RESEARCH PAPER

Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants

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ABSTRACT

Industry residues, phosphate fertilisers and wastewater as a source of irrigation have considerably increased levels of heavy metals in the soil, mainly cadmium (Cd^{2+}). To test the effects of a calcium (Ca^{2+}) treatment on Cd^{2+} accumulation and plant tolerance to this heavy metal, plants of two citrus genotypes, Cleopatra mandarin (CM) and Carrizo citrange (CC), were watered with increasing concentrations of Cd^{2+}, and phytochelatin (PC) and glutathione (GSH) content were measured. Both genotypes were able to synthesise PCs in response to heavy metal intoxication, although CM seems to be a better Cd^{2+} excluder than CC. However, data indicate that CC plants had a higher capacity for regenerating GSH than CM plants. In this context, the effects of Ca^{2+} treatment on Cd^{2+} accumulation, plant survival and PC, GSH and oxidised glutathione (GSSG) content were assessed. Data indicate that treatment with Ca^{2+} had two positive effects on citrus physiology: it reduced Cd^{+2} uptake into roots and also increased GSH content (even in the absence of Cd^{2+}). Overall, the data indicate that although Cd^{2+} exclusion is a powerful mechanism to avoid heavy metal build-up into photosynthetic organs, the capacity to maintain optimum GSH levels to feed PC biosynthesis could also be an important factor in stress tolerance.

INTRODUCTION

The onset of environmental pollution caused by the presence of metal elements can be associated with the Industrial Revolution that enormously expanded mine production in the early 20th century (Nriagu 1979). These pollutants, nowadays resulting from a growing number of diverse anthropogenic sources (industrial effluents and wastes, urban runoff, agricultural pesticides, phosphate fertilisers, mining, *etc.*), have progressively adversely affected many different ecosystems (Grant *et al.* 2008; Moreno-Jiménez *et al.* 2009). Among pollutants, metalloids such arsenic and selenium, and metals such as cadmium (Cd^{2+}), mercury and lead are of major concern with respect to plant exposure as well as human food chain accumulation (McLaughlin *et al.* 1999).

Cadmium is incorporated into agricultural soils through phosphate fertilisers, sewage sludge and atmospheric fallout from industrial and urban activities (Kirkham 2006). Cadmium is a non-essential element for plants, but is easily absorbed through roots because Cd^{2+} is taken up into plant cells *via* Fe^{2+}, Ca^{2+} and Zn^{2+} transporters/channels of low specificity (Clemens 2006). The Cd^{2+} build-up in roots of plants modifies cell homeostasis and causes a progressive reduction in photosynthesis and transpiration, decreasing water and nutrient uptake (di Toppi & Gabbrielli 1999). All these features are probably the major basis for Cd^{2+} toxicity (Singh & Tewari 2003). Part of the harmful effects produced by Cd^{2+} might also be explained by its ability to inactivate enzymes, possibly through reaction with the SH groups of proteins (Sharma & Dietz 2009). Furthermore, although Cd^{2+} does not participate directly in cellular redox reactions, its accumulation may disturb the redox balance. This intracellular redox environment is mainly controlled by the glutathione redox state, which is defined as the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG), and plays critical roles in maintaining cellular homeostasis and in various physiological functions (Boominathan & Doran 2003).

Plant responses to high concentrations of Cd^{2+} generally imply activation of the sulphur assimilation pathway to provide an enhanced supply of GSH for the biosynthesis of phytochelatins (PCs), which play a major role in metal sequestration (Na & Salt 2010; Cai *et al.* 2011). PCs, with a general structure of $(\gamma\text{-Glu-Cys})_n\text{–Gly}$ (n = 2–11), are rapidly synthesised using the cytoplasmic enzyme PC synthase in response to intoxication with heavy metals. Genetic studies have confirmed that GSH is pivotal for PC synthesis as GSH-deficient mutants of *Schizosaccharomyces pombe* and *Arabidopsis* are PC-deficient and hypersensitive to Cd^{2+} (Cobbett & Goldsbrough 2002; Lee *et al.* 2003).

Although citrus plants are generally sensitive to abiotic stress conditions, important differences among genotypes have been described in their response to high salinity (López-Climent *et al.* 2008) and soil flooding (Arbona *et al.* 2009). In terms of NaCl tolerance, it has been shown that citrus are more sensitive to Cl^- than to Na^+ ions (Moya *et al.* 2003). In this respect, Cleopatra mandarin (CM), a commercial citrus rootstock, is able to restrict Cl^- uptake to the aerial plant parts, whereas leaves of the Carrizo citrange (CC, another widely used rootstock) become rapidly intoxicated in the presence of high concentrations of ions (Moya *et al.* 2003). Nevertheless, CC shows effective antioxidant protection not only under high

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salinity but also under soil flooding conditions (Arbona *et al.* 2003, 2008).

Previous studies from our laboratory have demonstrated that citrus rootstocks are relatively tolerant to Cd^{2+} and did not show leaf damage after 2 months of being watered with 300 μM Cd^{2+} (López-Climent *et al.* 2011). In contrast, the same genotypes watered with a high Cd^{2+} concentration (3 mM) showed damage within a few days, significantly decreasing photosynthesis, transpiration and stomatal conductance. Moreover, citrus roots under different concentrations of Cd^{2+} either did not modify or reduced levels of abscisic, salicylic and jasmonic acids, independently of the different tolerance to Cd^{2+} observed in the studied genotypes. This fact clearly indicates that there is no specific hormonal response to the metal build-up in citrus genotypes. These so-called 'stress hormones' clearly did not respond to the Cd^{2+} stimulus, even though the same genotypes are able to accumulate large amounts of hormones in roots under water stress.

Calcium is involved in the regulation of plant cell metabolism and signal transduction (Yang & Poovaiah 2002) and has an important role in biotic and abiotic stress tolerance. Disturbances in Ca^{2+} content have been associated with toxicity of different heavy metals (Rodríguez-Serrano *et al.* 2009). Several works have indicated that Ca^{2+} alleviates Cd^{2+} toxicity in some plants (Suzuki 2005; Rodríguez-Serrano *et al.* 2009), but little information on the specific effect of Ca^{2+} in plant responses to stress induced by heavy metals can be found in the literature (Tian *et al.* 2011).

The objective of this work was to determine the effect of a Ca^{2+} treatment on plant physiology in two different genotypes of citrus watered with high concentrations of Cd^{2+}. The hypothesis tested was whether the effect of Ca^{2+} was restricted to counteracting Cd^{2+} uptake or whether there are other positive effects of this ion. For this, endogenous levels of GSH and PCs were determined in the two studied genotypes. Complementary, Cd^{2+} accumulation in roots and shoots were quantified to characterise the system.

MATERIAL AND METHODS

Plant material and sample collection

One-year-old seedlings of Carrizo citrange (*Poncirus trifoliata* L. Raf × *Citrus sinensis* L. Osb.; referred to as CC) and Cleopatra mandarin (*Citrus reshni* Hort. ex. Tan.; CM) were purchased from a commercial nursery. Immediately, the citrus rootstocks were transplanted into 2-l plastic pots with perlite as substrate. Plants were allowed to acclimate for 1 month in a greenhouse with a natural photoperiod, 25 ± 3 °C/18 ± 3 °C day/night temperature and 60–85% relative humidity. During this period, plants were watered three times a week with 0.5 l of a half-strength Hoagland solution (López-Climent *et al.* 2008).

For chemical analysis, only mature leaves at an intermediate position on the stem and young roots were harvested, washed with distilled water and immediately frozen in liquid nitrogen. The frozen material was ground to a fine powder using a pre-chilled mortar and pestle. Part of that tissue was stored at −80 °C, and the rest lyophilised. Each plant was processed as a biological replicate and therefore stored separately. For each of the analyses described below three independent extractions per plant were performed.

Treatments

Field concentrations of cadmium (preliminary experiment)
Thirty-three seedlings of each genotype (CC and CM) were separated into three different groups. One group was set as a control, and the other two were treated with increasing concentrations of $Cd(NO_3)_2$ in the watering solution (1 μM Cd^{2+} and 3 μM Cd^{2+}). Cd^{2+}-treated plants were watered three times a week with this nutrient solution plus $Cd(NO_3)_2$ to achieve the desired Cd^{2+} concentrations. Control plants were identically watered, but $Cd(NO_3)_2$ was omitted.

High concentrations of cadmium (Experiment I)
In this case, both CC and CM genotypes were used. Three groups of 14 plants per genotype were set: control plants, plants treated with 1.5 mM Cd^{2+} and plants treated with 3.0 mM Cd^{2+}. Plants were watered as in the preliminary experiment.

Low concentrations of cadmium (Experiment II)
Sixty-nine seedlings for each genotype were separated in three different groups. One group was set as a control and the other two were treated with increasing concentrations of $Cd(NO_3)_2$ in the watering solution (30 μM Cd^{2+} and 150 μM Cd^{2+}). Plants were watered as described above.

Cadmium and calcium treatment (Experiment III)
The effect of an increased concentration of Ca^{2+} in the watering solution on plant performance was tested in 48 plants of CC divided into four groups. Two of them were set as control (not treated with Cd^{2+}): one group was watered with the regular nutrient solution while the watering solution of the other group was supplemented with flocculated OCa (35%, W/W, Alcaplant; Codiagro S.A., Castellón, Spain) to achieve a Ca^{2+} concentration of 7.5 mM, as recommended by the manufacturer. The other two groups of plants were watered with the regular nutrient solution plus 1.5 mM·Cd^{2+}, supplemented or not with 7.5 mM·Ca^{2+}.

Leaf damage

In response to Cd^{2+} treatment, plants showed symptoms of leaf damage, such as vein yellowing and curling (see Supporting Information). The number of damaged leaves per plant was recorded regularly during the experimental period. Plants showing a percentage of damaged leaves equal to or above 50% were considered injured.

Cadmium determination

Cadmium concentration was determined using 1 g of tissue that was digested with 10 ml 35% nitric acid (Panreac S.A., Barcelona, Spain) for 3 h in an oven at 120 °C. Then extracts were filtered and Cd^{2+} concentration determined by inductively coupled plasma mass spectrometry (ICP-MS 7500cx, Agilent, Santa Clara, CA, USA).

Determination of total PC and GSH concentrations

Total non-protein SH (TNP-SH) compounds were extracted and assayed according to de Vos *et al.* (1992) Briefly, TNP-SH compounds were extracted by homogenising 0.5 g frozen plant

tissue with 2 ml 5% 5-sulphosalicylic acid (SSA) with 6.3 mM diethylenetriaminepenta acetic acid (DETAPAC) using a pre-chilled mortar, pestle and quartz sand. The homogenate was centrifuged, the supernatant collected and immediately used for assay of TNP-SH: 300 µl supernatant were mixed with 630 µl 0.5 M K_2HPO_4 (pH 7.5). The absorbance at 412 nm was read 2 min before and after the addition of 25 µl 6 mM DTNB solution (DTNBε = 13,600 $M^{-1} \cdot cm^{-1}$).

The DTNB GSSG reductase recycling procedure was used for determination of both total (GSH + GSSG) and oxidised glutathione (GSSG) levels (Anderson 1985). Recovery experiments of both GSH and GSSG allowed validation of the method for the citrus roots (see Supporting Information). PC production was evaluated as PC-SH levels by subtracting the amount of GSH from the amount of total non-protein SH compounds (de Vos *et al.* 1992).

Determination of different phytochelatins with HPLC-MS

This determination was performed according to the method described in Tennstedt *et al.* (2009). Briefly, 30 mg lyophilised tissue was mixed with 500 µl of a decomposition reagent (0.1 M HCl spiked with 5 µl 10 mM *N*-acetyl-L-cysteine) by ultrasonication for 20 min. After centrifugation (9000 *g*, 20 min, 4 °C), 50 µl of supernatant were incubated with 6 µl 20 mM *Tris*-(2-carboxyethyl) phosphine hydrochloride and 18 µl of 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid (EPPS)/DETAPAC buffer for 30 min at room temperature in darkness. For derivatisation, 72 µl EPPS/DETAPAC buffer and 10 µl 10 mM monobromobimane were mixed and incubated for 30 min at 45 °C. The reaction was stopped with 60 µl 1 M methanesulphonic acid. Before analysis, the sample was filtered using 0.45-µm membrane filters of polytetrafluoroethylene. The derivatives were determined using HPLC and fluorescence detection (FLD), as described in Döring *et al.* (2000). Identification of each PC and GSH was achieved by acquisition of the online spectra with a QTOF-MS operated in positive ionisation mode. Ion spray voltage was +5.5 Kv, scan rate 0.5 s^{-1}, declustering potential 1:50 V, declustering potential 2:15 V (see Supporting Information).

Statistical analysis

All data presented are means ± SE. Statistical analyses were performed using StatGraphics Plus (version 2.1.) for Windows (Statistical Graphics Corp., Warrenton, VA, USA). Differences between treatments were compared by means of the least significant difference (LSD) test ($P \leq 0.05$).

RESULTS

Leaf damage and root cadmium concentration

Citrus rootstocks exposed to increasing exogenous Cd^{2+} concentrations showed different responses to metal intoxication (see López-Climent *et al.* 2011). Different lines of evidence support that citrus plants showed a remarkable tolerance to Cd^{2+}. (i) Plants of both genotypes watered with Cd^{2+} concentrations that could be found in the field (1–3 µM) did not show any leaf injury for 1 month (Table 1). (ii) Plants watered with 30 µM Cd^{2+} did not show any leaf injury for 100 days (Fig. 1). (iii) Plants treated with 150 or 300 µM Cd^{2+} showed the first symptoms after 50 days but no plant deaths occurred throughout a 205-day experimental period. To assess the accumulation of Cd^{2+} and possible biosynthesis of PCs in roots, different experiments were performed using plants of two commercial citrus rootstocks, CC and CM.

In a preliminary experiment (Table 1), plants watered with very low exogenous Cd^{2+} concentrations (1 and 3 µM) did not show any leaf damage, although an initial accumulation of endogenous Cd^{2+} was observed in roots. Both Cd^{2+} basal levels and ion accumulation due to the exogenous treatment were higher in roots of CC than those of CM; however, under these conditions, no Cd^{2+} accumulation was detected in leaves of either of the two genotypes studied. In contrast, when watered with elevated exogenous Cd^{2+} concentrations (1.5 and 3.0 mM), all plants showed leaf damage symptoms within a few days (Fig. 1). At the beginning of the experimental period, the percentage of plants affected by the Cd^{2+} treatment was higher in CC than in CM. In general, plants watered with lower Cd^{2+} concentrations did not show any symptoms of damage, and only 7% of the CC plants watered with 1.5 mM Cd^{2+} were affected at the end of experimental period (Fig. 1).

Elevated Cd^{2+} concentrations in the irrigation solution (1.5 and 3.0 mM) increased endogenous Cd^{2+} content in roots (Fig. 2, lower part), with a similar pattern of increase in both genotypes. Very high endogenous levels of Cd^{2+} were detected in roots of both genotypes when the watering solution was supplemented with either 1.5 or 3.0 mM Cd^{2+}. At the end of the experimental period, root Cd^{2+} content was similar in plants of

Table 1. Effect of cadmium treatments on leaf damage, endogenous cadmium concentration and phytochelatin (PC) content in two genotypes of citrus.

	affected plants (%)		root Cd^{2+} (µg.g^{-1} DW)		leaf Cd^{2+} (µg.g^{-1} DW)		PCs (nM.g^{-1} DW)	
Treatments	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
CC								
CT	0 ± 0	0 ± 0	2.83 ± 0.76	3.00 ± 0.94	0.035 ± 0.006	0.040 ± 0.008	35.5 ± 4.5	40.8 ± 6.5
1 µM	0 ± 0	0 ± 0	7.04 ± 1.23*	12.18 ± 1.20*	0.046 ± 0.021	0.052 ± 0.022	52.3 ± 5.3*	73.3 ± 6.9*
3 µM	0 ± 0	0 ± 0	7.50 ± 2.36*	14.09 ± 1.32*	0.065 ± 0.024	0.070 ± 0.022	65.8 ± 4.2*	86.5 ± 5.4*
CM								
CT	0 ± 0	0 ± 0	1.42 ± 0.14	1.43 ± 0.14	0.027 ± 0.007	0.018 ± 0.089	21.5 ± 6.5	33.3 ± 2.4
1 µM	0 ± 0	0 ± 0	4.23 ± 0.83*	8.90 ± 1.14*	0.019 ± 0.004	0.020 ± 0.004	35.6 ± 1.5*	54.3 ± 4.5*
3 µM	0 ± 0	0 ± 0	4.47 ± 0.88*	9.35 ± 1.22*	0.038 ± 0.012	0.031 ± 0.014	38.7 ± 4.1*	65.3 ± 2.5*

Data are means of three replicates

*Statistical difference with respect to controls at $P \leq 0.05$.

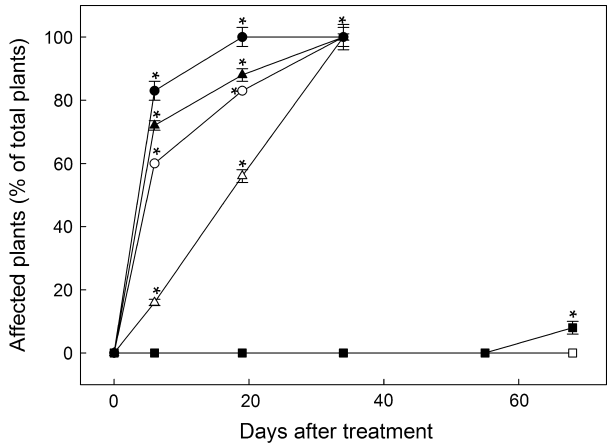

Fig. 1. Effect of cadmium treatments on leaf damage in two genotypes of citrus. Control plants and plants treated with 30 μM Cd^{2+} did not show any leaf symptoms during the whole experimental period (data not shown). Cd^{2+} treatments: 150 μM (□); 1.5 mM (△); and 3.0 mM (○). Black symbols represent CC plants and white ones CM plants. Data are means of three replicate experiments. *Statistical difference with respect to controls at $P \leq 0.05$.

both rootstocks. In contrast, Cd^{2+} concentration in leaves was much lower than in roots in both genotypes (Fig. 2, upper part). Whereas leaf Cd^{2+} concentration only reached 1.9 $\mu g \cdot g^{-1}$ in plants treated with 3.0 mM Cd^{2+}, the maximum endogenous concentration in roots was 10^4 times higher. Furthermore, Cd^{2+} accumulation in leaves was distinct between the two genotypes, CM being able to exclude even more Cd^{2+} from the aerial tissues. Therefore, when treated with 3.0 mM Cd^{2+}, endogenous leaf Cd^{2+} levels did not accumulate beyond 0.5 $\mu g \cdot g^{-1}$ in CM, whereas in CC, Cd^{2+} content reached 1.9 $\mu g \cdot g^{-1}$.

In the second experimental design, the two studied genotypes showed a different pattern of Cd^{2+} accumulation in roots when watered with moderate Cd^{2+} concentrations (30 and 150 μM; Fig. 3, lower part). After 68 days of treatment, CC roots accumulated the highest Cd^{2+} levels in both treatments, reaching 360.2 and 505.5 $\mu g \cdot g^{1}$ (30 and 150 μM Cd^{2+}, respectively). CM plants treated with a low Cd^{2+} concentration accumulated less Cd^{2+} in roots that CC plants (39% and 25%, respectively) throughout the whole experimental period. Furthermore, as in the first experiment, CM translocated Cd^{2+} to the aerial parts less efficiently than CC, and therefore CM exhibited lower endogenous Cd^{2+} content in leaves (Fig. 3, upper part). As described above, CM seems to be a better Cd^{2+} excluder than CC when watered with low Cd^{2+} concentrations. Control plants of both genotypes always contained low endogenous levels of Cd^{2+} in root and leaf tissue, ranging from 0.1 to 7.0 $\mu g \cdot g^{-1}$.

Effect of cadmium on total PCs in citrus rootstocks

In general, Cd^{2+} treatments had a significant effect on PC biosynthesis in the two citrus rootstocks. Plants from both genotypes watered with Cd^{2+} showed increases in these heavy metal-binding ligands, mostly proportional to the Cd^{2+} levels accumulated in the roots. Total PC content increased moderately in the roots of both genotypes when low Cd^{2+} concentrations were added to the irrigation solution (Table 1), according to the moderate accumulation of endogenous Cd^{2+} found.

When plants were treated with high Cd^{2+} concentrations (Fig. 4), PC levels increased from the beginning of the experimental period and reached much higher levels. After 6 days, plants watered with a solution supplemented with 1.5 mM Cd^{2+} had a higher PC content than plants watered with 3.0 mM Cd^{2+}. On day 19, both genotypes treated with 1.5 mM Cd^{2+} had the highest level of PCs, although concentrations in CM roots were significantly lower (Fig. 4). Thereafter, the concentration of PCs considerably decreased, even when Cd^{2+} concentration in the roots continued to increase. Moreover, during most of the experimental period, the highest PC concentrations were recorded in roots of the CC genotype. In control plants, root PC levels were similar in both genotypes, ranging from 30.0 to 102.0 $nM \cdot g^{-1}$.

The pattern of PC accumulation in plants treated with moderate Cd^{2+} concentrations (30and 150 μM Cd^{2+}) was very similar in the two genotypes (Fig. 4). After 10 days of treatment, CC plants exhibited a moderate increase in PC content, whereas CM plants showed levels similar to those determined in control plants. During the rest of the experimental period, plants of both genotypes watered with 30 or 150 μM Cd^{2+} increased root PC content.

Content of PC-2, PC-3 and PC-4

To evaluate possible differences in the accumulation of specific PCs, PC-2, PC-3 and PC-4 content in roots of both genotypes were determined (Fig. 5). GSH and GSSG content was also measured, as these compounds play an important role in the biosynthesis of PCs. First, it should be noted that root GSH and GSSG levels did not vary in plants treated with low concentrations of Cd^{2+} (see Supporting Information); however, when higher concentrations were used, root GSH and GSSG concentrations declined in all plants (Figs 5 and 7).

All PC levels increased in plants of both genotypes in response to Cd^{2+}, although higher levels of PCs were found in CC plants (Fig. 5). Levels of PC-2 and PC-3 in roots of both rootstocks were higher than those measured for PC-4. CM plants accumulated similar levels of PCs under the two different Cd^{2+} treatments, whereas CC plants had higher levels in response to treatment with 150 μM Cd^{2+}. Control plants of both genotypes had low PC levels in root tissue, ranging from 5.2 to 26.2 $nM \cdot g^{-1}$ throughout the experimental period.

Effect of calcium on leaf damage, cadmium uptake and GSH and PC levels

The effect of an increased concentration of Ca^{2+} in the watering solution on leaf damage and Cd^{2+} uptake in citrus was tested in CC and the results are shown in Fig. 6. As observed previously, when treated with 1.5 mM Cd^{2+} for 35 days, CC plants showed important leaf damage, and roots accumulated high levels of endogenous Cd^{2+}. The presence of high concentrations of exogenous Ca^{2+} prevented leaf injury and strongly reduced Cd^{2+} build-up in roots.

In this set of experiments, all plants treated with Cd^{2+} showed very similar increases in PC content regardless of root Cd^{2+} accumulation (Fig. 7). As observed in previous experiments, all plants watered with Cd^{2+} had higher levels of PC-3 than PC-2 in roots, whereas the lowest PC levels were reached for PC-4. Levels of the three PCs in control and Ca^{2+}-treated

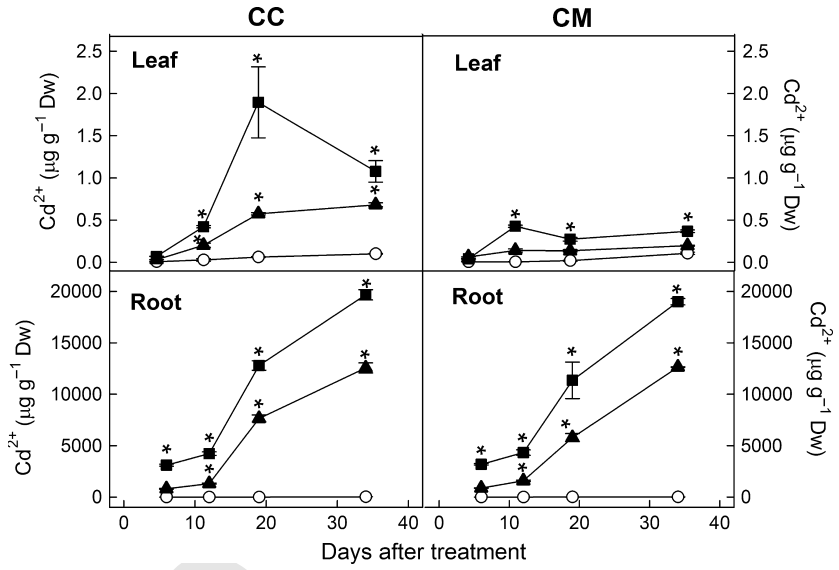

Fig. 2. Cadmium concentration in leaves and roots of citrus plants. Control plants (○); plants treated with 1.5 mM Cd^{2+} (▲); plants treated with 3.0 mM Cd^{2+} (■). Data are means ± SE. Error bars that are not visible are shorter than the height of the symbol. *Statistical difference with respect to controls at $P \leq 0.05$.

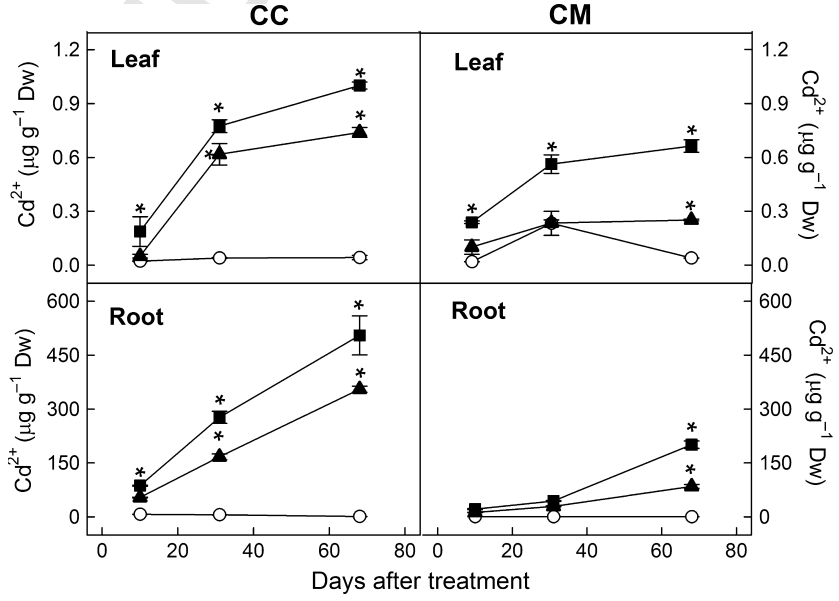

Fig. 3. Cadmium concentration in leaves and roots of two citrus plant varieties. Control plants (○); plants treated with 30 μM Cd^{2+} (▲); plants treated with 150 μM Cd^{2+} (■). Data are means ± SE. Error bars that are not visible are shorter than the height of the symbol. *Statistical difference with respect to controls at $P \leq 0.05$.

plants were very similar, ranging from 6.7 to 46.5 nM·g^{-1}. However, plants watered with a solution containing Ca^{2+} but no Cd^{2+}, had significantly increased GSH concentrations in comparison to control plants (1.62-fold). Furthermore, GSH content decreased in plants watered with 1.5 mM Cd^{2+} but the decrease was less drastic in those plants supplemented with 7.5 mM Ca^{2+}. GSSG content decreased in all treated plants when compared to controls. Treatment with added Ca^{2+} partially prevented the reduction in GSSG content caused by Cd^{2+}.

DISCUSSION

Despite the major concern of contamination of fruits and vegetables from metal accumulation in crops cultivated in soils containing Cd^{2+} (Grant *et al.* 2008), little information on the effect of this metal on citrus physiology can be found in the literature (Podazza *et al.* 2006). The present work, together with previous reports (López-Climent *et al.* 2011), indicates that citrus roots efficiently retain Cd^{2+}, avoiding its translocation to the shoots. Moreover, data demonstrate that Ca^{2+} supplied as a palliative treatment not only reduced root Cd^{2+} uptake but also increased GSH levels in control plants. Although further work is needed to understand metal distribution in shoots and fruits, this work can provide initial evidence for an effective treatment that could be of interest for citrus and other crops growing in contaminated soils.

To perform this and previous studies (López-Climent *et al.* 2011), two different levels of exogenous Cd^{2+} were used: (i) treatments with moderate concentrations of Cd^{2+} similar to those found in natural contaminated environments that could explain responses to heavy metal concentrations, and (ii) treatments with higher concentrations of the metal that caused visible symptoms in plants within a few weeks and allowed

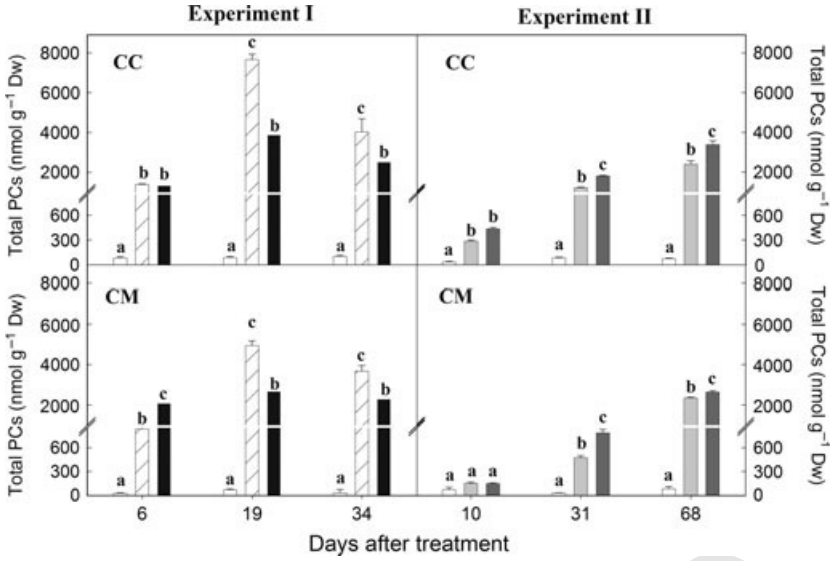

Fig. 4. Effect of cadmium treatments on total phytochelatin content in citrus roots. Different letters above bars denote statistical difference at $P \leq 0.05$. Cd^{2+} treatments: 30 μM (□); 150 μM (▩); 1.5 mM (▨); 3.0 mM (■); controls (□).

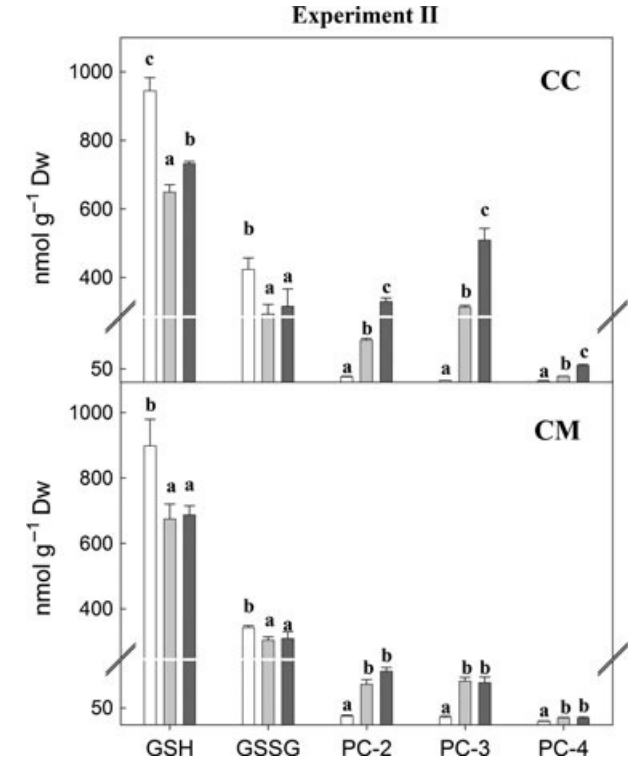

Fig. 5. Effect of cadmium treatments on GSH, GSSG, PC-2, PC-3 and PC-4 content in citrus roots. Different letters above bars denote statistical difference at $P \leq 0.05$. Metal treatments lasted for 31 days: 30 μM (□); 150 μM (▩); controls (□).

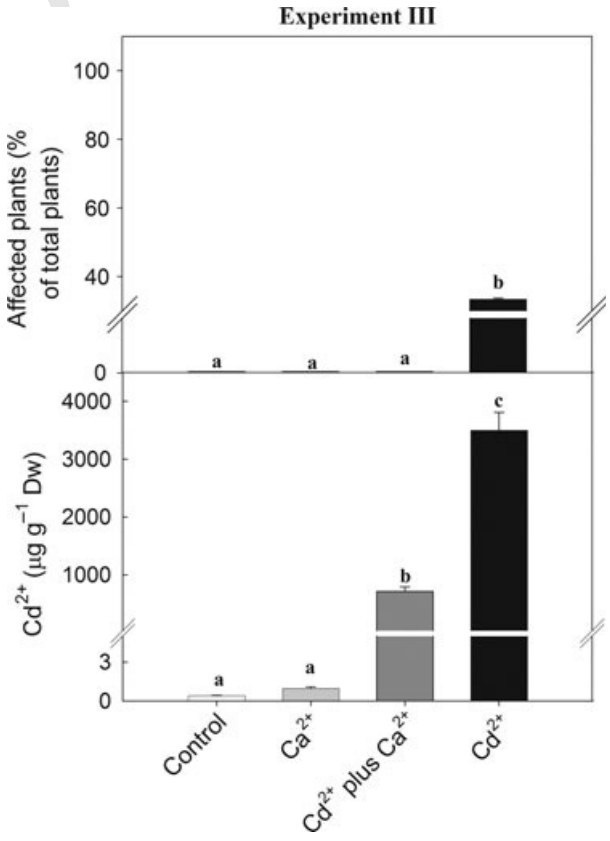

Fig. 6. Leaf damage and Cd^{2+} concentration in citrus roots under control conditions or treated for 30 days with 1.5 mM Cd^{2+}; 7.5 mM Ca^{2+} or 1.5 mM Cd^{2+} plus 7.5 mM Ca^{2+}. Bars followed with different letters denote statistical difference at $P \leq 0.05$.

establishment of a reproducible system (see Table 1, Fig. 1). It should be noted that the plants (1-year-old intact plants) watered with the lowest metal concentrations did not show any visible symptoms of Cd^{2+} toxicity for more than 2 months. Although it is assumed that plants would never encounter concentrations of Cd^{2+} as high as 1.5 and 3.0 mM Cd^{2+} in nature, the experiments provide valuable complementary information; other authors have previously used similar concentrations (Gratao *et al.* 2008).

Extremely low Cd^{2+} concentrations were found in leaves in comparison to roots. This was especially striking when high concentrations of exogenous Cd^{2+} were used. Taking in consideration the differences in exogenous Cd^{2+} concentration and root Cd^{2+} levels between the two experiments reported here, it was also surprising that levels of leaf Cd^{2+} were very

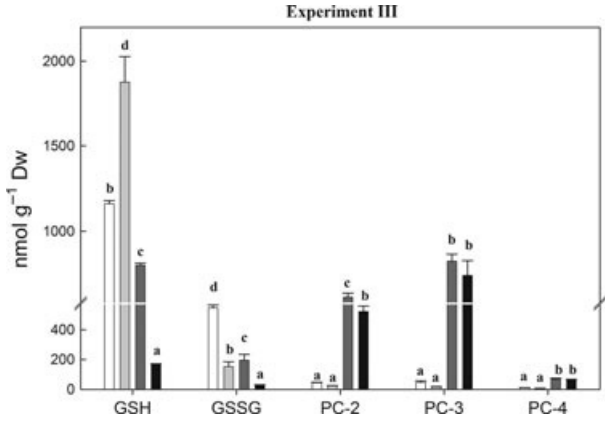

Fig. 7. GSH, GSSG, PC-2, PC-3 and PC-4 content in roots of citrus plants under control conditions (□) or treated for 30 days with 7.5 mM Ca^{2+} (▣); 1.5 mM Cd^{2+} plus 7.5 mM Ca^{2+} (▣); or 1.5 mM Cd^{2+} (■). Different letters above bars denote statistical difference at $P \leq 0.05$.

similar in plants in both situations (Figs 2 and 3). This can be explained, at least in part, by the drastic reduction in leaf transpiration that the high concentrations of Cd^{2+} caused (see López-Climent *et al.* 2011). Different processes can be involved in the Cd^{2+} transport from roots to shoots (López-Climent *et al.* 2011 and references therein), but due to the high levels of Cd^{2+} entering the roots, differences in transpiration rates can also influence Cd^{2+} translocation to the leaves. Interestingly, the low Cd^{2+} concentrations found in leaves in comparison to roots seem to support the hypothesis that most of the aerial damage observed in the different experimental designs was due to root malfunctioning (López-Climent *et al.* 2011). A similar situation was observed in citrus plants under continuous soil flooding (Arbona *et al.* 2008).

In other plant systems it has been shown that a Ca^{2+} amendment has a protective role against Cd^{2+} toxicity (Suzuki 2005; Rodríguez-Serrano *et al.* 2009). Evidence for Cd^{2+} uptake into plant cells *via* Ca^{2+} channels has come from electrophysiology studies showing that Ca^{2+} channels in guard cells are permeable to Cd^{2+} (Perfus-Barbeoch *et al.* 2002). Data presented in this work agree with previous reports and extend the findings to citrus genotypes. Increased concentrations of Ca^{2+} in the watering solution prevented Cd^{2+} accumulation (Fig. 6) in roots of CC. This result also opens the possibility of studying the use of Ca^{2+} as a palliative treatment in contaminated citrus-growing areas. Moreover, at the biochemical level, the most striking result is that Ca^{2+} treatment increased GSH concentrations (Fig. 7), a key metabolite in PC synthesis and therefore essential for toxic metal neutralisation. The importance of GSH as a precursor of PCs in roots is also supported by the fact that plants watered simultaneously with Cd^{2+} and Ca^{2+} had similar PC content to those treated only with the toxic metal, although their endogenous root Cd^{2+} concentration was much lower. Therefore, Ca^{2+} treatment had, at least, two positive effects: it reduced Cd^{2+} uptake and simultaneously increased endogenous GSH levels by promoting GSH synthesis and/or recycling mechanisms. There are several studies confirming that GSH-deficient mutants of *Arabidopsis thaliana* have impaired PC content and are hypersensitive to Cd^{2+} (Cobbett & Goldsbrough 2002; Lee *et al.* 2003). In rice and other species, a correlation between tolerance and increased glutathione content has been described (Na & Salt 2010; Cai *et al.* 2011). Moreover, in *Sedum alfredii*, a Cd hyper-accumulator native to China, Ca^{2+} treatment induced increases in GSH level under Cd^{2+} stress conditions (Tian *et al.* 2011).

Despite its evident role as a precursor of PCs, GSH has been shown to play a fundamental role in cellular events in different cells and tissues, including protection of organisms against oxidative stress (Sharma & Dietz 2009). In particular, in citrus under high salinity or soil flooding the key role of an active antioxidant system and the ability to recycle antioxidant metabolites, such as GSH and ascorbate, to prevent stress-induced oxidative damage has been demonstrated (Arbona *et al.* 2003, 2008). Therefore, the effect of Ca^{2+} treatment in increasing GSH levels would improve plant performance under any abiotic stress situation, as this molecule is a key antioxidant. This new finding contributes to explain part of the positive effect that Ca^{2+} had in citrus under salt stress (Bañuls *et al.* 1991) and opens new lines of study.

Previous reports have demonstrated that as a protection from heavy metal intoxication, plants have developed mechanisms to immediately inactivate metal ions entering the cytosol (Clemens 2006). PCs are rapidly induced *in vivo* by a wide range of heavy metal ions, including both cations, such as Cd^{2+}, copper and mercury, and anions such as arsenate. The enzyme is active only in the presence of heavy metal ions, but covers wide range of metal ions. Hara *et al.* (2005) proposed that citrus may have two classes of antioxidant binding proteins: an SH type of metal binding, like PCs, and a non-SH type of metal binding protein, like dehydrins. In this way, data presented herein demonstrate that Cd^{2+} stress caused PC accumulation in citrus roots even though they are not a hyper-accumulating species (López-Climent *et al.* 2011). Citrus plants watered with Cd^{2+} showed, within few days, an important increase in the concentration of these metabolites. This suggests that PCs effectively form complexes with the free Cd^{2+} in the cytosol to minimise the toxic effects of this metal in citrus roots, as reported in other plants (Clemens 2006; Cai *et al.* 2011). However, the high metabolic cost of PC synthesis implies that only plants able to trigger other mechanisms that can efficiently replace PC synthesis will be able to tolerate metal exposure (Lima *et al.* 2006).

Data on total PC content in roots of citrus treated with the lowest concentration of Cd^{2+} (Table 1) indicate that this protective mechanism is very sensitive to moderate changes in root Cd^{2+} concentration. Furthermore, data extracted from citrus treated with high Cd^{2+} exogenous concentrations (Fig. 4) showed that these plants have a certain limit to PC synthesis. Once this limit has been reached, citrus might not be able to synthesise more PCs in response to increased exogenous Cd^{2+}. This would increase free Cd^{2+} content in the root leading to increasing levels of this heavy metal in the intercellular spaces. In turn, Cd^{2+} would interact with SH groups essential for the enzyme reaction centre and stabilisation of the enzyme tertiary structure, inhibiting enzymatic activity and limiting the response of plants to excess Cd^{2+} (Sharma & Dietz 2009). This, together with a decline in transpiration and net photosynthetic (López-Climent *et al.* 2011), would cause collapse of the root and therefore plant death, as has been described in other plant species (Rodríguez-Serrano *et al.* 2009). Data also indicate that CC plants have a better capacity to synthesise PCs than CM

plants in response to Cd^{2+} stress; however, this makes no relevant contribution to heavy metal tolerance, as reported previously (López-Climent *et al.* 2011).

When citrus plants were watered with moderate Cd^{2+} concentrations, PC accumulation was correlated with the increase in root Cd^{2+} concentration in both genotypes. The pattern of Cd^{2+} and PC accumulation was similar in CM plants watered with either 30 or 150 μM Cd^{2+}, whereas CC plants showed increased PC synthesis along with a root Cd^{2+} build-up (Table 1, Figs 3 and 5). Moreover, in these experimental conditions, CC plants watered with Cd^{2+} (30 and 150 μM) showed GSH/GSSG ratios similar to control plants. These results reinforce the idea that CC plants have an efficient recycling system for GSH even under stress conditions (Arbona *et al.* 2003, 2008).

Overall, the results presented in this work indicate that Ca^{2+} treatment induced a considerable increase in endogenous levels of GSH in citrus plants, which can have positive effects in both facilitating PC synthesis and improving the antioxidant capacity of the cell. Moreover, as expected, Ca^{2+} treatment counteracted Cd^{2+} uptake. Data also show that citrus genotypes have some ability to tolerate elevated concentrations of Cd^{2+} in the soil or watering solution. Citrus roots efficiently retain Cd^{2+}, avoiding translocation to the aerial plant parts, which can have important commercial relevance. In this aspect, differences were found between the two studied genotypes: CM accumulated less Cd^{2+} in roots than CC when both genotypes were watered with low Cd^{2+} concentrations. In addition to the root differences, in all the experimental designs tested her, the amounts of Cd^{2+} translocated to the aerial part were lower in CM than in CC. It is also important to highlight the ability of CC plants to regenerate GSH because the availability of this metabolite could be vital for PC synthesis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplementary material S1. Identification of phytochelatins and thiol containing compounds by mass spectrometry

Supplementary material S2. Typical symptoms of cadmium toxicity in citrus

Supplementary material S3. Recovery assays were carried out for GSH and GSSG by spiking citrus root tissue (at the moment of sample grinding) with known amounts of standards of both analytes.

Supplementary material S4. Effect of cadmium treatments on GSH and GSSG content in roots of two genotypes of citrus.

REFERENCES

Anderson M.E. (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, **113**, 548–555.

Arbona V., Flors V., Jacas J., García-Agustín P., Gómez-Cadenas A. (2003) Enzymatic and non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive citrus rootstock, to different levels of salinity. *Plant Cell Physiology*, **44**, 388–394.

Arbona V., Hossain Z., López-Climent M.F., Pérez-Clemente R.M., Gómez-Cadenas A. (2008) Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. *Physiologia Plantarum*, **132**, 452–466.

Arbona V., López-Climent M.F., Perez-Clemente R.M., Gómez-Cadenas A. (2009) Maintenance of a high photosynthetic performance is linked to flooding tolerance in citrus. *Environmental and Experimental Botany*, **66**, 135–142.

Bañuls J., Legaz F., Primomillo E. (1991) Salinity–Calcium interactions on growth and ionic concentration of citrus plants. *Plant and Soil*, **133**, 39–46.

Boominathan R., Doran P.M. (2003) Cadmium tolerance and antioxidative defenses in hairy roots of the cadmium hyperaccumulator, *Thlaspi caerulescens*. *Biotechnology and Bioengineering*, **83**, 158–167.

Cai Y., Cao F., Cheng W., Zhang G., Wu F. (2011) Modulation of exogenous glutathione in phytochelatins and photosynthetic performance against Cd stress in the two rice genotypes differing in Cd tolerance. *Biological Trace Element Research*, **143**, 1159–1173.

Clemens S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie*, **88**, 1707–1719.

Cobbett C., Goldsbrough P. (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, **53**, 159–182.

Döring S., Korhammer S., Oetken M., Markert B. (2000) Analysis of phytochelatins in plant matrices by pre-column derivatisation, high-performance liquid chromatography and fluorescence detection. *Fresenius' Journal of Analytical Chemistry*, **366**, 316–318.

Grant C.A., Clarke J.M., Duguid S., Chaney R.L. (2008) Selection and breeding of plant cultivars to minimize cadmium accumulation. *Science of the Total Environment*, **390**, 301–310.

Gratao P.L., Monteiro C.C., Antunes A.M., Peres L.E. P., Azevedo R.A. (2008) Acquired tolerance of tomato (*Lycopersicon esculentum* cv Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321–333.

Hara M., Fujinaga M., Kuboi T. (2005) Metal binding by citrus dehydrin with histidine-rich domains. *Journal of Experimental Botany*, **56**, 2695–2703.

Kirkham M.B. (2006) Cadmium in plants on polluted soils: effects of soil factors, hyperaccumulation and amendments. *Geoderma*, **137**, 19–32.

Lee S., Moon J.S., Ko T.S., Petros D., Goldsbrough P.B., Korban S.S. (2003) Overexpression of Arabidopsis phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. *Plant Physiology*, **131**, 656–663.

Lima A.I.G., Pereira S.I.A., Figueira E.M.D.A.P., Caldeira G.C.N., De Matos Caldeira H.D.Q. (2006) Cadmium detoxification in roots of *Pisum sativum* seedlings: relationship between toxicity levels, thiol pool alterations and growth. *Environmental and Experimental Botany*, **55**, 149–162.

López-Climent M.F., Arbona V., Pérez-Clemente R.M., Gómez-Cadenas A. (2008) Relationship between salt tolerance and photosynthetic machinery performance in citrus. *Environmental and Experimental Botany*, **62**, 176–184.

López-Climent M.F., Arbona V., Pérez-Clemente R.M., Gómez-Cadenas A. (2011) Effects of cadmium on gas exchange and phytohormone contents in citrus. *Biologia Plantarum*, **55**, 187–190.

McLaughlin M.J., Parker D.R., Clarke J.M. (1999) Metals and micronutrients – food safety issues. *Field Crops Research*, **60**, 143–163.

Moreno-Jiménez E., Peñalosa J.M., Manzano R., Carpena-Ruiz R.O., Gamarra R., Esteban E. (2009) Heavy metals distribution in soils surrounding an abandoned mine in NW Madrid (Spain) and their transference to wild flora. *Journal of Hazardous Materials*, **162**, 854–859.

Moya J.L., Gómez-Cadenas A., Primo-Millo E., Talon M. (2003) Chloride absorption in salt-sensitive Carrizo citrange and salt-tolerant Cleopatra mandarin citrus rootstocks is linked to water use. *Journal of Experimental Botany*, **54**, 825–833.

Na G.N., Salt D.E. (2010) The role of sulfur assimilation and sulfur-containing compounds in trace element homeostasis in plants. *Environmental and Experimental Botany*, **72**, 18–25.

Nriagu J.O. (1979) Global inventory of natural and anthropogenic emissions of trace-metals to the atmosphere. *Nature*, **279**, 409–411.

Perfus-Barbeoch L., Leonhardt N., Vavasseur A., Forestier C. (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant Journal*, **32**, 539–548.

Podazza G., Rosa M., Gonzalez J.A., Hilal M., Prado F. E. (2006) Cadmium induces changes in sucrose partitioning, invertase activities, and membrane functionality in roots of Rangpur lime (*Citrus limonia* L Osbeck). *Plant Biology*, **8**, 706–714.

Rodríguez-Serrano M., Romero-Puertas M.C., Pazmino D.M., Testillano P.S., Risueno M.C., del Rio L.A. (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. *Plant Physiology*, **150**, 229–243.

Sharma S.S., Dietz K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*, **14**, 43–50.

Singh P.K., Tewari R.K. (2003) Cadmium toxicity induced changes in plant water relations and oxidative metabolism of *Brassica juncea* L. plants. *Journal of Environmental Biology*, **24**, 107–112.

Suzuki N. (2005) Alleviation by calcium of cadmium-induced root growth inhibition in Arabidopsis seedlings. *Plant Biotechnology*, **22**, 19–25.

Tennstedt P., Peisker D., Böttcher C., Trampczynska A., Clemens S. (2009) Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. *Plant Physiology*, **149**, 938–948.

Tian S.K., Lu L.L., Zhang J., Wang K., Brown P.H., He Z.L., Liang J., Yang X.E. (2011) Calcium protects roots of *Sedum alfredii* H. against cadmium-induced oxidative stress. *Chemosphere*, **84**, 63–69.

di Toppi L.S., Gabbrielli R. (1999) Response to cadmium in higher plants. *Environmental and Experimental Botany*, **41**, 105–130.

de Vos C.H.R., Vonk M.J., Vooijs R., Schat H. (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiology*, **98**, 853–885.

Yang T., Poovaiah B.W. (2002) Hydrogen peroxide homeostasis: activation of plant catalase by calcium/calmodulin. *Proceedings of the National Academy of Sciences USA*, **19**, 4097–4102.

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