



# Citrus rootstocks regulate the nutritional status and antioxidant system of trees under copper stress



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## ABSTRACT

Copper (Cu) deficiency and toxicity cause stresses in citrus orchards and limited information is available about which rootstocks and associated mechanisms would enhance plant resistance to such nutritional disorders. Therefore, this study evaluated the nutritional status and antioxidant system responses of citrus grafted onto selected rootstocks differing in horticultural performance [Swingle citrumelo (SW) or Rangpur lime (RL)], grown in nutrient solution with varying concentrations of Cu (0.015, 0.60 or 24.0  $\mu\text{M}$ ). The experiment was carried out in a greenhouse using young sweet orange trees. Once taken up, Cu mostly accumulated in roots (75% of total plant Cu content). Trees grafted onto RL were more responsive to enzyme activities related to oxidative stress and to nitrogen metabolism in leaves when grown in the presence of either the lowest or the highest Cu concentrations used. Those grown in 24.0  $\mu\text{M}$  Cu displayed decreased overall nutrient uptake and accumulation, with the exception of iron, which was predominantly found in roots. Cu/Zn superoxide dismutase activity in leaves was dependent upon signalling regulated by rootstocks, being lower in SW than in RL. Therefore, the use of appropriate rootstock varieties contributes to alleviate the effects of Cu stress on the metabolism and nutritional status of citrus plants.

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## 1. Introduction

Copper (Cu) participates in several physiological processes of higher plants, as a constituent of plastocyanin, a protein responsible for electron transport during photosynthesis and a cofactor of enzymes [e.g., Cu/Zn superoxide dismutase (SOD)] that scavenge reactive oxygen species (ROS) that cause oxidative stress (Ravet and Pilon, 2013; Yruela, 2009).

Cu-deficient plants exhibit a reduction in electron transport in photosystem I (PSI) due to decreases in plastocyanin synthesis (Ravet and Pilon, 2013) and the contents of chlorophylls and carotenoids (Yruela, 2009). Plants subjected to Cu excess suffer oxidative stress due to enhanced ROS production (via Haber-Weiss reaction; Ravet and Pilon, 2013), resulting from protein dysfunction associated with irreversible linkage between excess  $\text{Cu}^{2+}$  and sulfhydryl groups (Fernandes and Henriques, 1991). This causes

protein and enzyme degradation that affects cellular biochemistry and inhibits growth (Yruela, 2009).

Therefore, in either case, disorders of Cu nutrition affect plant physiological processes, wherein the main one is photosynthesis (Fernandes and Henriques, 1991; Ravet and Pilon, 2013). In order to scavenge ROS and alleviate their deleterious effects in cells, plants have a range of enzymes such as SOD, peroxidases and catalase (CAT), among others (Capaldi et al., 2015; Dourado et al., 2014; Gratão et al., 2005). SOD is the first enzyme in the detoxification of superoxide anions and can be found in several cellular compartments (Azevedo et al., 1998). They occur in three different molecular forms containing manganese (Mn; Mn-SOD), iron (Fe; Fe-SOD) or Cu and zinc (Zn; Cu/Zn-SOD) as enzymatic cofactors (Azevedo et al., 1998; Hippler et al., 2015).

In citrus orchards, Cu deficiency has been observed in young, vigorous plants (up to three years old) grown under selected soil conditions (e.g. high clay and/or organic matter content; high pH) and fertilized with high doses of nitrogen (N) (Mattos Jr. et al., 2010). Visual symptoms of deficiency are characterized by a canopy with young shoots developed into long branches, curved or

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“S-shaped”, with large, dark green and overdeveloped leaves (Alva and Chen, 1995).

Moreover, older plants are prone to receive frequent Cu applications as cupric fungicides to control citrus diseases, e.g., postbloom fruit drop, *Alternaria* brown spot, citrus black spot and citrus canker (Behlau et al., 2010). The healthy management of orchards is conducted preventively and according to the occurrence of diseases, the inputs of which can sum up 15–30 kg ha<sup>-1</sup> yr<sup>-1</sup> of Cu (Behlau et al., 2010; Fan et al., 2011; Silva Jr. et al., 2016). Most of the Cu sprayed onto leaves is deposited on the soil surface (Alva and Chen, 1995).

This scenario has become more important in the main citrus growing region of Brazil (noteworthy in the State of São Paulo), because of the increased area of recently replanted orchards (27 thousand ha), as well as changes in the current regulatory by-laws that disfavour the need to eradicate trees affected by citrus canker using chemical and cultural suppression of the disease in affected orchards, which can affect the majority of the local grown area (483 thousand ha). Citrus orchards in endemic areas with canker exhibit accumulation of Cu in the soil and consequently, increased availability for plant uptake, proportional to the age of tree plantings (Fan et al., 2011).

The use of rootstocks tolerant to adverse environmental conditions can contribute to the longevity of orchard trees as well to the sustainability of fruit production in the citrus industry. Despite the fact that a number of rootstock varieties has been already used by growers to provide tree tolerance either to biotic (Pompeu Jr. and Blumer, 2008, 2011) or abiotic stresses (Alva and Chen, 1995; Mattos Jr. et al., 2006; Mesquita et al., 2016; Syvertsen and Garcia-Sanchez, 2014; Zambrosi et al., 2013), we verified a lack of information about the response of those where either the lowest or highest availability of Cu in soils limit crop production.

In addition, since reciprocal grafting was proposed to extend current understanding on the responses of the antioxidant systems of plants under metal-stress (Arruda and Azevedo, 2009), signalling trends indicate that rootstocks distinctively activate related defence mechanisms in shoots (Gratão et al., 2015).

Therefore, supported by the fact that rootstocks play a role on plant growth and stress tolerance, as well by the need to characterize major genotype groups in citrus orchards (*Citrus* and *Poncirus* hybrid; Quaggio et al., 2004), we hypothesized that Cu availability in the rooting medium affects the activities of enzymes related to the antioxidant system in the plant shoot as well as nutrient assimilation. Since, such effects would likely occur under a rootstock dependent manner, the consequent impact on understanding how metal-stress and oxidative metabolism in leaves are influenced by roots become relevant to better use grafting to alleviate deleterious effects of abiotic stresses that impair tree horticultural responses.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Young sweet orange trees cv. Pera [*Citrus sinensis* (L.) Osbeck], grafted onto interstocked Swingle citrumelo [SW; *C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.] or Rangpur lime (RL; *C. limonia* Osbeck) were grown in pots with 11 L of nutrient solution (NS) in a greenhouse. The experiment was set up in a completely randomized, 2 x 3 factorial design, with two rootstock varieties (SW and RL) and three Cu concentrations in the NS (0.015, 0.60 and 24.0 μM; as CuSO<sub>4</sub>·5H<sub>2</sub>O), with four replications.

Six-year-old trees were cultivated in 4-L plastic bags filled with an organic substrate (80% pine bark, 5% carbonized materials and 15% vermiculite) fertilized with macro- and micronutrients, except Cu, for 120 days before transplant to the NS. Plants were adapted to

the new growing media for one week at 25% of the final NS concentration and then for two weeks at 50% of the final NS concentration. Plants were then maintained at the following concentrations, in mM: 12 N (80% N—NO<sub>3</sub>), 3.4 K, 0.4 P, 4.0 Ca, 25 Mg and 20 S, and the following, in μM: 41.6 B, 48.0 Fe, 8.2 Mn, 3.5 Zn and 1.3 Mo (modified from Zambrosi et al., 2013). Following the first flush of vegetative growth of plants (approximately 30 days after transplant), treatments were started by adding Cu at various concentrations to the NS, which was aerated continuously; the volumes of the containers were kept constant by addition of deionized water when necessary and renewed at intervals of approximately 15 days. The pH of NS was adjusted to 5.0–5.5 with 1 M KOH or 1 M H<sub>2</sub>SO<sub>4</sub>.

At 45 and 90 days after the start of Cu treatments, the indirect index of chlorophyll (formed after the start of the Cu concentration treatments applied) was determined in young leaves using a portable meter mod, SPAD-502 (Konica Minolta Holding Inc., Tokyo, Japan). The levels of total protein, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid peroxidation and the activities of the following enzymes: nitrate reductase (NRase; EC 1.6.6.1), superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POX; EC 1.11.1.7), were determined.

Furthermore, at the end of the 90-day period, plants were destructively harvested and separated into trunks, old branches and old leaves (grown before the initiation of treatments), new branches and new leaves (grown after treatment applications) and roots. Total leaf area was measured using the Leaf Area Integrator LI-3100 (LI-COR, Lincoln, NE, USA). Plant parts were washed in 0.01% detergent solution (v/v) to remove external contamination of the epidermis (Hippler et al., 2014) and the dry mass (DM) was determined by oven drying at 60–65 °C for 72 h. The plant material was ground to pass through a 200-mesh sieve and the concentrations of Cu and other nutrients were determined according to Bataglia et al. (1983) by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA). The nitrate (N—NO<sub>3</sub><sup>-</sup>) and ammonium levels (N—NH<sub>4</sub><sup>+</sup>) in plant parts were determined by steam distillation (Tedesco et al., 1995). The Cu partition (% of Cu) in each plant part was obtained from the product of DM and Cu concentration.

### 2.2. Nitrate reductase activity

Nitrate reductase (NRase) activity was determined by the *in vivo* method in new leaves as described by Dovic et al. (2014). The assay consists of incubation of 200 mg fresh weight (FW) of leaves in 100 mM of sodium phosphate buffer solution (pH 7.5) with 200 mM of KNO<sub>3</sub> and 1% *n*-propanol (w/v). The samples were vacuum filtered and kept in the dark at 40 °C for 30 min. The NO<sub>2</sub><sup>-</sup> was quantified by absorbance at 540 nm, with the addition of 1% sulfanilamide solution in 2.4 N HCl + 0.02% N-[1-naphthyl] ethylenediamine dihydrochloride (NED; w/v). The result was expressed in μmol NO<sub>2</sub><sup>-</sup> mg<sup>-1</sup> FW h<sup>-1</sup>.

### 2.3. Hydrogen peroxide and lipid peroxidation

The measurements of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation were performed from the same extraction, in which 500 mg of fresh leaves were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 5590 × g for 15 min at 4 °C (Alexieva et al., 2001). The supernatant was mixed with 100 mM of potassium phosphate buffer (pH 7.0) containing 1.0 M potassium iodide (1:1:4) and incubated in ice for 1 h in the dark, followed by 20 min at room temperature and the absorbance measured at 390 nm. The blank consisted of 0.1% TCA in the absence of leaf extract. The amount of H<sub>2</sub>O<sub>2</sub> was calculated using a standard curve generated

with known concentrations of  $\text{H}_2\text{O}_2$ . The result was expressed in  $\mu\text{mol g}^{-1}$  fresh weight.

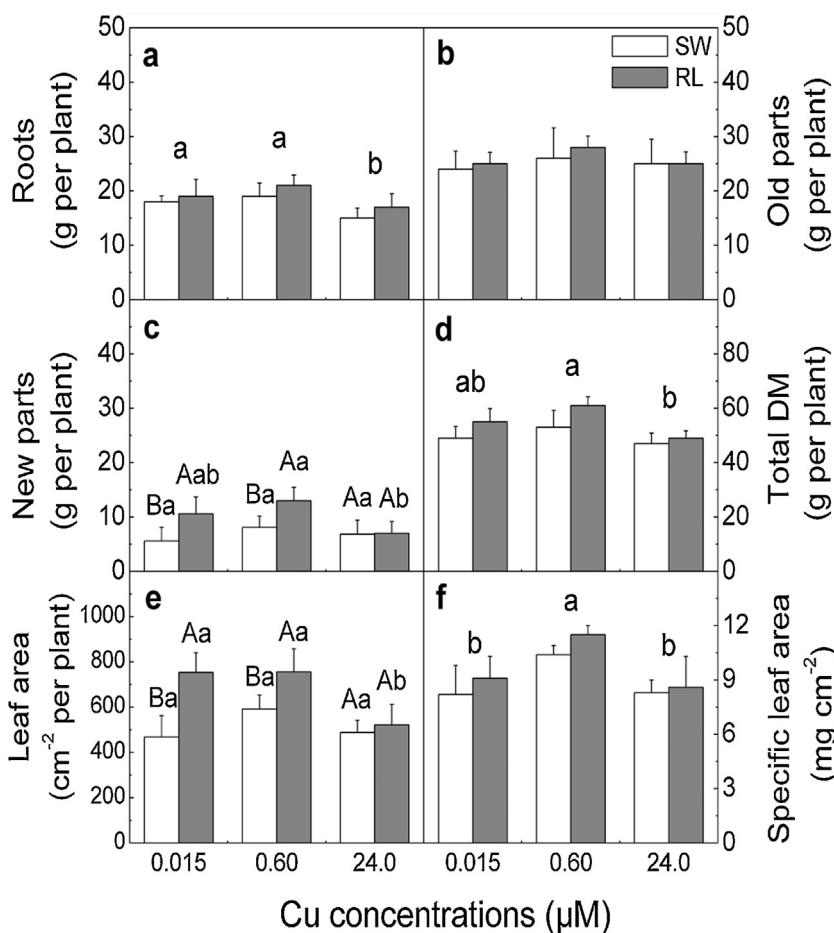
Lipid peroxidation was determined by the presence of malondialdehyde (MDA) according to Heath and Packer (1968). The supernatant (200 mL) from the TCA extraction was added to 1 mL of a solution containing 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA). The samples were incubated at  $95^\circ\text{C}$  for 30 min and then quickly cooled in ice to stop the reaction. The samples were centrifuged for 5 min at  $12,100 \times g$  and the absorbance of the supernatant was measured at 535 and 600 nm. The absorbance of the formed TBA reactive substances was determined at 535 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated using an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ . The result was expressed in  $\mu\text{mol g}^{-1}$  fresh weight.

#### 2.4. Protein content and antioxidant enzyme activities

One gram of fine leaf powder was homogenized in 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 4% (w/v) polyvinylpyrrolidone (PVPP) (Hippler et al., 2015). The suspension was centrifuged at  $12,100 \times g$  at  $4^\circ\text{C}$  for 35 min, and the supernatant was stored at  $-80^\circ\text{C}$  for further analysis. The total protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

The SOD activity staining was performed as described by Durado et al. (2014). Electrophoresis was carried out under non-denaturing conditions in 12% Polyacrylamide gel electrophoresis (PAGE) with equal amounts of protein ( $50 \mu\text{g}$ ) loaded onto each gel lane. After non-denaturing PAGE separation, the gel was rinsed in distilled deionized water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium (NBT), and 0.3% N,N,N',N',N'-tetramethylethylenediamine (TEMED). One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control for activity. After 30 min, the gels were rinsed with distilled deionized water and then illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. SOD isoenzyme characterization was performed as described by Azevedo et al. (1998). Briefly, SOD isoenzymes were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide (KCN) and 5 mM  $\text{H}_2\text{O}_2$ . Densitometry analysis of SOD bands was performed according to Tewari et al. (2006).

CAT activity was determined according to Kraus et al. (1995) with modifications (Azevedo et al., 1998). The reaction was initiated by addition of  $20 \mu\text{L}$  of plant extract in a reaction mixture containing 1.0 mL of 100 mM potassium phosphate buffer (pH 7.5) and  $2.5 \mu\text{L}$   $\text{H}_2\text{O}_2$  (30% solution) at  $25^\circ\text{C}$ . The enzyme activity was determined by following the decrease in absorbance at 240 nm, which represents the decomposition of  $\text{H}_2\text{O}_2$ , for 1 min against a plant extract-free blank. CAT activity was calculated using an extinction coefficient of  $39.4 \text{ M}^{-1} \text{ cm}^{-1}$ .



**Fig. 1.** Dry mass (a, b, c, and d) and leaf area (e and f) of sweet orange trees grafted onto Swingle citrumelo (SW) or Rangpur lime (RL), grown with varying [Cu] after 90 days in nutrient solution. Legend: Old parts = old twigs + old leaves; New parts = new twigs + new leaves; Vertical lines represent standard error of the mean ( $n = 4$ ); Cu rates: means ( $n = 4$  or 8) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means ( $n = 4$  or 12) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).

APX activity was determined by the method of Nakano and Asada (1981), by monitoring the rate of ascorbate oxidation at 290 nm and 30 °C. The reaction medium contained 50 mM phosphate buffer (pH 7.0), with 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The reaction was started by the addition of ascorbate. A molar extinction coefficient of 2.8 mM<sup>-1</sup> cm<sup>-1</sup> was used in calculations.

POX activity was determined following the method of Kar and Mishra (1976). The assay mixture contained 25 mM phosphate buffer (pH 6.8) containing 20 mM pyrogallol and 20 mM H<sub>2</sub>O<sub>2</sub>. The samples were incubated at room temperature for 1 min and 0.5% H<sub>2</sub>SO<sub>4</sub> (v/v) was added to stop the reaction. The activity was estimated by measuring the absorbance at 420 nm for 1 min, and a molar extinction coefficient of 2.47 mM<sup>-1</sup> cm<sup>-1</sup> was used in calculations. CAT, APX and POX activities were expressed as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein.

## 2.5. Statistics

A two-way ANOVA was performed to evaluate the effect of Cu concentration in the nutrient solution and citrus rootstocks on the evaluated variables, with a level of significance of  $\alpha = 0.05$ . When the interaction of copper levels and rootstock varieties (Cu\*RT) was significant, means were compared using the Tukey test at 5% level of significance.

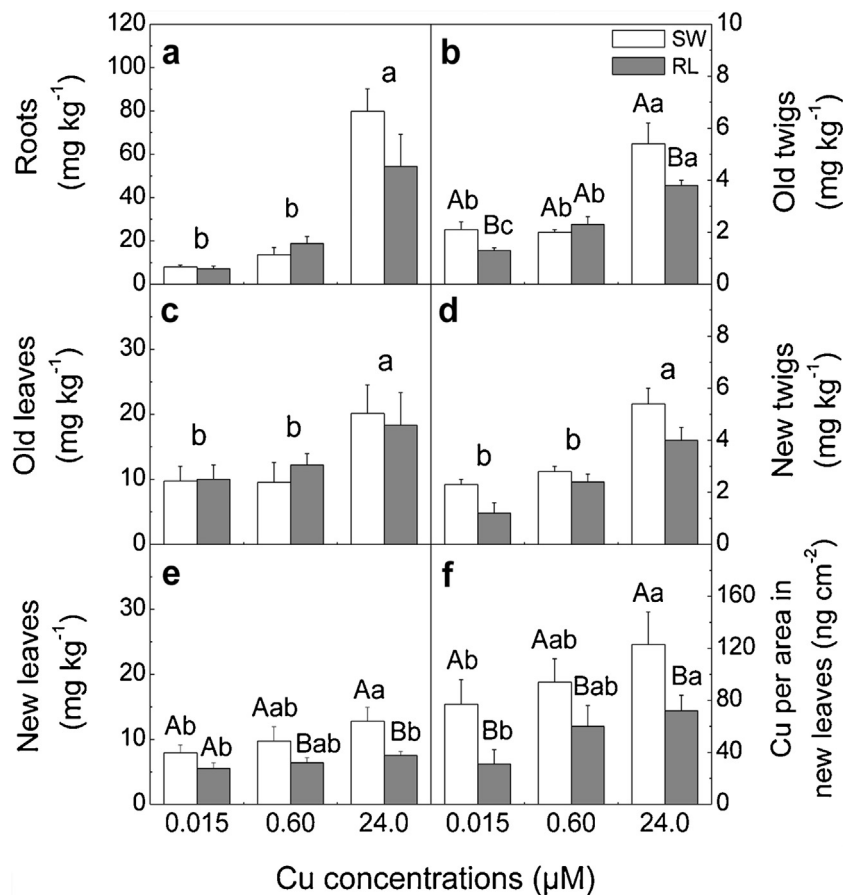
## 3. Results

### 3.1. Plant biomass production

The growth of plants increased with Cu concentration over the range of 0.015–0.60  $\mu\text{M}$  Cu in the NS and then decreased from 0.60 to 24.0  $\mu\text{M}$  Cu, with roots being less responsive to the treatment effects than new plant parts (twigs and leaves) (Fig. 1). The plants grafted on SW responded more positively to Cu availability in the NS in the first case (45% increase in DM of new parts) and exhibited superior tolerance to metal stress in the second case (16% decrease in DM of new parts) when compared to those on RL rootstock (23% increase and 46% decrease in DM, respectively). Similar effects were observed for leaf area of plants with those on SW again exhibiting better growth than RL under varying Cu concentrations in the NS (Fig. 1). Moreover, specific leaf weight was higher (11.0 mg m<sup>-2</sup>) at 0.60  $\mu\text{M}$  of Cu when compared with the lower and higher nutrient concentrations in the NS (Fig. 1).

### 3.2. Copper content and plant nutritional status

Cu concentrations in all plant tissues increased with Cu levels in the NS, with those on SW exhibiting higher levels in roots and new leaves when compared with the RL (Fig. 2). Moreover, the nutrient concentration in roots was increased 10-fold in the NS Cu concentration ranged of 0.015–24.0  $\mu\text{M}$ . Likewise, the Cu concentration in leaves increased approximately 2-fold (Fig. 2).



**Fig. 2.** Copper concentrations in plant parts of young sweet orange trees grafted onto Swingle citrumelo (SW) or Rangpur lime (RL), grown with varying [Cu] after 90 days in nutrient solution. Vertical lines represent standard error of the mean ( $n = 4$ ); Cu rates: means ( $n = 4$  or  $8$ ) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means ( $n = 4$  or  $12$ ) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).

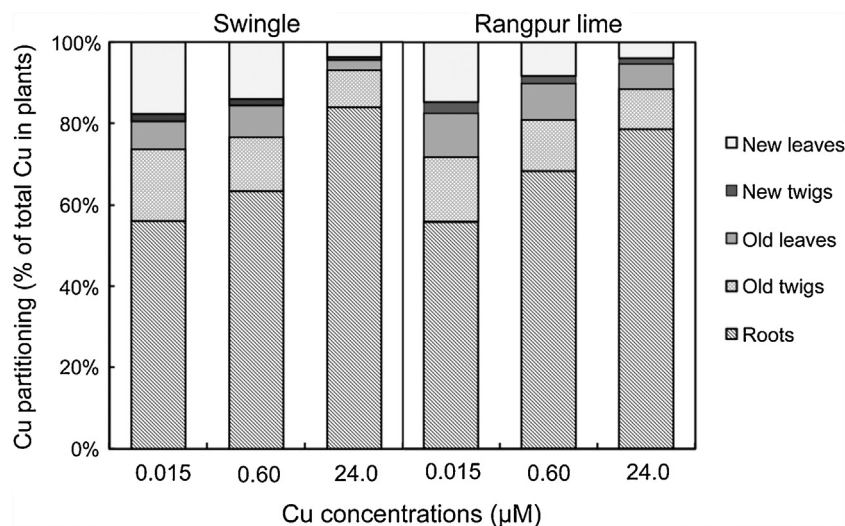


Fig. 3. Copper partitioning in sweet oranges grown for 90 days with varying Cu concentrations in nutrient solution.

Noteworthy, differences between rootstocks were more pronounced when the Cu contents of new leaves were expressed on the basis of leaf area, i.e., trees grafted on SW presented  $96 \text{ ng cm}^{-2}$  of Cu, much higher than those on RL, which displayed  $53 \text{ ng cm}^{-2}$  of Cu ( $p < 0.01$ ), indicating a greater potential to accumulate Cu in leaves of SW.

In accordance with the Cu concentrations found in plant parts, roots accumulated more Cu than twigs and leaves, with 56% and 80% more in the roots of plants grown in the presence of  $0.015 \mu\text{M}$  Cu and  $24.0 \mu\text{M}$  Cu in the NS, respectively (Fig. 3). Conversely, the concentrations of other nutrients were lower in roots (N—NO<sub>3</sub>, total N, Ca, B, Mn and Zn) and leaves (P, Ca, S, Fe and Mn) with increased Cu availability in the NS (Fig. 4). Plants also exhibited higher concentrations of N—NO<sub>3</sub> in roots when grown in  $0.015$  and  $24.0 \mu\text{M}$  Cu, independent of rootstock, and the same was true in the leaves of plants grown in  $0.015 \mu\text{M}$  of Cu for those grafted onto SW and with  $24.0 \mu\text{M}$  of Cu for those grafted onto RL (Fig. 4). Where the iron (Fe) concentrations in roots and leaves were concerned, the plants exhibited a peculiar response to treatment: a 25% increase in root Fe concentration occurred in parallel with a decrease of the same magnitude in leaves, whilst the concentrations of other micronutrients decreased in both plant parts in the presence of excess Cu (Fig. 4).

### 3.3. Estimated leaf chlorophyll content and nitrate reductase activity

The SPAD values and NRase activity, measured at 45 and 90 days after treatments were initiated, were similar for both tree/rootstock combinations (Fig. 5). However, trees grafted on RL exhibited higher than those grafted on SW and were also more sensitive to the varying Cu concentration in the NS, the response to which was only optimal with  $0.60 \mu\text{M}$  Cu (Fig. 5).

### 3.4. Hydrogen peroxide and lipid peroxidation

Plants grown in  $24.0 \mu\text{M}$  Cu exhibited higher concentrations of H<sub>2</sub>O<sub>2</sub> in their leaves than those grown in  $0.60 \mu\text{M}$  Cu at 45 and 90 days, regardless of the rootstock (Fig. 6a and b). Additionally, lipid peroxidation was higher in plants grown at lower and higher Cu concentrations at 45 days (Fig. 6c) and only at the highest concentration of Cu at 90 days (Fig. 6d).

### 3.5. Protein content and antioxidant enzymes activities

The total protein content of leaves was reduced by 15% in plants grown in  $24.0 \mu\text{M}$  Cu in both PE (data not shown). At 45 days, the increase in Cu in the NS caused an increase in the activity of Cu/Zn-SOD III (Fig. 7a and b), but the presence of the Cu/Zn-SOD II isoform was only detected at the highest concentration of Cu, particularly for trees grafted onto RL (Fig. 7a and b). At the same concentration of Cu, trees on SW exhibited increased activity of Mn-SOD (I and II) and Fe-SOD, whereas plants on RL exhibited a reduction in the activity of these isoforms (Fig. 7a and b). At 90 days, the total SOD activity observed was lower than at 45 days (Fig. 7c and d).

CAT activity after 45 days was higher in plants grafted on RL and grown in  $0.015$  and  $24.0 \mu\text{M}$  Cu, but did not vary with Cu in NS in plants grafted on SW (Fig. 8a). After 90 days, CAT activity was higher in plants grown in  $24.0 \mu\text{M}$  Cu, regardless of rootstock (Fig. 8b).

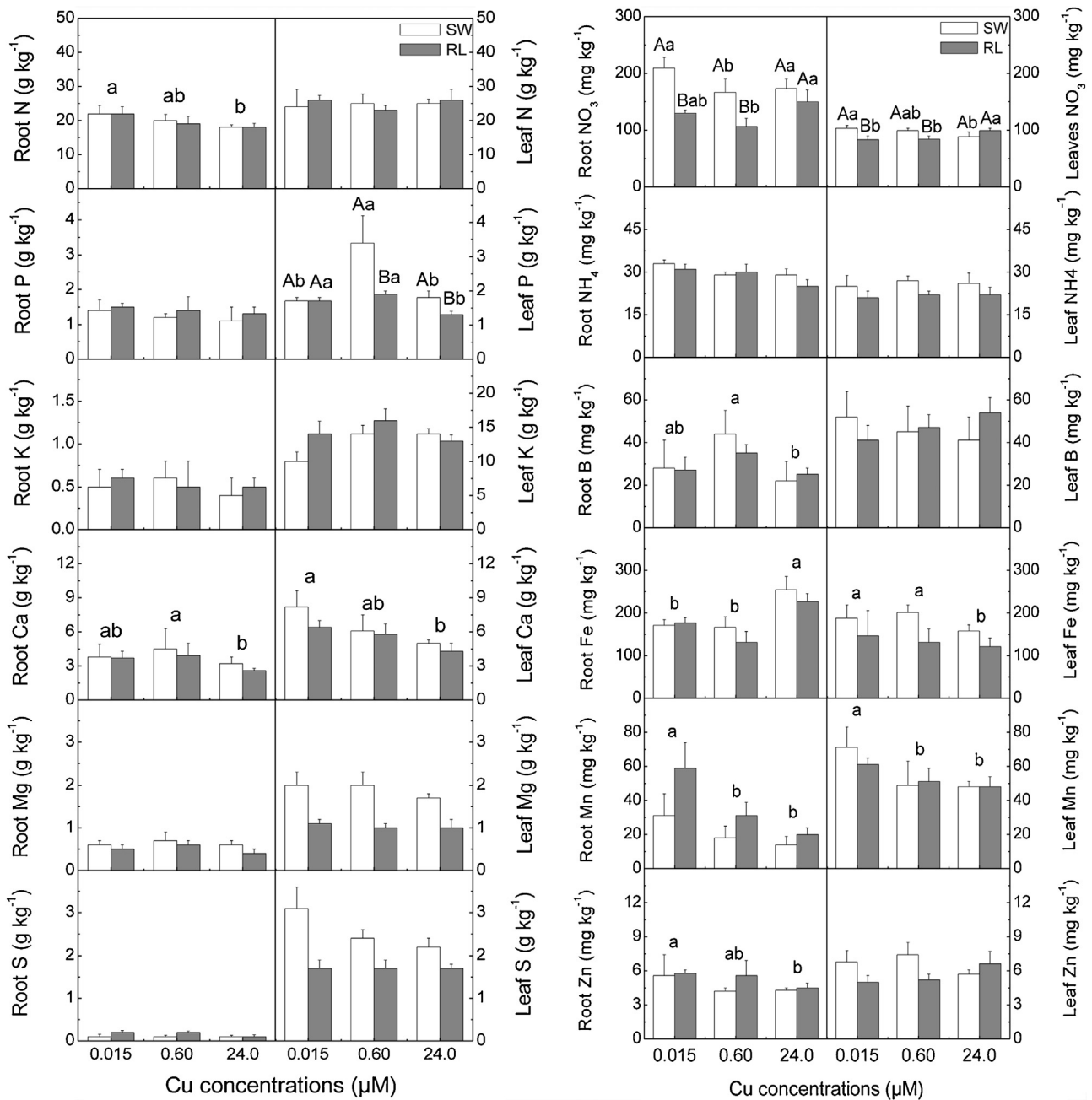
The activities of APX and POX were higher when plants were grown in  $24.0 \mu\text{M}$  Cu compared with  $0.60 \mu\text{M}$  of Cu in the NS (Fig. 8c–f); such differences were more dramatic at 45 days (APX =  $30.4 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  protein; POX =  $17.6 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  protein) compared with 90 days ( $p < 0.05$ ; APX =  $17.3 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ ; POX =  $10.2 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  protein) (Fig. 8c–f).

## 4. Discussion

### 4.1. Plant biomass production and Cu uptake and partitioning

Nutritional disorders caused by Cu reduce the horticultural performance of citrus (Alva and Chen, 1995; Zambrosi et al., 2013), in which deficiency and toxicity commonly occur in young and bearing citrus orchards. In this context, our study evaluated the responses of sweet orange trees grafted onto two rootstock varieties that have demonstrated distinct characteristics to variation in the Cu supply in commercial orchards.

Few changes were observed in the biomass production of citrus grown in Cu at the lowest concentration ( $0.015 \mu\text{M}$ ), indicating that low Cu availability over the study period did not limit growth. At the highest Cu concentration ( $24.0 \mu\text{M}$ ), plants exhibited reduced growth of new parts, particularly roots (Fig. 1), thus showing increased sensitivity to an excess of the nutrient. Excess



**Fig. 4.** Macronutrients concentration of roots (R) and new leaves (NL) of young sweet oranges grafted onto Swingle citrumelo (SW) or Rangpur lime (RL), at 90 days with varying Cu concentrations in nutrient solution. Vertical lines represent standard error of the mean ( $n = 4$ ); Cu rates: means ( $n = 4$  or 8) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means ( $n = 4$  or 12) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).

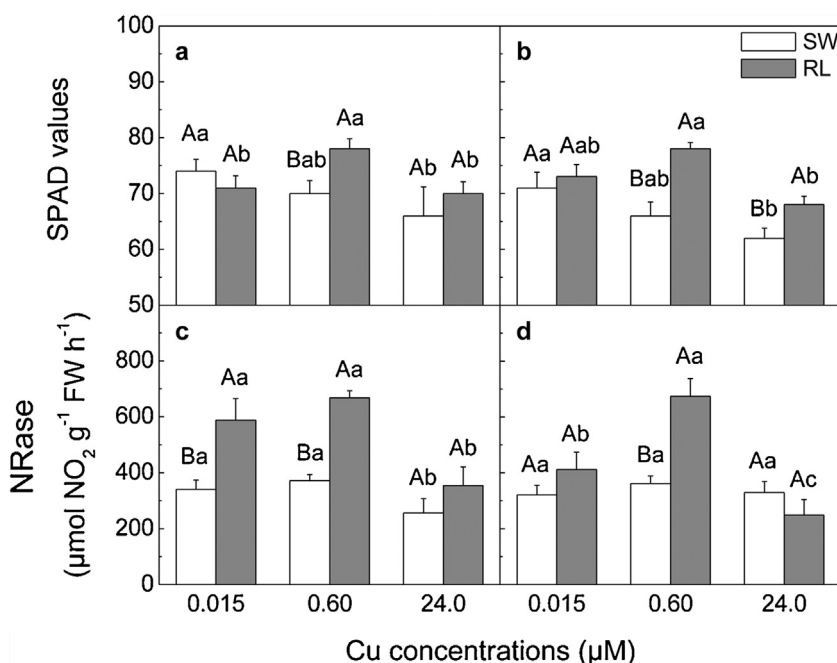
Cu affects cell division and the production of auxin, thereby reducing cell extension (Yuan et al., 2013b), lignification and tissue formation as a result of impaired cell wall and plasma membrane integrity, which is associated with leakage of cellular compounds and nutrients (Arduini et al., 2005). In addition, excess Cu interferes with the biosynthesis and activity of the photosynthetic apparatus due to changes in protein composition and pigments of the photosynthetic membrane (Yruela, 2009), which affects carbohydrate production. These deleterious effects can occur faster due to excess Cu when compared with, for instance, cadmium (Cd), given the rapid incorporation of Cu into cellular metabolic pathways (Arduini et al., 1995).

The highest proportion of Cu was observed in roots, and increased with increasing concentration of the nutrient in the NS

( $p < 0.01$ ; Fig. 3), reaching a level of up to 7-fold greater ( $67 \text{ mg kg}^{-1}$ ) than that found in the new leaves ( $9.8 \text{ mg kg}^{-1}$ ). The accumulation of most of the Cu taken up by plants in the roots represents a strategy to enable biomass production of plants grown in an excess of Cu substrates (Rouphael et al., 2008).

The accumulation of Cu at the roots in a proportion of up to 60-fold higher than the shoots (Zambrosi et al., 2013) indicates that this is a major mechanism of tolerance of *Citrus* to an excess of the metal. On the contrary, the exclusion of Cu by the roots appears not to be comparatively important for this gender, since no differences were observed with respect to the partitioning of the element in plants grafted on either rootstock (Fig. 2).

The occurrence of the highest Cu accumulation in the roots indicates that the Cu content of leaves may not be a good general



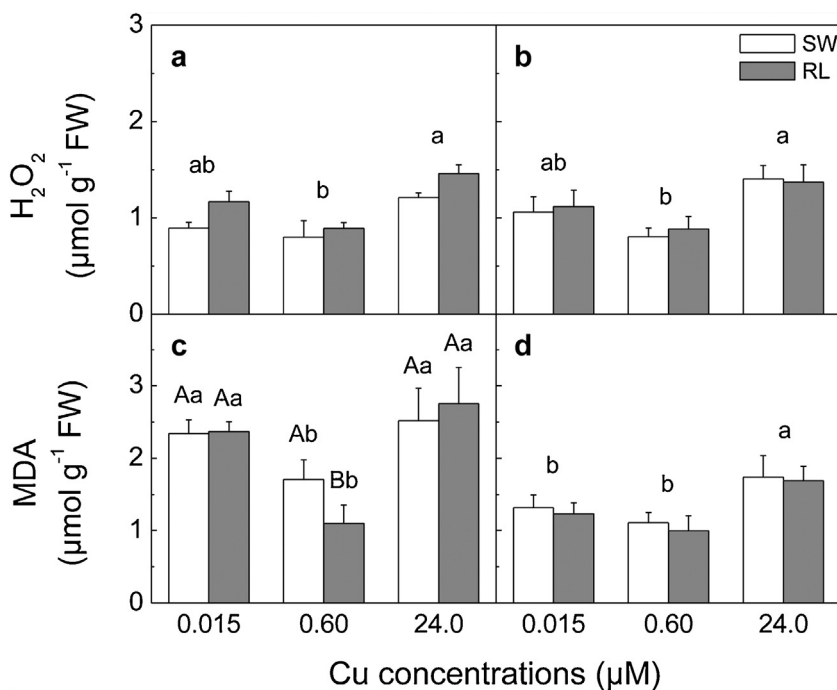
**Fig. 5.** SPAD readings and nitrate reductase (NRase) activity in leaves of young sweet oranges grafted on Swingle citrumelo (SW) or Rangpur lime (RL), at 45 (a and c) and 90 (b and d) days with different Cu concentrations in nutrient solution. Vertical lines represent standard error of the mean (n=4). Cu rates: means (n=4 or 8) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means (n=4 or 12) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).

indicator of high Cu content in plants ( $R^2=0.31$ ;  $p>0.05$ ), as expected in citrus orchards managed with frequent copper-containing fungicides (Fan et al., 2011). In our study, trees grafted on SW exhibited higher Cu concentrations on a leaf area basis ( $\text{ng cm}^{-2}$  of Cu) when compared with those grafted on RL (Fig. 2), with the greatest difference observed when  $24.0 \mu\text{M}$  Cu was included in the NS (Fig. 2). This is a key aspect to be taken into

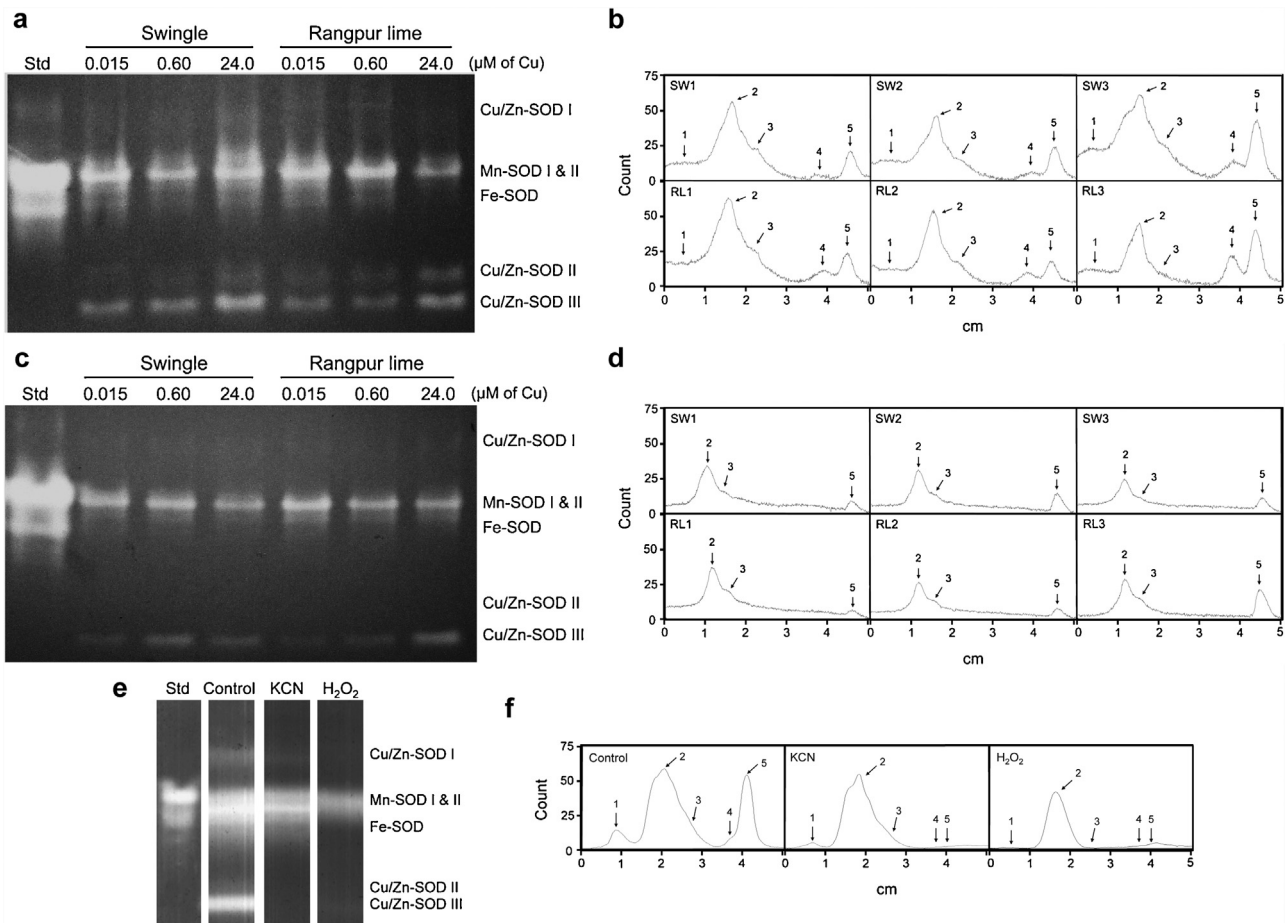
consideration in future studies for Cu and eventually to be tested for other metal elements.

#### 4.2. Nutritional status under Cu stress

In general, Cu excess inhibits the absorption and assimilation of nutrients by plants (Rouphael et al., 2008; Zambrosi et al., 2013) as



**Fig. 6.** Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid peroxidation (MDA) in leaves of young sweet oranges grafted on Swingle citrumelo (SW) or Rangpur lime (RL), at 45 (a and c) and 90 (b and d) days with different Cu concentrations in nutrient solution. Vertical lines represent standard error of the mean (n=4). Cu rates: means (n=4 or 8) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means (n=4 or 12) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).



**Fig. 7.** Activity in Polyacrylamide gel electrophoresis (PAGE 12%) and densitogram of superoxide dismutase (SOD) activity in leaves of young sweet oranges grafted on Swingle citrumelo or Rangpur lime, at 45 (A and B) and 90 days (C and D) with different Cu concentrations in nutrient solution. Isoforms of SOD determined in 12% PAGE (e) and densitogram (f) of sweet orange leaves. Gel legend: Std = Bovine SOD standard; Control = without any inhibitor; KCN = 2 mM potassium cyanide (Cu/Zn-SOD inhibitor); 5 mM hydrogen peroxide (Cu/Zn-SOD and Fe-SOD inhibitor); Densitogram legend: Plants grafted on Swingle citrumelo (SW1 = 0.015  $\mu$ M of Cu; SW2 = 0.60  $\mu$ M of Cu; and SW3 = 24.0  $\mu$ M of Cu) or Rangpur lime (RL1 = 0.015  $\mu$ M of Cu; RL2 = 0.60  $\mu$ M of Cu; and RL3 = 24.0  $\mu$ M of Cu); 1 = Cu/Zn-SOD I; 2 = Mn-SOD I and II; 3 = Fe-SOD; 4 = Cu/Zn-SOD II; and 5 = Cu/Zn-SOD III.

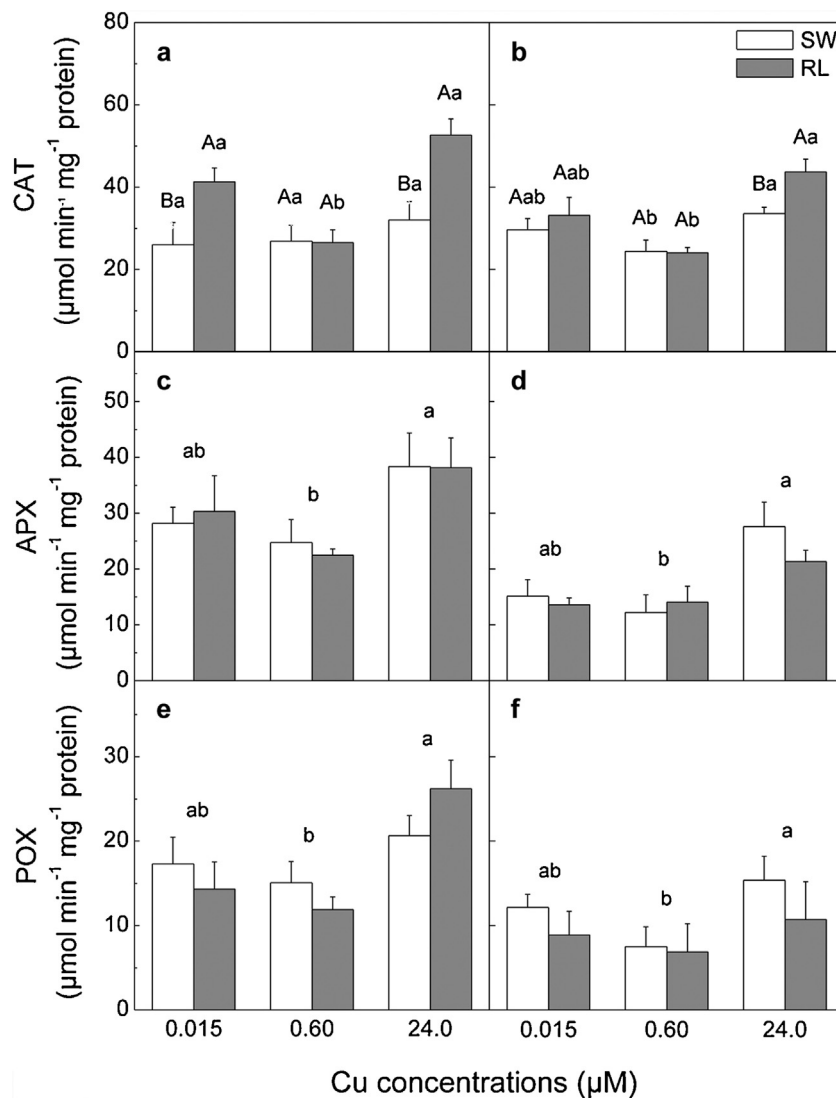
an indirect and/or cascade effect response caused by the toxicity of the element on growth. In the specific case of N, we observed direct interference of the absorption and assimilation of inorganic N in plants due to a reduction in the activity of NRase, the first enzyme in the metabolic pathway of nitrate ( $\text{NO}_3^-$ ) reduction into the cell (Andrews et al., 2013).

Similarly, orange trees grafted onto RL, grown in an excess of Cu, exhibited reduced NRase activity (Fig. 5a and b), which caused  $\text{NO}_3^-$  accumulation in leaves and roots (Fig. 4). Inhibition of this enzyme occurs due to the high affinity of  $\text{Cu}^{+2}$  with the active site of NRase, which would cause metabolic impairment of the enzyme (Hall, 2002; Xiong et al., 2006), or degradation mediated by the formation of ROS in plant cells (Martins et al., 2012). With impairment in absorption and assimilation of N (Fig. 4), protein levels and stability (Martins et al., 2012) as well as the chlorophyll content decreases (SPAD; Fig. 5c and d), which possibly explains the reduction in the photosynthetic activity of plants (Tanyolac et al., 2007). This effect would then reduce the production of sugars that are the source of energy for the activity of various enzymes (Alaoui-Sossé et al., 2004), including NRase (Fig. 5 a and b).

Our study also demonstrated that the absorption of Zn and Mn by plants was affected by excess Cu (Fig. 4) because high external concentrations of metals in the NS can cause damage to the plasma membrane followed by ion leakage from cells (Hall, 2002) and interfere directly with the synthesis and/or activity of specific

membrane transporters, such as members of the ZIP family, which are associated with the transport of other metallic nutrients, such as Zn (Hall and Williams, 2003). Copper could also play a role in the reduction of nutrient uptake by plant roots (Fig. 4) by altering the activity of  $\text{H}^+$ -ATPases and the proton pumping capacity of the plasma membrane, consequently affecting ion transport (Janicka-Russak et al., 2012).

Orange trees apparently demonstrated the ability to accumulate metals in roots as a major mechanism for detoxification and/or tolerance of excess concentrations in the NS, which did not discriminate between Fe and Cu (Fig. 4; Hall, 2002). In plants, Cu deficiency can increase the transcript level of genes related to Fe acquisition by roots, as ferric reductase oxidase, in response to a transcriptional regulator related with Cu homeostasis named as SPL7 (Squamosa Promoter binding protein-Like7; Bernal et al., 2012; Waters et al., 2014). However, there is still a lack of information about the interaction of these nutrients under Cu toxicity conditions, markedly for woody plants. Indeed, in citrus groves with excess Cu in the soil, the occurrence of Fe-deficiency symptoms in young leaves has been observed (Alva and Chen, 1995), visually characterized by generalized chlorosis of leaf blades and greener mid-veins. Based on the increased Fe concentration in root tissues and decreased concentrations in leaves, we may argue that Fe uptake was not impaired, as was observed for Mn and Zn (Fig. 4).



**Fig. 8.** Catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX) activities in leaves of young sweet oranges grafted on Swingle citrumelo (SW) or Rangpur lime (RL), at 45 (a, c and e) and 90 days (b, d and f) with different Cu concentrations in nutrient solution. Vertical lines represent standard error of the mean ( $n = 4$ ). Cu rates: means ( $n = 4$  or 8) followed by different lowercase letters are significantly different by Tukey's test ( $p < 0.05$ ). Rootstocks: means ( $n = 4$  or 12) followed by different uppercase letters are significantly different by Tukey's test ( $p < 0.05$ ).

From the above, it may even be argued that for the maintenance of cellular homeostasis in the case of metals of high reducing capacity, such as Cu, Mn and Fe, their concentration in the form of free ions in cells should be reduced, mostly by chelation with proteins or metallochaperones, such as histidine and nicotianamine (Fernandes and Henriques, 1991; Yruela, 2009). On the other hand, it is also possible that excess Cu interferes with the transport of Fe from roots to shoots through the xylem vessels by inactivation of specific transporters (Puig et al., 2007). However, further understanding on the mechanisms of plant inhibition of Cu and Fe transport to shoots is still required, once we can identify what other important characteristics plant use to alleviate the damages caused by Cu excess.

#### 4.3. Antioxidant enzyme system to scavenge excess ROS under Cu stress

Although plants grafted onto RL were more sensitive during growth when exposed to high concentrations of Cu, increases in  $H_2O_2$  and MDA concentrations in leaves were found regardless of rootstock (Fig. 6). While  $H_2O_2$  acts as a signal in response to plant

stress, it becomes toxic at high concentrations in the cell, producing the hydroxyl radical ( $OH^\bullet$ ), one of the most reactive species, which causes damage to membranes (Capaldi et al., 2015; Gr  o et al., 2005; Soares et al., 2016). In response to the increase in ROS, plants require an efficient antioxidant system to alleviate plant stresses. In this case, the elimination of the superoxide radical ( $O_2^{\bullet-}$ ) is largely carried out by SOD (Azevedo et al., 1998; Gr  o et al., 2015; Rout and Sahoo, 2013), the distinct isoforms of which exhibited differing activities in the leaves of plants grafted onto different rootstocks and exposed to different concentrations of Cu (Fig. 7).

When the supply of Cu was limited to 0.015  $\mu M$ , rootstocks decreased their activity of Cu/Zn-SOD over both periods evaluated (Fig. 7), which possibly resulted from the requirement of plants for Cu for use in plastocyanin, an important electron transfer protein in photosystem II (Yamasaki et al., 2008). In *Arabidopsis thaliana* under conditions of low Cu supply, Yamasaki et al. (2008) reported an increase in Fe-SOD activity in the chloroplasts as a compensatory mechanism in the elimination of  $O_2^{\bullet-}$  radical, despite a reduction in Cu/Zn-SOD activity. In our study, citrus plants displayed increased activity of Mn-SOD, a mitochondrial SOD

isoform, to compensate for the reduction in Cu/Zn-SOD activity (Fig. 7). Analyses of the expression of genes encoding Cu/Zn-SOD (*CSD1* and *CSD2*) or Cu chaperones for SOD (*CCS1*) or microRNAs such as miR397 and miR398, that regulate the post-transcription of Cu/Zn-SOD and Fe-SOD, will contribute to the better understanding of the balance between the regulation of these two SOD isoforms either in a Cu deficiency or excess condition (Paul et al., 2015; Waters et al., 2012). Furthermore, microRNAs-Cu-homeostasis related seems to be important to reduce the biosynthesis of other Cu proteins or enzymes, which enhances the Cu availability for plastocyanin biosynthesis (Paul et al., 2015). However, for both *Arabidopsis* (Yamasaki et al., 2008) and *Citrus*, such responses did not appear to be sufficient to reduce the effect of oxidative stress on plant metabolism caused by Cu stress.

Both Cu/Zn-SOD and Mn-SOD activity were important to scavenge  $O_2^{\bullet-}$  in plants exposed to high levels of Cu. However, in young sweet-oranges grafted onto SW, when exposed to high Mn availability in the soil, the activities of the Cu/Zn-SOD isoforms were reduced, which resulted in chlorophyll degradation (Hippler et al., 2015). Since the SOD isoforms are localized in different cell compartments, rootstocks could influence the responses of plants to Cu excess in specific locations of leaf cells. In this case, the effect of rootstocks on plant signalling would be regulated by proteins, hormones (e.g. auxin and abscisic acid) and other molecules (e.g.  $H_2O_2$ ) transported from roots to shoots (Gratão et al., 2015; Soares et al., 2016; Yuan et al., 2013a). Thus, we could hypothesize that trees grafted on RL would possibly present greater detoxification of ROS in thylakoid membranes and the cytoplasm, where Cu/Zn-SOD is found. On the other hand, plants on SW possibly exhibit a different mechanism, probably related to reducing the toxic effects of excess Cu in cells, such as a chelation or vacuolar compartmentalization in roots, resulting in a reduction of damage that compromises metabolism in the plant canopy. In addition to the above, sweet oranges grafted onto SW demonstrated a higher capacity to recover normal photosynthetic metabolism, as was verified in plants exposed to low night temperatures (Machado et al., 2013).

Indeed, the SW is a hybrid between grapefruit and trifoliate (Castle et al., 1988), whose rootstock variety presents distinct horticultural characteristics, such as diseases tolerance (Pompeu Jr. and Blumer, 2011), higher nutritional demands (Mattos Jr. et al., 2006; Mesquita et al., 2016; Quaggio et al., 2004) and physiological responses to temperature stress conditions (Machado et al., 2013) compared to the RL rootstock. A role of grafting in signal transduction between roots and shoots and the consequent decrease in adverse effects caused by heavy metals in plants was proposed (Rouphael et al., 2008; Savvas et al., 2013) and confirmed in tomato plants exposed to excess Cd (Gratão et al., 2015). In the latter, non-grafted plants displayed increased activities of antioxidant enzymes in both tissues, whereas grafted ones exhibited differing trends that demonstrated signalling responses from the rootstocks on the activation of defence mechanisms in shoots.

The activities of CAT, APX and POX (Fig. 8) were similar to those observed for  $H_2O_2$  and MDA contents (Fig. 6). The balance between such enzyme activities and SOD is important to determine the effect of ROS levels in cells, since the peroxidases are responsible for the modulation of ROS signalling in response to stresses, while CAT would be responsible for removing  $H_2O_2$  formed by SOD in a  $O_2^{\bullet-}$  detoxification reaction (Azevedo et al., 1998; Gratão et al., 2015). Stresses caused by deficiency or excess of Cu ( $H_2O_2$  and MDA; Fig. 6) resulted in significant increases in the activities of SOD, CAT and peroxidases (APX and POX), which can be considered evidence of ROS production increased by nutritional Cu disorders in plants (Rout and Sahoo, 2013).

We observed that the deficient or excessive availability of Cu impairs the growth and nutritional status of orange trees. *Citrus* plants preferentially accumulate Cu in the roots, and the rootstocks interfere with biochemical responses of the plant canopy, as in the activity of enzymes related to oxidative stress and nitrogen metabolism in leaves, with those grafted on RL being more prone to both conditions. Under conditions of Cu toxicity, a reduction was observed in the absorption and accumulation of other nutrients, except for Fe, which also accumulated in roots. The activity of Cu/Zn-SOD and CAT are dependent upon rootstock signalling, in which plants grafted onto SW exhibited lower activities in leaves when grown with low and high availability of Cu in the nutrient solution. Therefore, we were able to demonstrate that the use of rootstock varieties can represent a first line agronomic practice to ensure better plant growth under Cu stress conditions, of which the Swingle citrumelo, and presumably hybrids from the *Poncirus* group will likely limit damages caused to plant growth and consequently induce better horticultural performance of citrus orchards.

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