

Temporal dynamic responses of roots in contrasting tomato genotypes to cadmium tolerance

Karina Lima Reis Borges ¹ · Fernanda Salvato ^{1,2} · Berenice Kussumoto Alcântara ³ ·
Rafael Storto Nalin ⁴ · Fernando Ângelo Piotto ¹ · Ricardo Antunes Azevedo ¹

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Abstract Despite numerous studies on cadmium (Cd) uptake and accumulation in crops, relatively little is available considering the temporal dynamic of Cd uptake and responses to stress focused on the root system. Here we highlighted the responses to Cd-induced stress in roots of two tomato genotypes contrasting in Cd-tolerance: the tolerant Pusa Ruby and the sensitive Calabash Rouge. Tomato genotypes growing in the presence of 35 μM CdCl₂ exhibited a similar trend of Cd accumulation in tissues, mainly in the root system and overall plants exhibited reduction in the dry matter weight. Both genotypes showed similar trends for malondialdehyde and hydrogen peroxide accumulation with increases when exposed to Cd, being this response more pronounced in the sensitive genotype. When the antioxidant machinery is concerned, in the presence of Cd the reduced glutathione content was decreased in roots while ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione S-transferase (GST) activities were increased in the presence of Cd in the tolerant genotype. Altogether these results suggest APX, GR and GST as the main players of the antioxidant machinery against Cd-induced oxidative stress.

Keywords Antioxidants · Cadmium · Heavy metals · Oxidative stress · *Solanum lycopersicum*

Introduction

Heavy metals occur naturally in the soil and can also be released into the environment from anthropogenic activities such as mining, phosphate fertilizers applications and disposal of agro-industrial wastes (Gallego et al. 2012). Due to the increase of agricultural areas contaminated by heavy metals, cadmium (Cd) toxicity is getting worse every year. However, the number of plant species that have some tolerance degree is restricted (Bianconi et al. 2013).

The knowledge about interaction between plants and heavy metals is important to reduce the probability of the introduction of these toxic elements into the food chain (Azevedo et al. 2012; Benavides et al. 2005; Teklić et al. 2013). Such interaction can be understood as the ability that some plant species have to uptake and accumulate heavy metals, associated with the effects caused by these elements in the plant development and metabolism (Kumar et al. 2013). Plant tolerance to Cd is also a relevant issue due to the negative effects on food production and quality, allied to the risks to animal and human health caused by the consumption of contaminated products (Bianconi et al. 2013; Teklić et al. 2013). It is estimated that more than 80% of Cd contamination occurs by ingestion of vegetables and cereals (Egan et al. 2007).

In addition to the well-known negative effects on plant growth, Cd is considered a toxic metal due to its high mobility, disturbing basic physiological and metabolic processes such as the uptake and transport of water and nutrients, photosynthesis and respiration (Arasimowicz-

✉ Ricardo Antunes Azevedo
raa@usp.br

¹ Laboratory of Plant Genetics and Biochemistry, Genetics Department, “Luiz de Queiroz” College of Agriculture, Piracicaba, SP, Brazil

² Laboratory of Molecular Physiology of Plants, Institute of Biology, State University of Campinas, Campinas, SP, Brazil

³ Federal University of Acre, Rio Branco, AC, Brazil

⁴ Laboratory of Statistical Genetics, Genetics Department, “Luiz de Queiroz” College of Agriculture, Piracicaba, SP, Brazil

Jelonek et al. 2011; Benavides et al. 2005; Nogueirol et al. 2016). The root system is the main way for Cd^{2+} ions uptake by plants from the soil, where they are quickly translocated by the xylem to the shoot (Lux et al. 2011). In this context, roots play a significant role in the active uptake of metallic ions and the Cd effects on plant growth may be variable among species (Wei et al. 2012), ecotypes (Li et al. 2009) or cultivars (Kubo et al. 2011). Some plants have the ability to tolerate and accumulate high concentrations of Cd in the shoots, being designated as hyperaccumulators (Milner and Kochian 2008). Nonetheless, in the large majority of plant species Cd accumulates mainly in the root system with only limited amounts being translocated to the upper parts of the plants (Pereira et al. 2002; Vitória et al. 2001).

According to Dalcorso et al. (2010) there are different signaling pathways for heavy metal stress responses. Cd can induce oxidative damage in plants by increasing the production of reactive oxygen species (ROS), which are extremely cytotoxic to all organisms (Gratão et al. 2005; Soares et al. 2016) and whose yield should be minimized (Perez-Chaca et al. 2014; Xu et al. 2010). ROS can cause protein oxidation, DNA damage and lipid peroxidation, being the latter considered the most drastic effect of heavy metals to the plant cells (Gratão et al. 2005). Plant cells have a number of protective mechanisms to eliminate or to reduce ROS (Cuyper et al. 2016; Wang et al. 2015), one of them involving the activation of the enzymatic antioxidant system, which operates by the sequential and simultaneous actions of enzymes including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.11.1.9), among many others (Alves et al. 2017; Gratão et al. 2015). Another potential mechanism includes non-enzymatic compounds, such as low molecular weight thiols that show high affinity for toxic metals (Yadav 2010). One of the most active low molecular weight biological thiol is glutathione (GSH), which is a vital part of the antioxidant machinery of plant cells against oxidative stress playing multifaceted roles by contributing to the cellular defense (Anjum et al. 2012; Mendoza-Cozatl et al. 2011). For instance, Tausz et al. (2004) have suggested the use of GSH as a marker for oxidative stress in plants. An optimum level of GSH pool, required for normal metabolic activities, is maintained in plants cells growing under non-stressful conditions (Cuyper et al. 2001; Jozefczak et al. 2012; Kandziora-Ciupa et al. 2016). Additionally, the concentration and redox state of GSH are important in transducing oxidative signals originating from ROS in order to activate the antioxidative response (Ball et al. 2004). Cd shows a high affinity for thiols, especially GSH, which is a primary target for free Cd-ions (Cuyper et al. 2010), therefore under Cd-stress with depletion of GSH pool, some enzymes from the GSH metabolic pathway such as glutathione

S-transferase (GST, EC 2.5.1.18) operate in plant tolerance responses to oxidative stress induced by heavy metals (Ghelfi et al. 2011; Gill and Tuteja 2010).

Most of the information available about Cd responses in plants was originated from studies with model plants such as the Cd-hyperaccumulator *Thlaspi caerulescens* (Lombi et al. 2002) and Cd-tolerant plants such as *Arabidopsis halleri* (Weber et al. 2006; Zhao et al. 2006), whereas there is less information about Cd accumulation and its effects on cell physiology, especially for economically important commercial crops. A large amount of tomato (*Solanum lycopersicum*) is produced in greenhouse, frequently using reutilization of water, therefore subjected to an increased risk of heavy metal contamination by the use of Cd-contaminated water (López-Millán et al. 2009). Studies about the effects caused by Cd-exposure on plant development and antioxidant system of cultivated plants have been carried out based on the evaluation after a certain period of exposure (Alves et al. 2017; Gratão et al. 2008a, 2009, 2015; Monteiro et al. 2011; Nogueirol et al. 2016; Pompeu et al. 2017). Despite numerous studies on Cd uptake and accumulation in crops, not much information is available when the temporal dynamic of Cd uptake and responses to Cd-induced stress focused on the root system is concerned. Therefore, the study of temporal dynamic of antioxidant response plus root development may help to highlight potential tolerance mechanisms, which in turn involves necessary time for plants to recognize the stress and unleash machinery to cope with Cd-induced stress in tissues as well as the signaling to shoots. In this work, we investigated the temporal dynamic of Cd uptake and response in roots to Cd-induced stress of two tomato genotypes previously characterized as having different degrees of Cd-tolerance. For this, plant growth evaluations were performed paralleled with the characterization of the responses of the enzymatic antioxidant and non-enzymatic defense systems in tomato roots treated with Cd.

Material and methods

Plant material, growth conditions and treatments

Two tomato genotypes with differential tolerance degrees to Cd-induced stress were selected based on previous studies (Piotto et al. 2014): *Solanum lycopersicum* cv. Calabash Rouge (CR) and *Solanum lycopersicum* cv. Pusa Ruby (PR), characterized as sensitive and tolerant to Cd, respectively. Seeds were sterilized with 5% hydrogen peroxide (H_2O_2) for 15 min and washed with double distilled-deionized water, and then, seeds were sown in trays with vermiculite substrate and maintained with Peters commercial solution (JR Peters Inc. 20-20-20 at 1 g L^{-1}) applied

during 20 d in order to maintain seedling development. The seedlings were cultivated in a glasshouse under the following conditions: an average mean temperature of 27 °C, 11.5 h/13 h (winter/summer) photoperiod, and 250–350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR irradiance (natural radiation reduced with a reflecting mesh; Aluminete-Polysack Industrias Ltda, Leme, Brazil). Twenty-day-old seedlings were transferred from the trays to a hydroponic system (10-L trays) containing a 10% ionic strength solution (Hoagland and Arnon 1950), pH 6.5 checked daily, and the total volume maintained at a constant level by using distilled-deionized water. Plants were maintained in hydroponics for 24 h as an adaptation period in order to mitigate stress generated by seedling transplanting, and also to increase nutrient concentration from 10 to 50% ionic strength. This procedure (gradual increase of salt concentration) was carried out in order to diminish plant stress due to an increased content of salts in solution. For stress induction a 35 μM cadmium chloride (CdCl_2) treatment was used and plants were sampled in three time periods predetermined in preliminary experiments: 1, 4 and 7 d after Cd addition, designated as T1, T2 and T3, respectively. Moreover, T0 samples (day zero treatment) were collected representing the initial condition before applying the stress. The concentration chosen resembles the limiting amount of Cd (3.6 mg of Cd per kg of dried weight) allowed in agricultural soils in São Paulo State, Brazil (CETESB 2016). Four biological replicates composed of three plants each were collected. The control was cultivated in trays without Cd application (–Cd) whilst Cd treatments were represented as +Cd. Plant roots and shoots for enzymatic assays and stress indicators were collected and immediately frozen in liquid nitrogen and subsequently stored at -80°C for further analysis. Additionally, four biological replicates composed of four plants each were collected for dry matter weight measurements.

Dry matter weight and Cd content

Roots and shoots were dried at 55°C for 5 d to reach a constant weight followed by precision weighing. Plant tissues were ground to pass a 200-mesh sieve and Cd concentration was determined by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA) after nitro-perchloric digestion (Bataglia et al. 1983).

Quantification of stress indicators

The measurements of the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were performed in the same extraction, where powdered tissues (200 mg of fresh weight) were homogenized in a pestle and mortar with 20% (w/v) of insoluble polyvinylpyrrolidone (PVPP) and 2 mL of 0.1% trichloroacetic acid (TCA). For MDA the homogenate

was centrifuged at $10,000 \times g$ for 10 min and 250 μL of the supernatant was added to 1 mL of 0.5% 2-thiobarbituric acid (TBA) and 20% TCA solution and incubated in a water bath at 95°C for 30 min. The concentration of MDA was calculated from the absorbance at 535 nm by using the absorbance coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$, following a correction for unspecific turbidity determined by the absorbance at 600 nm (Heath and Packer 1968).

H_2O_2 was measured spectrophotometrically after reaction with KI as described by Alexieva et al. (2001). The reaction mixture consisted of 200 μL of 0.1% TCA leaf and root extracts supernatants, 200 μL of 100 mM potassium phosphate buffer and 800 μL of 1 M KI. The reaction was developed for 1 h in darkness at room temperature and absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2 .

Enzyme extraction and activity determination

Tomato roots (1 g of fresh weight) were homogenized in a pestle and mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylene diaminetetracetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble PVPP (Azevedo et al. 1998). The homogenate was centrifuged at $10,000 \times g$ for 30 min and the supernatant was stored in separate aliquots at -80°C for further enzymatic assays. The total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard (Bio-Rad Protein Dye Reagent).

SOD assay

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was carried out as reported by Constantine and Ries (1977) by the inhibition of 4-nitroblue tetrazolium (NBT) chloride photoreduction. The assay was carried out using the following reaction mixture: 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM riboflavine, 50 mM methionine, 1 mM NBT, 10 mM EDTA and 15 μL of plant extract. The reaction mixture was illuminated ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C for 10 min and the absorbance measured at 560 nm. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. SOD activity of the extracts was expressed as U mg^{-1} protein.

CAT assay

Catalase (CAT, E.C. 1.11.1.6) activity was determined as described by Kraus et al. (1995) with modifications as described by Azevedo et al. (1998). CAT was assayed spectrophotometrically at 25°C in a 10 mL reaction mixture

containing 100 mM potassium phosphate buffer (pH 7.5) and 25 μL H_2O_2 (30% solution). The reaction was initiated by the addition of 25 μL plant extract and determined by monitoring H_2O_2 degradation at 240 nm over 1 min. CAT activity was calculated using an extinction coefficient for H_2O_2 of $39.4\text{ mM}^{-1}\text{ cm}^{-1}$ and results was expressed as $\mu\text{mol}^{-1}\text{ min}^{-1}\text{ mg}$ protein.

APX assay

Ascorbate peroxidase (APX, E.C. 1.11.1.11) activity was determined by the addition of 50 μL plant extract to 1 mL of a medium containing 80 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbate, 1 mM EDTA and 1 mM H_2O_2 (Nakano and Asada 1981). APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. APX activity was expressed as $\mu\text{mol}^{-1}\text{ min}^{-1}\text{ mg}$ protein.

GR assay

Glutathione reductase (GR, E.C. 1.11.1.9) activity was assayed at 30 °C in a mixture consisting of 1 mL 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM oxidized glutathione (GSSG) and 1 mM NADPH (Smith et al. 1988). The reaction was started by the addition of 30 μL of plant extract. The rate of reduction of GSSG was followed in a spectrophotometer by monitoring the increase in absorbance at 412 nm over a 1 min period. GR activity was expressed as $\mu\text{mol min}^{-1}\text{ mg}^{-1}$ protein.

GST assay

Glutathione S-transferase (GST, E.C. 2.5.1.18) was assayed by spectrophotometer using the method of Habig and Jakoby (1981) with some modifications (Ghelfi et al. 2011). The assay mixture contained 100 mM potassium phosphate buffer (pH 6.5), 100 mM reduced glutathione (GSH), 40 mM 1-chloro-2,4-dinitrobenzene (CDNB) prepared in 100% ethanol and 25 μL of plant extract, in 1 mL final volume. Reactions were initiated with addition of the CDNB substrate. The enzymatic formation of 2,4-dinitrophenyl-S-glutathione at 340 nm was monitored for 1 min. GST activity was calculated using an extinction coefficient of $9.6\text{ mM}^{-1}\text{ cm}^{-1}$ and the results were expressed as $\mu\text{mol}^{-1}\text{ min}^{-1}\text{ mg}$ protein.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out using 12% polyacrylamide gels, with a 4% stacking gel, as previously described (Laemmli 1970). Sodium dodecyl sulfate (SDS) was

omitted for non-denaturing gels. Electrophoresis was carried out at 4 °C at a constant current of 15 mA per gel during 3 h for gels stained for SOD activity, 22 h for CAT activity and 4 h for GR activity. Equal amounts of protein (30 μg) were loaded onto each gel lane. Densitometry was performed using ImageJ software, version 1.47 (National Institutes of Health, USA; <https://imagej.nih.gov/ij>) with relative percentage calculated based on the activity of the respective standard in each gel.

SOD activity staining

SOD activity staining was carried out as described by Beauchamp and Fridovich (1971) and optimized by Azevedo et al. (1998). After 3 h non-denaturing-PAGE separation, the gel was rinsed in distilled-deionized water and incubated in the dark, at room temperature, in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM riboflavin, 0.1 mM nitroblue tetrazolium, 1 mM EDTA, and 3% N,N,N,N-tetramethylethylenediamine (TEMED). After 30 min, the gels were rinsed with distilled water and then illuminated in water until the achromatic bands of SOD activity were visible on a purple-stained gel. SOD isoenzymes were distinguished and classified by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM H_2O_2 , according to Azevedo et al. (1998).

CAT activity staining

CAT activity staining in native PAGE gels was determined as described by Medici et al. (2004). The gels were incubated at room temperature in 0.003% H_2O_2 for 10 min and developed in a 1% (w/v) FeCl_3 and 1% $\text{K}_3\text{Fe}(\text{CN})_6$ (w/v) solution for 10 min. The relative percentage was calculated based on the activity of the standard in the gel (CAT from bovine liver, Sigma C-100 lot 20K7010) as described by Alcântara et al. (2015).

GR activity staining

GR activity staining in native PAGE gels was determined as described by Lee and Lee (2000). The gels were incubated in the dark for 30 min at room temperature in 50 mL of a reaction mixture containing 250 mM Tris buffer (pH 7.5), 3.4 mM GSSG, 0.5 mM NADPH, 50 mg of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 10 mg of 2,6-dichloro-N-(4-hydroxyphenyl)-1,4-benzo-quinoneimine sodium salt (DPIP). The relative percentage was calculated based on the activity of the standard in the gel (GR from yeast, Sigma G3664) as described by Alcântara et al. (2015).

Glutathione content

The reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were determined spectrophotometric according to Anderson (1985) with some modifications. Fresh tissues (100 mg) were homogenized in a pestle and mortar with 5% sulfosalicylic acid (1 mL). The homogenate was centrifuged at $10,000 \times g$ at 4°C during 20 min and 200 μL of the supernatant was added to 1.8 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 100 μL of 3 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). The mixture was kept in the dark for 5 min at room temperature and the absorbance was monitored at 412 nm. Next, the same cuvettes received 100 μL of 0.4 mM NADPH and 2 μL GR (205 U mg^{-1}) and the mixtures were kept in the dark during 20 min. At the end of this period the absorbance was measured at 412 nm. The GSH content was calculated from a standard curve constructed using concentrations of GSH over the 0–1 mM range.

Statistical analysis

All experiments were performed in completely random design. Data from figures represent established linear, quadratic or cubic regressions, showing the trends of the measured parameters during the experimental period in Cd-treated and untreated (control) plants, whereas the Time was considered as independent variable. The choice of the best model was based on Akaike's Information Criterion (AIC) (Akaike 1974). Data from tables represent means from four biological replicates ($n = 4$) \pm standard deviation (SD). Means corresponding to the tomato genotypes were compared to the respective control plants within each time period (T1, T2 or T3) by Student's *t*-test. Differences were considered significant when $p \leq 0.05$. All analyses

were performed using the R software version 2.15.1 (R 2015).

Results

Cadmium accumulation in tomato tissues and impact on plant growth

Tomato genotypes growing in the presence of $35 \mu\text{M}$ CdCl_2 exhibited a similar trend of Cd accumulation in tissues, mainly in the root system (Fig. 1a), with greater increases of this metal until T2 and following a more constant tendency of Cd increments until T3 in both genotypes. Regarding the shoot, the highest accumulation was observed at T2 for the tolerant genotype PR and at T3 for the sensitive genotype CR (Fig. 1b). Although the amounts of Cd in the roots have been similar in both genotypes over time, it is evident that after 7 d of Cd-exposure (T3), the CR genotype accumulated more Cd in the shoots than PR.

Cd exposure inhibited the growth of tomato plants with an overall reduction in the dry matter weight of plants during the time length of experiment (Fig. 2). Both genotypes presented a similar pattern of growth decrease caused by Cd exposure, with larger declines in the sensitive genotype. The root growth rate over time was similar for both genotypes in the absence of Cd (Fig. 2a). On the other hand, the roots exposed to Cd exhibited lower accumulation of dry matter due to the presence of the metal, with CR plants exhibiting lower growth rate than PR. In other words, the sensitive genotype had its development more affected by Cd than the tolerant genotype. Similarly to what was observed in the roots, shoot growth was also reduced in the presence of Cd (Fig. 2b). There was a small trend of increased dry mass accumulation in the PR genotype when compared to

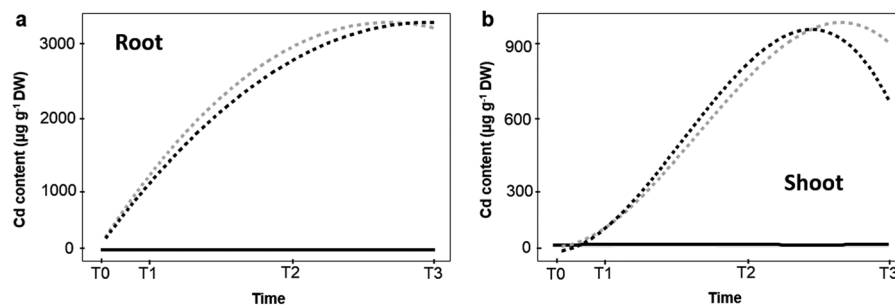


Fig. 1 Cd accumulation ($\mu\text{g g}^{-1}$ dry weight) in tissues of tomato genotypes. Mean values in roots (a) and shoots (b) are shown. Solid gray lines represent control condition of sensitive genotype Calabash Rouge in root ($R^2 = 0.30$, $p < 0.01$) and shoot ($R^2 = 0.60$, $p < 0.01$). Solid black lines represent control condition of tolerant genotype Pusa Ruby in root ($R^2 = 0.24$, $p = 0.11$) and shoot ($R^2 = 0.30$, $p = 0.01$). Dotted gray lines represent stress condition of sensitive genotype in

root ($R^2 = 0.95$, $p < 0.01$) and shoot ($R^2 = 0.93$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype in root ($R^2 = 0.96$, $p < 0.01$) and shoot ($R^2 = 0.95$, $p < 0.01$). Time indicate 1, 4 and 7 d growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. DW dry weight

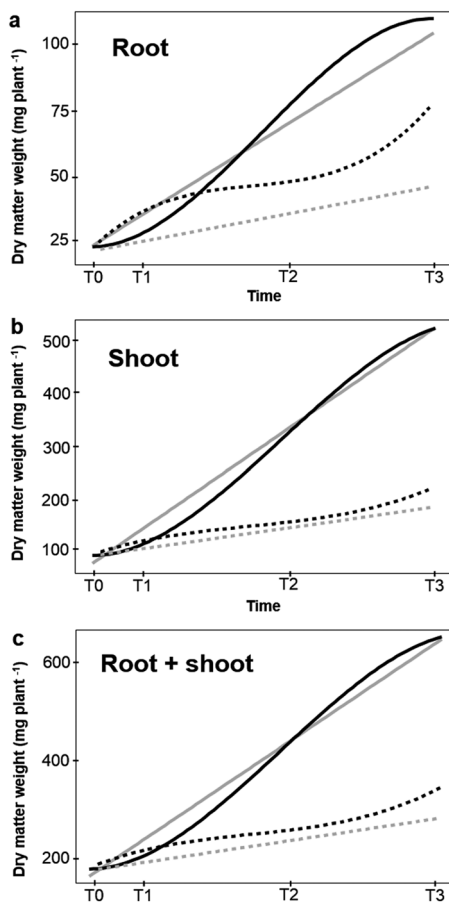


Fig. 2 Impact of Cd on plant growth expressed as dry matter weight (mg plant^{-1}) in tomato tissues. Mean values in roots (a), shoots (b) and root + shoot (c) are represented. Solid gray lines represent control condition of sensitive genotype Calabash Rouge in root ($R^2 = 0.91$, $p < 0.01$), shoot ($R^2 = 0.94$, $p < 0.01$) and root + shoot ($R^2 = 0.94$, $p < 0.01$). Solid black lines represent control condition of tolerant genotype Pusa Ruby in root ($R^2 = 0.96$, $p = 0.11$), shoot ($R^2 = 0.96$, $p = 0.01$) and root + shoot ($R^2 = 0.97$, $p < 0.01$). Dotted gray lines represent stress condition of sensitive genotype in root ($R^2 = 0.51$, $p < 0.01$), shoot ($R^2 = 0.74$, $p < 0.01$) and root + shoot ($R^2 = 0.72$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype in root ($R^2 = 0.94$, $p < 0.01$), shoot ($R^2 = 0.97$, $p < 0.01$) and root + shoot ($R^2 = 0.97$, $p < 0.01$). Time indicate 1, 4 and 7 d growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress

CR in stressed plants (Fig. 2c), although the overall trend of shoot development under Cd-induced stress has been practically the same (Fig. 2b).

Lipid peroxidation level

The increase in MDA content was gradual in plants exposed to Cd and first detected in roots of the tolerant genotype PR (Fig. 3a). MDA accumulation in PR roots treated with Cd was always greater compared to CR and followed by a linear accumulation until the end of the experiment period. The highest MDA contents were detected in shoots after

Cd-exposure, notably at T2 in CR genotype (Fig. 3b). Both genotypes showed a similar trend for MDA accumulation when exposed to Cd, increased after T1 and decreased after T2 in shoots, being more pronounced in the sensitive genotype (CR).

H_2O_2 content presented a trend of gradual increases for both genotypes and tissues (Fig. 3c, d), with major contents detected in roots of both genotypes between T2 and T3 of Cd exposure (Fig. 3c). The sensitive genotype presented higher contents of H_2O_2 mainly after T2 in both tissues. When shoots are concerned, higher H_2O_2 contents were detected between T2 and T3, following a decrease tendency at the last time (Fig. 3d). Roots and shoots growing without Cd presented similar patterns of H_2O_2 accumulation.

Antioxidant responses to Cd-induced stress

A trend of reduction in SOD activity in root tissue was observed in both genotypes when exposed to Cd, mainly in the tolerant one (Fig. 4a). Similar results for the tolerant genotype were observed for CAT activity (Fig. 4b), whilst the sensitive genotype exhibited no alteration in the enzyme activity pattern. APX activity was increased over time in Cd exposed roots of both genotypes with similar trends of increase found in both genotypes, with higher activity in tolerant roots (Fig. 4c). An early response of APX induced by Cd was detected at T1 in both genotypes, reaching a peak at T2 followed by a downward trend after 7 days of exposure to the metal (T3). GR activity was shown to be gradually increased during the time of exposure to Cd, with a similar trend exhibited by APX activity (Fig. 4d). GR was induced by Cd reaching the maximum activity at T2 for both genotypes showing at least 58% increase when compared to the controls. At T3, GR activity in roots of both genotypes remained higher than in the controls, suggesting enzyme activation induced by Cd. As well as GR, GST activity was stimulated by Cd earlier in CR roots (T1), whose high activity was maintained in both genotypes (Fig. 4e), followed by a downward trend, exhibiting lower values between T2 and T3 for both genotypes.

The activity of antioxidant enzymes of tomato roots grown over 7 days in the presence of Cd were also estimated based on the separation of isoenzymes by non-denaturing PAGE (Fig. 5). The different bands of activity were identified using isoform selective staining (data not shown) (Vitória et al. 2001). Five SOD isoenzymes were detected: Mn/SOD (SOD I and II), Fe/SOD (SOD III) and Cu-Zn/SOD (SOD IV and V). SOD I and SOD IV bands were apparently not altered during Cd stress in both genotypes, while SOD II appeared to be induced by Cd in CR roots (Fig. 5). SOD III became more evident in the presence of Cd at T2, but it is hardly noticeable in control roots of both genotypes. SOD V was barely noticed in PR roots,

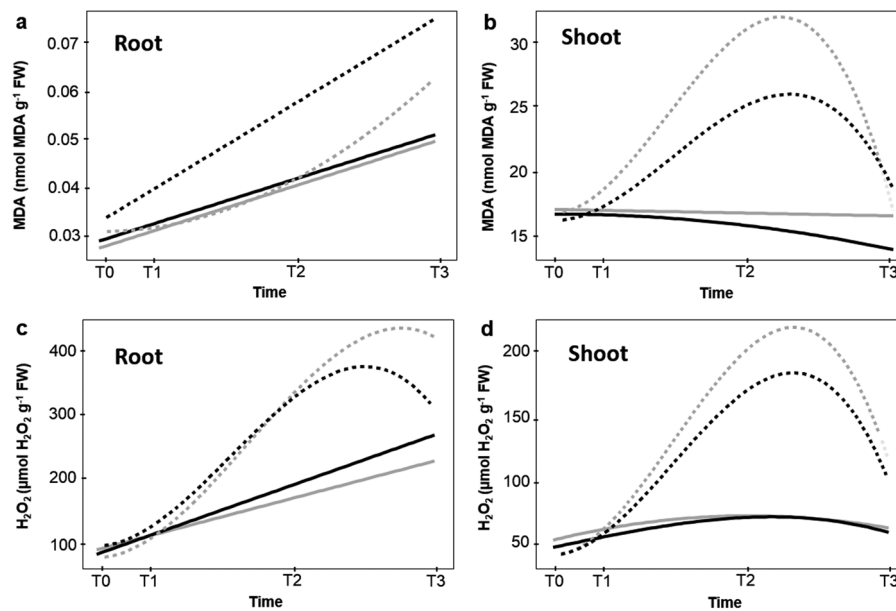


Fig. 3 Oxidative damage induced by Cd-exposure in tomato tissues expressed as MDA content (nmol g^{-1} fresh weight) and H_2O_2 ($\mu\text{mol g}^{-1}$ fresh weight). Mean values of MDA in roots (**a**) and shoots (**b**), H_2O_2 in roots (**c**) and shoots (**d**) are shown. Solid gray lines represent control condition of sensitive genotype Calabash Rouge in root (**a** $R^2 = 0.64$, $p < 0.01$; **c** $R^2 = 0.65$, $p < 0.01$) and shoot (**b** $R^2 = 0.68$, $p < 0.01$; **d** $R^2 = 0.54$, $p < 0.01$). Solid black lines represent control condition of tolerant genotype Pusa Ruby in root (**a** $R^2 = 0.75$, $p < 0.01$; **c** $R^2 = 0.55$, $p < 0.01$) and shoot (**b** $R^2 = 0.52$, $p < 0.01$; **d** $R^2 = 0.54$,

$p < 0.01$). Dotted gray lines represent stress condition of sensitive genotype in root (**a** $R^2 = 0.64$, $p < 0.01$; **c** $R^2 = 0.84$, $p < 0.01$) and shoot (**b** $R^2 = 0.73$, $p < 0.01$; **d** $R^2 = 0.57$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype in root (**a** $R^2 = 0.52$, $p < 0.01$; **c** $R^2 = 0.53$, $p < 0.01$) and shoot (**b** $R^2 = 0.80$, $p < 0.01$; **d** $R^2 = 0.73$, $p < 0.01$). Time indicate 1, 4 and 7 d growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. FW fresh weight

whilst in CR it was unaltered after Cd exposure. When CAT activity is concerned, only one CAT isoenzyme was found (Fig. 5) and its activity was apparently not altered by Cd treatment over time in both genotypes (Fig. 5; Table 1). Three GR isoenzymes were detected in both genotypes with a gradual increase on band intensity of GR III after Cd exposure (Fig. 5). In general, the pattern found in the gels is in accordance with the results measured spectrophotometrically (Fig. 4).

Under Cd exposure a reduction trend of GSH and increased of GSSG were detected in the sensitive roots at T1 and T2, whereas the opposite were observed in the tolerant genotype (Table 2). However, the GSSG/GSH ratio for the tolerant genotype was lower under stress (T1), indicating higher initial GSH content in the roots. At T3 increases of GSH content followed by decreases of GSSG were observed for both genotypes (Table 2).

Discussion

PR and CR tomato genotypes were previously characterized as Cd-tolerant and Cd-sensitive genotypes, respectively (Piotto et al. 2014). In the present work, using the same genotypes, we aimed at focusing on temporal dynamic response of the antioxidant system to Cd-induced stress in

order to shed some light on possible mechanisms involved in Cd tolerance in these tomato genotypes. It is well known that Cd accumulation varies considerably among plant species and also among cultivars within species (Gratão et al. 2008a). Accumulation of Cd in tomato tissues notably occurred within the first 24 h of Cd exposure (T1) and continued over time, mainly in roots (Fig. 1a). Similar results have been reported for the large majority of plant species studied (Gallego et al. 2012) including maize (Anjum et al. 2015), soybean (Perez-Chaca et al. 2014), radish (Vitória et al. 2001), *Crotalaria juncea* (Pereira et al. 2002) and tomato (Gratão et al. 2008a; López-Millán et al. 2009). The root system is the first tissue of contact with the metal and through which it enters the plant system, suggesting that roots may be the first place where defense mechanisms against stress occurs, for instance by heavy metal immobilization in the vacuoles (Mendoza-Cozatl et al. 2011). Vitória et al. (2001) working with radish suggested that Cd-binding proteins would be produced in the roots to control Cd eventual stress. The increase of Cd allocation in roots after 7 d of Cd-exposure (T3) explains a possible mechanism used by the plants to protect the shoots against excess of Cd, since it can affect photosynthesis (Bashri and Prasad 2015; Khan et al. 2007). When Cd accumulation in tomato is concerned, the majority of the studies have confirmed that Cd accumulates in larger

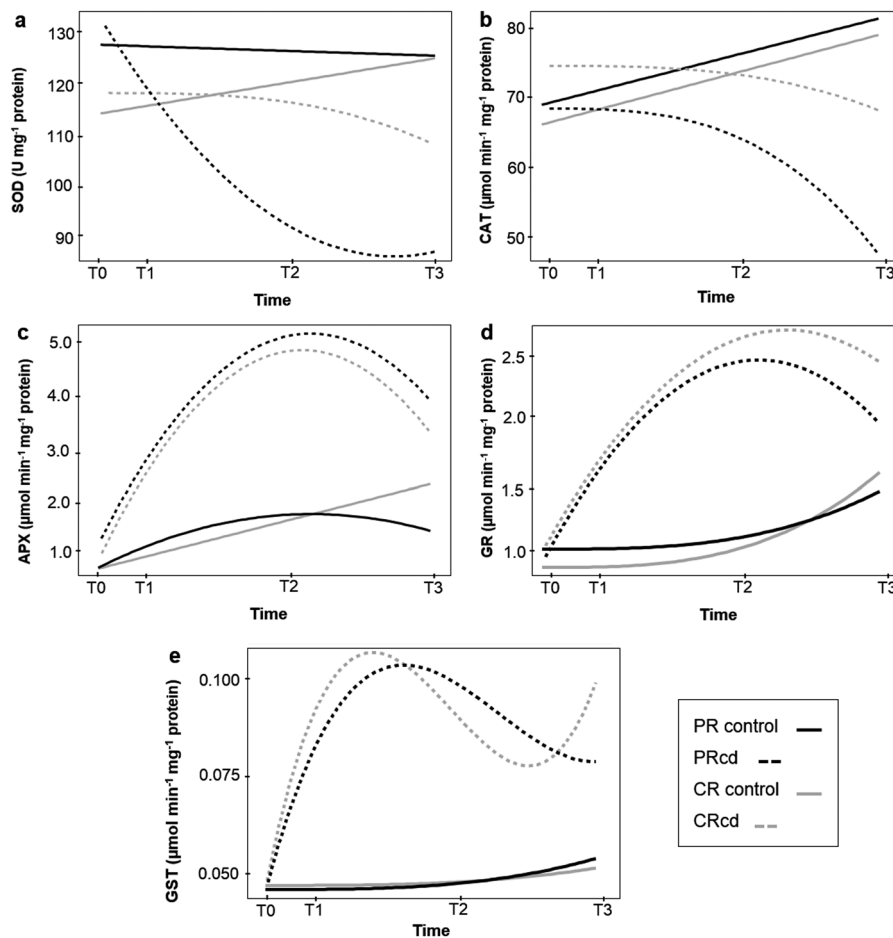


Fig. 4 Specific activity of the antioxidant enzymes in tomato roots under Cd-induced stress. **a** superoxide dismutase (SOD), **b** catalase (CAT), **c** ascorbate peroxidase (APX), **d** glutathione reductase (GR), **e** glutathione S-transferase (GST). Dotted and solid lines represent treatment with Cd and control conditions respectively. Solid gray lines represent control condition of sensitive genotype Calabash Rouge (**a** $R^2 = 0.04$, $p = 0.04$; **b** $R^2 = 0.23$, $p = 0.45$; **c** $R^2 = 0.47$, $p < 0.01$; **d** $R^2 = 0.75$, $p < 0.01$; **e** $R^2 = 0.65$, $p = 0.55$). Solid black lines represent control condition of tolerant genotype Pusa Ruby (**a** $R^2 = 0.04$, $p = 0.46$; **b** $R^2 = 0.11$, $p = 0.16$; **c** $R^2 = 0.27$, $p < 0.01$; **d** $R^2 = 0.22$, p

< 0.01 ; **e** $R^2 = 0.36$, $p < 0.01$). Dotted gray lines represent stress condition of sensitive genotype (**a** $R^2 = 0.02$, $p = 0.42$; **b** $R^2 = 0.04$, $p = 0.47$; **c** $R^2 = 0.56$, $p < 0.01$; **d** $R^2 = 0.71$, $p < 0.01$; **e** $R^2 = 0.78$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype (**a** $R^2 = 0.68$, $p < 0.01$; **b** $R^2 = 0.45$, $p < 0.01$; **c** $R^2 = 0.46$, $p = 0.02$; **d** $R^2 = 0.78$, $p < 0.01$; **e** $R^2 = 0.71$, $p < 0.01$). Time indicate 1, 4 and 7 d growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. FW fresh weight

amounts in the roots (Hediji et al. 2010), which can be related to the avoidance of translocation, but one report has also shown that Cd could also accumulate in the fruits, which is dependent upon the Cd exposure system used (Gratão et al. 2008a). According to Lux et al. (2011) tolerant plants develop extracellular barriers and can store Cd-chelates in the vacuoles, which might be occurring in tomato roots. In our study, we observed at T1 a rapid metal increase in roots tissue, whilst shoots exhibited less Cd accumulation in both tomato genotypes. Accordingly, high proportion of Cd in the soluble fraction as well as in its chemical form could limit root-to-shoot translocation of Cd, resulting in lower Cd levels in the shoots (Su et al. 2014). Gratão et al. (2015) reported low Cd accumulation in shoots of the Micro-Tom (MT) tomato cultivar and suggested it

may be driven by transpiration. In another study with *Vaccinium myrtillus* L. (bilberry) and *Vaccinium vitis-idaea* L. (lingonberry) growing in contaminated soils, Cd accumulated especially in the shoots, which appears to be a strategy in evergreen plant species (Kandziora-Ciupa et al. 2017). Apparently effective root-to-shoot translocation of metals indicates a tolerance strategy that may be employed by the PR genotype, once this genotype exhibited lower Cd accumulation in shoots than the CR genotype (Fig. 1b), confirming that tolerant genotypes have lower Cd content in the shoots than sensitive genotypes (Hossain et al. 2012).

Growth inhibition was observed in tomato genotypes (Fig. 2). The sensitive genotype exhibited a remarkable growth decrease during the stress period, suggesting that the existence of intracellular detoxification or defense strategies

Fig. 5 Activity staining of CAT, SOD and GR following non-denaturing polyacrylamide gel electrophoresis of tomato roots. The sensitive genotype Calabash Rouge (CR) and the tolerant Pusa Ruby (PR) after 1, 4 and 7 d growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. The treatments are with Cd (+Cd) and without Cd (-Cd). I, II, III, IV and V represents SOD (I-V) and GR isoforms (I-III) in the respective gels

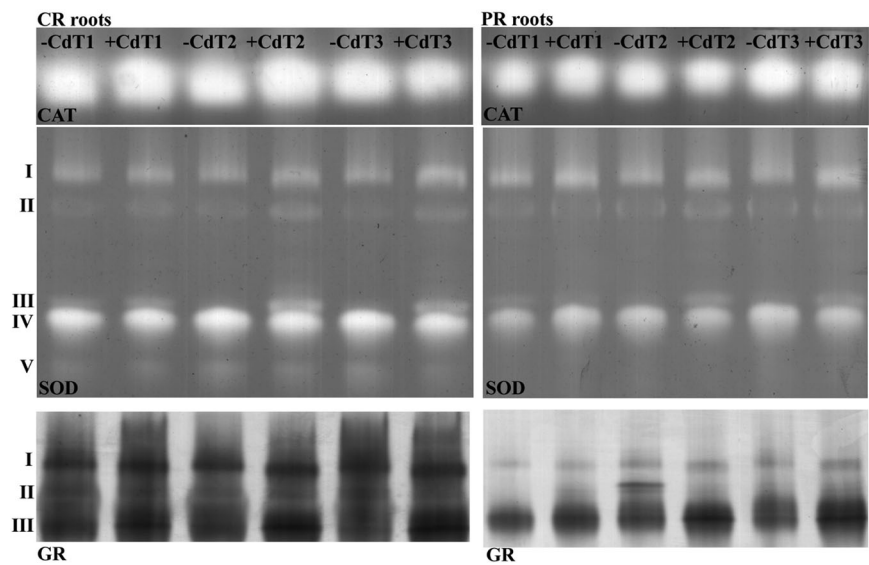


Table 1 Densitometric analysis of catalase (CAT) in non-denaturing PAGE. Activities are presented as a percent (%) relative to a bovine liver CAT activity standard not shown in Fig. 5

Genotype	Time	Treatment	CAT
Calabash Rouge (sensitive)	T1	-Cd	70.0
		+Cd	75.1
	T2	-Cd	67.4
		+Cd	74.9
	T3	-Cd	71.6
		+Cd	65.7
Pusa Ruby (tolerant)	T1	-Cd	69.1
		+Cd	75.4
	T2	-Cd	72.8
		+Cd	71.9
	T3	-Cd	84.7
		+Cd	77.1

may be different from those acting in the tolerant genotype. These findings are in accordance with Lu et al. (2013) who reported that root and shoot biomasses of peanut cultivars were mainly affected after Cd exposure. In this work, similar growth rates for both genotypes were observed in the absence of Cd, whilst plants exposed to Cd showed impaired growth induced by metal exposure. At T3, reduction of total biomass (Fig. 2c) by 65 and 54% for CR and PR respectively, were observed suggesting that PR plants may have better mechanisms to cope with Cd-induced stress, controlling its growth and development even under stress.

The effect of Cd toxicity can be indirectly determined by evaluating MDA accumulation, a product of lipid peroxidation in plant tissues. Our results confirmed that Cd may increase the rate of lipid peroxidation and membrane

Table 2 Glutathione forms in plant roots (%)

Genotype	Time	Treatment	GSH	GSSG	GSSG/GSH
Calabash Rouge (sensitive)	T1	-Cd	71.22	28.78	0.40
		+Cd	69.03*	30.97*	0.45
	T2	-Cd	52.52	47.48	0.90
		+Cd	45.39*	54.61*	1.20
	T3	-Cd	46.72	53.28	1.14
		+Cd	83.77*	16.23*	0.19
Pusa Ruby (tolerant)	T1	-Cd	69.13	30.87	0.45
		+Cd	76.65*	23.35*	0.30
	T2	-Cd	42.36	57.64	1.36
		+Cd	43.59	56.41	1.29
	T3	-Cd	53.87	46.13	0.86
		+Cd	87.02*	12.98*	0.15

GSH (reduced glutathione), GSSG (oxidized glutathione) and GSSG/GSH ratios. The sensitive genotype Calabash Rouge (CR) and the tolerant Pusa Ruby (PR) after 1, 4 and 7 d growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. The treatments are with Cd (+Cd) and without Cd (-Cd)

Values represent the means of 4 replicates ($n = 4$)

* $p \leq 0.05$ by Student's *t*-test

damage since MDA levels were altered in the Cd-stressed plants of both genotypes and tissues analyzed (Fig. 3a, b). Similar increases of MDA have been observed after Cd-exposure in maize (Anjum et al. 2015), wheat (Khan et al. 2007), rice (Sebastian and Prasad 2014) and tomato (Gratão et al. 2008a). ROS generation may affect the auxin physiology that consequently leads to a reduction of plant biomass (Bashri and Prasad 2015). Increased lipid peroxidation induced by Cd was observed in the tomato MT *sitiens* mutant (ABA-deficient mutant), which was more pronounced in the roots of the mutant than in the wild-type

(MT), suggesting that ABA deficiency amplifies the stress response to Cd (Pompeu et al. 2017). Analyzing shoots at T2, we observed a peak of stress considering the changes in MDA content for both genotypes (Fig. 3b), with increases by 56 and 42% for CR and PR, respectively. This may be an indication of different levels of shoot capability to cope with Cd-induced stress leading to the increased MDA contents observed, particularly in CR, which faced a higher level of oxidative stress and in accordance with the activities of antioxidant enzymes, that showed different mechanisms triggered in Cd-sensitive and Cd-tolerant genotypes (Fig. 4).

An analysis over time suggests that H_2O_2 production was increased in plants treated with Cd, especially in roots, for both tomato genotypes (Fig. 3a). A major increase of H_2O_2 content was detected in roots of the CR genotype at T3, which appears to be related to enzyme activities observed in the root tissue (Fig. 4) since this genotype at T3 showed higher SOD activity compared with PR genotype resulting in higher H_2O_2 content (Fig. 3c). Comparing shoots in the presence of Cd we observed a decrease of H_2O_2 content at T3 in both genotypes, which may be a strategy to protect photosynthetic function from Cd-induced oxidative stress (Dixit et al. 2001), by activation of antioxidant enzymes and other protective mechanisms. Stress indicators (MDA and H_2O_2) exhibited higher values for the CR genotype under Al-stress cultivated in sandy and clay soils (Nogueirol et al. 2015). In accordance with these authors, once H_2O_2 plays a role in cellular expansion facilitating the expanding abilities of the cell wall, maybe growth could restrict the greater impact of H_2O_2 , as shown by the more pronounced growth of CR plants. Higher H_2O_2 contents in CR roots and shoots at T2 and T3 under Cd-induced stress were observed, but in contrast, lower growth rate of CR genotype at T3 was observed with root growth decreased by 2-fold when compared with the PR genotype (Fig. 2). This evidence reinforces the higher tolerance degree exhibited by the PR genotype as originally observed and reported by Piotto et al. (2014) when testing tomato cultivars in the presence of Cd.

To gain a better insight into the possible role of oxidative stress induced by Cd we focused on some antioxidant enzymes such as SOD, CAT, APX, GR and GST. SOD specific activity decreased in the presence of Cd over time only in tolerant roots (Fig. 4a) and accordingly, lower H_2O_2 concentrations were found in Cd-treated plants in the PR genotype (Fig. 3b). Previous reports showed variable responses of SOD activity in plants exposed to Cd (Gratão et al. 2008a, 2015; Semane et al. 2007; Tamas et al. 2016). Although no change in SOD total enzymatic activity was observed, PAGE analysis showed changes in some SOD isoenzymes which were time-, treatment- and genotype-dependent (Fig. 5). Five SOD isoenzymes (SOD I, II, III, IV and V) were observed in PAGE of tomato roots. SOD III (classified as Fe/SOD) was activated in the presence of Cd

when compared to the control at T3, but was barely noticeable in control roots for both genotypes. This result is very similar to that reported by Nogueirol et al. (2016) who observed enhancement in SOD III activity in CR roots following increase in Cd concentration. Jozefczak et al. (2014) observed Fe/SOD activity in roots of *A. thaliana* only after 3 d of Cd exposure, whereas Cu-Zn/SOD (SOD IV and V) activity was reduced in a time- and dose-dependent manner in roots starting after 24 h exposure. No visible changes in the activity of SOD I and SOD II isoenzymes were detected in tomato roots (Fig. 5).

The accumulation of H_2O_2 , a strong oxidant, is prevented in plant cells either by CAT and a number of peroxidases or by the ascorbate-glutathione cycle where APX reduces it to H_2O (Gratão et al. 2005). Gratão et al. (2008b) studying antioxidant responses of tobacco to Cd-stress reported increased CAT activity in the second half of the experimental period, which was accompanied by a shift in the activity of peroxidases that operated during the Cd stress applied to the cells. In this work, CAT specific activity decreased at T3 in PR roots (Fig. 4b), suggesting that the detoxification of H_2O_2 was not prevented by CAT activity in both genotypes. Increased activity of APX in stressed roots was noticed mainly in the tolerant genotype at all time-points of the experiment (Fig. 4c), suggesting that it is actively working to break the H_2O_2 produced in excess. The later response of CAT to Cd in PR roots appeared to have been compensated by the increased activity of APX, which in our study appears to be a main player in the defense response to the oxidative stress in tomato roots cells.

GR was activated in Cd-treated tomato roots of both genotypes studied, with higher proportions at T2 (Fig. 4d). Cd stress induced the enzymatic activity in both genotypes earlier (T1), while at T2 the maximum activity of GR was observed in both genotypes, remaining higher in stressed plants until the end of the experiment (Fig. 4d). In gel analysis (Fig. 5), the GR III isoenzyme exhibited similar behavior of the spectrophotometer analysis, suggesting that this specific isoenzyme might have been the main contributor to the overall change in GR activity. We hypothesize that perhaps sensitive roots were responding to Cd-induced stress earlier than the tolerant roots and so the GR III was activated first in this sensitive genotype (CR). Increasing GR activity has strengthened the defense mechanism in wheat roots, protecting them against Cd-induced oxidative stress (Yannarelli et al. 2007), which agrees with the data obtained for the tomato genotypes. According to Ding et al. (2009) GR plays an important role in the regeneration of GSH prior to incorporation into phytochelatin (PCs), protecting against oxidative stress and maintaining the ascorbate pool as well. Our work suggests that GR and APX together may be key players against oxidative stress in tomato genotypes exposed to Cd.

The detoxification activity of GST has been reported mainly related to oxidative stress, xenobiotics and heavy metals (Kumar et al. 2013). GST activity was stimulated by Cd-stress particularly after T2 for both genotypes (Fig. 4e), exhibiting the same activity pattern of GR, possibly indicating that there was a balance in GSH utilization by these antioxidant enzymes. It also indicates that Cd caused a rapid induction of both GR and GST (as compared to SOD and CAT) with GST probably operating in the detoxification process using GSH as a substrate. In the present study, the activities of APX, GR and GST were increased in the roots of both genotypes treated with Cd. GR and GST showed distinct behavior than APX in tomato genotypes, and whilst GR and GST were activated in both genotypes at T1, APX was activated firstly in the tolerant genotype. These results appear to indicate that Cd-induced stress increased the activity of some antioxidant enzymes, which may represent a secondary defensive mechanism against oxidative stress that are not as direct as primary defensive responses such as PCs production and vacuolar compartmentalization (Sanità di Toppi and Gabbriellini 1999). GSH-conjugate formation is mediated by GST, an enzyme that contains active sites that bind GSH to cytotoxic compounds such as MDA (Blair 2010). This may explain why at T1 differences were not observed in MDA content of CR roots (Fig. 3a), which might be quickly conjugated by GST (Fig. 4e).

At the earlier stage of Cd exposure (T1) GSH increased while GSSG decreased in the tolerant roots, which are in accordance to the GR and GST results (Fig. 4d, e). Since GR activity is responsible for maintaining GSH pools in the cells (Jozefczak et al. 2012) converting GSSG into GSH, we believe that GR activity was the main responsible for the reduction of GSSG/GSH ratio at T3 in both genotypes after Cd exposure. We observed an initial enhancement of the GSH pool in PR roots, which might allow the conjugation of oxidative stress metabolites. In accordance with Gill et al. (2013) GSH is an abundant compound in plants that directly scavenges ROS and may protect enzyme thiol groups. Under heavy metal stress it is well established in the literature that regulation of GSH biosynthesis undergoes with significant changes, such as increase of GSH oxidation and PCs production, resulting in a depletion of cellular GSH levels and consequently, the feedback inhibition is released (Jozefczak et al. 2012). Cd exposure increased GSSG/GSH ratio in *A. thaliana*, which was accompanied by enhanced GR and APX activities (Semane et al. 2007). At T2 a decrease in GSH content was observed only in roots of the sensitive genotype (Table 2), which might be due to its utilization as substrate for GST enzyme. Another hypothesis suggests that a redox signal generated by a decreased GSH pool in the cells in combination with an altered GSSG/GSH ratio may increase the antioxidant capacity (Jozefczak et al. 2014). Thus, for tomato roots we can suggest that the

decreased GSH content could be due to increased antioxidant activity in tomato roots, especially of APX, GR and GST.

Summarizing, tomato genotypes differing in Cd-tolerance grown in media containing 35 μM CdCl₂ exhibited metal accumulation in roots and shoots tissues in both genotypes, but with increased Cd accumulation over time mainly in roots. Since PR (tolerant genotype) exhibited higher growth rate as compared to sensitive genotype, PR appears to have a more efficient mechanism to cope with Cd-induced stress. Regarding to antioxidant machinery, Cd induced decreased GSH content in roots whilst increased the activities of APX, GR and GST, which together may be the main players against oxidative stress in Pusa Ruby genotype exposed to Cd. Therefore, different tomato genotypes can employ distinct strategies to cope with Cd-induced challenges to the plant development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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