

Potential of hydrogen (pH) differentially modulates cadmium stress response in abscisic acid-deficient *sitiens* tomato mutant

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ABSTRACT: Uptake of nutrients and cadmium (Cd) are dependent upon many factors, including plant species, ions concentration and pH. Tolerance to Cd-induced oxidative stress can be modulated by phytohormones such as abscisic acid (ABA), which induce the production of reactive oxygen species, activating proteins and enzymes involved in stress response and possibly stress tolerance. The present study aimed to evaluate the biochemical variations induced by Cd in ABA-deficient *sitiens* tomato mutant (*sit*) and its wild-type counterpart, Micro-Tom (MT), grown at different pH conditions. The plants were cultivated in nutrient solution (pH 5, 6 and 7; 20-days) and were then further grown over a 48-h period in 0 or 50 μM CdCl_2 at pH 6. Before Cd addition, the concentrations of nitrogen, sulfur, copper, iron and zinc were determined and variations in nutrients

concentrations were observed. After Cd addition, *sit* roots grown at pH 5 and 7 did not exhibit differences in ascorbate peroxidase (APX) activity in 0 or 50 μM CdCl_2 , and *sit* root grown at pH 6 exhibited lower glutathione reductase (GR) activity in the presence of Cd. Moreover, *sit* shoot grown at pH 5 showed decreased activities of superoxide dismutase (SOD), Mn-SOD II and Cu/Zn-SOD V, in 0 and 50 μM CdCl_2 . The results indicated that pH modulates the plant nutrition in a complex way and may involve multiple ABA signaling pathways. Likewise, ABA status seems to be related with the Cd-translocation within the plant, suggesting that Cd, ABA and pH responses cannot be evaluated as isolated systems.

Key words: antioxidant enzymes, Micro-Tom, mineral nutrition, oxidative stress, *Solanum lycopersicum*.

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INTRODUCTION

Cadmium (Cd) is a heavy metal present in the environment in trace concentrations. It can be introduced into the food-chain through anthropogenic activities such as wastewater usage for irrigation, sewage sludge application and fertilizers applications (Gratão et al. 2005; Cuyppers et al. 2010; Borges et al. 2018). The high mobility of Cd in the soil-plant system can affect biochemical mechanisms, causing plasma membrane rupture and serious disturbances in physiological processes such as photosynthesis, respiration, plant-water relations, uptake and distribution of macro- and micronutrients, plant growth reduction and/or even cell death (Gallego et al. 2012; Moradi and Ehsanzadeh 2015; Alves et al. 2017). Cd can also increase the production of reactive oxygen species (ROS), resulting in extracellular superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) accumulation. Excess of ROS is dangerous mainly due to reactions with lipids, proteins and nucleic acids, resulting in membrane damage, enzyme inactivation and DNA breakage or mutations, which can result in severe effect on cell viability (Carvalho et al. 2018a; 2018b; Gaion et al. 2018). The ability of a plant to improve its ROS scavenging capacity may be a key element in stress tolerance. The enzymatic mechanisms include the action of superoxide dismutase (SOD, EC 1.15.1.1) that catalyzes $O_2^{\cdot-}$ into H_2O_2 , which is subsequently detoxified into water (H_2O) by catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) (Alves et al. 2016; Noctor et al. 2018).

Cd-induced oxidative stress can be modulated by phytohormones such as auxin (Alves et al. 2017), gibberellin (Cai et al. 2015), cytokinin (Bezrukova et al. 2016), ethylene (Gratão et al. 2015), jasmonic acid (Yan et al. 2015) and abscisic acid (Pompeu et al. 2017). Abscisic acid (ABA) regulates a wide range of processes in plants, including seed development and dormancy, transition from the vegetative to the reproductive phase, stomatal closure, and responses to a variety of environmental stresses (Galpaz et al. 2008; Harrison et al. 2011; Akpinar et al. 2012; Harrison 2012). Under stressful conditions such as drought, low temperature, high salinity or heavy metals presence, large amounts of ABA are synthesized to increase the plant stress tolerance (Cutler et al. 2010; Stroiński et al. 2013; Wang et al. 2016; Dar et al. 2017). However, despite the information available on the rapid response and prominent role in plant adaptation to abiotic stresses, only a limited number of articles are

available in the literature on the metabolic mechanisms through which ABA acts inducing Cd tolerance. ABA signaling has been considered as an important factor in Cd-signal transduction. A rapidly increase on ABA's level under stressful conditions triggers response mechanisms via several ABA-responsive genes, ultimately leading to physiological changes such as the rapid closing of stomata (Asgher et al. 2015; Bücken-Neto et al. 2017).

In addition to phytohormones, an optimum potential of hydrogen (pH) range allows increased availability of essential nutrients for the growth and development of plants, resulting in increased synthesis of phytochelatin and glutathione (GSH), increased tolerance and protection of metabolism and physiological processes, as well as alleviation of the toxicity induced by Cd (Nazar et al. 2012; Asgher et al. 2015; Wang et al. 2016). In general, plants present a better development in a pH ranging from 5 to 6.5. These values enable all elements to be available to plants. When pH is higher than 6.5, certain nutrients such as phosphorous, calcium, iron and manganese can precipitate. On the other hand, a pH lower than 4 can affect the cellular membranes integrity (Braccini et al. 1999; Furlani et al. 1999). It is important to bear in mind that cellular pH is sensitive to the external pH, hence changes in the root medium pH affect plant water uptake. In the same way, ABA also affects root water flow, an effect that is pH dependent (Kamaluddin and Zwiazek 2004). Moreover, ABA is also involved in the root-to-shoot communication process. It is also associated with stomatal conductance (Saradadevi et al. 2017), can cause a suppression of transpirational flow and consequent restriction of root-to-shoot translocation of ions (Bücken-Neto et al. 2017).

The Micro-Tom (MT) tomato cultivar has natural genetic variations and a number of hormonal mutants produced with deficient enzyme activities, such as the ABA-deficient mutant *sitiens* (*sit*), which is deficient in the functional enzyme activity at the final step leading to ABA biosynthesis (Harrison et al. 2011). Hormonal mutants are excellent plant genotypes to investigate the role of hormones on stress response. Thus, the objective of this study was to characterize the biochemical variations induced by Cd in the *sit* mutant and its wild-type counterpart MT, grown at different pH conditions, with regard to the modulation of the antioxidant enzymatic responses and potential tolerance induced by the interaction between pH and ABA.

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MATERIALS AND METHODS

Plant material and treatments

In a greenhouse with an average mean temperature of 28 °C, relative humidity of 75%, and a 13 h photoperiod (springer/summer), seeds of tomato lines, (*Solanum lycopersicum* L.) cv Micro-Tom (MT) and ABA-deficient *sitiens* mutant (*sit*), were sown in trays containing expanded vermiculite supplemented with Hoagland and Arnon (1950) nutrient solution (20% ionic strength) at pH 6. After a period of 20 days post germination, the plants were transferred to 12 L trays containing Hoagland's nutrient solution (10%) at pH 5, 6 and 7. On the fourth day, the solution was changed to 50% ionic strength. During 20 days, 3 sets of 24 MT plants and 24 *sit* plants were grown in nutrient solution at pH 5, 6 and 7. All treatments were performed in two replicates with a total of 48 plants evaluated by treatment (pH). At the end of this period, the nutrient solution was replaced again but keeping the concentration of 50% ionic strength and supplemented with 0 and 50 µM CdCl₂, and maintained at pH 6. The pH 6 was used in studies on Cd toxicity in tomato in hydroponic systems (Piotto et al. 2014; 2018). The Cd-concentration chosen was based on previous results with MT (Gratão et al. 2012, 2015; Pompeu et al. 2017). Forty-eight hours after Cd addition, roots and shoots were collected and stored for further biochemical analysis. All analyses were performed in three replicates.

Dry mass production, mineral elements and Cd concentration in plant tissues

Before the addition of CdCl₂, a quarter of the MT and *sit* plants grown over the 20 days period in nutrient solution with pH 5, 6 and 7 were separated into roots and shoots, dried in a forced circulation oven at 65 °C for 72 h, and subsequently ground in a mortar with a pestle. Nitrogen (N) concentration in the samples was determined by sulfuric acid digestion, followed by distillation and titration; sulfur (S) by turbidimetrically; and copper (Cu), iron (Fe) and zinc (Zn) by atomic absorption spectrophotometry. For Cd determination, roots and shoots were dried in a forced circulation oven at 65 °C for 72 h, subsequently ground in a mortar with a pestle, and the concentration determined by ICP-OES (Malavolta et al. 1997).

Lipid peroxidation and H₂O₂ concentrations

Lipid peroxidation was measured by estimating the concentration of thiobarbituric acid reactive substances (TBARS). Plant tissue was ground with 20% (w/v) polyvinylpyrrolidone (PVPP) and 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was added to a solution of 0.5% (w/v) 2-thiobarbituric acid (TBA) and 20% (w/v) TCA. The mixture was incubated in a dry bath at 95 °C for 30 min. The homogenate was then maintained in an ice bath for 10 min and centrifuged at 10,000 g for 10 min. The concentration of malondialdehyde (MDA) equivalents was determined spectrophotometrically between 535 and 600 nm. Data were calculated using an extinction coefficient of $1.55 \times 10^{-5} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Gomes Junior et al. 2006). The H₂O₂ concentration was determined according to Monteiro et al. (2012). Plant samples were homogenized in 0.1% (w/v) TCA and the homogenate was centrifuged at 10,000 g for 20 min at 4 °C. After centrifugation, 100 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide (KI) were added. The reaction mixture was incubated on ice for 1 h, the absorbance was read at 390 nm and the H₂O₂ concentration was determined using known concentrations of H₂O₂ as standard (Monteiro et al. 2012).

Enzyme extraction and protein determination

The plant material was homogenized (2:1 volume/fresh weight) in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 4% (w/v) insoluble PVPP. The homogenate was centrifuged at 10,000 g for 30 min and the supernatant was stored in separate aliquots at -80 °C (Gratão et al. 2015). The concentration of total proteins was determined by the Bradford method (1976) using the Bovine Serum Albumin (BSA) as standard.

Superoxide dismutase assay (SOD, EC 1.15.1.1)

SOD activity was determined through native PAGE in 12% polyacrylamide gels as described by Vitoria et al. (2001). After non-denaturing-PAGE separation, the gels were rinsed in distilled water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM riboflavin, 1 mM EDTA, 0.1 mM nitroblue →

tetrazolium and 0.3% *N,N,N',N'*-tetramethylethylenediamine. The gels were rinsed with distilled-deionized 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 5 mM H₂O₂ or 2 mM potassium cyanide.

Catalase assay (CAT, EC 1.11.1.6)

CAT activity was assayed at 25 °C in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5) and 25 µL H₂O₂ (30% solution). The reaction was initiated by the addition of 25 µL of protein extract and the activity determined by following the decomposition of H₂O₂ as changes in absorbance at 240 nm over 1 min (Alves et al. 2017). CAT activity was expressed in micromole per minute per milligram protein.

Glutathione reductase assay (GR, EC 1.6.4.2)

GR activity was measured using a spectrophotometer (412 nm) at 30 °C in a mixture consisting of 1 mL of 100 mM potassium phosphate buffer (pH 7.5), 500 µL of 3 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 100 µL of 2 mM NADPH, 100 µL of 20 mM oxidized glutathione (GSSG) and 50 µL of protein extract. GR activity was estimated by reduction of GSSG and was expressed in micromole per minute per milligram protein (Gratão et al. 2015).

Ascorbate peroxidase assay (APX, EC 1.11.1.11)

APX activity determination was measured spectrophotometrically in a reaction consisting of 40 µL protein extract to 1 mL of an assay mixture containing 50 mM ascorbate, 0.1 mM EDTA and 0.1 mM H₂O₂. APX activity was measured by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C over 1 min (Alves et al. 2017). APX activity was expressed in micromole per minute per milligram protein.

Statistical analysis

The exploratory and descriptive analyses of the data were performed by the evaluation of the residues studied by

the Shapiro-Wilks test, by graphical analysis of the studied residues, by boxplot and quantile-quantile graphs, by the evaluation of the relationship between mean and variance of the data and by the Box-Cox transformation. Thus, the logarithmic transformations for root and shoot dry mass, shoot MDA concentration, root and shoot CAT activity, and shoot GR activity were determined. Reverse transformation was applied for shoot Fe concentration, and quadratic transformation for root GR activity. The other variables met the assumptions of the analysis of variance. The transformations were considered for the analysis of the data, and the graphs presented the untransformed values. Afterwards, the data were submitted to analysis of variance (ANOVA) in factorial scheme 3 × 2 × 2 (pH × Genotype × Cd concentration) and Tukey's test ($p \leq 0.05$). Analyses were performed using software R and SAS. The results were expressed as the mean and standard error of the mean (\pm SEM).

RESULTS AND DISCUSSION

The uptake of a chemical element in hydroponic cultivation systems is proportional to its concentration near the roots, being influenced by factors such as salinity, oxygenation, temperature, photoperiod and pH. The importance of the pH in the nutrient solution is to maintain these elements available to the plants, whilst the increased of acidity or alkalinity may result in damage to the cell membrane permeability, allowing the release of ions. In fact, we clearly observed differences in nutrient uptake in MT tomato plants grown in nutrient solution with pH 5, 6 and 7 (Table 1). However, the mechanisms by which the pH modulates the nutrition of plants varies and may also depend on hormones, such as ABA. Among the genotypes, under the same pH conditions, *sit* shoots exhibited the highest concentrations of N and Zn (pH 6 and 7), and Cu (pH 5, 6 and 7), whereas *sit* roots showed the highest concentrations of Zn (pH 7) (Table 1). Under different pH conditions, MT roots exhibited higher concentrations of S (pH 5 and 7), Fe (pH 6 and 7) and Zn (pH 6), whereas MT shoots showed the highest concentrations of N (pH 5), S (pH 6 and 7), Fe (pH 6) and Zn (pH 7) (Table 1). Differences in nutrient uptake were also observed in *sit* plants. Roots and shoots of *sit* plants exhibited higher concentrations of Zn (pH 6), S and Fe (pH 7), whilst *sit* shoots grown at pH 5 showed higher Fe concentration (Table 1). The different

Table 1. Tissue concentrations of nitrogen (N), sulfur (S), copper (Cu), iron (Fe) and zinc (Zn) in plants of Micro-Tom (MT) and *sitiens* (*sit*) grown over a 20 days period in nutrient solution with different pH conditions (5, 6 and 7).

Genotype	Treatment	N (g·kg ⁻¹)	S (g·kg ⁻¹)	Cu (mg·kg ⁻¹)	Fe (mg·kg ⁻¹)	Zn (mg·kg ⁻¹)
Root						
MT	pH 5	32.74 ± 2.34 ^A	*3.12 ± 0.08 ^A	*38.20 ± 0.72 ^A	2340.67 ± 119.72 ^B	92.77 ± 1.07 ^B
	pH 6	31.59 ± 2.37 ^A	2.91 ± 0.06 ^B	*38.73 ± 0.59 ^A	*2620.00 ± 40.63 ^A	*104.67 ± 1.33 ^A
	pH 7	32.50 ± 2.20 ^A	2.97 ± 0.07 ^{AB}	37.50 ± 0.50 ^A	2735.67 ± 44.84 ^A	90.68 ± 0.68 ^C
<i>sit</i>	pH 5	32.37 ± 2.41 ^A	2.77 ± 0.11 ^B	37.10 ± 0.82 ^A	2249.00 ± 53.11 ^B	91.35 ± 0.42 ^B
	pH 6	29.80 ± 1.98 ^A	2.94 ± 0.10 ^{AB}	36.61 ± 0.60 ^A	2380.00 ± 23.26 ^B	101.98 ± 0.13 ^A
	pH 7	31.17 ± 0.57 ^A	3.05 ± 0.09 ^A	37.83 ± 0.38 ^A	2843.00 ± 32.05 ^A	*93.03 ± 0.35 ^B
Shoot						
MT	pH 5	*45.90 ± 0.66 ^a	3.45 ± 0.28 ^b	11.37 ± 0.60 ^a	*161.00 ± 3.61 ^b	24.83 ± 0.47 ^b
	pH 6	41.40 ± 0.74 ^b	3.65 ± 0.18 ^{ab}	12.30 ± 0.41 ^a	*202.17 ± 1.26 ^a	24.52 ± 0.23 ^b
	pH 7	42.02 ± 0.37 ^b	3.74 ± 0.07 ^a	12.22 ± 0.25 ^a	*155.09 ± 0.86 ^c	26.46 ± 0.56 ^a
<i>sit</i>	pH 5	43.76 ± 0.86 ^b	3.25 ± 0.09 ^b	*13.23 ± 0.69 ^a	142.67 ± 3.06 ^a	24.80 ± 0.74 ^c
	pH 6	*43.84 ± 0.42 ^b	3.45 ± 0.21 ^{ab}	*13.45 ± 0.22 ^a	131.17 ± 2.04 ^b	*29.87 ± 0.32 ^a
	pH 7	*45.89 ± 0.72 ^a	3.68 ± 0.22 ^a	*13.54 ± 0.54 ^a	141.23 ± 1.47 ^a	*28.71 ± 0.29 ^b

*Significantly higher than those of the other genotype, under the same pH conditions. Uppercase letters indicates roots, lowercase indicates shoots, and compare means of different pH conditions, for the same genotype. Means with different letters differ from each other (Tukey, $p \leq 0.05$).

pH conditions tested did not interfere in the uptake of N and Cu by the roots of MT and *sit*. Such multifaceted responses triggered in *sit* ABA-deficient mutant in different pH suggest that the way how pH modulates plant nutrition is complex and may involve multiple ABA signaling pathways, once ABA is involved in the root-to-shoot communication process and the effect of ABA on water flow is pH dependent (Kamaluddin and Zwiazek 2004), which can restrict the root-to-shoot translocation of ions.

In a recent study, the *sit* tomato mutant exhibited altered biochemical and morpho-anatomical responses to Cd exposure (Pompeu et al. 2017). For example, *sit* plants submitted to 100 μM CdCl₂ at pH 6 for 96 h accumulated more Cd in the roots when compared to MT plants. However, an increase in vacuole number in MT roots was observed in the presence of Cd (Pompeu et al. 2017). Thus, the important role of ABA appears to be related to a reduction in Cd accumulation in tomato (Pompeu et al. 2017). In this work, we have been able to understand and discuss a bit more the interaction among pH, Cd and ABA. MT roots absorbed a higher Cd concentration (Table 2), while *sit* shoots also exhibited higher concentration of the metal when grown at pH 5 and 6 (Table 2). In other words, *sit* absorbs less Cd but the translocation of the metal to the shoots is higher. In the *sit* mutant this feature seems to be dependent upon root morpho-anatomy and ABA-deficiency. This mutant does not show a reduction in root cell size when

compared to the MT plants grown in 100 μM CdCl₂ (96 h, at pH 6), even with the higher accumulation of Cd (Pompeu et al. 2017). On the other hand, under this condition the mutant increases leaf cell size, especially in the palisade parenchyma (Pompeu et al. 2017). It is interesting to note that root morphology alterations occur naturally in the *sit* tomato mutant (Monteiro et al. 2012). In addition, ABA can induce stomatal closure (Buckley 2017; Bucker-Neto et al. 2017). Thus the *sit* mutant could remain with the stomata open, increasing the transpiration and consequently the transport and accumulation of Cd in the shoots.

Although Cd may also induce a decrease in growth, reduction of photosynthesis, enzymatic and metabolic alterations, changes in stomata and electron transport (Mondal et al. 2013), our results showed that plants of MT and *sit* exhibited increases in biomass production in the presence of Cd (Table 2). Such results have been reported for MT roots and shoots, and *sit* grown in 10 and 100 μM CdCl₂ at pH 6 for a 96 h-period (Pompeu et al. 2017). The concentration of the metal and the time of exposure appear to be related to the slight increase in plant growth, as reported for sugarcane seedlings (Fornazier et al. 2002) and coffee cell suspension cultures (Gomes Junior et al. 2006). This indicates a hormetic effect, which does not happen at much higher concentrations, as generally observed in studies with Cd. For instance, the MT tomato genotype, when cultivated over a 40-day period in 1 mM CdCl₂,

Table 2. Dry mass (g) and Cd concentration ($\text{mg}\cdot\text{g}^{-1}$) in roots and shoots of Micro-Tom (MT) and *sitiens* (*sit*) grown in nutrient solution at pH 5, 6 and 7, and after exposure to nutrient solution with Cd (0 and 50 μM CdCl_2 ; 48-h; pH 6).

Genotype	Treatment	Dry mass (g)		Cd concentration ($\text{mg}\cdot\text{g}^{-1}$)
		0 μM CdCl_2	50 μM CdCl_2	
Root				
MT	pH 5	*0.142±0.016 ^{Ba}	*0.210±0.018 ^{Aa}	*0.734±0.014 ^b
	pH 6	0.121±0.039 ^{Bb}	0.142±0.007 ^{Ab}	*0.718±0.016 ^b
	pH 7	*0.167±0.011 ^{Ba}	*0.186±0.009 ^{Aa}	*0.940±0.037 ^a
<i>sit</i>	pH 5	0.095±0.013 ^{Bb}	0.103±0.015 ^{Ab}	0.496±0.019 ^b
	pH 6	*0.144±0.044 ^{Ba}	*0.165±0.044 ^{Aa}	0.513±0.008 ^b
	pH 7	0.073±0.009 ^{Ba}	0.095±0.005 ^{Ab}	0.739±0.020 ^a
Shoot				
MT	pH 5	*0.683±0.040 ^{Bb}	*0.783±0.047 ^{Ab}	0.130±0.014 ^b
	pH 6	0.672±0.043 ^{Bb}	0.775±0.031 ^{Ab}	0.125±0.014 ^b
	pH 7	*0.770±0.044 ^{Ba}	*0.992±0.024 ^{Aa}	0.199±0.012 ^a
<i>sit</i>	pH 5	0.497±0.035 ^{Bb}	0.548±0.035 ^{Ab}	*0.214±0.008 ^a
	pH 6	0.632±0.064 ^{Ba}	0.700±0.072 ^{Aa}	*0.207±0.017 ^a
	pH 7	0.497±0.008 ^{Bb}	0.618±0.020 ^{Ab}	0.210±0.006 ^a

*Significantly higher than those of the other genotype, under the same pH conditions. Uppercase letters compare means of different Cd concentrations. Lowercase letters compare means of different pH conditions, for the same genotype. Means with different letters differ from each other (Tukey, $p \leq 0.05$).

exhibited a major growth reduction of all tissues analyzed (Gratão et al. 2012).

We have also observed that *sit* plants under Cd stress altered, dependent on pH, a range of biochemical parameters related to the oxidative stress system. In this study, Cd-treatment induced lipid peroxidation, measured as MDA concentration, in roots of MT and *sit* grown at pH 5 and 6 (Fig. 1a). On the other hand, based on the induction of lipid peroxidation by H_2O_2 , plant roots exhibited increased H_2O_2 concentration when grown at pH 7 (Fig. 1c), while in shoots of MT and *sit* the H_2O_2 accumulation occurred at pH 6 for both genotypes (Fig. 1d). These results add more information to help clarifying the interaction between ABA and H_2O_2 . But the oxidative stress based on these compounds is still complex due to the fact that ABA can induce the synthesis of H_2O_2 under stressful conditions, and can also stimulate the antioxidant defense system in plants, yielding more peroxidase activity to deal with the oxidative stress condition established (Choudhary et al. 2012; Choudhury et al. 2017). Moreover, we observed that Cd did not alter SOD activity in plants roots (Figs. 2a and 2b), indicating that the alteration in H_2O_2 concentration does not appear to be associated with the level of SOD enzyme activity.

Differently, in MT shoots of plants grown at pH 5 and 7 an increase of the Fe-SOD III activity in the presence of

Cd was observed (Fig. 2b), whereas *sit* shoot grown at pH 5 exhibited decreased activities of Mn-SOD II and Cu/Zn-SOD V isoenzymes in both 0 and 50 μM CdCl_2 (Fig. 2d). SOD activity under Cd-stress can increase, remain constant or decline, depending on plant species, tissue, stage of development, and time-length of exposure (Cuyper et al. 2010; Nazar et al. 2012; Khan et al. 2016). Moreover, other oxidases such as the glycolate oxidase may also increase the H_2O_2 concentration (Noctor et al. 2018). Therefore, these different responses suggest a correlation among genotype, Cd-induced stress and different pH conditions. In a similar manner, in response to lipid peroxidation induced by the increase of the H_2O_2 concentration under Cd-stress, the different pH conditions tested also interfered with the enzymatic activities (Fig. 3). Although Cd induced the increase of the H_2O_2 concentration in *sit* roots grown at pH 7 (Fig. 1c), roots grown at pH 5, 6 and 7 did not show changes in CAT activities in the presence of the metal (Fig. 3a). On the other hand, *sit* roots grown at pH 5 and 7 did not exhibit differences in APX activity in both presence and absence of Cd (Fig. 3e). However, *sit* root grown at pH 6 exhibited lower GR activity in Cd presence, when compared to the other pH tested (Fig. 3c).

Compared to MT, *sit* exhibited altered MDA and H_2O_2 concentrations, SOD, CAT, GR and APX activities, revealing multifaceted responses when grown at pH 5, 6 and 7. Certainly

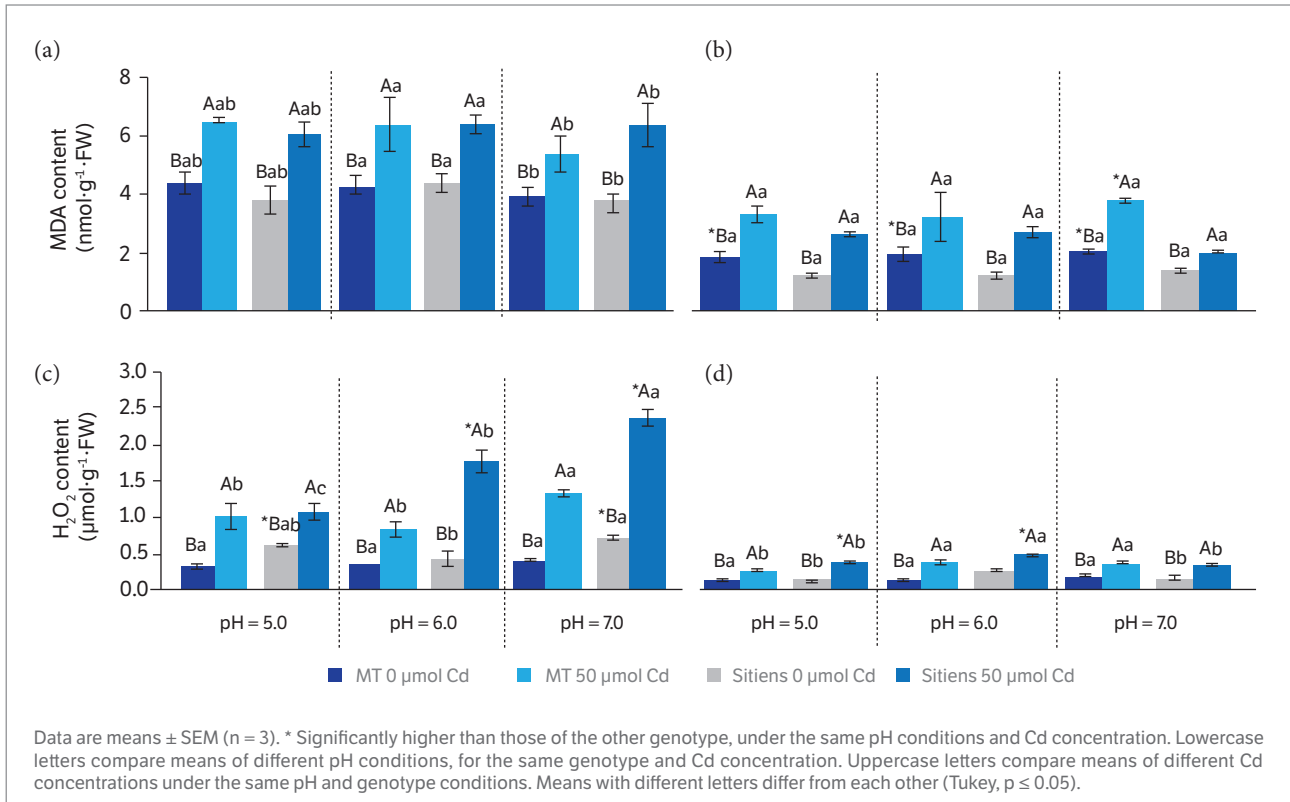


Figure 1. Lipid peroxidation measured as malondialdehyde (MDA) concentration (nmol·g⁻¹ fresh weight) and hydrogen peroxide (H₂O₂) concentration (μmol·g⁻¹ fresh weight) in roots (a and c) and shoots (b and d) of Micro-Tom (MT) and *sitiens* (*sit*) plants grown in nutrient solution with pH 5, 6 and 7, and after exposure to nutrient solution with Cd (0 and 50 μM CdCl₂; 48-h period; pH 6).

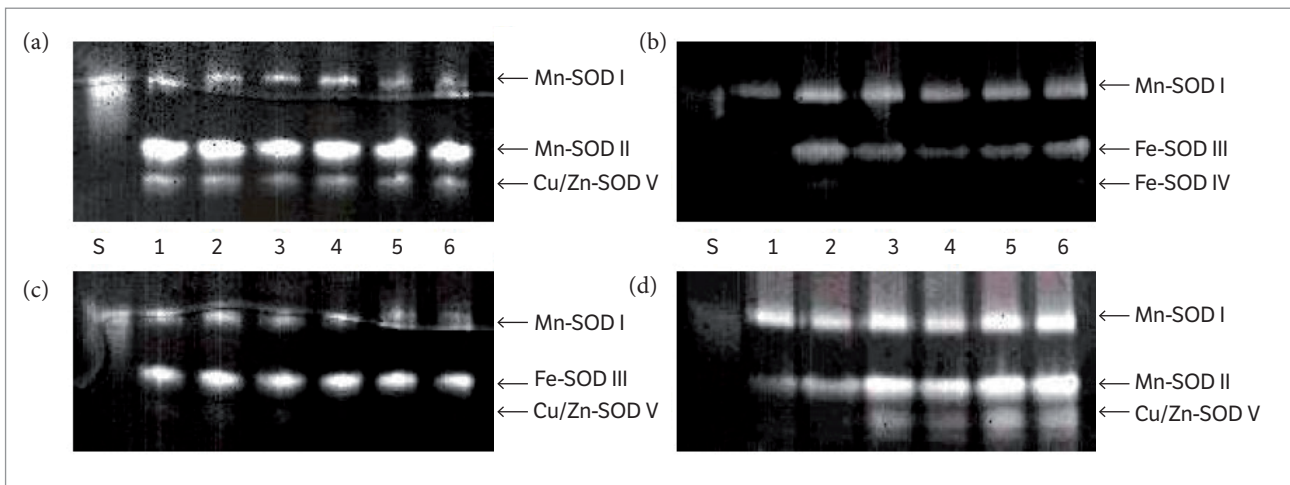


Figure 2. Superoxide dismutase (SOD) activity staining following non-denaturing polyacrylamide gel electrophoresis of roots and shoots extracts isolated from Micro-Tom (MT) and *sitiens* (*sit*) plants grown in nutrient solution with different pH (5, 6 and 7), and after exposure to nutrient solution with Cd (0 and 50 μM CdCl₂; 48-h period; pH 6). The lanes listed in (a) MT root, (b) MT shoot, (c) *sit* root and (d) *sit* shoot are: S = bovine SOD standard; 1 = 0 μM Cd, pH 5; 2 = 50 μM Cd, pH 5; 3 = 0 μM Cd, pH 6; 4 = 50 μM Cd, pH 6; 5 = 0 μM Cd, pH 7; 6 = 50 μM Cd, pH 7.

this is regulated in *sit* plants by multiple altered oxidative stress signals, which can be modulated by ABA. This modulation is very complex because the changes in *sit* at different pH conditions go beyond the ABA signaling dependent on pH,

since *sit* plants exhibit natural adverse water relations and altered growth, such as increased transpiration, and thus reduced leaf expansion (Mäkelä et al. 2003). Although this can explain, at least in part, the antioxidant enzymes alterations

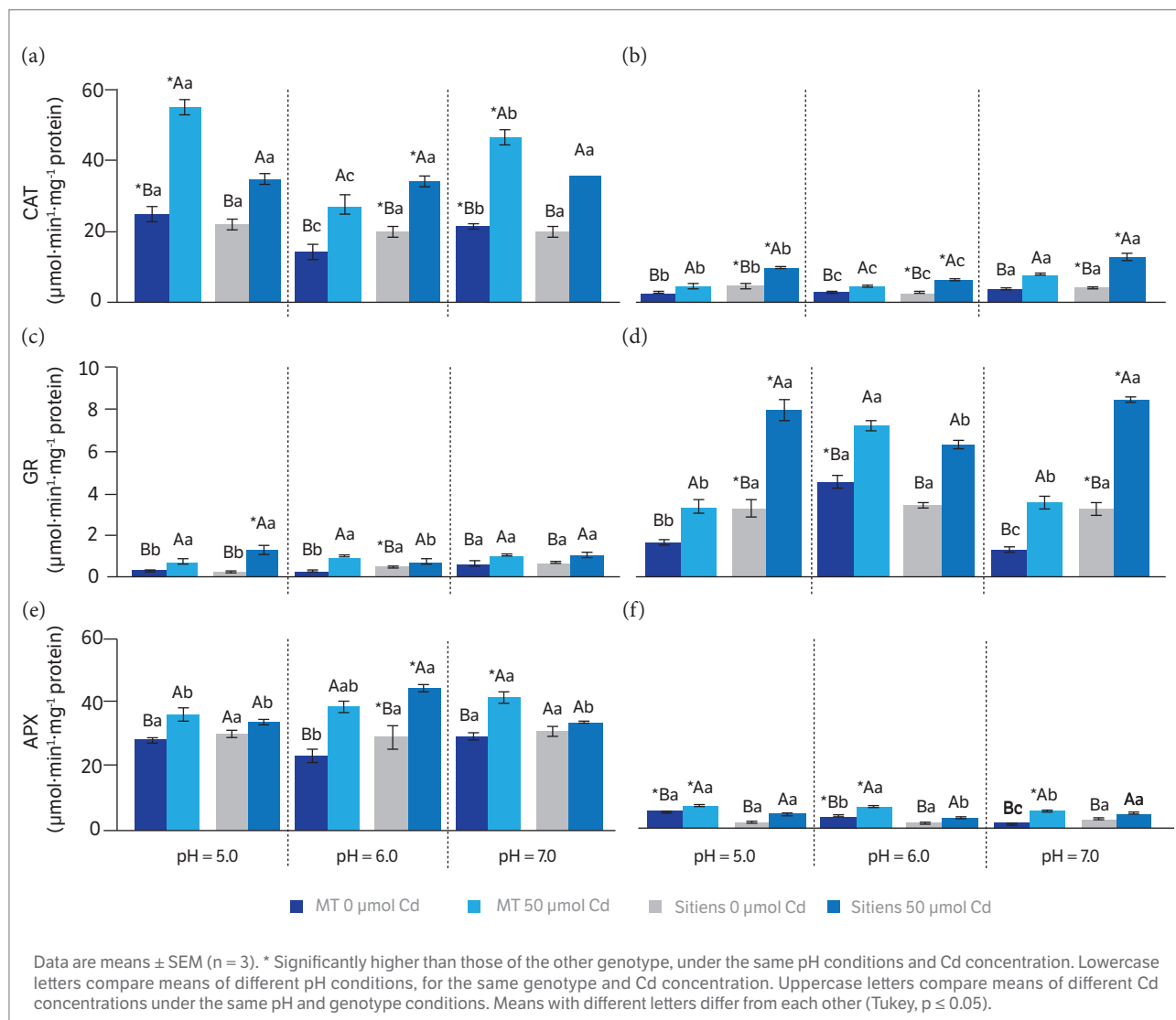


Figure 3. Total activities ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\cdot\text{protein}$) of Catalase (CAT), Glutathione reductase (GR) and Ascorbate peroxidase (APX) in roots (a, c and e) and shoots (b, d and F) of Micro-Tom (MT) and *sitiens* (*sit*) plants grown in nutrient solution with different pH (5, 6 and 7), and after exposure to nutrient solution with Cd (0 and 50 μM CdCl_2 ; 48-h; pH 6).

observed in response to the Cd treatments (Fig. 3), by using grafting experiments it was shown that this issue may avoid confounding effects of water and ABA status (Holbrook et al. 2002). When control tomato scions were grafted onto both *sit* and control rootstocks and then exposed to water stress, the mutant root system accumulated more biomass than the control root system (Holbrook et al. 2002), suggesting that ABA deficient roots had a greater sink strength independent of the shoot water status, providing some support for another role for ABA during growth, including under another abiotic stress condition. Thus, this indicates that the *sit* mutant can more convincingly provide hormonal information from adverse plant growth conditions.

CONCLUSION

Varying pH conditions, deficiency in ABA production and Cd presence resulted in several responses of the plant defense system. Herein, our data indicated that the antioxidant responses of *sit* plants to Cd stress were ABA-dependent and modulated by pH conditions. Although such interactions are yet not fully understood, plants responses to Cd, ABA and pH should be taken into account from an integrative perspective. Such complex interactions may clearly influence *sit* plants growth. Therefore, ongoing research using reciprocal grafting between *sit* and MT are being carried out in order to further elucidate these interactions. The recent reports by Gratão

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et al. (2015) and Alves et al. (2017) using Cd treatments in grafted tomato have indicated the complex cross talk and stress signaling among plant organs and hormones. ABA is already well known as a stress hormone and its mode of action is not completely established.

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Pompeu G. B., Carvalho R. F., Gratão P. L. and Azevedo R. A.; Writing, Pompeu G. B., Ambrosano G. B., Vilhena M. B., Carvalho R. F., Gratão P. L., Andrino F. G., Lira S. P. and Azevedo R. A.

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