Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 163: 399–413. 2018

Photosynthesis is differently regulated during and after copper-induced nutritional stress in citrus trees

Franz W.R. Hippler[a,b,*], Veronica L. Dovis[a], Rodrigo M. Boaretto[a], Jose A. Quaggio[c], Ricardo A. Azevedo[b], Lorraine E. Williams[d] and Dirceu Mattos-Jr[a,*]

[a]Centro de Citricultura Sylvio Moreira, Instituto Agronômico, Rod. Anhanguera, Km 158, CP 04, CEP 13490-970, Cordeirópolis, SP, Brazil
[b]Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, CP 9, CEP 13418-900, Piracicaba, SP, Brazil
[c]Centro de Solos e Recursos Ambientais, Instituto Agronômico, Av. Barão de Itapura, 1481, CP 28, CEP 13020-902, Campinas, SP, Brazil
[d]Biological Sciences, University of Southampton, Life Sciences Building 85, Highfield Campus, SO17 1BJ, Southampton, UK

Correspondence
*Corresponding authors,
e-mail: franz@ccsm.br
e-mail: ddm@ccsm.br

Received 9 October 2017;
revised 28 March 2018

doi:10.1111/ppl.12746

Antioxidant enzymatic responses in citrus leaves under Cu-induced stress depend on rootstock genotypes. However, there is a lack of information about how woody plants recover growth capacity after exposure to elevated Cu and if growth is affected by the redistribution of the metal to new vegetative parts and consequently whether photosynthesis is affected. Therefore, the biomass of plants and Cu concentrations in new leaf flushes were determined in young citrus trees grafted onto contrasting rootstocks [Swingle citrumelo (SW) and Rangpur lime (RL)]. Photosynthetic rate, chlorophyll fluorescence and antioxidant enzymatic systems were evaluated in plants previously grown in nutrient solution with Cu varying from low to high levels and with no added Cu. Both rootstocks exhibited reduced plant growth under Cu toxicity. However, trees grafted onto RL exhibited better growth recovery after Cu excess, which was dependent on the modulation of antioxidant enzyme activities in roots and leaves that maintained the integrity of the photosynthetic apparatus. In contrast, plants grafted onto SW exhibited a lower photosynthetic rate at the lowest available Cu concentration. Although the highest accumulation of Cu occurred in citrus roots, the redistribution of the nutrient to new vegetative parts was proportional to the Cu concentration in the roots.

Introduction

Copper (Cu) nutritional disorders cause impairment of plant growth and citrus production losses. Copper deficiency is observed in nursery and non-bearing citrus trees supplied with high nitrogen (N) rates or grown in soils with high pH and organic matter/clay content (Mattos Jr et al. 2010, Hippler et al. 2018a). On the other hand, Cu excess has been associated with metal accumulation in soils, which results from continuous applications of Cu-based fungicides for plant protection (Komárek et al. 2009, Fan et al. 2011). Amounts of Cu applied in citrus orchards are likely to increase because of the enhanced incidence of citrus canker in growing areas as observed in Florida (United States) and São Paulo state (Brazil) after the suspension of eradication

Abbreviations – $\Delta F/F'_M$, effective quantum yield of PSII; AEF, alternative electron flow; APX, ascorbate peroxidase; CAT, catalase; C_i, internal CO_2 concentration; ETR, apparent electron transport rate; F_V/F_M, maximum quantum yield of PSII; g_S, stomatal conductance; MDA, lipid peroxidation; P_N, net photosynthetic rate; PPFD, photosynthetic photon flux density; PS, photosystem; qNP, non-photochemical quenching; qP, photochemical quenching; RL, Rangpur lime; ROS, reactive oxygen species; SOD, superoxide dismutase; SW, Swingle citrumelo.

programs against disease affecting trees (Behlau et al. 2016).

Under Cu-induced stress conditions, caused either by deficiency or toxicity, reactive oxygen species (ROS) increase in plants (Hippler et al. 2016, 2018a). The accumulation of ROS in cells affects membranes and the cell wall by degrading proteins, lipids and DNA, consequently affecting the cellular metabolism (Cuypers et al. 2011, 2016). Photosynthesis is one of the most highly disturbed processes influenced by ROS; photosynthesis suboptimal functioning impacts growth and hence the fruit yield of trees (Syvertsen and Garcia-Sanchez 2014, Zandalinas et al. 2017). Plants generally contain enzymatic and non-enzymatic antioxidant systems to provide tolerance to biotic and abiotic stresses and consequently alleviate damages caused by ROS (Juraniec et al. 2013, Piotto et al. 2014). Superoxide dismutase (SOD, EC 1.15.1.1) is the first enzyme that reduces the superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). Then, the excess of H_2O_2 in cells is eliminated by catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) or guaiacol peroxidase (Anjum et al. 2016).

In woody plants, such as citrus, different rootstock genotypes differentially regulate the absorption and distribution of nutrients into the shoots (Martínez-Ballesta et al. 2010), they also influence the activity of antioxidant enzymes in the leaves by regulating root-signaling processes (Hippler et al. 2016). The antioxidant system is recognized to be directly involved in heavy metal tolerance of plants (Gratão et al. 2015, Borges et al. 2018). In this context, the role of rootstocks and scions on the root-to-shoot communication and increased plant tolerance to cadmium (Cd) toxicity were recently characterized (Gratão et al. 2015). For example, the activities of antioxidant enzymes in roots of grafted tomato plants improved tomato tolerance to metal excess, indicating the importance of root-to-shoot stress signaling (Gratão et al. 2015). Under Cu excess, sweet orange trees grafted onto Rangpur lime [RL; *Citrus limonia* (L.) Osbeck] had higher enzyme activities in leaves than those grafted onto Swingle citrumelo [SW; *Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.]. However, information about responses of the antioxidant enzyme system in roots of contrasting citrus rootstocks under Cu-induced stress remains unclear. The efficiency of the enzyme antioxidant system is a key component to maintain the integrity of the photosynthetic apparatus and leaf gas exchange for high fruit yield of citrus trees (Syvertsen and Garcia-Sanchez 2014). Understanding the efficacy of the antioxidant enzymes of RL and SW rootstocks in alleviating oxidative stress might support future strategies of citrus trees management in fields subjected to Cu nutritional disorders, because those rootstocks present 80% of the Brazilian citrus orchards.

Because rootstocks regulate Cu compartmentation in roots to maintain plant homeostasis (Zambrosi et al. 2013, Hippler et al. 2018b), they are also expected to affect Cu redistribution to new vegetative organs. The phloem mobility of metal micronutrients, such as manganese (Mn) and zinc (Zn) in citrus trees, is very low (Hippler et al. 2015). However, there is still a need of information to elucidate the significance of Cu redistribution in relation to the development of new growing parts of woody plants. A comprehensive understanding on how rootstocks modulate physiological and biochemical aspects, as well as plant development, under deficiency or toxicity of Cu will contribute to the selection of suitable rootstocks genetic material supporting the sustainability of plant production. Therefore, this work aimed (1) to evaluate the performance and integrity of the photosynthetic apparatus, as well as the activity of antioxidant enzymes in response to Cu deficiency or toxicity in citrus trees grafted onto two rootstocks with contrasting horticultural characteristics and (2) to assess the Cu redistribution in citrus trees and their growth capacity after suspension of the metal-nutrient supply.

Materials and methods

Plant material and growth conditions

One-year-old sweet orange trees [*Citrus sinensis* (L.) Osbeck cv. Valencia] grafted onto SW [*C. paradisi* Macf. × *P. trifoliata* (L.) Raf.] or RL [*C. limonia* (L.) Osbeck] were grown in a greenhouse. Plants were transferred from organic substrate to pots (one plant per pot) containing 11 l of nutrient solution as described by Hippler et al. (2016). Plants were adapted to the hydroponic condition for 2 weeks at 25% of the concentration of the full nutrient solution and subsequently for 2 weeks at 50% of the full nutrient solution. Then, plants were maintained at the following concentration (mM): 12 N (80% N-NO_3), 0.4 P, 3.4 K, 4.0 Ca, 25 Mg and 20 S, plus (μM): 41.6 B, 48.0 Fe, 8.2 Mn, 3.5 Zn and 1.3 Mo (modified from Zambrosi et al. 2013). The experiment was set up in a completely randomized, 2 × 3 factorial design, with two rootstock genotypes (SW and RL) and three Cu concentrations in the nutrient solution (low, 0.015 μM $CuSO_4 \cdot 5H_2O$; medium, 0.60 μM $CuSO_4 \cdot 5H_2O$; high, 24.0 μM $CuSO_4 \cdot 5H_2O$), with five replications. Treatments of Cu concentrations in the nutrient solution started after 45 days of plant adaptation to the full nutrient solution, when the first vegetative flush started sprouting (herein identified as old parts; Fig. S1). The nutrient solution was aerated continuously, and the

volumes of the containers were kept constant by adding deionized water when necessary and were renewed at intervals of approximately 15 days. The pH of the nutrient solution was adjusted to 5.0–5.5 with additions of 1 M KOH or 1 M H_2SO_4.

When plants exhibited the second vegetative flush of physiologically mature leaves (identified as new parts), 110 days after starting treatments with Cu, photosynthesis was evaluated (new leaves) and biochemical analyses (roots and new leaves, period 1) were conducted to assess the effects of Cu treatments. The new parts (twigs and leaves) formed after starting the Cu treatments were destructively collected, and leaf area was measured (LI-3100C; LI-COR, Lincoln, NE). Plant material was washed and dried at 58–60°C to determine the mass production, and Cu and Fe tissue concentrations were determined by inductively coupled plasma optical emission spectrometry (Perkin Emler 5100 PC, Norwalk, CT) after nitro-perchloric digestion according to Bataglia et al. (1983).

In period 2, we evaluated the growth capacity and the redistribution of Cu in plants after interrupting the metal-nutrient supply by changing the plants to a solution without Cu (<0.001 µM Cu; Fig. S1). For this reason, just after the evaluations in period 1, containers and root surfaces were passed through a washing process before starting with the Cu-deprived solution to minimize a residual effect of Cu on the root surface. Then, roots and containers were rinsed with distilled water (dH_2O), quickly immersed in a solution of 200 mM ethylenediaminetetraacetic acid (EDTA) and again rinsed with dH_2O. At 140 days after the first evaluation period (250 days after the start of treatments in period 1), the second new vegetative flush was physiologically mature (period 2; Fig. S1). At this point, evaluation of photosynthesis (new leaves) and sampling of roots and new leaves for biochemical analysis were repeated (period 2). In period 2, trees were destructively collected and separated into coarse (>3 mm Ø) and fibrous roots (≤3 mm Ø), twigs, and leaves from the older part (existing before the start of the treatments) and the newer part (grown after starting the treatment of period 2) for the determination of dry mass (DM) production and Cu and Fe concentrations in plant tissues (Bataglia et al. 1983). The accumulation and partition of Cu were estimated based on DM and the nutrient concentration in plant parts.

Gas exchange and chlorophyll *a* fluorescence measurements

The net photosynthetic rate (P_N), stomatal conductance to water vapor (g_S) and internal CO_2 concentration (C_i) were determined in sun-exposed and recently expanded leaves in the middle third of twigs from the new part. The evaluations were performed on a clear day between 9:00 and 11:00 with an infrared gas analyzer open system LI-6400 (LI-COR, Lincoln, NE) equipped with an integrated fluorescence chamber head (LI-6400-40, LI-COR), at ambient temperature (vapor pressure deficit of 1.32 kPa in period 1 and 1.94 kPa in period 2), at 40 Pa CO_2 partial pressure and under artificial photosynthetic photon flux density (PPFD) 800 µmol m^{-2} s^{-1} at the leaf level.

Steady-state (F'_o) and maximum (F'_M) fluorescence yield were assessed in light-adapted leaf tissues, whereas minimum (F_O) and maximum (F_M) fluorescence yields were carried out in dark-adapted (over-night) leaf tissues. F_M and F'_M were measured after a light saturation pulse [$\lambda < 710$ nm, PPFD approximately 10 000 µmol (photon) m^{-2} s^{-1}, 0.8 s]. The variable fluorescence yield in both dark-adapted ($F_V = F_M - F_O$) and light-adapted ($F'_V = F'_M - F'_o$) leaves was calculated. The maximum quantum yield of photosystem II (PSII) [$F_V/F_M = (F_M - F_O)/F_M$], the effective quantum yield of PSII [$\Delta F/F'_M = (F'_M - F_S)/F'_M$], the alternative electron flow [AEF = $(\Delta F/F'_M)/(P_N/[\text{PPFD} \times 0.84])$], the photochemical quenching coefficient [qP = $(F'_M - F_S)/(F'_M - F'_o)$] and the non-photochemical quenching coefficient [qNP = $(F_M - F'_M)/(F_M - F'_o)$] were calculated (Schreiber et al. 1994). The apparent electron transport rate [ETR = $\Delta F/F'_M \times \text{PPFD} \times 0.84 \times 0.5$] was calculated according to Genty et al. (1989). The ratio ETR/P_N was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 assimilation rate.

Hydrogen peroxide, lipid peroxidation and antioxidant enzyme activities

The measurements of the hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) contents were performed from the same extraction, in which 500 mg of fresh mass of leaves (new parts) or fibrous roots (≤3 mm Ø) 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). For H_2O_2 content, the supernatant was mixed with 100 mM potassium phosphate buffer (pH 7.0), and 1.0 M potassium iodide (1:1:4) and incubated at 4°C for 1 h in darkness and then for 20 min at 25°C before measuring the absorbance at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve with known concentrations of H_2O_2. The MDA was determined according to Heath and Packer (1968). To the supernatant sample, 1 ml of a solution containing 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA) was added, and then incubated at 95°C

for 30 min followed by a quick cooling at 4°C to stop the reaction. The samples were recentrifuged for 5 min at 12 100 *g*, and 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 mM^{-1} cm^{-1}.

For protein quantification and enzyme activities, 1 g of fine leaves or fibrous root powder was homogenized in 5.0 ml of 100 mM potassium phosphate buffer (pH 7.5), with 3 mM dithiothreitol, 1 mM EDTA and 4% (w/v) polyvinylpolypyrrolidone (Gratão et al. 2015). The suspension was centrifuged at 12 100 *g* at 4°C for 35 min, and the supernatant was stored at −80°C for further analysis. The total protein content was determined using bovine serum albumin as a standard (Bradford 1976).

SOD activity staining was carried out according to Beauchamp and Fridovich (1971), with modifications (Hippler et al. 2015). Electrophoresis was carried out under non-denaturing conditions in 12% polyacrylamide gel (PAGE) with 50 µg proteins per lane for leaves and 75 µg for root samples. One unit of bovine liver SOD (Sigma, St. Louis, MO) was used as a positive control of activity. After non-denaturing PAGE separation, the gel was 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 and 0.3% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. 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 and 5 mM H_2O_2. CAT activity was determined according to Kraus et al. (1995) with modifications (Azevedo et al. 1998) and APX was determined by the method of Nakano and Asada (1981), both as described in Hippler et al. (2016). CAT activity was calculated by using an extinction coefficient of 39.4 M^{-1} cm^{-1} and APX by 2.8 mM^{-1} cm^{-1}.

Statistical analysis

To analyze the studied factors and their interactions, ANOVA was used at $P < 0.05$. When the interaction between Cu levels and citrus rootstocks (Cu × RT) was significant, means were compared using the Tukey test ($\alpha = 0.05$).

Results

Plant growth under different Cu treatments

After 110 days of exposure to different levels of Cu (period 1), the highest concentration of Cu in the nutrient solution (24.0 µM Cu) reduced the biomass production of twigs and leaves (new parts) and leaf area, compared to the medium level of Cu (0.60 µM Cu; Fig. 1). Trees grafted onto RL exhibited higher DM of twigs and young leaves, especially in the highest concentration of Cu, they also exhibited greater leaf area at concentrations of 0.60 and 24.0 µM Cu, both compared to those grafted onto SW (Fig. 1).

In period 2, the orange trees grafted onto RL exhibited lower biomass production and leaf area upon Cu-deprivation compared when previously grown in 0.015 µM Cu (Fig. 1). Plants that received 24.0 µM Cu in period 1 had greater biomass production in the period 2, with a total DM similar to those grown in 0.60 µM Cu (Fig. 1). The biomass production of trees grafted on SW did not differ among the Cu concentrations, but the smallest leaf area was observed under the highest concentration of Cu (Fig. 1).

Cu uptake and distribution in grafted citrus plants

The uptake and distribution of Cu in citrus plants grafted onto contrasting rootstocks were assessed at the end of period 1, when levels of the nutrient varied in the nutrient solution, as well as in period 2, when the same plants were grown without Cu. The Cu concentration in the new vegetative flush (leaves and twigs) of trees on both rootstocks increased proportionally to the Cu concentration in the nutrient solution in both periods (Fig. 2). However, in period 1, trees grafted onto RL accumulated more Cu in new parts of the plants, such as fibrous roots, new leaves and new twigs, when grown on 24.0 µM Cu, compared to those grafted onto SW (Fig. 3).

At the end of the period 2 (without Cu supply), plants previously grown in 24.0 µM Cu exhibited the highest metal accumulation and partition into the roots, about 3.4-fold more than in old twigs, whereas those grown in 0.015 µM Cu showed similar partitioning of the metal between the old twigs and roots, which represented approximately 80% of the total Cu in the plant (Fig. 3). Although plants in both rootstocks exhibited higher Cu partition into the roots, metal accumulation in those grafted onto RL was similar for both fibrous and coarse roots, while in the same plants grafted onto SW, the metal accumulation occurred mainly in the coarse roots (Fig. 3). No differences in Cu partition were seen for other plant parts (Fig. 3).

The Fe concentration increased in roots and decreased in leaves with the highest concentration of Cu in the nutrient solution (Fig. 4), as observed previously (Hippler et al. 2016). Furthermore, in this study, we demonstrated that plants accumulated more Fe in the coarse roots of SW (5.1 mg of Fe) compared to those of RL (2.2 mg of Fe,

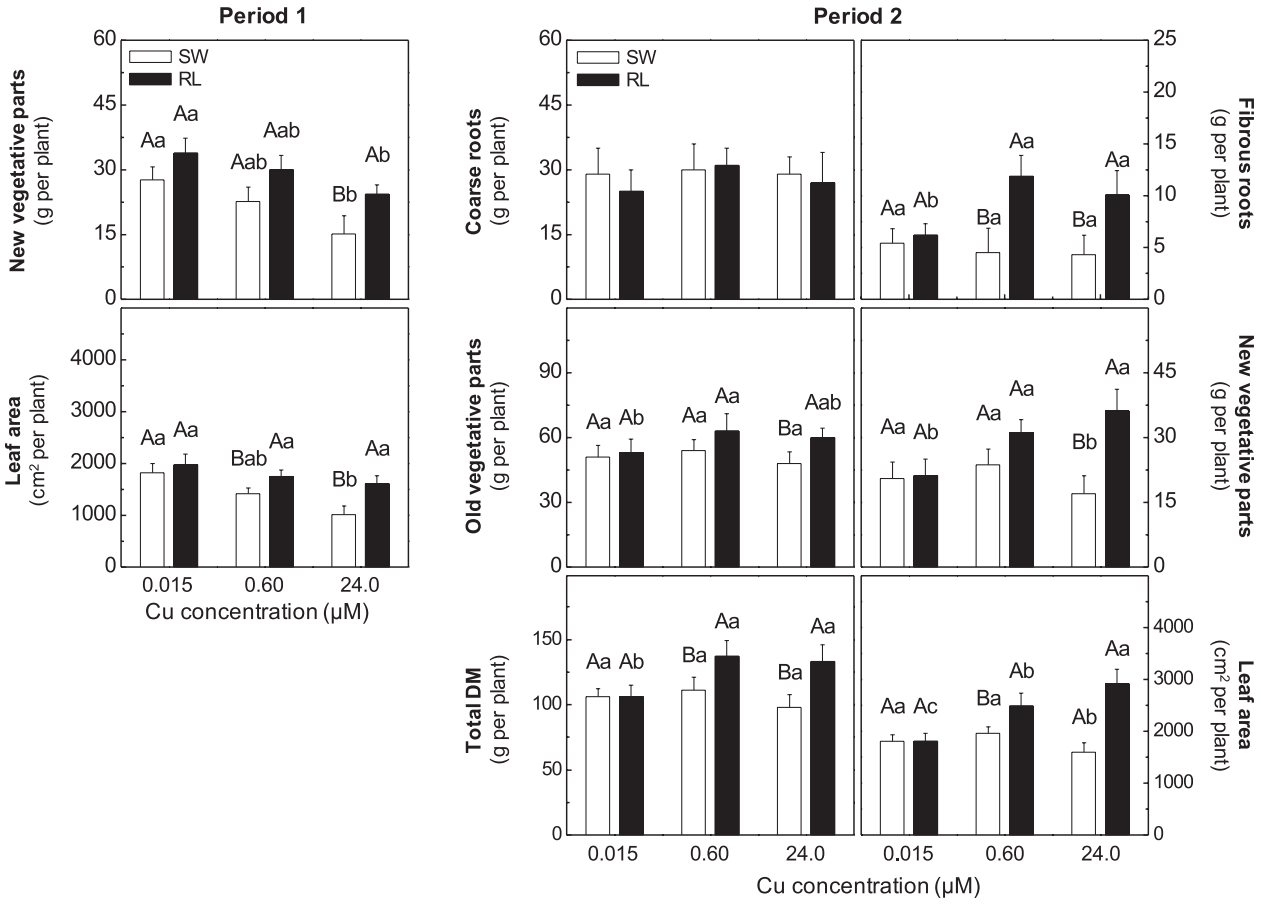

Fig. 1. Dry mass of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different copper (Cu) levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Coarse roots >3 mm Ø; fibrous roots ≤3 mm Ø; Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

$P < 0.01$). On the other hand, we observed the opposite for fibrous roots: 5.1 mg of Fe for RL and 3.3 mg of Fe for SW (Fig. 4).

Leaf gas exchange and chlorophyll fluorescence in citrus plants during and after Cu-induced stress

In the first period, the photosynthetic rate (P_N), stomatal conductance (g_S), apparent ETR, and instantaneous carboxylation efficiency (P_N/C_i) were higher in trees grown in 0.60 μM Cu, especially those grafted onto RL (Fig. 5). The internal CO_2 concentration (C_i) varied with Cu concentrations only in trees grafted onto RL, with the highest C_i being in those grown in 24.0 μM Cu (225 μmol mol^{-1}) and the lowest grown in 0.60 μM Cu (155 μmol mol^{-1}; Fig. 5). In period 2, trees grafted onto SW and grown in nutrient solution containing 0.015 or 0.60 μM Cu were more sensitive to the limitation of the nutrient than those grafted onto RL, as verified by the lower values of P_N, g_S and P_N/C_i, and the highest values of ETR/P_N (Fig. 5). Plants grafted onto RL and grown in 24.0 μM Cu presented similar values of P_N, g_S, and C_i in period 1 and period 2, while trees grafted onto SW had increased values after the period 1 (Fig. 5).

In period 1, no differences in the potential quantum yield of PSII (F_V/F_M), or for photochemical (qP) or qNP (Fig. 6) were monitored for varying Cu concentrations. Nevertheless, the effective quantum yield of PSII ($\Delta F/F'_M$) was lower in plants grown either at the lowest or at the highest Cu concentration in the nutrient solution (Fig. 6). Furthermore, the AEF were higher in trees grafted onto SW grown in both 0.015 or 24.0 μM Cu compared to those grown in 0.60 μM Cu, whereas the AEF for trees grafted onto RL was only higher in 0.15 μM Cu, compared to the other Cu concentrations (Fig. 6). In the second period, plants grown in 24.0 μM Cu exhibited higher values of $\Delta F/F'_M$, qP and qNP, mainly when compared to those grown in 0.015 μM Cu (Fig. 6), in which trees grafted onto RL with the lowest Cu concentration exhibited lower qP than those grafted onto SW (Fig. 6).

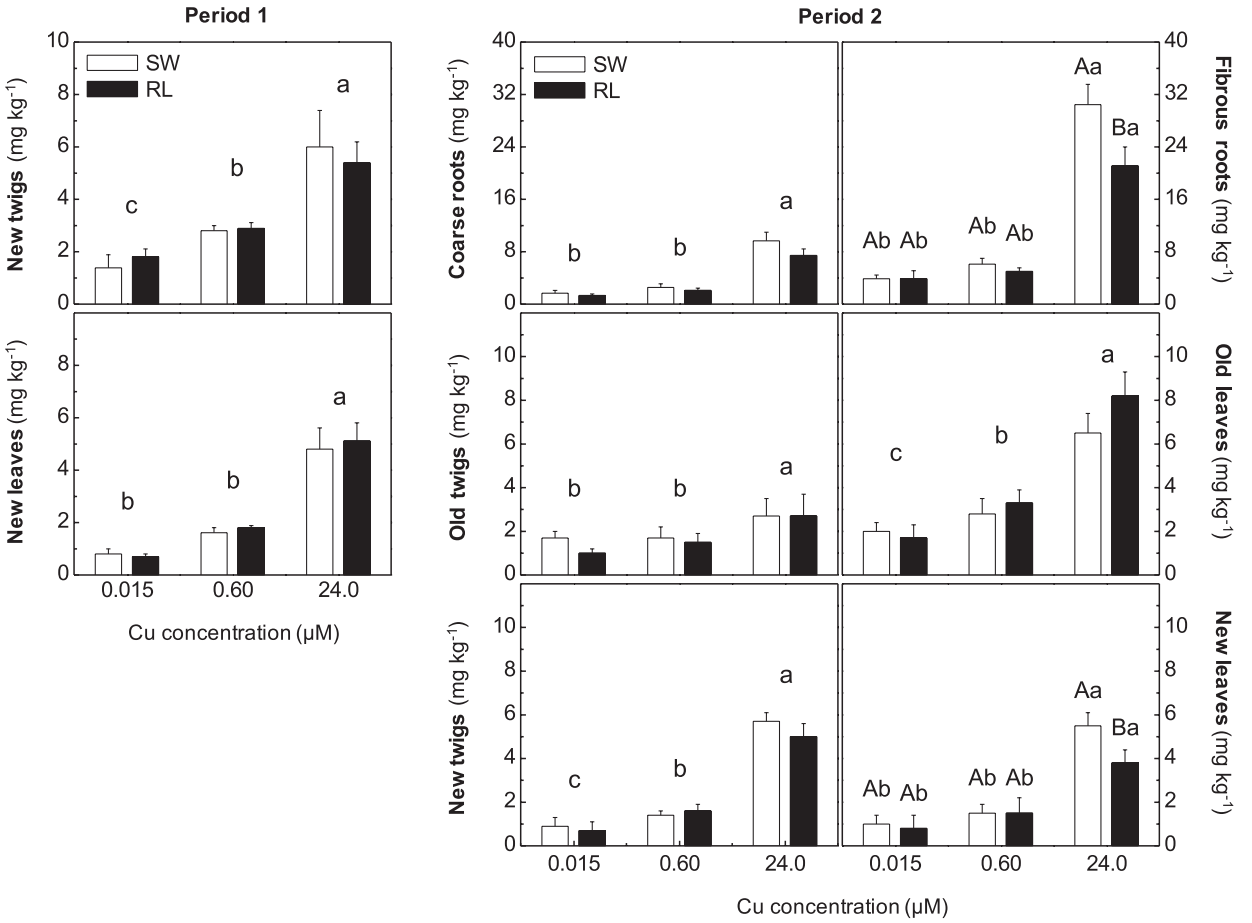

Fig. 2. Copper (Cu) concentrations in young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Coarse roots >3 mm Ø; fibrous roots ≤3 mm Ø; Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

The highest value of AEF was observed in plants grafted onto SW grown in the lowest Cu concentration, whereas those grafted onto RL had an AEF value only higher when trees were grown in 24.0 μM Cu (Fig. 6).

Oxidative stress levels induced by Cu-stress and antioxidant enzyme responses of citrus rootstocks

The H_2O_2 and MDA contents in the first period were higher in leaves and roots of plants grown in 24.0 μM Cu; this was also observed at a lower intensity in plants grown in solution with low availability of Cu (0.015 μM Cu) compared to those grown in solution with adequate Cu concentration (Fig. 7). In period 2, plants grafted onto SW grown in the lower and higher Cu concentrations presented similar H_2O_2 and MDA contents than during period 1, while the H_2O_2 concentration in leaves and roots and MDA in leaves of plants grafted onto RL was reduced when the Cu supply in the nutrient solution was suspended (Fig. 7). However, plants grown in 0.015 μM Cu, for both rootstocks, presented an increase in H_2O_2 levels in roots and MDA in leaves (Fig. 7).

The activity of the SOD isoforms differed between the Cu concentrations and rootstocks, both in the leaves and the roots (Fig. 8). Moreover, the Cu/Zn-SOD activity in the leaves increased according to the Cu concentration in solution. For orange trees grafted onto SW, an increase in activity was observed for the isoform Cu/Zn-SOD III, while for trees grafted onto RL, both Cu/Zn-SOD II and III increased activity (Fig. 8A). Seven isoforms of SOD were identified in roots of both rootstock genotypes: three Fe-SOD, two Mn-SOD and two Cu/Zn-SOD (Fig. 8). Increases in the activity of Cu/Zn-SOD, especially the Cu/Zn-SOD II in both rootstocks, were correlated with increases of Cu concentration in the nutrient

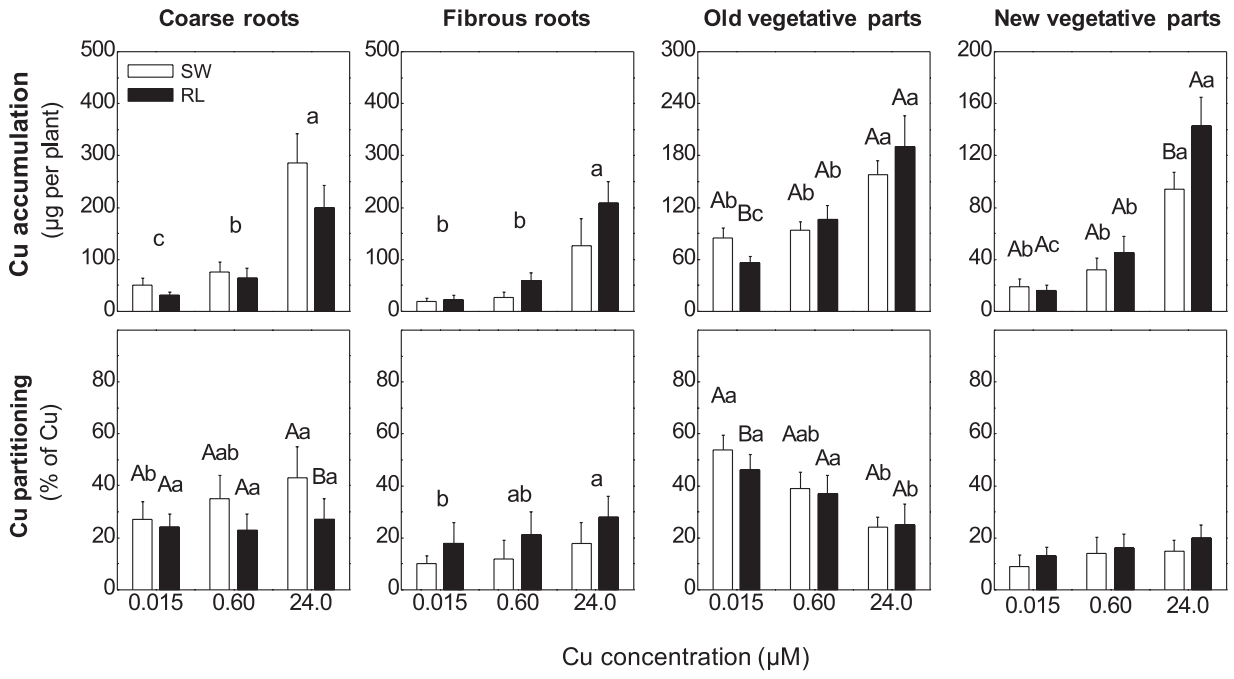

Fig. 3. Accumulation and partitioning of copper (Cu) in sweet orange trees grafted onto SW or RL at the end of period 2 (after 140 days without Cu supply). Coarse roots >3 mm Ø; fibrous roots ≤3 mm Ø; Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

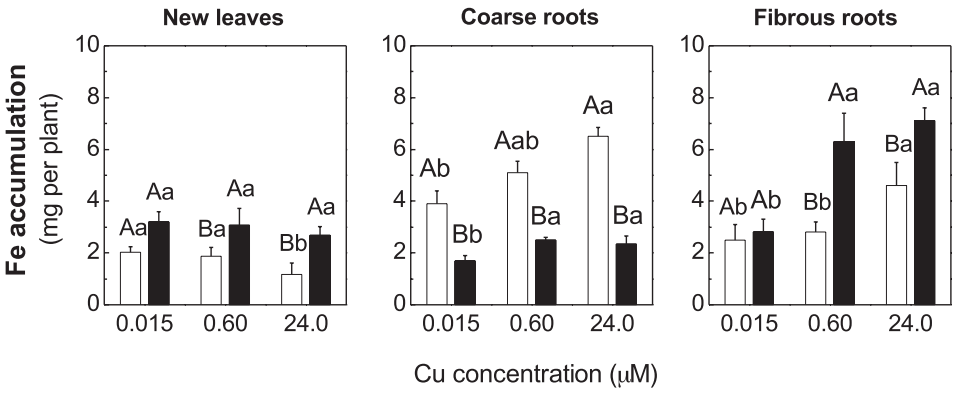

Fig. 4. Accumulation of iron (Fe) in new leaves, course roots and fibrous roots of sweet orange trees grafted onto SW or RL at the end of period 2 (after 140 days without Cu supply). Coarse roots >3 mm Ø; fibrous roots ≤3 mm Ø; Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

solution (Fig. 8). On the contrary of what observed for Cu/Zn-SOD, the Fe-SOD activity in roots was reduced with increased Cu concentration in the nutrient solution (Fig. 8B). In period 2, the SOD activity was reduced in leaves and roots compared to the first period (Fig. 8C, D). Trees grown in 0.015 µM Cu in the nutrient solution exhibited higher activities of Mn-SOD I and II isoforms in leaves independently of the rootstocks used (Fig. 8). Furthermore, the activity of Cu/Zn-SOD III was observed only in SW-trees grown in 0.015 µM Cu and RL-trees grown in 24.0 µM Cu (Fig. 8C). In roots, the activity of Cu/Zn-SOD increased with Cu concentrations in the first period, and this was higher for trees grafted onto RL (Fig. 8). Trees grafted onto SW exhibited higher activities of Mn-SOD I and II than those grown in 0.015 and 24.0 µM Cu, while in trees grafted onto RL, enzyme activity was only detected for the Mn-SOD II for all Cu treatments (Fig. 8).

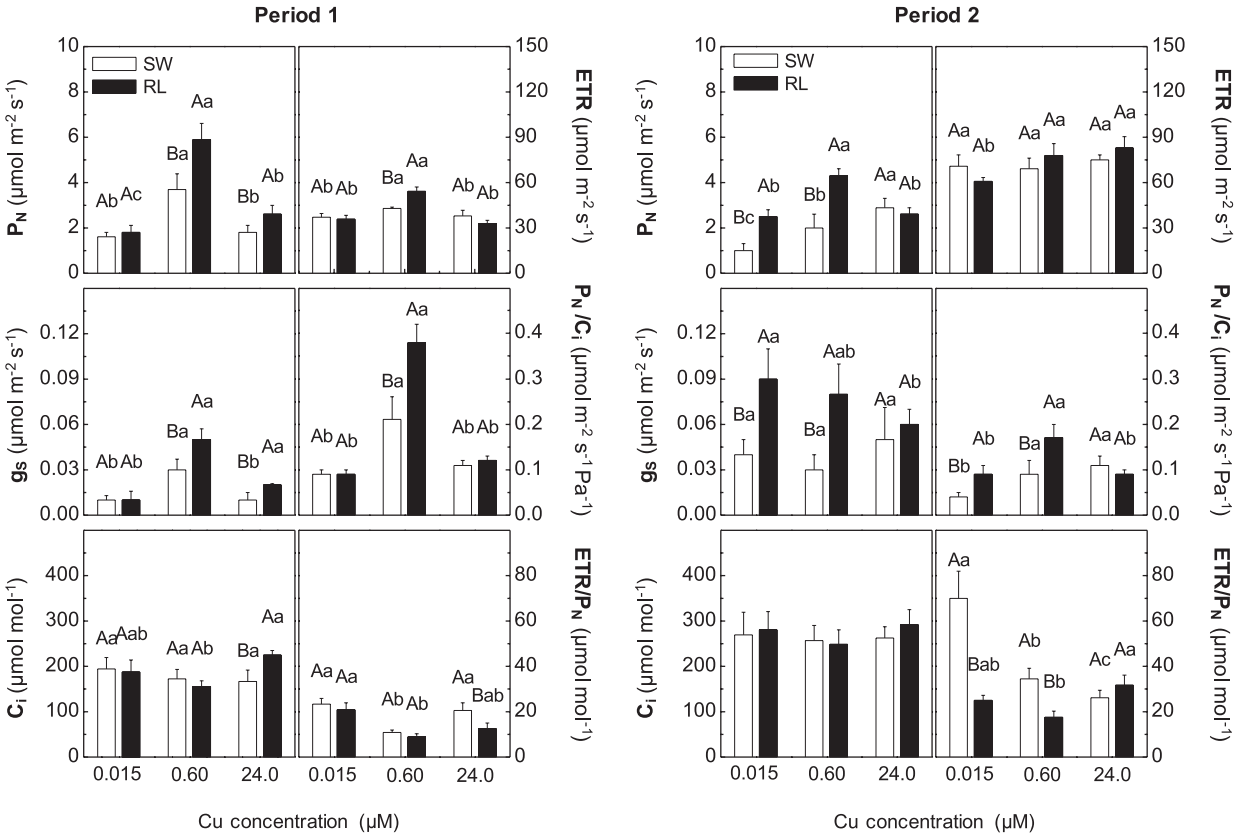

Fig. 5. Photosynthetic rate (P_N), stomatal conductance (g_S), internal CO_2 concentration (C_i), apparent ETR, instantaneous carboxylation efficiency (P_N/C_i) and ratio between ETR and P_N (ETR/P_N) in leaves of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

The CAT activity in leaves and roots in period 1 was higher when plants were grown in the lowest and highest Cu concentrations (Fig. 9). However, with the highest concentration of Cu, the greatest CAT activity was verified in leaves of RL and in roots of SW (Fig. 9). In period 2, CAT activity in leaves of trees grafted onto RL and grown in 24.0 μM Cu was higher than those grafted onto SW (Fig. 9). In the roots, the activity of CAT varied only in trees grafted onto RL that were grown with the highest concentration of Cu (Fig. 9).

For both rootstocks in the first period, the APX activity in leaves was higher in plants grown in 24.0 μM Cu, followed by those grown in 0.015 μM Cu, while APX activity increased in roots only at the highest concentration of Cu (Fig. 9). In period 2, trees grafted onto SW showed no variation in the activity of APX, while RL-trees had an increased APX activity in the leaves when grown in solution with the highest concentration of Cu and a higher activity in roots when grown in either 0.015 or 24.0 μM Cu (Fig. 9).

Discussion

Excess Cu directly damages the integrity of cellular ultrastructure in citrus trees (Zambrosi et al. 2013), and responses of antioxidant enzyme activities in plant leaves are dependent on the rootstock genotypes used (Hippler et al. 2016). In this study, we determined the effect of rootstocks with different horticultural characteristics and nutritional demand (Mattos Jr et al. 2010, Zambrosi et al. 2013) on responses of the photosynthetic rate, antioxidant activity and redistribution of metals within the vegetative parts of citrus trees exposed to Cu concentrations in two growth periods (Fig. 10). In period 1, trees were grown in low, medium or high concentrations of Cu in the nutrient solution (period 1), and the main effects of excess Cu were characterized. In the second period, when the same trees were grown without Cu addition in the nutrient solution (period 2), metal redistribution to new plant parts was assessed, and the effects of Cu deficiency were most pronounced

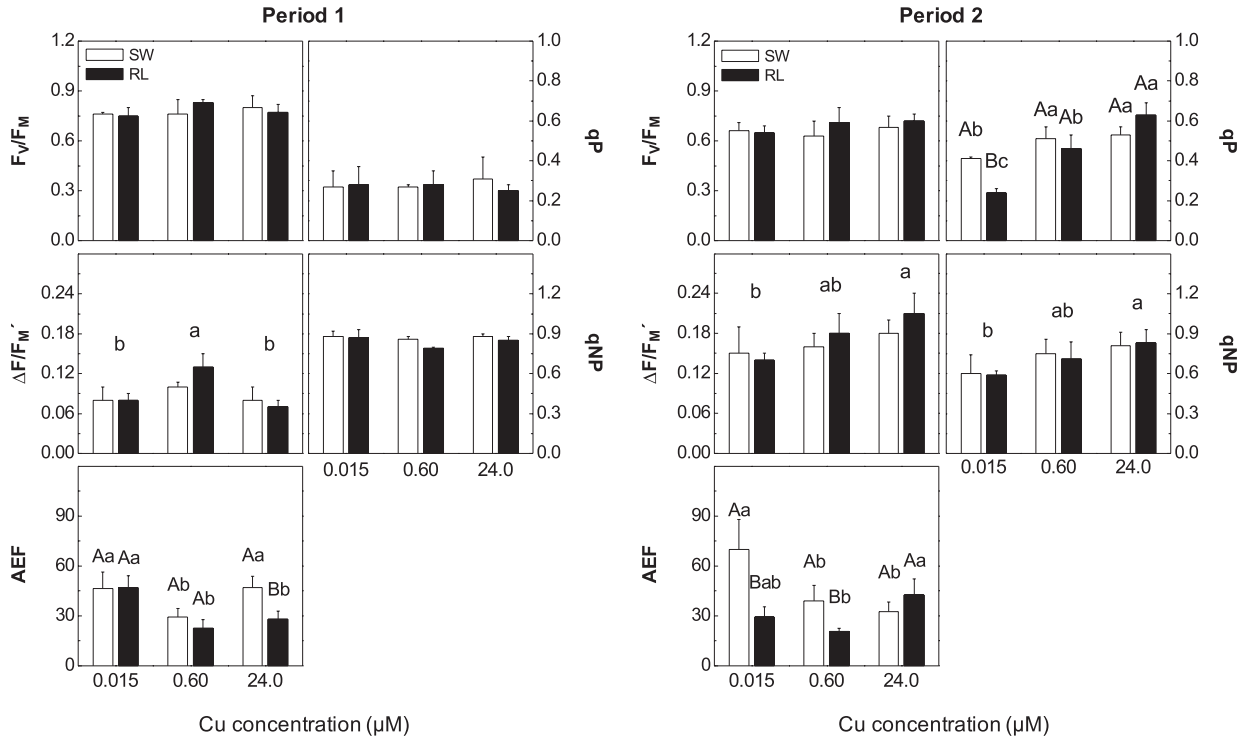

Fig. 6. The potential (F_V/F_M) and effective ($\Delta F'/F_M'$) quantum yield of PSII, the AEF, and the photochemical (qP) and q_NP in leaves of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution or after an additional 140 days (period 2) without Cu supply. Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

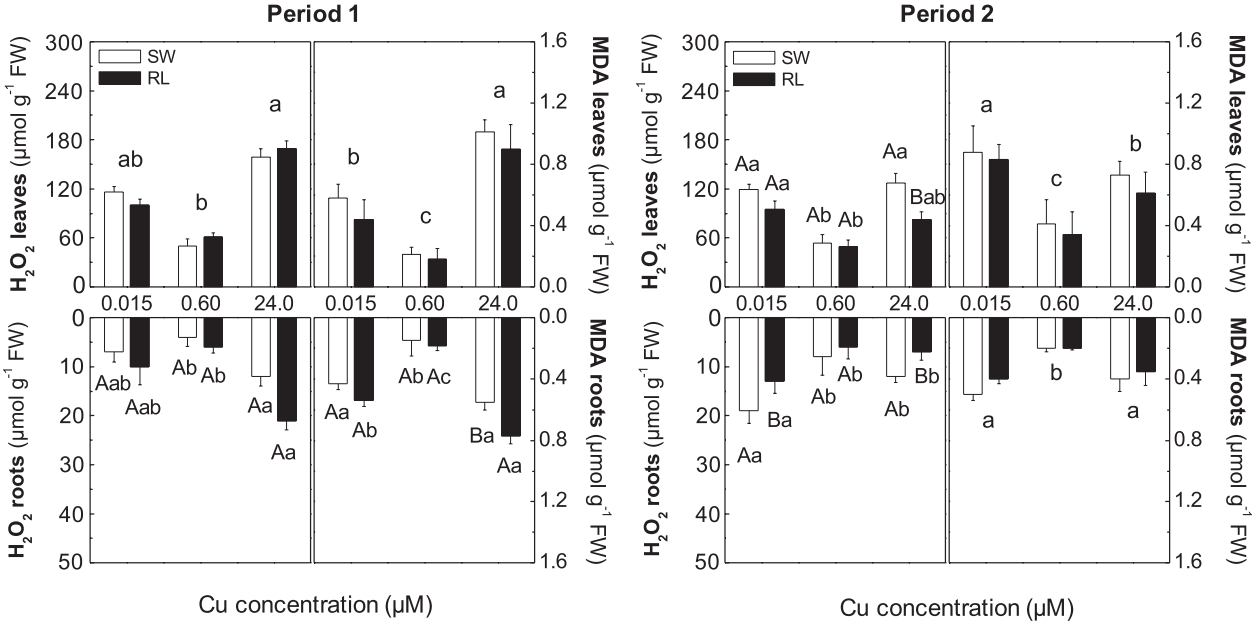

Fig. 7. Hydrogen peroxide (H_2O_2) and MDA in leaves and roots of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

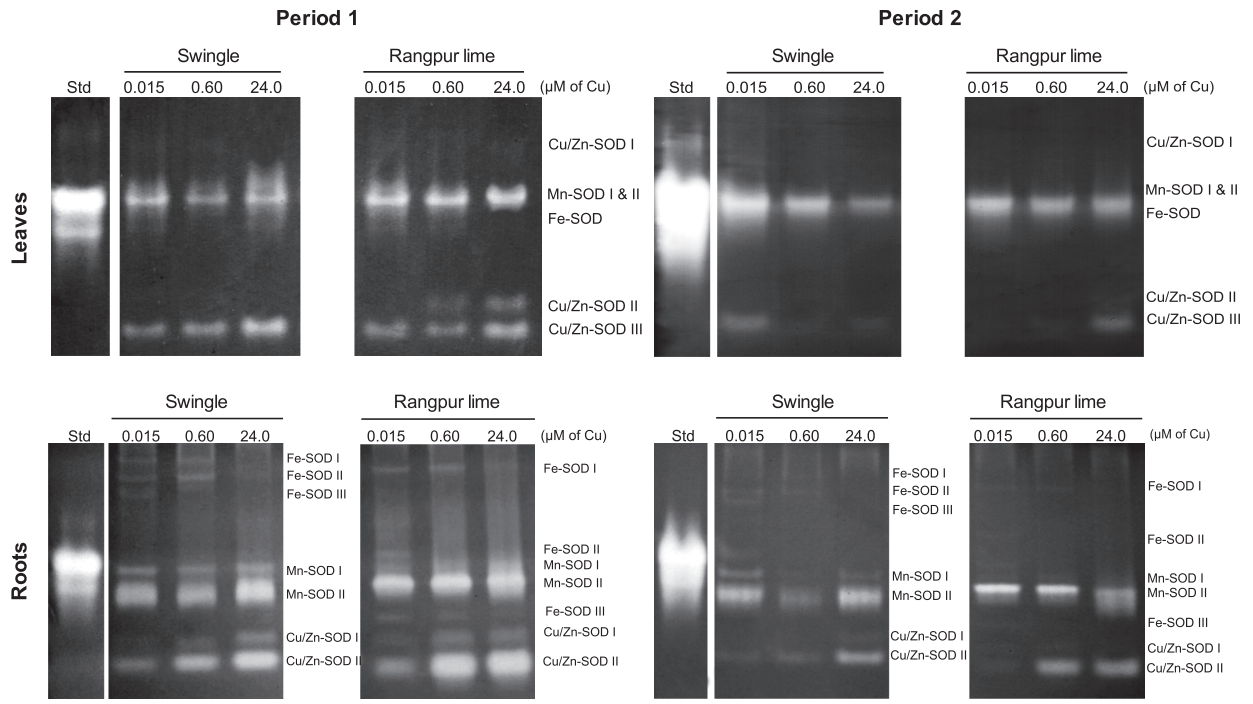

Fig. 8. SOD activity in PAGE electrophoresis (12%) in leaves and roots of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Std, bovine SOD standard.

because of the extended period with limited nutrient availability (Fig. 10).

Plant growth, Cu uptake, distribution and redistribution

Plants grown on the lowest and highest concentrations of Cu decreased in plant biomass (Fig. 1) because of direct damage caused to photosynthesis (Fig. 5). Although trees grafted onto SW exhibited lower variation in biomass production compared to those grafted onto RL in period 1 (Fig. 1), trees grafted onto RL grown in 24.0 μM Cu exhibited higher recovery of growth of new vegetative flushes in period 2, when plants were grown without the Cu supply in the nutrient solution (Fig. 1). On the other hand, trees grown in 0.015 μM Cu exhibited lower biomass production of new vegetative flushes and lower leaf area at the end of period 2, compared to those grown in medium and high Cu concentrations (Fig. 1), which was probably because of the lower nutrient reserve in these trees that reduced P_N (Fig. 5).

The accumulation of Cu occurred mainly in the roots, even after a period without the nutrient supply (period 2); this ranged from 40 to 60% of total nutrient in the plants grown in 0.015 and 24.0 μM Cu, respectively (Fig. 3). Up to 80% of Cu in plants is likely partitioned to the roots when Cu supply is not suspended, as reported previously (Hippler et al. 2016, 2018a). The rootstocks accumulated different levels of Cu between coarse and fibrous roots, with a main accumulation in the coarse roots for trees grafted onto SW, while Cu accumulation was evenly distributed within each root class for trees grafted onto RL (Fig. 3). Apple trees exposed to a high Cu level showed that Cu mobility to the canopy was most limited by accumulation of Cu in fibrous roots (Wang et al. 2016). In our study, trees grafted onto RL produced more fibrous roots than those grafted onto SW (Fig. 1) and the former consequently accumulated more Cu (Fig. 3), which could be related with superior capacity of these plants to recover plant growth after an initial condition of excess Cu (Fig. 1).

Even though roots limited Cu transport to the canopy (Fig. 2), as a tolerance mechanism (Hippler et al. 2016, 2018b), the root system also represented the main endogenous pool of this nutrient for the new vegetative flush growth (Fig. 3). Copper redistributed to the new vegetative parts represented 10–15% of the basal Cu within the plant (Fig. 3). Copper redistribution from the old organs to the new vegetative organs is most likely to occur in citrus trees when they are well supplied with the nutrient at the root level. Therefore, considering the low mobility of metal micronutrients in the phloem of woody plants (Hippler et al. 2018b), our work emphasizes the

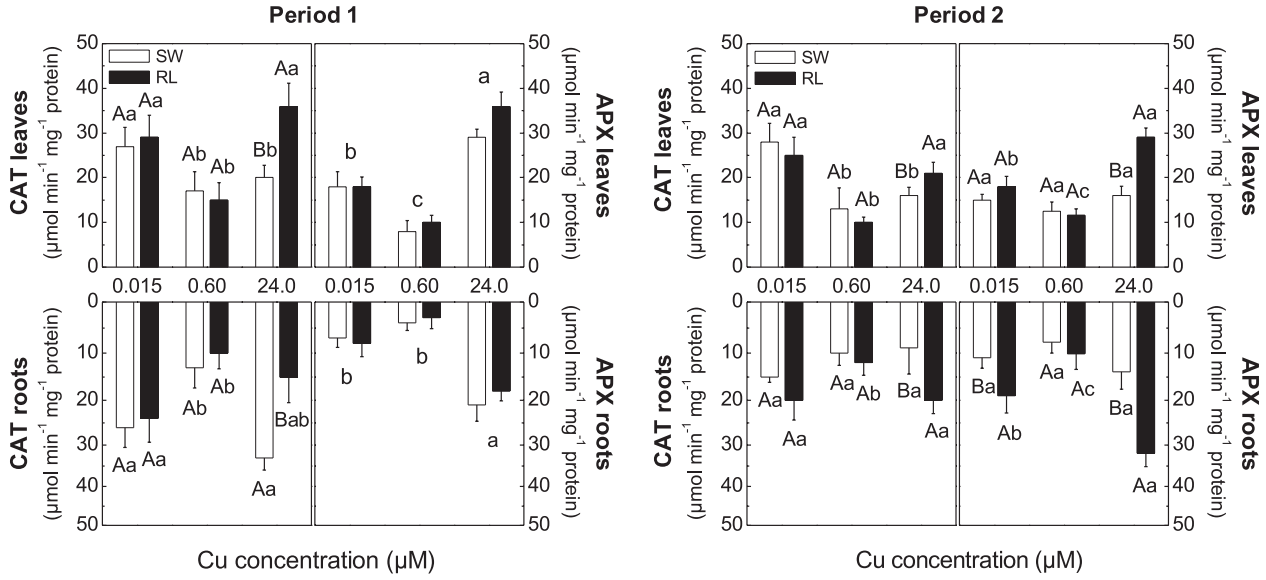

Fig. 9. CAT and APX activities in leaves and roots of young sweet oranges grafted onto SW or RL after 110 days (period 1) with different Cu levels in the nutrient solution and after an additional 140 days (period 2) without Cu supply. Cu rates and rootstocks means followed by different lowercase or uppercase letters, respectively, are significantly different by the Tukey test ($P < 0.05$).

importance of an adequate soil management plan to produce well-nourished plants by supplying nutrients not only to the canopy but also to the roots.

Gas exchange measurements and photochemistry activity

In this work, ETR was more affected in period 1 compared to period 2 (Fig. 5). Under Cu deficiency or toxicity conditions, the electron transport between the PSs is most affected (Tanyolaç et al. 2007, Hippler et al. 2018a). Metal toxicity causes the photo-oxidation and dissipation of electrons, resulting in excessive formation of ROS (Gururani et al. 2015). We monitored high levels of ROS in PSII, after impairment of the electron transport from PSII to PSI, which would further affect the integrity of chlorophyll and photosynthetic activity of plants (Gururani et al. 2015).

In period 1, the reduction of g_S was followed by a decrease in P_N, both caused by the Cu-induced stress (Fig. 5). However, in period 2, the reduction of P_N was possibly not caused by the control of stomatal opening, because no change in g_S and C_i were verified for different Cu levels (Fig. 5). Damage to the antenna complex in PSII, which is responsible for receiving photons and converting it into energy for photosynthetic machinery (Baker et al. 2007), likely occurred, as suggested by the reduction of P_N/C_i and the lower integrity of chlorophyll *a* (Fig. 6).

At the end of period 2, trees grafted onto SW exhibited greater sensitivity of photosynthesis to Cu deficiency, as indicated with lower values of P_N and P_N/C_i in 0.015 and 0.60 μM Cu, compared to those grafted onto RL (Fig. 5). Moreover, the reduction of P_N and the constant values of ETR indicated that the number of electrons (e^-) per CO_2 molecule assimilated was higher in SW grown in low Cu levels (ETR/P_N; Fig. 5). In this case, the e^- excess causes the increase of ROS formation and consequently reduces the integrity of the photosynthetic apparatus. The highest values of AEF found in trees grafted onto SW grown in 0.015 μM in both periods and those grafted onto RL grown in 0.015 μM Cu in period 1 and 24.0 μM Cu in period 2 (Fig. 6) suggest that a greater proportion of electrons was directed to other sinks, such as photorespiration, Mehler's reaction or nitrogen metabolism, instead of the photosynthetic process per se (Miyake 2010). To reduce photo-oxidative damage and to dissipate excess energy (e^-) into heat, plants have a qNP system, which reduces the concentration of the excited state of chlorophyll (Yruela 2013, Gururani et al. 2015). Variations of qP and qNP were observed in period 2 in trees grown in 0.015 μM Cu, suggesting that the photoprotective system effectively reduced the damage to PSII only under the lowest level of Cu. However, the increase in qP observed only in trees grafted onto SW grown in 0.015 μM Cu (Fig. 6) indicated a less efficient absorption of photons during photosynthesis (Yruela 2013), because more fluorescence was quenched by the photochemical process.

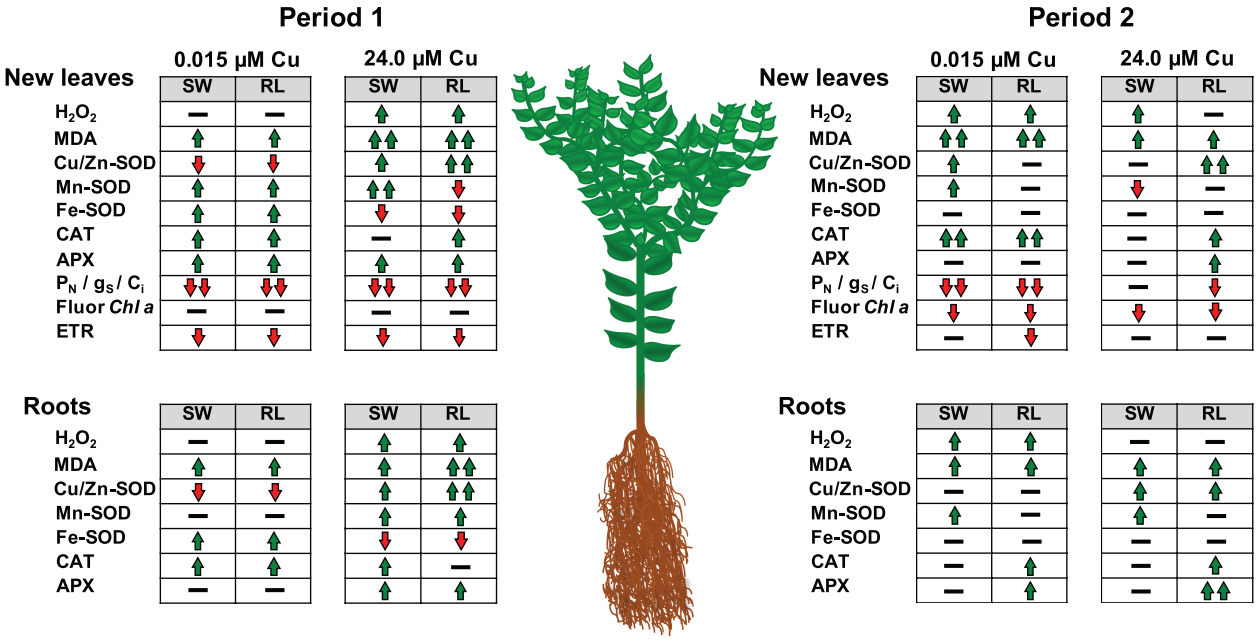

Fig. 10. General view of the antioxidant enzymatic system in leaves and roots and the photosynthetic apparatus in leaves of young sweet orange plants, grafted onto two contrasting rootstocks (SW or RL) and grown in three different Cu concentrations (low, 0.015 µM; medium, 0.60 µM; high, 24.0 µM Cu) in period 1 and without Cu supply in period 2. Arrows represent upregulated (up arrow), downregulated (down arrow) or no alteration (horizontal bar) from the control plants (0.60 µM Cu). APX, ascorbate peroxidase; CAT, catalase; Cu/Zn-SOD, Mn-SOD and Fe-SOD, isoforms of SOD; ETR, apparent electron transport rate; Fluor *Chl a*, fluorescence of chlorophyll *a* measurements; H_2O_2, hydrogen peroxide content; MDA, lipid peroxidation; $P_N/g_S/C_i$, leaf gas exchange measurements.

This decreased the production of photoassimilates necessary to achieve highest yield capacity (Syvertsen and Garcia-Sanchez 2014).

ROS and antioxidant enzyme system

The nutritional disorders caused either by deficiency or excess of Cu in plants promoted ROS accumulation, as indicated by the H_2O_2 and MDA levels observed in leaves and roots (Fig. 7). Accumulation of ROS, such as OH^- and H_2O_2, in plant tissues increases MDA content, and consequently the degradation of membranes and biomolecules (Piotto et al. 2014, Choudury et al. 2016). Accumulation of H_2O_2 and MDA was higher in trees exposed to the highest concentration of Cu in period 1 (Fig. 7). However, in period 2 the trees grafted onto SW exhibited higher H_2O_2 concentrations in roots when grown on low metal concentration. This demonstrated greater sensitivity of this rootstock for the Cu-limited condition (Fig. 7). On the other hand, trees grafted onto RL exhibited a greater detoxification ability of ROS in the second period (Fig. 7), which was demonstrated by the increased activities of CAT and APX in both leaves and roots (Fig. 9). This greater efficiency in the elimination of ROS may also be related to the increased production of fibrous roots by RL (Fig. 1). These roots are more active when compared to the coarse roots because of the higher absorption and assimilation of nutrients or other organic compounds, and enzymatic activity (Wang et al. 2016).

In the case of ROS production, SOD activity was likely important in minimizing damage caused by either deficiency or excess of Cu, especially because the activities in leaves and roots of the diverse isoforms were shown to be dependent on the citrus rootstock, with the main differences being observed in Mn-SOD and Cu/Zn-SOD activities (Fig. 8). Although Cu/Zn-SOD activity has been considered dispensable for photosynthetic activity and the development of *Arabidopsis thaliana* after exposure to high concentrations of Cu and light intensity (Cohu et al. 2009), this isoform was shown to be important in the recovery of citrus plants to an excess condition of the metal, particularly for those grafted onto RL (Fig. 8). In leaves of citrus trees, the activity of Cu/Zn-SOD was more responsive to the different supply of Zn sources, while Mn-SOD and Fe-SOD slightly varied with different supplies of Mn (Hippler et al. 2015).

Under abiotic stress conditions, the balance of the activities of SOD, APX and CAT in plants is essential for determining the levels of $O_2^{\bullet-}$ and H_2O_2 as well as other ROS, because a compensatory mechanism is needed if the balance of protective enzymes is modified (Azevedo

et al. 1998, Prasch and Sonnewald 2015, Choudury et al. 2016). In this context, H_2O_2 scavenging was carried out by the activity of both CAT and APX in period 1 (Fig. 9). CAT activity was then regulated by the citrus rootstock: the activity increased in leaves of trees grafted onto RL and roots of SW (Fig. 9). In this case, the CAT activity in leaves of RL at higher Cu concentration appeared to be more effective to maintain the photosynthetic activity, as indicated by the largest values of P_N, g_S and C_i (Fig. 5).

In period 2, trees grafted onto RL exhibited an increase in the activities of CAT of roots and APX of leaves and roots when grown in 24.0 μM Cu allowing greater plant regrowth than those grafted onto SW (Figs 1 and 10). Similar to citrus trees, increased activities of SOD followed by APX in sugarcane were important to prevent damages to the photosynthetic machinery of plants under drought stress (Sales et al. 2013). This emphasizes the importance of understanding the balance between activities of APX and CAT enzymes, and possibly other peroxidases not analyzed in this study, which generally have a different role in the elimination of H_2O_2 (Gratão et al. 2015, Anjum et al. 2016, Hippler et al. 2018a).

Copper and iron interactions in citrus trees

Although three Fe-SOD isoforms were identified in roots, no activity was observed in trees grown in 24.0 μM Cu in period 1 (Fig. 8). Plants exposed to high levels of metals such as Cu (Lequeux et al. 2010, Mattos Jr et al. 2010), Zn (Kendziorek et al. 2014), nickel (Ni) (Kuhmar et al. 2015) or cadmium (Cd) (Gratão et al. 2015) has shown decreases in Fe levels of shoots/leaves and increase in roots. In this case, Fe is likely to become unavailable to plant growth, as confirmed by the reduction in the Fe-SOD activity of both rootstocks, even given the greater accumulation of nutrients in roots when plants were grown in the highest concentration of Cu (Fig. 8).

At the end of period 2, the redistribution of Fe from roots to canopy in trees that were grown in 24.0 μM Cu was greater in plants grafted onto RL than SW (Fig. 4). Plants have mechanisms that may limit the distribution of heavy metals in vegetative parts (Hall 2002), with histidine and nicotianamine identified to be important Cu-chelators in *Brassica carinata* (Irtelli et al. 2009) and with phytochelatins for Cd in citrus seedlings (López-Climent et al. 2014). On the other hand, tomato plants overexpressing *AtHMA4*, a Zn export protein involved in the control of the root-to-shoot metal translocation, showed limited Fe translocation from roots (Kendziorek et al. 2014). Therefore, chelating or transport limiting mechanisms would likely reduce Fe activity in the cells more significantly in trees grafted onto SW. The chelation probably mainly occurs in citrus roots, where it primarily accumulates (Figs 2 and 3).

In conclusion, our data indicate that rootstocks regulate root-to-shoot communication under and after stress caused by Cu nutritional disorders. Regardless of the rootstock, citrus trees are sensitive to Cu excess. However, the trees grafted onto RL exhibited higher antioxidant enzymes activities in both roots and leaves upon Cu toxicity, which resulted in a greater integrity of the PS, a better assimilation of CO_2 and a better plant growth. Even though trees grafted onto SW exhibited lower efficiency of the antioxidant enzymatic system, this rootstock likely limited the transport and redistribution of Cu from the roots more than RL, which also limited Fe transport to the plant canopy. Because both rootstock genotypes of citrus used in this study accumulate more Cu in roots to maintain Cu homeostasis, redistribution of this metal from roots to new vegetative flushes is critical for plant growth under low availability of Cu. This emphasizes the importance of an enhanced nutritional management with adequate Cu supply to roots to support plant nutrient demand in new growth parts.

Author contributions

F. W. R. H., D. M. J., J. A. Q. and R. M. B. designed the study. F. W. R. H. performed the experiment and laboratorial analysis. F. W. R. H. and V. L. D. performed photosynthetic evaluations. F. W. R. H. analyzed the data and F. W. R. H. and D. M. J. wrote the manuscript. R. M. B., R. A. A., V. L. D. and L. E. W. revised the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements – The authors thank São Paulo Research Foundation (FAPESP, grant #2015/13572-8 and #2012/13917-7). We also thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), which granted R. M. B., R. A. A. and D. M. J. fellowships.

References

Alexieva V, Sergiev I, Mapelli E, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and trees markers in pea and wheat. Plant Cell Environ 24: 1337–1344

Anjum NA, Sharma P, Gill SS, Hasanuzzaman M, Khan EA, Kachhap K, Mohamed AA, Thangavel P, Devi GD, Vasudhevan P, Sofo A, Khan NA, Misra AN, Lukatkin AS, Singh HP, Pereira E, Tuteja N (2016) Catalase and ascorbate peroxidase – representative H_2O_2-detoxifying heme enzymes in plants. Environ Sci Pollut Res 23: 19002–19029

Azevedo RA, Alas RM, Smith RJ, Lea PJ (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air ozone fumigation, in leaves and roots of wild-type and catalase-deficient mutant of barley. Physiol Plant 104: 280–292

Baker NR, Harbinson J, Kramer DM (2007) Determining the limitations and regulation of photosynthetic energy transduction in leaves. Plant Cell Environ 30: 1107–1125

Bataglia OC, Furlani AMC, Teixeira JPF, Furlani PR, Gallo JR (1983) Método de análise química de Plantas. Instituto Agronomico, Campinas

Beauchamp CH, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287

Behlau F, Fonseca AE, Belasque Jr J (2016) A comprehensive analysis of the Asiatic citrus canker eradication programme in São Paulo state, Brazil, from 1999 to 2009. Plant Pathol 65: 1390–1399

Borges KLR, Salvato F, Alcântara BK, Nalin RS, Piotto FÂ, Azevedo RA (2018) Temporal dynamic responses of roots in contrasting tomato genotypes to cadmium tolerance. Ecotoxicology 27: 245–258

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principles of protein dye-binding. Anal Biochem 72: 248–254

Choudury FK, Rivero RM, Blumwald E, Mittler R (2016) Reactive oxygen species, abiotic stress and stress combination. Plant J 90: 856–867

Cohu C, Abdel-Ghany SE, Reynolds KAG, Onofrio AM, Bodecker JR, Krimbel JA, Niyogi KK, Pilon M (2009) Copper delivery by the copper chaperone for chloroplast and cytosolic copper/zinc-superoxide dismutases: regulation and unexpected phenotypes in an *Arabidopsis* mutant. Mol Plant 2: 1336–1350

Cuypers A, Karen S, Jos R, Kelly O, Els K, Tony R, Nele H, Nathalie V, Suzy VS, Frank VB, Yves G, Jan C, Jaco V (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. J Plant Physiol 168: 309–316

Cuypers A, Hendrix S, Amaral dos Reis R, De Smet S, Deckers J, Gielen H, Jozefczak M, Loix C, Vercampt H, Vangronsveld J, Keunen E (2016) Hydrogen peroxide, signaling in disguise during metal phytotoxicity. Front Plant Sci 7: 470

Fan J, He Z, Ma LQ, Stoffella PJ (2011) Accumulation and availability of copper in citrus grove soils as affected by fungicide application. J Soil Sediment 11: 639–648

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll. Biochim Biophys Acta 990: 87–92

Gratão PL, Monteiro CC, Tezotto T, Carvalho RF, Alves LR, Peters LP, Azevedo RA (2015) Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. Biometals 28: 803–816

Gururani MA, Venkatesh J, Tran LSP (2015) Regulation of photosynthesis during abiotic stress-induced photoinhibition. Mol Plant 8: 1304–1320

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53: 1–11

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189–198

Hippler FWR, Boaretto RM, Quaggio JA, Azevedo RA, Mattos Jr D (2015) Towards soil management with Zn and Mn: estimates of fertilization efficacy of *Citrus* trees. Ann Appl Biol 166: 484–495

Hippler FWR, Cipriano DO, Boaretto RM, Quaggio JA, Gaziola SA, Azevedo RA, Mattos Jr D (2016) *Citrus* rootstocks regulate the enzymatic and nutritional status and antioxidant system of trees under copper stress. Environ Exp Bot 130: 42–52

Hippler FWR, Boaretto RM, Dovis VL, Quaggio JA, Azevedo RA, Mattos-Jr D (2018a) Oxidative stress induced by Cu nutritional disorders in *Citrus* depends on nitrogen and calcium availability. Sci Rep 8: 1641

Hippler FWR, Petená G, Boaretto RM, Quaggio JA, Azevedo RA, Mattos-Jr D (2018b) Mechanisms of copper stress alleviation in Citrus trees after metal uptake by leaves or roots. Environ Sci Pollut Res 25: 13134–13146 https://link.springer.com/article/10.1007%2Fs11356-018-1529-x

Irtelli B, Petrucci WA, Navari-Izzo F (2009) Nicotianamene and histidine/proline are, respectively, the most importeat copper chelators in xylem sap of *Brassica carinata* under conditions of copper deficiency and excess. J Exp Bot 60: 269–277

Juraniec M, Lequeux H, Hermans C, Willems G, Nordborg M, Schneeberger K, Salis P, Vromant M, Lutts S, Verbruggen N (2013) Towards the discovery of novel genetic component involved in stress resistance in *Arabidopsis thaliana*. New Phytol 201: 810–824

Kendziorek M, Barabasz A, Rudzka J, Tracz K, Mills RF, Williams LE, Antosiewicz DM (2014) Approach to engineer tomato by expression of *AtHMA4* to enhance Zn in the aerial parts. J Plant Physiol 171: 1413–1422

Komárek M, Vaněk A, Chrastny V, Szakova J, Kubova K, Drahota P, Balik J (2009) Retention of copper originating from different fungicides in contrasting soil types. J Hazard Mater 166: 1395–1402

Kraus TE, McKersie BD, Fletcher RA (1995) Paclobutrazol-induced tolerance of wheat leaves to paraquat may involve increased antioxidant enzyme activity. J Plant Physiol 145: 570–576

Kuhmar P, Rouphael Y, Cardarelli M, Colla G (2015) Effect of nickel and grafting combination on yield, fruit quality, antioxidative enzyme activities, lipid peroxidation, and

mineral composition of tomato. J Plant Nutr Soil Sci 178: 848–860
Lequeux H, Hermans C, Lutts S, Verbruggen N (2010) Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. Plant Physiol Biochem 48: 673–682
López-Climent MF, Arbona V, Pérez-Clemente RM, Zandalinas SI, Gómez-Cadenas A (2014) Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. Plant Biol 16: 79–87
Martínez-Ballesta MC, Alcaraz-López C, Muriez B, Mota-Cadenas C, Carvajal M (2010) Physiological aspects of rootstock–scion interactions. Sci Hort 127: 112–118
Mattos Jr D, Ramos UM, Quaggio JA, Furlani PR (2010) Nitrogênio e cobre na produção de mudas de citros em diferentes porta-enxertos. Bragantia 69: 135–147
Miyake C (2010) Alternative electron flows (water–water cycle and cyclic electron flow around PSI) in photosynthesis: molecular mechanisms and physiological functions. Plant Cell Physiol 51: 1951–1963
Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22: 867–880
Piotto FA, Tulmann-Neto A, Franco MG, Boaretto LF, Azevedo RA (2014) Rapid screening for selection of heavy metal-tolerant plants. Crop Breed Appl Biotechnol 14: 1–7
Prasch CM, Sonnewald U (2015) Signaling events in plants: stress factors in combination change the picture. Environ Exp Bot 114: 4–14
Sales CRG, Ribeiro RV, Silveira JAG, Machado EC, Martins MO, Lagôa AMMA (2013) Superoxide dismutase and ascorbate peroxidase improve the recovery of photosynthesis in sugarcane plants subjected to water deficit and low substrate temperature. Plant Physiol Biochem 73: 326–336
Schreiber U, Bilger W, Neubauer C (1994) Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In: Schulze ED, Caldwell MM (eds) Ecophysiology of Photosynthesis. Springer, Berlin, pp 49–70
Syvertsen JP, Garcia-Sanchez F (2014) Multiple abiotic stresses occurring with salinity stress in citrus. Environ Exp Bot 103: 128–137
Tanyolaç D, Ekmekçi Y, Ünalan S (2007) Changes in photochemical and antioxidant enzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. Chemosphere 67: 89–98
Wang QY, Liu JS, Hu B (2016) Integration of copper subcellular distribution and chemical forms to understand copper toxicity in apple trees. Environ Exp Bot 123: 125–131
Yruela I (2013) Transition metals in plant photosynthesis. Metallomics 5: 1090–1109
Zambrosi FCB, Mesquita GL, Tanaka FAO, Quaggio JA, Mattos Jr D (2013) Phosphorous availability and rootstock affect copper-induced damage to the root ultra-structure of *Citrus*. Environ Exp Bot 95: 25–33
Zandalinas SI, Belfagón D, Arbona V, Gómez-Cadenas A (2017) Regulation of citrus responses to the combined action of drought and high temperatures depends on the severity of water deprivation. Physiol Plant 162: 427–438

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Diagram of experimental treatments that consisted on two evaluation periods.