and our results, roots are more affected than shoots and leaves because they are the first target of Cd effects and have a high capacity for retaining Cd (Jastrow and Koeppe, 1980; Meuwly and Rauser, 1992; Punz and Sieghardt, 1993; Wójcik and Tukendorf 1999). In agreement with these findings, we demonstrated that Cd-exposed roots accumulated 92% of the total metal ion taken up. However, Sandalio et al. (2001) reported that roots are less affected than aerial parts. These authors consider that root sensitivity to Cd could be explained by its ability to accumulate Cd in a non-active form and/or Cd mobility, both are associated with phytochelatins (Gong et al. 2003). According to Rauser (1995), an explanation for the high Cd content detected in Rangpur lime roots could be the presence of Cd-binding peptides in them. However, Cohen et al. (1998) reported that pea roots can accumulate Cd in the apoplast by ionic interactions with carboxyl and/or sulphydryl groups existing in the cell wall and into vacuole, together with phytochelatins. The same authors also reported

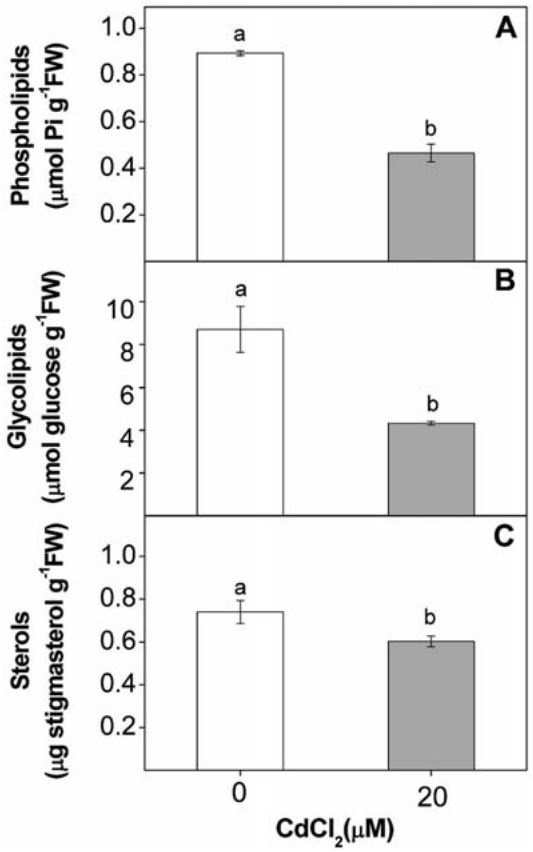

Fig. 5 Cadmium effect on Rangpur lime root lipid content. (**A**) Phospholipids; (**B**) glycolipids; (**C**) sterols. Each value is the mean ± SD of four separate measurements. Different letters indicate significant differences between means ($p < 0.05$) by the Tukey test.

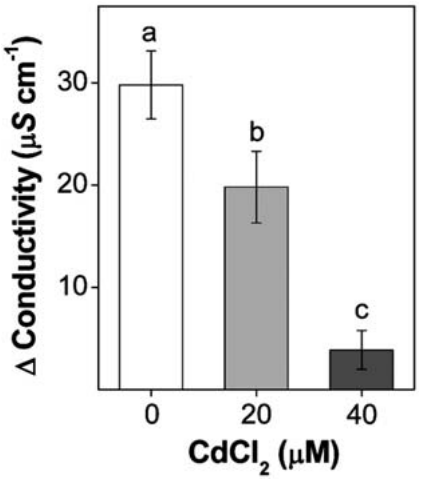

Fig. 6 Cadmium effect on Rangpur lime root membrane conductivity. Values are the mean ± SD of four separate measurements. Different letters indicate significant differences between means ($p < 0.05$) by the Tukey test.

the existence of two components in Cd uptake by pea roots: a linear component associated with Cd apoplastic influx and a second saturable element associated with a transporter-mediated Cd influx across the plasma membrane.

Fig. 7 Histochemical localisation of lipid peroxidation in Cd-unexposed (right) and 40 µM Cd-exposed (left) Rangpur lime roots. Bar = 2 cm.

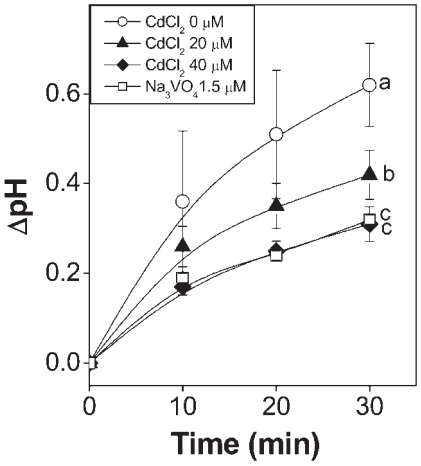

Fig. 8 Cadmium effect on temporal evolution of proton extrusion in Rangpur lime root. Each value is the mean ± SD of four separate measurements. Different letters indicate significant differences between means ($p < 0.05$) by the Tukey test.

Cadmium effects on carbohydrate synthesis and metabolism have been reported in several species (Greger et al., 1991; Somashekaraiah et al., 1992; Moya et al., 1993; Sandalio et al., 2001). Thus, Moya et al. (1993) demonstrated an increase in carbohydrate levels in Cd-exposed rice shoots; while Greger and co-workers (Greger and Lindberg, 1986; Greger et al., 1991; Greger and Bertell, 1992) found that Cd supplies decreased or increased soluble sugar and starch contents in sugar beet, depending on which nutrients plus Cd were supplied to the plants. Nonetheless, despite the available literature on Cd phytotoxicity on carbohydrate metabolism, information regarding Cd effects on sucrose content and cell distribution is scarce and unclear. Cadmium effects on sucrose partitioning into the cells in Rangpur lime roots was analysed through apoplastic and symplastic sucrose content. Results obtained in Cd-unexposed roots showed that sucrose symplastic content was predominant over sucrose apoplastic content. Nevertheless, in Cd-exposed roots, a strong increment (12-fold) in the apoplastic sucrose level was observed (Fig. **3**). The highest sucrose content, observed in Cd-exposed root apoplasts, could be explained by: a) a decrease in sucrose cellular metabolism or b) a reduction in sucrose cell flux.

The first assumption proposes that decreasing carbohydrate utilisation for growth is more pronounced than decreased CO_2 fixation, resulting in an increase in carbohydrate accumulation (Greger and Bertell, 1992). Due to the important role of invertases in sucrose metabolism (Kingston-Smith 1999; Sturm, 1999), the increase observed in apoplastic sucrose content can be attributed to a reduction in intracellular invertase activities. Intracellular invertases are classified as neutral or alkaline and vacuolar or acidic (Sturm, 1999). Neutral or alkaline invertases seem to be confined to the cytoplasm, they appear to be sucrose-specific, and are involved in sucrose hydrolysis to supply free hexoses to cell metabolism (Lee and Sturm, 1996; Sturm, 1999; Sturm and Tang, 1999). Vacuolar invertases are involved in phloem unloading, in storage organs, and in other metabolic processes (Wu et al., 1993; Sturm et al., 1995; Sturm, 1999; Roitsch et al., 2000). In this sense, our results demonstrated that Cd treatment induced a significant

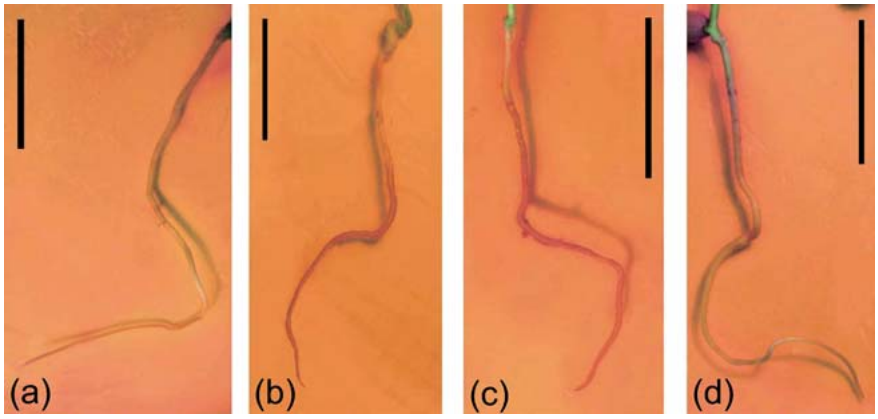

Fig. 9 Proton extrusion visualisation in Rangpur lime roots with or without $CdCl_2$. (**a**) $CdCl_2$ 0 µM (control); (**b**) $CdCl_2$ 20 µM; (**c**) $CdCl_2$ 40 µM; (**d**) Na_3VO_4 1.5 µM. Bar = 5 cm.

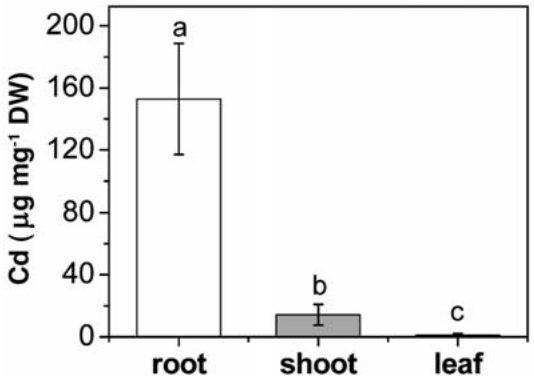

Fig. 10 Cadmium content of root, leaf, and shoot of Rangpur lime seedlings. Each value is the mean ± SD of six separate measurements.

decrease (– 80%) in neutral invertase level while vacuolar invertase level did not show any change (Fig. **4**). However, an interaction between Cd and plasmodesmatal functionality cannot be discarded (Citovsky et al., 1998)

The second assumption could be explained either through an alteration in the sucrose/H^+-symport due to a reduction of H^+-ATPase plasma membrane activity (Michelet and Boutry, 1995), or by a decrease in sucrose hydrolysis due to changes in cell wall-bound invertase activity. Plasma membrane H^+-ATPase generates proton-motive force to provide energy for sugar transport (sucrose or hexoses) into the cell. H^+-ATPase also supports normal H^+/K^+ exchange that regulates intracellular pH and maintains the acidic apoplastic pH (Michelet and Boutry, 1995; Sanità di Toppi and Gabbrielli, 1999; Winch and Pritchard, 1999; Roitsch et al., 2003). Root plasma membrane H^+-ATPase has been demonstrated to function as a primary nutrient transporter by pumping protons out of the cell (proton extrusion) (Michelet and Boutry, 1995). On the other hand, cell wall-bound invertases catalyse the irreversible cleavage of apoplast sucrose released via sucrose transporters (Roitsch et al., 2000). The resulting hexoses are then imported into the sink cell by monosaccharide transporters (Roitsch et al., 2000, 2003). Cell wall-bound invertases are aploplastic key regulators of sucrose level via enzymological properties. Sucrose level is limiting because the K_m value for hexose transporters is in the µM range, and cell wall-bound invertase K_m values are in the mM range (Roitsch et al., 2003). Additionally, several studies have demonstrated that cell wall-bound invertases are regulated by highly specific inhibitory proteins (Weil et al., 1994; Greiner et al., 1998; Hothorn et al., 2004). These invertase inhibitors have been shown to be heat-stable, non-glycosylated and to affect invertase activity in a pH-dependent manner (Weil et al., 1994). In relation to these facts, we found a significant reduction in plasma membrane H^+-ATPase activity and cell wall-bound invertase level in Cd-exposed roots of Rangpur lime (Figs. **4, 8**). Cadmium also inhibits proton extrusion. These results agree with Fodor et al. (1995), Hall (2002), Nocito et al. (2002), and Iivonen et al. (2004), who demonstrated that Cd toxicity also reduces plasma membrane H^+-ATPase activity in wheat and sunflower roots. We confirmed that the plasma membrane H^+-ATPase is involved in proton extrusion by adding a specific inhibitor (Na_3VO_4) to the medium. Results showed that H^+-ATPase activity decreases until reaching the value obtained with 40 µ $CdCl_2$ (Fig. **8**). On the other hand, because cell wall-bound and neutral invertases play a key role in establishing and maintaining sink metabolism in developing roots, it is possible to expect a decrease in activities leading to an apoplastic sucrose content increment, as observed in Cd-exposed roots. Additionally, the apoplastic/symplastic sucrose ratio is an indicator of sucrose cellular metabolism and flux (Pollock and Farrar, 1996). In our research, this ratio was 0.03 in Cd-unexposed roots and 0.89 in Cd-exposed roots, resulting in disturbed sucrose partitioning.

Cadmium has also been associated with plasma membrane functionality alterations (Das et al., 1997; Sandalio et al., 2001; Hsu and Kao, 2003; Yeh et al., 2004). Membrane function alteration represents the most important target in Cd toxicity (Hernández and Cooke, 1997; Hall, 2002). To assess this, it is necessary to know the level of changes that Cd can induce in lipid composition and plasma membrane integrity in plant tissues. The plasma membrane is the first functional structure in contact with Cd, and its lipid composition changes with environmental variations. Plasma membrane lipid composition controls membrane permeability and its efficiency as a semipermeable barrier (Meharg, 1993). Membrane lipid composition changes may affect fluidity and intrinsic membrane protein activities (Ros et al., 1990; Hernández and Cooke, 1997; Quartacci et al., 2001; Liang et al., 2005). Our studies determined a significant decrease in content and composition alterations in lipids in Cd-exposed roots (Fig. **5**). The highest decreases were observed in phospholipid and glycolipid (polar lipids) contents, which have also been reported in tomato, pea, and *Thlaspi ochroleucum* (Ouzounidou et al., 1992; Hernández and Cooke, 1997; Ouariti et al., 1997). These authors considered that the decrease in polar lipid content through peroxidative breakdown is produced via lipoxygenase activity, which is Cd-induced. Similarly, our results showed an *"in vivo"* lipidic peroxidation in Cd-exposed roots (Fig. **7**). A high phospholipid/sterol ratio has been associated with increased plasma membrane fluidity (Palta et al., 1993). In Rangpur lime roots, this ratio is significantly decreased in Cd-exposed roots, indicating membrane alterations. Plasma membrane integrity of Cd-exposed roots was also analysed using $\Delta_{conductivity}$ measurements. Results obtained suggest severe injury to the plasma membrane and transport/integrity ratio.

In conclusion, according to our results, the high apoplastic sucrose level observed in Cd-exposed Rangpur lime roots could be explained, at least partially, by a direct effect of Cd on the sucrose transporter and/or sucrose-related enzymes, or by an indirect metal ion effect. In the latter case, alteration of plasma membrane functionality, inhibition of H^+-ATPase activity and apoplastic pH unbalancing may be responsible. However, further experiments are required to achieve greater insight into sucrose partitioning and metabolism under Cd toxicity.

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