Annals of Applied Biology ISSN 0003-4746

RESEARCH ARTICLE

Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress

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Keywords
Antioxidant enzymes; cadmium; heavy metals; lipid peroxidation; oxidative stress; tomato.

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Received: 1 October 2008; revised version accepted: 14 October 2008.

doi:10.1111/j.1744-7348.2008.00299.x

Abstract

The effects of varying concentrations of cadmium (Cd) on the development of *Lycopersicon esculentum* cv. Micro-Tom (MT) plants were investigated after 40 days (vegetative growth) and 95 days (fruit production), corresponding to 20 days and 75 days of exposure to $CdCl_2$, respectively. Inhibition of growth was clearly observed in the leaves after 20 days and was greater after 75 days of growth in 1 mM $CdCl_2$, whereas the fruits exhibited reduced growth following the exposure to a concentration as low as 0.1 mM $CdCl_2$. Cd was shown to accumulate in the roots after 75 days of growth but was mainly translocated to the upper parts of the plants accumulating to high concentrations in the fruits. Lipid peroxidation was more pronounced in the roots even at 0.05 mM $CdCl_2$ after 75 days, whereas in leaves, there was a major increase after 20 days of exposure to 1 mM $CdCl_2$, but the fruit only exhibited a slight significant increase in lipid peroxidation in plants subjected to 1 mM $CdCl_2$ when compared with the control. Oxidative stress was also investigated by the analysis of four key antioxidant enzymes, which exhibited changes in response to the increasing concentrations of Cd tested. Catalase (EC 1.11.1.6) activity was shown to increase after 75 days of Cd treatment, but the major increases were observed at 0.1 and 0.2 mM $CdCl_2$, whereas guaiacol peroxidase (EC 1.11.1.7) did not vary significantly from the control in leaves and roots apart from specific changes at 0.5 and 1 mM $CdCl_2$. The other two enzymes tested, glutathione reductase (EC 1.6.4.2) and superoxide dismutase (SOD, EC 1.15.1.1), did not exhibit any significant changes in activity, apart from a slight decrease in SOD activity at concentrations above 0.2 mM $CdCl_2$. However, the most striking results were obtained when an extra treatment was used in which a set of plants was subjected to a stepwise increase in $CdCl_2$ from 0.05 to 1 mM, leading to tolerance of the Cd applied even at the final highest concentration of 1 mM. This apparent adaptation to the toxic effect of Cd was confirmed by biomass values being similar to the control, indicating a tolerance to Cd acquired by the MT plants.

Introduction

In addition to an adequate supply of water (Kirda *et al.*, 2007; Latini *et al.*, 2007; Monti *et al.*, 2007; Tambussi *et al.*, 2007), nitrogen (Kingston-Smith *et al.*, 2006; Lea & Azevedo, 2006, 2007; Andrews *et al.*, 2007; Barbanti *et al.*, 2007; Lea *et al.*, 2007; Samborski *et al.*, 2008) and other minerals and elements (Dobbss *et al.*, 2007), for maximum rates of growth, plants also require a soil environment that is free of toxic compounds. Plants are constantly subjected to several different abiotic and biotic stresses (Hasdai *et al.*, 2006; Cabrera-Bosquet *et al.*, 2007;

Di Caterina *et al.*, 2007; Gorai & Neffati, 2007; Secchi *et al.*, 2007; Wei *et al.*, 2006), and it is now unquestionable that environmental pollution is far greater than had been previously assumed and that many heavy metals are capable of remaining in the environment for a long time, endangering the natural environment, affecting soil fertility and human health (Fontes & Alleoni, 2006; Przedpelska & Wierzbicka, 2007). Contamination with cadmium (Cd) is a serious problem facing the industrialised world leading to considerable losses in plant productivity and hazardous health effects (Mendes *et al.*, 2006; Gratão *et al.*, 2008*a*) but may not be a problem for the few Cd-hyperaccumulator plant species reported (Tolrà *et al.*, 2006). Cd is toxic to humans, animals and plants even at low doses (Mobin & Khan, 2007; Wahid & Ghani, 2008), affecting plant metabolism and inducing oxidative stress (Ferreira *et al.*, 2002; Benavides *et al.*, 2005; Gomes-Junior *et al.*, 2006*a*; Groppa *et al.*, 2007; Stobrawa & Lorenc-Plucinska, 2007). However, the intensity of the stress varies among plant species and tissues and is dependent on the Cd concentration and duration of exposure (Benavides *et al.*, 2005; Groppa & Benavides, 2008).

Under normal growth conditions, the production of reactive oxygen species (ROS) occurs in cells; however, adverse environmental factors that disrupt the cellular homeostasis may cause oxidative damage to proteins and DNA and lipid peroxidation, which in turn causes severe damage to cell membranes (Pitzschke *et al.*, 2006). Once formed, ROS must be detoxified as efficiently as possible to minimise such damage. The detoxification mechanisms in plants are complex and involve an array of non-enzymatic and enzymatic mechanisms capable of preventing the cascades of uncontrolled oxidation (Gratão *et al.*, 2005; Pitzschke *et al.*, 2006). Plant ROS-scavenging mechanisms include the action of some key enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), which dismutates $O_2^{\bullet -}$ to H_2O_2. Subsequently, H_2O_2 may be detoxified to H_2O by ascorbate peroxidase (EC 1.11.1.11), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9), among other peroxidases (Gratão *et al.*, 2005; Hassan, 2006; Passardi *et al.*, 2007; Hassan *et al.*, 2008). In addition, for the detoxification of H_2O_2, phenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases [guaiacol peroxidase (GPOX), EC 1.11.1.7] (Sakihama *et al.*, 2002). The ascorbate–glutathione cycle is closed by regeneration of reduced glutathione (GSH) from oxidised glutathione (GSSG) by glutathione reductase (GR, EC 1.6.4.2) using NAD(P)H as a reducing agent (Moller *et al.*, 2007).

Although information focused on the oxidative stress induced by Cd in plants has been available, particularly in the past few years (Gomes-Junior *et al.*, 2006*a*; Lin *et al.*, 2007; Liu *et al.*, 2007; Maksymiec *et al.*, 2007; Quartacci *et al.*, 2007; Wang *et al.*, 2008), it is still difficult to draw general conclusions. This is because of the enormous variation in responses that have been observed, making it difficult to establish the critical toxic metal concentrations in soils that allow normal plant growth. Model systems have been widely used to explore biological phenomena, and for this purpose, tomato can be considered an excellent plant material for biochemical and physiological analyses (Lima *et al.*, 2004). In addition, tomato can also be used as a model crop for gene transfer studies for other species with flesh berry fruits (Sun *et al.*, 2006; Ahsan *et al.*, 2007). The tomato miniature cultivar named 'Micro-Tom' (MT) has been proposed as a genetic model based on the small size, growth at a high density and production of viable fruits and seeds in containers using 50–100 mL of substrate, all within a life cycle ranging between 70 and 100 days (Meissner *et al.*, 1997). Moreover, MT can be grown in laboratories with the same minimum facilities needed for *Arabidopsis thaliana*.

The aim of this work was to study the effect of Cd on the lipid peroxidation and antioxidant responses of MT plants. However, instead of following the approach that has been extensively used to study heavy metal-induced stress, which is normally carried out by subjecting seedlings for a short period of time (few days) to high concentrations of a metal (acute treatment), we have grown MT plants to maturity under varying concentrations of Cd and also to increasing concentrations of Cd (chronic treatment). The results obtained not only demonstrated that MT is naturally quite tolerant to Cd when compared with other plant species, but tolerance may be acquired by growth in increasing metal concentrations and that antioxidant enzymes are directly involved in such a mechanism.

Materials and methods

Plant material

The MT cultivar of tomato (*Lycopersicon esculentum*) was kindly provided by Dr A. Levy (Weizmann Institute of Science, Rehovot, Israel). Seeds were sown in boxes containing a mixture of 1:1 (by volume) commercial pot mix (Plantmax HT Eucatex, São Paulo, Brazil) and vermiculite, supplemented with 1 g of NPK (Nitrogen-Phosphorus-Potassium) 10:10:10 and 4 g lime L^{-1} of mixture. After the first true leaves appeared, seedlings were transplanted to 1 L Leonard pots filled with sand and polystyrene (4:3) and Hoagland's nutrient solution (750 mL). Twenty-day-old plants (two per pot) were selected and grown further in the same medium, but containing 0 (control), 0.05, 0.1, 0.2, 0.5 and 1 mM $CdCl_2$

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(acute treatment). An extra set of plants was subjected to stepwise increasing concentrations of Cd (0.05, 0.1, 0.2, 0.5 and 1 mM $CdCl_2$) (chronic treatment). For this extra treatment, the plants were grown in each concentration of $CdCl_2$ for 15 days and the solution replaced sequentially by the higher concentration. The experiments were carried out in a glasshouse under natural daylight (May–August 2006 and 2007) with temperatures in the range of 20–30°C. The Hoagland solution with or without $CdCl_2$ was changed weekly, and the total volume was completed with water once a week. After periods of 40 and 95 days of growth since germination, corresponding to 20 and 75 days of exposure to $CdCd_2$, respectively, roots, leaves and fruits were collected, washed in distilled–deionised water and stored at −80°C for further analysis.

Cadmium content

Quantitative Cd analysis was carried out using energy disperse X ray (EDX). Samples of roots, leaves and fruits were dried at 60°C for 7 days, and 0.2 g of fine power obtained following grinding in a mortar and pestle set. Biological samples of each organ with increasing concentrations of Cd were used to establish standard calibration curves. The samples were irradiated for 150 s, using a Shimadzu EDX 720 system (São Paulo, Brazil), with 50 kV applied on the rhodium tube. For all studies, matrices with 2048 dependent variables (columns) were constructed (energy values), and the independent variable was the Cd concentration. The modelling and prediction tools for date exploration were performed with the use of the chemometrics package Pirouette, version 3.11 (Infometrix, Bothell, WA, USA).

Lipid peroxidation

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substances (TBARS) following the method of Heath & Packer (1968). The concentration of malondialdehyde (MDA) equivalents was calculated using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1}.

Extraction and analysis of antioxidant enzymes

The following steps were carried out at 4°C unless stated otherwise. The roots, leaves and fruits of MT plants were homogenised (2:1, buffer volume : fresh weight) in a mortar with a pestle in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid, 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinylpolypyrrolidone (Azevedo *et al.*, 1998). The homogenate was centrifuged at 10 000 *g* for 30 min, and the supernatant was stored in separate aliquots at −80°C prior to CAT, GR, GPOX and SOD analyses.

Catalase assay

Catalase activity was assayed spectrophotometrically at 25°C in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5), which contains 2.5 μL H_2O_2 (30% solution) prepared immediately before use. The reaction was initiated by the addition of 15 μL of plant extract, and the activity was determined by monitoring the removal of H_2O_2 at 240 nm over 1 min against a plant extract-free blank (Gomes-Junior *et al.*, 2007). CAT activity is expressed as μmol min^{-1} mg^{-1} protein.

Glutathione reductase assay

Glutathione reductase activity was assayed spectrophotometrically at 30°C in a mixture containing 3 mL of 100 mM potassium phosphate buffer (pH 7.5), which contains 1 mM 5,5″-dithiobis(2-nitrobenzoic acid), 1 mM GSSG and 0.1 mM NADPH. The reaction was started by the addition of 50 μL of enzyme extract. The rate of reduction of GSSG was followed by monitoring the increase in absorbance at 412 nm over 2 min (Gomes-Junior *et al.*, 2006*b*). GR activity is expressed as μmol min^{-1} mg^{-1} protein.

Guaiacol peroxidase assay

Guaiacol peroxidase activity was determined as described by Gomes-Junior *et al.* (2006*b*). One enzyme activity unit (U) of GPOX corresponds to an increase of 0.001 in absorbance per min per mg protein. The reaction medium contained 250 μL phosphate–citrate buffer (sodium phosphate dibasic 0.2 M : citric acid 0.1 M) pH 5.0, 150 μL enzyme extract and 25 μL 0.5% guaiacol, which was vortex shaken and incubated at 30°C for 15 min. The reaction was stopped by quickly cooling in an ice water bath, followed by the addition of 25 μL of 2% sodium metabisulphide solution. The reaction mixture was held for 10 min, and the GPOX activity was evaluated by monitoring the absorbance at 450 nm.

Polyacrylamide gel electrophoresis and superoxide dismutase activity staining

Electrophoretic analysis was carried out under non-denaturing condition in 10% polyacrylamide gels, followed by SOD activity staining as described by Vitória *et al.* (2001), with equal amounts of protein being loaded

onto each gel lane. Electrophoresis buffers and gels were prepared as described by Gratão *et al.* (2008*b*), except that SDS was excluded.

Determination of protein concentration

Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The experimental design was randomised with plants from three replicate pots being extracted separately for each treatment/time interval, and the results are expressed as mean and standard error of mean (±SEM) of three independent replicate enzyme assays of each extract for TBARS content, CAT, GR and GPOX activity, and two independent replicates for Cd accumulation measurements.

Results

Plant growth and cadmium accumulation

When the MT plants were grown in $CdCl_2$ concentrations ranging from 0.05 to 1 mM, the concentrations of 0.2, 0.5 and 1 mM caused inhibition of growth based on dry weight accumulation in the leaves and fruits, whereas the extra set of plants that were subjected to gradual increasing concentrations of Cd exhibited growth rates practically identical to the control (Fig. 1A and Fig. 1B and Fig. 2). It was also observed that Cd was only detected in the roots after 75 days of growth and that there was a general increase in Cd accumulation with the increase in external $CdCl_2$ concentration (Table 1). However, leaves accumulated high Cd concentrations after 20 days of growth, which appeared to be reduced after 75 days. High Cd accumulation was also observed in the fruits with the largest amount being detected in the plants subjected to increasing concentrations of $CdCl_2$ (Table 1).

Lipid peroxidation

Cadmium-induced lipid peroxidation as determined by TBARS content was observed in all organs but was more pronounced in the roots after 20 days of treatment with $CdCl_2$ and in all treatments after 75 days of growth, with the exception of the extra set of plants that were subjected to increasing concentrations of $CdCl_2$, which exhibited a content only slightly higher than the control (Fig. 3A). In leaves, 1 mM $CdCl_2$ caused a considerable

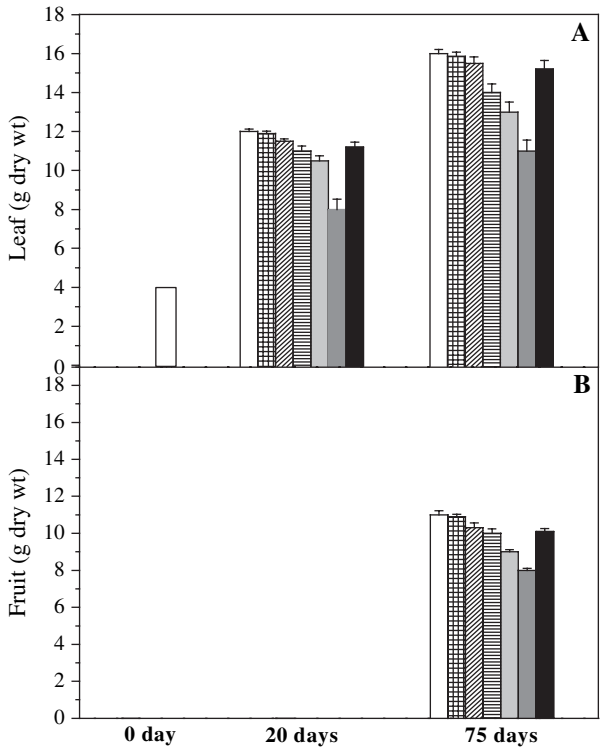

Figure 1 Leaves (A) and fruits (B) dry mass (g dry wt) of MT plants grown over a 75-day period in the presence of $CdCl_2$. Control, 0 $CdCl_2$ (□), 0.05 mM $CdCl_2$ (▦), 0.1 mM $CdCl_2$ (▨), 0.2 mM $CdCl_2$ (▤), 0.5 mM $CdCl_2$ (▩), 1 mM $CdCl_2$ (■) and 0.05–1 mM $CdCl_2$ (■). Values are the means of three replicates ±SEM.

accumulation of TBARS even after 20 days, whereas the other treatments only induced lipid peroxidation after 75 days, which was still much reduced when compared with the 1 mM concentration (Fig. 3B). A small

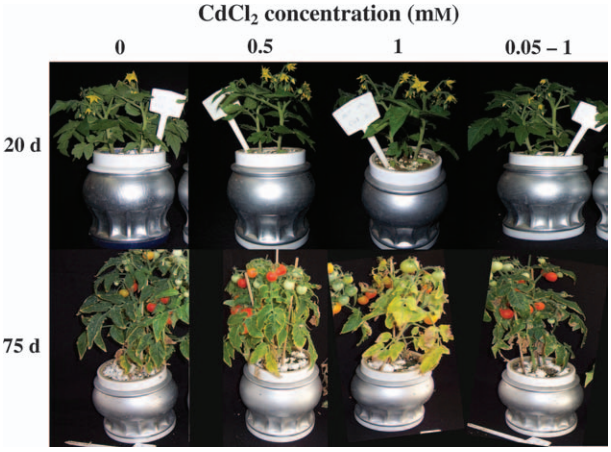

Figure 2 MT plants grown for 20 and 75 days in increasing concentrations of $CdCl_2$ as described in the *Materials and Methods*.

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Table 1 Cadmium (Cd) accumulation (μmol g^{-1} dry weight) during plant development. Micro-Tom tomato plants were subjected to increasing $CdCl_2$ concentrations and samples analysed after 20 and 75 days of treatment. The ±SEM values were all below 3%[a]

Organ	$CdCl_2$ Concentration (mM)	20 days	75 days
Root	0.05	ND	0.40
	0.10	ND	0.50
	0.20	ND	0.75
	0.50	ND	2.50
	1.00	ND	3.05
	0.05–1.0	ND	1.45
Leaf	0.05	2.50	0.20
	0.10	2.25	0.65
	0.20	2.55	1.00
	0.50	2.85	1.55
	1.00	3.10	2.30
	0.05–1.0	2.85	1.50
Fruit	0.05	NF	2.25
	0.10	NF	2.40
	0.20	NF	2.50
	0.50	NF	2.75
	1.00	NF	3.30
	0.05–1.0	NF	5.20

ND, not detected; NF, no fruits formed.
[a]Cd was not detected in any of the control organ samples subject to 0 $CdCl_2$ treatment.

significant increase in lipid peroxidation was detected in fruits sampled from the 1 mM $CdCl_2$ treatment, while for all other treatments, including the extra set of plants that were subjected to gradual increasing concentrations of $CdCl_2$, the TBARS content did not differ statistically from the control treatment (Fig. 3C).

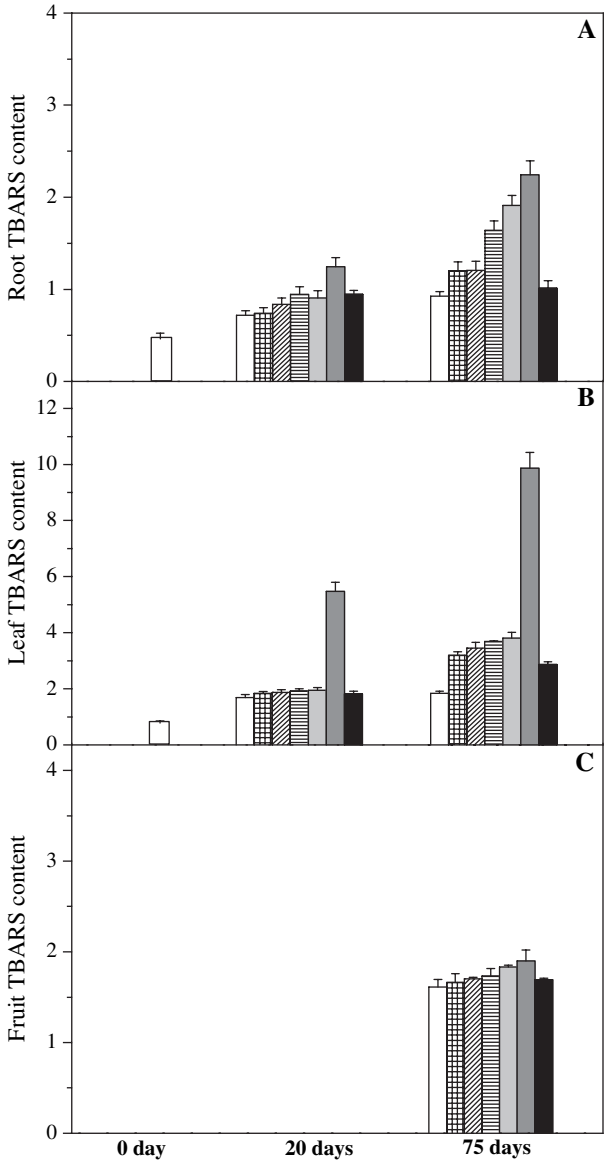

Figure 3 Lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS) content (nmol g^{-1} fresh weight) of roots (A), leaves (B) and fruits (C) of MT plants grown over a 75-day period in the presence of $CdCl_2$. Control, 0 $CdCl_2$ (□), 0.05 mM $CdCl_2$ (▦), 0.1 mM $CdCl_2$ (▨), 0.2 mM $CdCl_2$ (▤), 0.5 mM $CdCl_2$ (▩), 1 mM $CdCl_2$ (■) and 0.05–1 mM $CdCl_2$ (■). Values are the means of three replicates ±SEM.

Antioxidant enzymes activities

Analysis of CAT clearly showed Cd-induced increases in total activity in the three organs tested (Fig. 4). In roots and leaves, the lowest $CdCl_2$ concentrations induced high levels of CAT activity, whereas at the highest $CdCl_2$ treatments, CAT activity was very similar to that of the control. However, CAT activity in the fruit tissue was consistently higher in all $CdCl_2$ treatments when compared with the control (Fig. 4C).

The total activity of GPOX also exhibited changes in response to the $CdCl_2$ treatment, but such changes were not uniform or linear (Fig. 5). GPOX activity was shown to be stimulated by 0.5 and 1 mM $CdCl_2$ after 20 days in the roots (Fig. 5A) and in the leaves by 1 mM $CdCl_2$ after 20 and 75 days of treatment (Fig. 5B), but in most treatments, GPOX activity was within the same range or below that observed for the control (Fig. 5A and Fig. 5B). Fruits from plants grown on low concentrations of Cd also exhibited GPOX activity below the control levels; however, those grown on 0.5 and 1 mM $CdCl_2$ and the extra set of plants that were subjected to increasing concentrations of Cd exhibited a doubling in GPOX activity when compared with the control (Fig. 5C).

Analysis of total GR activity revealed clear trends for all three organs analysed but different from each other (Fig. 6). GR activity in the roots and leaves was reduced

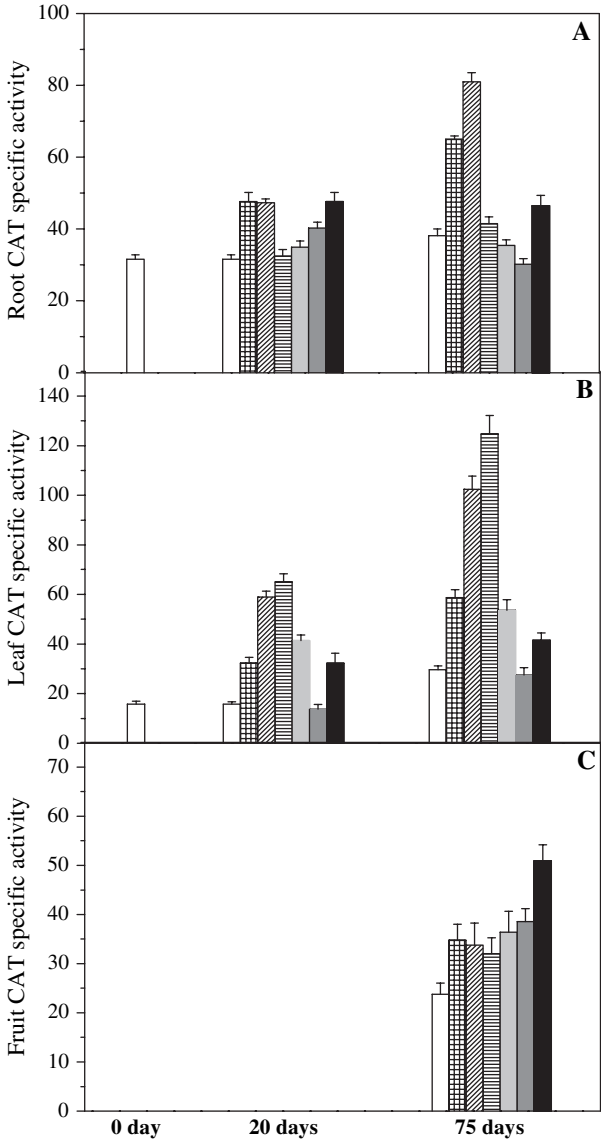

Figure 4 Specific activity of catalase (CAT) (μmol min^{-1} mg^{-1} protein) in the roots (A), leaves (B) and fruits (C) of MT plants grown over a 75-day period in the presence of $CdCl_2$. Control, 0 $CdCl_2$ (□), 0.05 mM $CdCl_2$ (▦), 0.1 mM $CdCl_2$ (▨), 0.2 mM $CdCl_2$ (▤), 0.5 mM $CdCl_2$ (▩), 1 mM $CdCl_2$ (■) and 0.05–1 mM $CdCl_2$ (■). Values are the means of three replicates ±SEM.

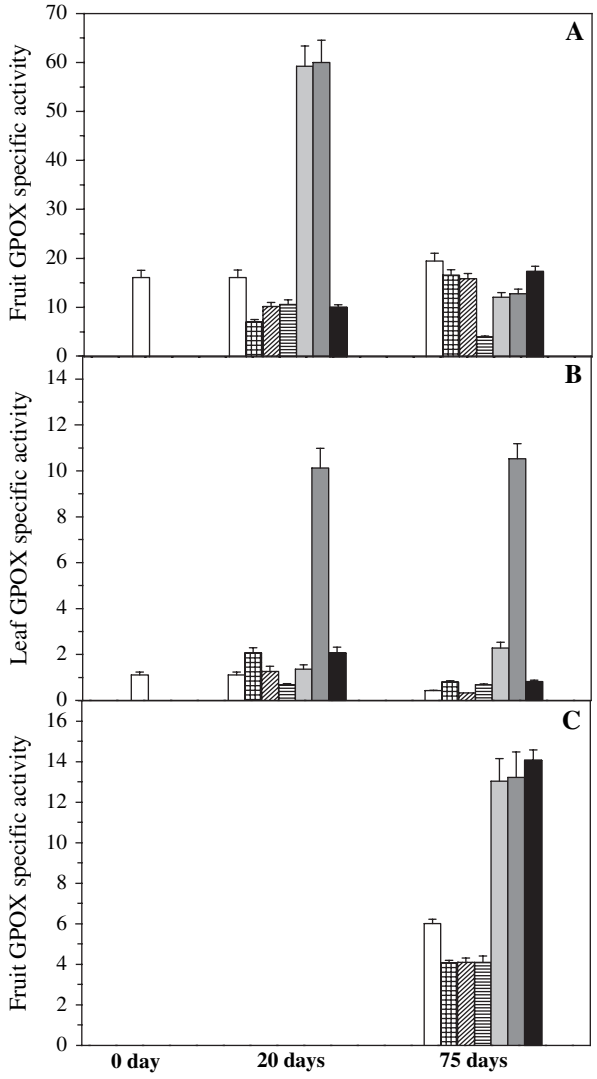

Figure 5 Specific activity of guaiacol peroxidase (GPOX) (U) in the roots (A), leaves (B) and fruits (C) of MT plants grown over a 75-day period in the presence of $CdCl_2$. Control, 0 $CdCl_2$ (□), 0.05 mM $CdCl_2$ (▦), 0.1 mM $CdCl_2$ (▨), 0.2 mM $CdCl_2$ (▤), 0.5 mM $CdCl_2$ (▩), 1 mM $CdCl_2$ (■) and 0.05–1 mM $CdCl_2$ (■). Values are the means of three replicates ±SEM.

by more than 50% after 75 days, irrespective of the treatment, when compared with the 0 day control and the majority of GR activities determined after 20 days of growth (Fig. 6A and Fig. 6B). However, GR activity in the fruits was increased in all $CdCl_2$ treatments with the exception of the lowest concentration used and increased by more than twofold in fruits from the extra set of plants that were subjected to increasing concentrations of $CdCl_2$ (Fig. 6C).

The activity of SOD in extracts of plants grown for 75 days was determined based on the separation of isoenzymes by non-denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 7). SOD activity staining revealed the existence of three isoenzymes in leaves (bands I, II and III) (Fig. 7A), but only two isoenzymes were observed in roots (Fig. 7B) and fruits (Fig. 7C), which corresponded to SOD bands I and III from leaves (Fig. 7B). Although a densitometric analysis was not carried out, it is possible to observe that a continuous decrease in band intensity was observed for concentrations above 0.1 mM $CdCl_2$ in leaves (Fig. 7A) and above 0.2 mM $CdCl_2$ in roots (Fig. 7B)

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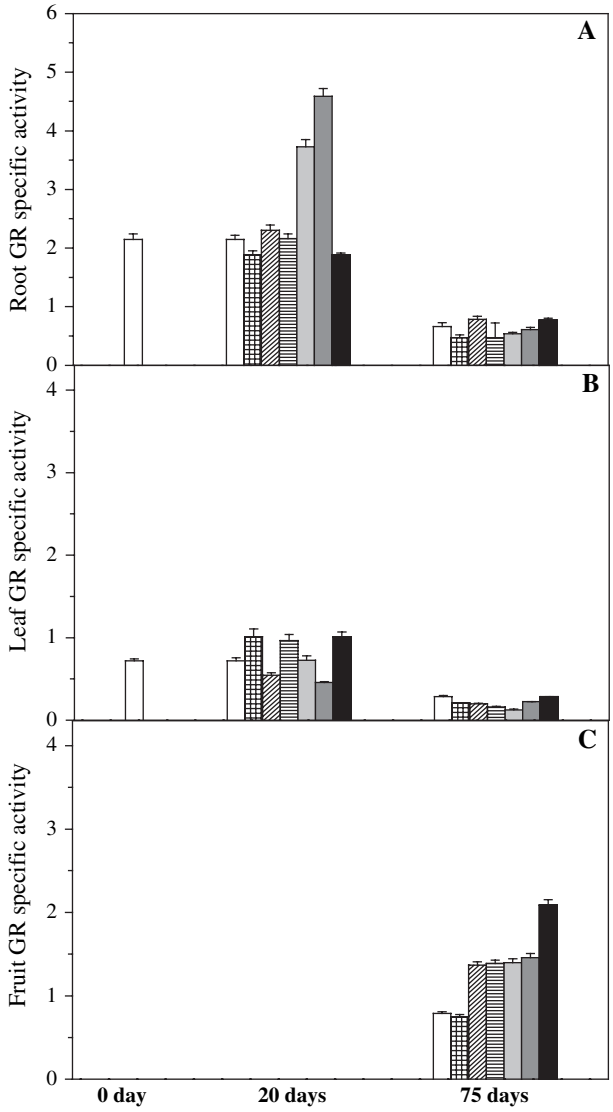

Figure 6 Specific activity of glutathione reductase (GR) (μmol min^{-1} mg^{-1} protein) in the roots (A), leaves (B) and fruits (C) of MT plants grown over a 75-day period in the presence of $CdCl_2$. Control, 0 $CdCl_2$ (▭), 0.05 mM $CdCl_2$ (▦), 0.1 mM $CdCl_2$ (▨), 0.2 mM $CdCl_2$ (▤), 0.5 mM $CdCl_2$ (▩), 1 mM $CdCl_2$ (▩) and 0.05–1 mM $CdCl_2$ (■). Values are the means of three replicates ±SEM.

and fruits (Fig. 7C), while the extra set of plants that were subjected to gradual increasing concentrations of Cd exhibited bands of control level intensity in all organs (Fig. 7). For most treatments, the activity changes observed were similar for all bands and could not be attributed to specific changes for any particular isoenzyme. However, SOD II, which is present only in leaves, appeared to maintain the same level of activity, consequently accounting for a higher percentage of the total SOD activity at 0.5 and 1 mM $CdCl_2$ treatments. Whereas in the extra set of plants that were subjected to gradual increasing concentrations of $CdCl_2$, SOD II was reduced when compared with 0.5 and 1 mM $CdCl_2$ (Fig. 7A).

Discussion

Cadmium is a non-essential element in plant nutrition that can inhibit growth (Mediouni *et al.*, 2006; Liu *et al.*, 2007) and stimulate ROS production, resulting in several metabolic perturbations (Yakimova *et al.*, 2006; Durcekova *et al.*, 2007). Under high levels of ROS, the cellular homeostasis can be disrupted causing oxidative damage of macromolecules that may involve induction of lipid peroxidation, thus causing severe damage to cell membranes (Gratão *et al.*, 2005; Pitzschke *et al.*, 2006).

Although information on long-term exposure to heavy metals have been reported (Dong *et al.*, 2006; Djebali *et al.*, 2008), the large majority of studies published on the effect of heavy metals on plant growth and on the biochemical responses to the eventual oxidative stress have been carried out using short-term treatments with metal concentrations normally above that found in polluted soil (acute treatment), leading to a rapid response by the plant system. We have tried a different approach combining acute and chronic treatments by exposing tomato plants to a range of $CdCl_2$ concentrations varying from 0.05 to 1 mM, most of which are higher than those normally observed in polluted soils. The experimental treatments were carried out for up to 75 days when mature fruits (95 day-old plants) were produced. Such an approach in association with another uncommon strategy of subjecting plants to a stepwise increase in Cd concentration during plant development has allowed us to investigate whether tomato plants can adapt to high concentrations of Cd over a period of time. The MT tomato line is a very useful plant model for such a purpose because the plant is fully developed with the production of mature fruit after approximately 100 days.

According to our results, Cd inhibited growth (Figs 1 and 2) and induced oxidative stress detected as an increase in TBARS content, with the highest levels of lipid peroxidation being always detected in all organs of plants that had been grown in 1 mM $CdCl_2$ (Fig. 3). Furthermore, the data obtained for Cd accumulation was surprising because Cd was mainly translocated to the upper parts of the plants, including the fruits where it reached the highest values observed in this study (Table 1). Cd is known to accumulate mainly in the roots system in the majority of plant species studied so far (Vitória *et al.*, 2001; Pereira *et al.*, 2002). Such high concentrations can stimulate the synthesis of phytochelatins, which can sequester Cd in the vacuole

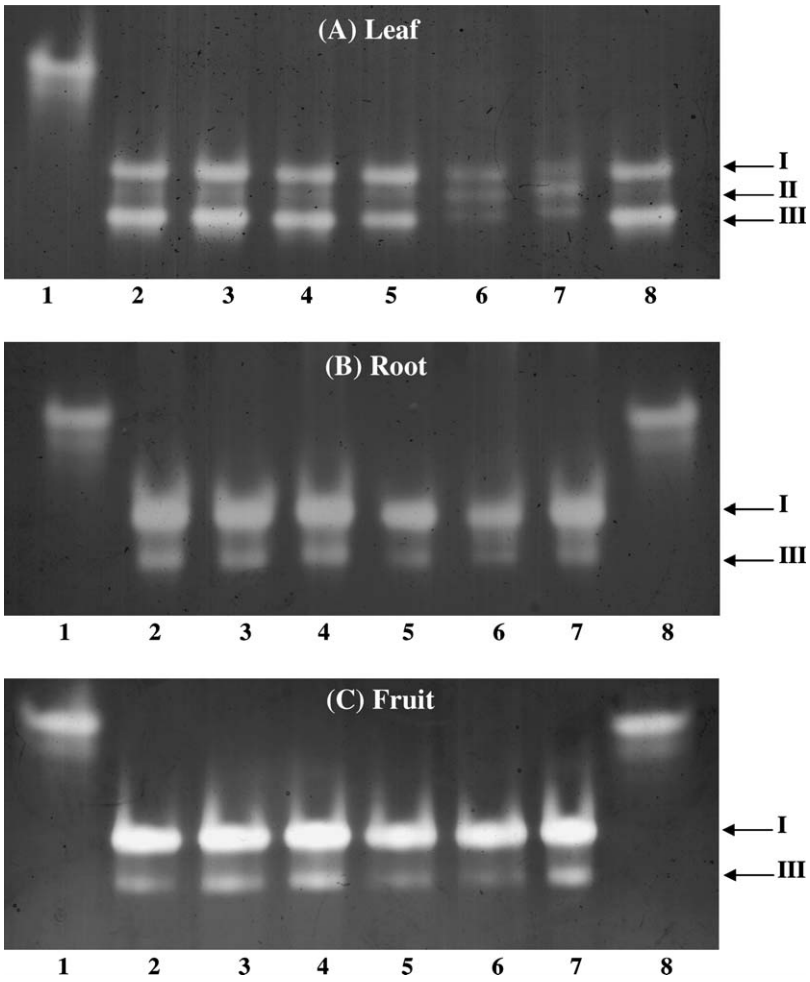

Figure 7 Activity staining for superoxide dismutase (SOD) following native polyacrylamide gel electrophoresis of extracts of leaves (A), roots (B) and fruits (C) isolated from MT plants grown over a 75-day period in the presence of $CdCl_2$. (A), lane 1, bovine SOD standard; lane 2, control 0 $CdCl_2$; lane 3, 0.05 mM $CdCl_2$; lane 4, 0.1 mM $CdCl_2$; lane 5, 0.2 mM $CdCl_2$; lane 6, 0.5 mM $CdCl_2$; lane 7, 1.0 mM $CdCl_2$ and lane 8, 0.05–1 mM $CdCl_2$. (B), lanes 1 and 8, bovine SOD standard; lane 2, control 0 $CdCl_2$; lane 3, 0.05 mM $CdCl_2$; lane 4, 0.1 mM $CdCl_2$; lane 5, 0.2 mM $CdCl_2$; lane 6, 0.5 mM $CdCl_2$; lane 7, 0.05–1 mM $CdCl_2$. (C), lanes 1 and 8, bovine SOD standard; lane 2, control 0 $CdCl_2$; lane 3, 0.05 mM $CdCl_2$; lane 4, 0.1 mM $CdCl_2$; lane 5, 0.2 mM $CdCl_2$; lane 6, 0.5 mM $CdCl_2$; lane 7, 0.05–1 mM $CdCl_2$.

(Wójcik *et al.*, 2005). Different parts of the plants have different capacities to accumulate Cd because of the localisation of specific ligands with affinity to bind to a particular metal (Prasad & Freitas, 2000). The accumulation of Cd in the fruits is particularly important because of the eventual problem with the food chain. In this study, Cd was shown to accumulate to high concentrations independent of the $CdCl_2$ treatment but was considerably higher in tomato fruits from the plants subjected to stepwise increasing $CdCl_2$ concentrations.

The majority of studies on Cd-induced stress have concentrated on the use of seedlings and cell cultures, with a much lower number on plants that have been allowed to grow to maturity. The literature for tomato has shown that Cd appears to accumulate mainly in the roots (Ben Ammar *et al.*, 2008; Delperee & Lutts, 2008) but can be translocated to the aerial parts of the plant, including fruits (Shentu *et al.*, 2008). The MT cultivar has a different pattern of development, and the combination of chronic and acute treatments revealed that Cd in MT plants is mainly translocated to the upper parts of the plants (Table 1). It is also interesting to see that after 75 days of growth, Cd is clearly present in all organs, but the concentrations in leaves are lower than those observed at 20 days of growth. After 75 days of growth, the concentrations of Cd in the fruits are the highest of the three organs, suggesting that following the beginning of fruit development Cd is translocated

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preferentially to this organ. The combination of the different treatments, plant cultivar and growth condition may help explain the different behaviour observed for the accumulation of Cd in MT plants. Even within the same plant species, Cd has been shown to accumulate in different concentrations in the same organ depending on the cultivar (Alexander *et al.*, 2006; Zheng *et al.*, 2008).

Cadmium does not appear to generate free radicals directly, but it does elevate lipid peroxidation, contributing to a process of oxidative damage (Gratão *et al.*, 2005). MDA, the most frequently used indicator of lipid peroxidation, is one of several low-molecular-mass products formed through the decomposition of primary and secondary lipid peroxidation products (Dewir *et al.*, 2006). Cd has been shown to cause an increase in the amount of MDA in rice leaves (Hsu & Kao, 2007*a*,*b*), tomato seedlings (Mediouni *et al.*, 2006), *Phaseolus coccineus* (Skorzynska-Polit & Krupa, 2006), *Bacopa monnieri* L. (Mishra *et al.*, 2006), *Lemna polyrrhiza* (John *et al.*, 2007) and *Brassica juncea* (Mobin & Khan, 2007), probably as a result of increased H_2O_2 production. The toxicity of ROS explains the evolution of complex arrays of non-enzymatic and enzymatic detoxification mechanisms in plants capable of quenching ROS (Pauly *et al.*, 2006). We have analysed the activities of some of the antioxidant enzymes in response to Cd treatment, and distinct responses were observed among the organs analysed.

As already stated in the *Introduction*, the enzymes evaluated in this study are important in the antioxidant responses of plants to stress. Such responses to Cd and other heavy metals have received a great deal of attention in recent years and have been shown to vary considerably among plants species, organs and tissues and are dependent on the Cd concentration and duration of exposure. For the sake of conciseness, detailed information about the extensive list of reports is not presented in this study (for reviews, see Benavides *et al.*, 2005 and Gratão *et al.*, 2005).

In this study, CAT activity in the three tomato plant organs increased consistently in the roots and leaves in response to the two lowest $CdCl_2$ concentrations (0.05 and 0.1 mM) and in the extra set of plants subjected to a stepwise increase in $CdCl_2$ from 0.05 to 1 mM, while all $CdCl_2$ treatments induced CAT activity increases in the fruits (Fig. 4). In a recent short-term study of more than 4 days, 0.2 and 0.4 mM $CdCl_2$ was shown to stimulate CAT activity by more than 100% in the leaves of two Cd accumulator species but to reduce activity in non-accumulating tobacco (Wang *et al.*, 2008). Thus, it would appear that CAT is an important enzyme in the defence of tomato plants against Cd because the activity of the other enzymes tested, for example GPOX and GR, exhibited varied responses to Cd in the three organs analysed. SOD, which detoxifies $O_2^{\bullet -}$, did not exhibit any increase in activity in response to Cd stress but, on the contrary, exhibited reduced activity with the increase in Cd concentration. Although we have not measured H_2O_2 or other ROS in this study, increases are likely to have occurred based on the responses of the antioxidant enzymes that were measured. GPOX also exhibited some increase in activity, but only at the highest Cd concentrations tested and in leaves and fruits (Fig. 5), further indicating that CAT may be the main player in the defence response to Cd in MT plants. The lack of consistent significant increases in GR activity in response to $CdCl_2$, particularly in the leaves, suggests that increased synthesis of phytochelatins, which requires GSH, may not have occurred. However, some reports have demonstrated that cell lines and tomato plants growing in higher concentrations of Cd were able to synthesise phytochelatins (Gupta & Goldsbrough, 1991; Mediouni *et al.*, 2006). Therefore, it is necessary that future research investigates in more detail the synthesis of phytochelatins and even other antioxidant systems and metabolites, such as glutathione, ascorbate, amino acids, polyamines and organic acids, which may also have been altered in response to Cd (Benavides *et al.*, 2005; Dong *et al.*, 2007; Groppa & Benavides, 2008) in MT plants. In the fruits, GR activity was increased, suggesting that the fruit tissue was more significantly affected by Cd. This would probably be expected because the development of the fruit only occurred a short time before the plants were sampled, whereas the leaves and roots were subjected to Cd for a much longer period. A reduction in the intensities of the SOD activity bands I and III was detected in response to the $CdCl_2$ treatments, while the extra SOD band (II), present only in the leaves, appeared to be little affected.

However, the most striking results were those obtained for the plants subjected to gradual stepwise increasing concentrations of $CdCl_2$ (0.05, 0.1, 0.2, 0.5 and 1 mM $CdCl_2$) for 75 days. Based on dry weight production, the plants became tolerant to the Cd applied, even at the final highest concentration of 1 mM (Fig. 2). This result indicated an adaptation to the effect of the metal, which was also confirmed by lipid peroxidation (TBARS content), that was similar to the control (Fig. 3). Furthermore, the responses obtained for all the enzymes analysed also indicated specific alterations, for instance, CAT (Fig. 4), GPOX (Fig. 5), GR (Fig. 6) and SOD (Fig. 7) activity levels in roots and leaves were similar to those of the control. However, in the fruit, the stepwise increase in Cd induced the highest levels of the four enzyme activities when compared with the other Cd treatments, indicating a higher level of antioxidant response. As already mentioned above, the increased antioxidant

enzyme activities in the stepwise treatment are in full agreement with Cd accumulation in the fruits of these plants, which exhibited the highest Cd accumulation among all organs and treatments (Table 1). Lipid peroxidation in the fruits varied little with increased Cd concentration, indicating that the antioxidant enzymes may be playing a key role in this organ when compared with the roots and leaves, where other defence systems may be more important. In addition to SOD, other antioxidant enzymes are also present as isoenzymes and future studies should include non-denaturing PAGE analyses for isoenzymes because it is not possible at this stage to establish whether the major increase in CAT activity is because of the presence of an individual CAT isoenzyme. Furthermore, these isoenzymes are located in different cell compartments and specific alterations for a particular isoenzyme may help correlate the induced stress to a specific physiological phenomenon. An ongoing more comprehensive study on this aspect and others is being conducted by our group with hormonal mutants in the model system MT, representing a complementary study concerning the integration of environmental and endogenous signals of phytotoxicity and resistance to stress factors.

It is important to note that the fruit organ was only present at the later stage of development and consequently subjected for a shorter period to the Cd treatment, which could possibly explain the higher levels of enzymes activity and lipid peroxidation seen consistently for the 1 mM and stepwise treatments. As the cells in the fruit had to respond more rapidly to the induced stress, in the case of the stepwise treatment, a more strong response was obtained that led to the highest tolerance to the metal when compared with the other treatments detected by fruit dry weight (Fig. 1B). Once again, it is important to mention the high levels of Cd accumulated in the fruits of plants subjected to the stepwise treatment. Such a distinct behaviour may also be observed between short-term and long-term stress effects, which can be partially compensated for by acclimation, adaptation and repair mechanisms and high stress or chronic stress events, which can cause considerable damage and may eventually lead to cell and plant death (Lichtenthaler, 1996). At the beginning of stress when plants are suddenly confronted with a critical situation, they will activate metabolic pathways, repair processes and long-term metabolic and morphological adaptations of the general adaptation syndrome (McKersie & Leshem, 1994), representing a generalised effort by the organism to adapt itself to new conditions (Leshem & Kuiper, 1996).

In conclusion, the gradual increase in $CdCl_2$ concentration over a period of time appeared to adapt the plants to the toxic effects of Cd, but it also led to a significant higher accumulation of Cd in the fruits. The information available concerning plants subjected to increasing concentrations of Cd and other metals should provide a better understanding of the mechanisms of detoxification, which may help integrate biochemical genetics with plant breeding to produce stress-tolerant plants for detoxification or phytoremediation programmes of polluted environments by heavy metals.

Acknowledgements

This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Grant number 04/08444-6). R. A. A. and P. L. G. would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for the fellowship and scholarship granted.

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