

Mechanisms of copper stress alleviation in *Citrus* trees after metal uptake by leaves or roots

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Abstract

Nutritional disorders caused by copper (Cu) have affected citrus orchards. Since Cu is foliar sprayed as a pesticide to control citrus diseases, this metal accumulates in the soil. Thereby, we evaluated the effects of Cu leaf absorption after spray of different metal sources, as well as roots absorption on growth, nutritional status, and oxidative stress of young sweet orange trees. Two experiments were carried out under greenhouse conditions. The first experiment was set up with varying Cu levels to the soil (nil Cu, 0.5, 2.0, 4.0 and 8.0 g of Cu per plant as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), whereas the second experiment with Cu application via foliar sprays (0.5 and 2.0 g of Cu per plant) and comparing two metal sources ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ or Cu(OH)_2). Copper was mainly accumulated in roots with soil supply, but an increase of oxidative stress levels was observed in leaves. On the other hand, Cu concentrations were higher in leaves that received foliar sprays, mainly as Cu(OH)_2 . However, when sulfate was foliar sprayed, plants exhibited more symptoms of injuries in the canopy with decreased chlorophyll contents and increased hydrogen peroxide and lipid peroxidation levels. Copper toxicity was characterized by sap leakage from the trunk and twigs, which is the first report of this specific Cu excess symptom in woody trees. Despite plants with 8.0 g of Cu soil-applied exhibiting the sap leakage, growth of new plant parts was more vigorous with lower oxidative stress levels and injuries compared to those with 4.0 g of Cu soil-applied (without sap leakage). With the highest level of Cu applied via foliar as sulfate, Cu was eliminated by plant roots, increasing the rhizospheric soil metal levels. Despite citrus likely exhibiting different mechanisms to reduce the damages caused by metal toxicity, such as responsive enzymatic antioxidant system, metal accumulation in the roots, and metal exclusion by roots, excess Cu resulted in damages on plant growth and metabolism when the metal was taken up either by roots or leaves.

Keywords Copper-based pesticides · Toxicity symptoms · Sap leakage · Oxidative stress · Metal homeostasis

Introduction

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Copper (Cu) is a key element for citrus production either as a nutrient or a defensive indispensable for healthy management of groves affected by diseases that decrease fruit yield and quality (Behlau et al. 2010; Silva Jr. et al. 2016). Copper pesticides have been used to control citrus canker, citrus black spot, *Alternaria* brown spot, and postbloom fruit drop (Silva Jr. et al. 2016). Moreover, in the north of Paraná and São Paulo states, the major citrus fruit production regions in Brazil where more than 190 million trees are grown, the amounts of Cu-based products applied can reach 30 kg ha^{-1} of Cu once the incidence of citrus canker has exponentially increased in groves in the last few years (Behlau et al. 2017).

Copper hydroxide (Cu(OH)_2), copper oxychloride ($\text{ClCu}_2\text{H}_3\text{O}_3$), cupric oxide (CuO), cupric hydroxy carbonate

($\text{Cu}_2(\text{OH})_2\text{CO}_3$), and copper sulfate (CuSO_4) are the most efficient and commonly Cu-based pesticides used in the agriculture (Husak 2015). Since these fungicides work as a protective film, in general, the Cu is insoluble and these solutions may also contain some adjuvants that make the metal to stand under the leaf or fruit surfaces for long periods of time (Behlau et al. 2010; Husak 2015). However, part of this Cu applied as pesticides is taken up by trees, mostly through the leaves and depending on the product source (Smoleň 2012), while the remaining portion is washed out by rainfall from the plant canopy to the soil (Fan et al. 2011).

Copper does not present significant mobility along the soil profile (Komárek et al. 2009), mostly in those from the tropical regions that are often characterized by low pH and high contents of 1:1 clay types such as kaolinites and iron or aluminum oxides (Hippler et al. 2014), which minerals adsorb the metal to their colloidal surface (Fontes and Gomes 2003; Bradl 2004). Increased accumulation of Cu in the soil surface (0–30 cm of the soil depth, Fan et al. 2011) affects plant growth (Sonmez et al. 2006; Martins et al. 2014) and adversely impacts the environment (Komárek et al. 2009; Husak 2015). In a previous study, we demonstrated that citrus rootstocks regulate the enzymatic antioxidative system in leaves to alleviate stress caused by excess Cu in the canopy (Hippler et al. 2016). However, information about how Cu affects tree development under toxic levels when absorbed by either leaves or roots is scarce.

In this work, we hypothesized that Cu leaf sprayed results in lower metal amount absorbed by plants when compared to equivalent levels supplied to the soil, which the former still varies with the metal-based source used. Furthermore, Cu taken up in excess causes oxidative stress damages in leaves, once roots, seem to present an important homeostasis mechanism that reduces the metal activity in the plant (Mattos-Jr. et al. 2010; Hippler et al. 2016, 2018). Thereby, the aim of this study was to evaluate the effects of Cu in citrus young trees after leaf sprays and soil supply on plant growth, nutritional status, and in antioxidant enzymatic processes of young sweet orange trees.

Materials and methods

Two experiments were carried out in a greenhouse with 1-year-old sweet orange trees (*Citrus sinensis* (L.) Osbeck cv. Valencia), grafted onto Swingle citrumelo (*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.), grown in pots containing 20 dm³ of soil. A loamy fine sand soil (10% of clay, 3% of silt, and 87% of sand); pH (CaCl_2) of 4.6; and containing 13 g dm⁻³ of organic matter and 0.3 mg dm⁻³ of Cu (diethylenetriaminepentaacetic acid—

triethanolamine (DTPA-TEA) pH 7.3; van Raij et al. 2001) was used.

Same management treats were performed in both experiments during the initial stages of plant growth. Plant fertilization was conducted by applying 750 mL of solution fortnightly with the following concentrations: 136.0 mg KH_2PO_4 L⁻¹, 365.2 mg KNO_3 L⁻¹, 1019.4 mg $\text{Ca}(\text{NO}_3)_2$ L⁻¹, 114.7 mg CaCl_2 L⁻¹, 492.9 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ L⁻¹, 100 mg Fe-EDDHA L⁻¹, 5.1 mg H_3BO_3 L⁻¹, 3.6 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ L⁻¹, 8.0 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ L⁻¹, and 0.5 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ L⁻¹ (Hippler et al. 2015, with modifications). From the planting to the harvesting of the plants, these provided the following total amounts of nutrients: 17.9 g of N, 12.2 g of K, 3.4 g of P, 15.5 g of Ca, 4.9 g of Mg, 1.9 g of S, 1.0 g of Fe, 0.95 g of B, 0.65 g of Mn, 0.9 g of Zn, and 0.12 g of Mo.

The Cu treatments started after 120 days of planting into the pots, when the first vegetative flush was physiologically mature. The first experiment was composed by levels of Cu applied to the soil (0.5, 1.0, 2.0, 4.0, and 8.0 g per plant of Cu) as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The second experiment was set up in a 2 × 2 factorial design with two sources ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ or $\text{Cu}(\text{OH})_2$) and two levels of Cu (0.5 and 2.0 g per plant) applied via foliar weekly during the first 60 days and biweekly until the end of the experiment.

Adsorption isotherms of copper to the soil

Adsorption isotherms for Cu in the soil used in this study were characterized by the *batch* method (Sposito 1982; Hippler et al. 2014). A soil sample, air-dried, was sieved to pass a 10-mesh sieve. One gram of soil and 10 mL of 0.01 M CaCl_2 containing Cu, as chloride, in the follow concentrations: 0.25, 1.0, 4.0, 16, 64, and 256 mg L⁻¹ were added to 15-mL plastic flasks. The sets were shaken for 72 h in a horizontal shaker at 150 rpm and then centrifuged for 10 min at 905×g. Supernatants were filtrated to quantify Cu concentrations in a mass spectrometer coupled to a plasma source (ICP-OES, PerkinElmer, mod. Optima, Norwalk, USA). To estimate the amounts of the metal adsorbed on the soil, the following equation was used $[\text{]}_{\text{ads}} = [V_{\text{solution}} \times (C_0 - C_e)] / M_{\text{soil}}$; in which $[\text{]}_{\text{ads}}$ is the metal adsorbed (mg kg⁻¹); V_{solution} is the volume of the solution (L); C_0 is the initial concentration of the metal in the solution (mg L⁻¹); C_e is the final concentration of the metal in the equilibrium solution (mg L⁻¹); and M_{soil} is the mass of the soil sample (kg). The Langmuir model was used to quantify the metal adsorption capacity of soils (Sposito 1982; Hippler et al. 2014). The non-linearized form of the isotherm was fitted ($R^2 = 0.95$; $p < 0.01$), which described the maximum adsorption capacity ($b_L = 631.3$ mg kg⁻¹) and the energy of adsorption ($K_L = 0.089$ L mg⁻¹) of Cu (Bradl 2004; Mouta et al. 2008).

Chlorophyll content

At 7, 90, and 180 days after the beginning of the Cu treatments, physiologically mature leaves were collected from the first, second, and third vegetative flushes, respectively, and the total chlorophyll (*Chl*) amount was evaluated according to Barnes et al. (1992), with modifications (Hippler et al. 2015). In glass vials, 50 mg of fresh leaf tissue were collected, cut into small strips, and placed with 7.0 mL of dimethyl sulfoxide. The vials were heated in a water bath at 68 °C for approximately 4 h (until tissue depigmentation). The absorbance (*A*) of extracts was read in a spectrophotometer (600 Plus, Femto, São Paulo, Brazil) at 646 and 663 nm, and the determination of $Chl = 7.49 \times A_{663} + 20.34 \times A_{646}$ was performed according to Barnes et al. (1992).

Hydrogen peroxide, lipid peroxidation, and antioxidant enzymes

Such as for chlorophyll measurements, physiologically mature leaves were collected from the first, second, and third vegetative flushes and frozen at –80 °C for further measurements of hydrogen peroxide (H_2O_2) content and lipid peroxidation (MDA), as well as the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX).

The measurement of the H_2O_2 and lipid peroxidation content were performed from the same extraction, in which 500 mg of fresh mass of leaves was homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and 4% (w/v) polyvinylpolypirrolidone (PVPP) 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 in 4 °C for 1 h in darkness and after 20 min in 25 °C. The samples had the absorbance measured at 390 nm and calculated using a standard curve with known concentrations of H_2O_2 . The lipid peroxidation was determined by the presence of malondialdehyde (MDA) 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 re-centrifuged 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. Using an extinction coefficient of 155 $mM^{-1} cm^{-1}$, the amount of MDA was calculated.

For protein quantification and enzyme activities, 1 g of fine leaves 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) PVPP (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 as according to Beauchamp and Fridovich (1971), with some modifications (Hippler et al. 2016). Electrophoresis was carried out under non-denaturing condition in 12% polyacrylamide gels (PAGE) with 50 µg protein for leaves. 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, and 0.3% N,N,N,9,N9-tetramethylethylene-diamine. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control of activity. SOD isoenzyme characterization was performed as described by Azevedo et al. (1998), in which they were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM H_2O_2 .

The activity of CAT was determined according to Kraus et al. (1995) with modifications (Azevedo et al. 1998). The reaction was initiated by addition of 20 µL of plant extract in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and H_2O_2 (30% solution) at 25 °C. The enzyme activity was determined by following the decrease in absorbance at 240 nm, which represents the decomposition of H_2O_2 , for 1 min against a plant extract-free blank. CAT activity was calculated using an extinction coefficient of 39.4 $M^{-1} cm^{-1}$.

The activity of APX 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_2O_2 . The reaction started by the addition of ascorbate. A molar extinction coefficient of 2.8 $mM^{-1} cm^{-1}$ was used in calculations.

Plant growth and nutritional status

At 180 days after the Cu treatments started, plants were destructively harvested. Leaf area was measured using a foliar area integrator LI-3100 (LI-COR, Lincoln, USA). The leaves and twigs were separated and divided into vegetative flushes. All plant parts were carefully washed in detergent solution 0.08% (v/v), rinsed twice in distilled water, and then dried at 58–60 °C until constant weight was reached. The plant materials were ground (200-mesh sieve) and the concentration of Cu and Fe were measured after nitro-perchloric digestion according to Bataglia et al. (1983), by plasma emission spectrometry (ICP-OES, PerkinElmer 5100 PC, Norwalk, CT, USA).

During the experiment, the plants of the treatments with 8.0 g per plant of Cu to the soil and those treatments with foliar spray showed leakage of sap content from the trunk and twigs. The leakage material was collected periodically and stored at -8°C until the end of the experiment. In this moment, the extracts were dried at 53–55 °C, weighted, and dissolved in HCl 0.2 N solution at 1:50 (w/v) ratio. The extracts were filtered (3.0-μm pore size) and the concentration of inorganic-N (N-NO₃ and N-NH₄), P, K, Ca, Mg, S, B, Fe, Mn, Zn, and Cu in ICP-OES (PerkinElmer, mod. Optima, Norwalk, USA) were quantified.

Soil samples were also collected using an auger for evaluation of Cu availability extracted with DTPA-TEA (pH 7.3) according to van Raij et al. (2001). The soil from pots that received Cu applications was sampled in two different depths (0–8 and 8–20 cm), while in the case of plants that received Cu sprays on the leaves, the soil was sampled at 0–8-cm depth as well at adjacent portion to the roots (rhizospheric soil).

Statistical analysis

Both experiments were set up in a randomized design with five replicates (plants) per treatment. In the first experiment, regressions with simple correlation analysis were used to describe relationships between variables. In the second experiment, descriptive statistics was applied and the data were analyzed using analysis of variance (ANOVA) as incomplete factorial design evaluating the interactions between control and Cu-supply treatments (average of sources and levels) and source \times rate interaction was

significant, means were compared using Tukey test at 5% level of significance.

Results

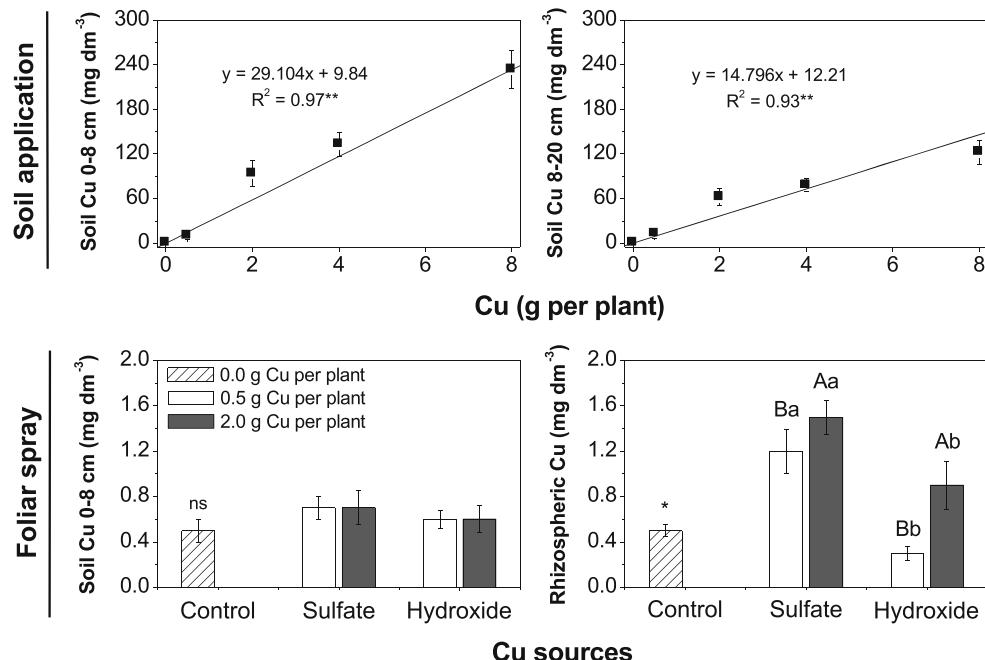
Copper concentration in the soil

Increases on Cu concentrations were observed in the soil depth layers of 0–8 and 8–20 cm with metal supply, with maximum levels reaching, respectively, 234 and 123 mg dm⁻³ for plants that received 8.0 g of Cu via soil (Fig. 1). Cu sprayed onto the leaves did not increase Cu soil availability (0–8 cm, Fig. 1), even though it enhanced the availability of this nutrient in the rhizosphere, mainly for the plants that received sulfate source (Fig. 1).

Plant nutritional status

Levels of total Cu in the trees increased according to the Cu doses applied to the soil (Fig. 2). Roots exhibited the highest Cu concentrations of 470 mg kg⁻¹ when the metal was applied to the soil, while the same increased up to 18 mg kg⁻¹ when trees were foliar sprayed with the sulfate source (Fig. 2). Moreover, Cu concentration in leaves and twigs from the second and third vegetative flushes grown after tree planting increased when the metal was foliar sprayed, being higher for those that received hydroxide source (Fig. 2). Among plant parts, old twigs were the only one to exhibit increased Cu concentration with the highest doses of the metal applied

Fig. 1 Copper availability in two soil depth layers and adjacent to the roots (rhizospheric) with sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO₄ or Cu(OH)₂). Vertical lines represent standard deviation of the mean ($n = 5$); Soil application experiment:
** $p < 0.01$. Foliar spray experiment: control versus Cu supply treatments, ns = not significant ($p > 0.05$), and * $p < 0.05$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)



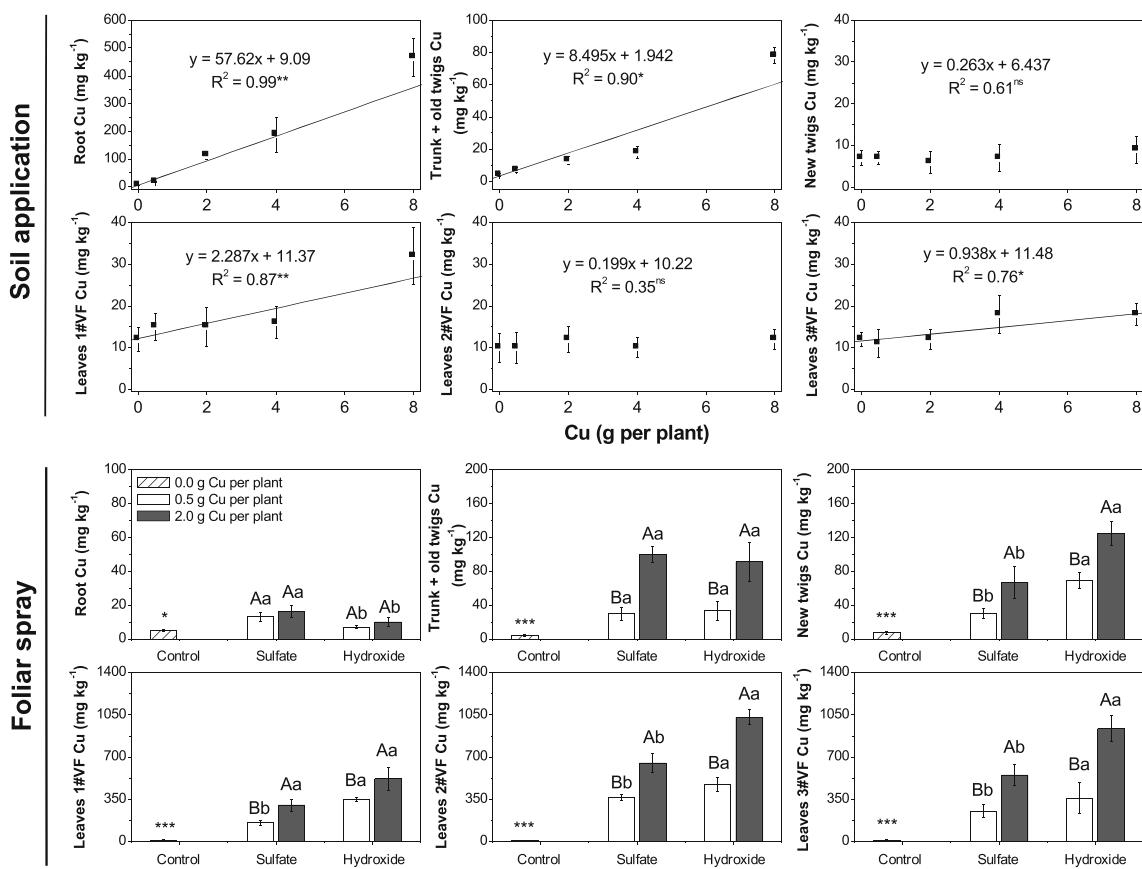


Fig. 2 Copper tissue concentrations in sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO_4 or $\text{Cu}(\text{OH})_2$). Legend: VF = recent and mature vegetative flush; 1#, 2#, and 3# indicates 7, 90, and 180 days after beginning the experiment. Vertical lines represent standard deviation of the mean ($n=5$). Soil application experiment: ns = not significant ($p > 0.05$), * $p < 0.05$, and ** $p < 0.01$. Foliar spray

either to the soil (8.0 g per plant) or to the leaves (2.0 g per plant, Fig. 2).

The sap leaked from the trunk and twigs (Fig. 2) in plants that received 8.0 g of Cu via soil or via leaf sprays, irrespective to the Cu sources, presented measured concentrations of nutrients (Table 1). Potassium and Mg were higher in the first case, whereas P, Ca, S, and Cu were higher in leaf sprayed plants.

Iron concentrations in roots increased with the increase of Cu levels of tested treatments, regardless whether the same were sprayed to the leaves or supplied to the soil, when compared with the control plants (Fig. 3). No differences were verified for Fe concentration on other plant parts.

Biomass production

Root dry weight (DW) decreased with increased Cu doses applied to the soil (Fig. 4). When Cu was foliar sprayed, root DW reduced in trees with the highest dose of Cu sprayed as sulfate (Fig. 4). In both experiments, the increment of the Cu

Cu sources

experiment: control versus Cu supply treatments, ns = not significant ($p > 0.05$); * $p < 0.05$, and *** $p < 0.001$; Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

doses supplied either via foliar spray or via soil reduced the total DW of trees (Fig. 4).

Dry weights of leaves and twigs of the new part were not affected by the Cu application to the soil, while leaf area of these (from the second and third vegetative flushes) decreased in the plants that received the highest dose of the metal (8.0 g per plant) (Fig. 4). Moreover, the supply of the highest dose of Cu as hydroxide also reduced the leaf area of the second and third vegetative flushes compared to other Cu-foliar sprayed treatments (Fig. 4).

Copper toxicity symptoms in trees

Trees that received 8.0 g of Cu via soil exhibited epidermal cell disruption in the scion-rootstock intersection of the trunk (Fig. 5a), as well as in the rootstock or scion part (Fig. 5b). These initial cell disruptions developed to form greater ruptures with the plant growth, which likely affected the bark (periderm and living phloem), vascular cambium, and sapwood tissues (Fig. 5c). In the same

Table 1 Nutrient concentrations in the sap leakage from trunk and twigs of sweet orange trees 180 days after Cu application via soil or leaf sprays (CuSO_4 or $\text{Cu}(\text{OH})_2$)

| Cu treatment Cu per plant (g) | $\text{N}-\text{NO}_3$ mg L^{-1} | $\text{N}-\text{NH}_4$ | P | K | Ca | Mg | S | Cu | Fe | Zn | Mn $\mu\text{g L}^{-1}$ | B |
|--|--|------------------------|---------------|--------------|--------------|-------------|---------------|----------------|---------------|----------------|----------------------------|--------------|
| Soil application of CuSO_4 | | | | | | | | | | | | |
| 8.0 | $3.8 \pm 0.6^{\$}$ | 4.0 ± 1.2 | 0.6 ± 0.2 | 100 ± 18 | 277 ± 30 | 76 ± 9 | 3.8 ± 0.5 | 1.5 ± 0.3 | 2.9 ± 0.8 | 9.7 ± 2.3 | 18 ± 9 | 60 ± 11 |
| Foliar application of CuSO_4 | | | | | | | | | | | | |
| 0.5 | 2.7 ± 0.4 | 4.7 ± 0.7 | 3.0 ± 0.9 | $9 \pm <1$ | 410 ± 26 | 36 ± 1 | 8.9 ± 2.2 | 10.3 ± 1.3 | 2.4 ± 1.1 | 11.9 ± 2.0 | 40 ± 10 | 60 ± 10 |
| 2.0 | 3.0 ± 0.2 | 4.3 ± 0.3 | 2.0 ± 0.9 | 9 ± 3 | 405 ± 12 | 55 ± 11 | 3.8 ± 0.3 | 15.5 ± 1.7 | 3.2 ± 1.7 | 10.2 ± 1.8 | 15 ± 6 | 75 ± 10 |
| Foliar application of $\text{Cu}(\text{OH})_2$ | | | | | | | | | | | | |
| 2.0 | 3.1 ± 0.3 | 4.9 ± 0.8 | 1.7 ± 0.6 | 8 ± 2 | 459 ± 14 | 41 ± 4 | 6.9 ± 2.2 | 10.3 ± 0.4 | 1.7 ± 0.4 | 5.8 ± 2.9 | 18 ± 8 | 50 ± 7.1 |

[§] Standard deviation of the mean ($n=4$)

trees, the sap leakage occurred in the middle part of twigs (Fig. 5d), as well as in leaf buds causing tissue death (Fig. 5e). In the trees subjected to Cu foliar sprays, high metal accumulation also resulted in sap leakage from the leaf buds of new twigs (Fig. 5f).

Chlorophyll contents

The amount of *Chl* in leaves of the first and second vegetative flushes reduced with the increase of Cu doses applied to the soil, but not in those grown with the third one

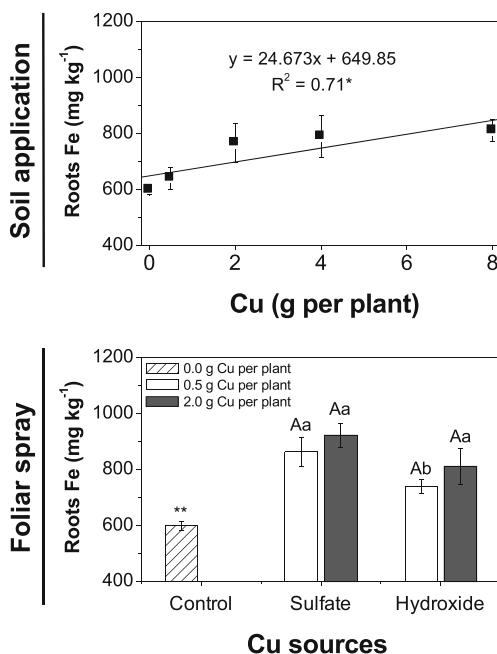


Fig. 3 Iron (Fe) concentration in roots of sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO_4 or $\text{Cu}(\text{OH})_2$). Vertical lines represent standard deviation of the mean ($n=5$); Soil application experiment: * $p < 0.05$. Foliar spray experiment: control versus Cu supply treatments: ** $p < 0.01$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

(Fig. 6). A decrease of *Chl* content was also observed in trees with sulfate foliar sprayed, mainly at the highest dose (Fig. 6).

Hydrogen peroxide and lipid peroxidation levels

Contents of H_2O_2 and MDA in the leaves of the first vegetative flush (7 days after starting Cu treatments) increased according to the increment of the Cu doses applied to the soil (Fig. 7). However, the same in leaves of the second (90 days) and third (180 days) vegetative flushes decreased in trees with the highest level of Cu applied via soil, whereas the greatest levels of H_2O_2 and MDA were observed in trees with 4.0 g Cu per plant (Fig. 7).

Trees that received Cu foliar sprays exhibited higher concentrations of H_2O_2 and MDA in leaves of all vegetative flushes when compared to the control ones (Fig. 7). However, trees that received 2.0 g per plant Cu exhibited the highest concentrations of H_2O_2 and MDA in leaves of the second and third vegetative flushes, mostly when applied as sulfate source (Fig. 7).

Antioxidant enzyme activities

Superoxide dismutase activity increased with applications of Cu, mainly the Cu/Zn-SOD isoforms when compared to the control plants that displayed the lower SOD activity in leaves of both evaluated periods (Fig. 8). Even 7 days after the beginning of the Cu treatments, the activity of the isoforms Mn-SOD I and II and Cu/Zn-SOD II and III were higher than in the control plants (Fig. 8a). In the leaves of the second vegetative flush, the SOD activity was higher in those plants that received either 4.0 g of Cu via soil for the Mn-SOD and Cu/Zn-SOD isoforms in the first experiment or received 2.0 g of Cu sprayed to the leaves as sulfate for the Cu/Zn-SOD II and III in the second experiment (Fig. 8b). In the third vegetative flush, all plants that received the Cu treatments in both

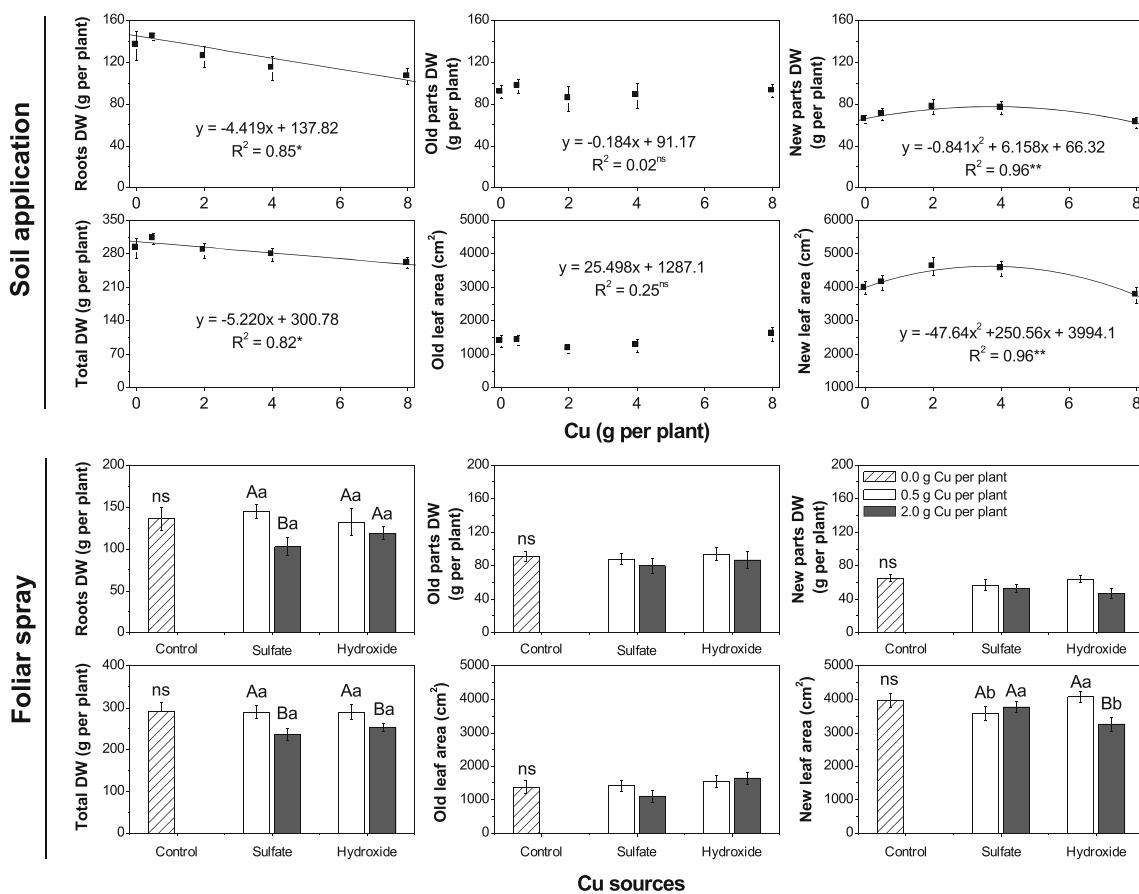


Fig. 4 Dry weight (DW) of sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO_4 or $\text{Cu}(\text{OH})_2$). Legend: Old parts = trunk + old twigs + old leaves. New parts = new twigs + new leaves. Old leaves = leaves from the first vegetative flush. New leaves = leaves from the second and third vegetative flushes. Vertical lines represent standard deviation of the mean ($n = 5$). Soil application experiment: ns = not significant ($p > 0.05$), * $p < 0.05$, and ** $p < 0.01$. Foliar spray experiment: control versus Cu supply treatments: ns = not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

experiments exhibited a higher SOD activity compared to the control.

Similar as verified with the H_2O_2 and MDA contents and SOD activity, in the first vegetative flush, 7 days after the Cu treatments began, the application of 8.0 g per plant of Cu via soil increased the activities of CAT and APX up to 29 and 44 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, respectively, followed by trees with 4.0 g per plant of Cu via soil (Fig. 9). However, the activities of CAT and APX were apparently reduced in plants with 8.0 g per plant via soil in the second and third vegetative flushes, being the highest activities observed in those with 4.0 g Cu per plant (Fig. 9).

When Cu was foliar applied, CAT activity was higher in the leaves of trees with the highest dose of Cu sprayed as sulfate (Fig. 9), whereas the APX activities in the periods evaluated were similar to the CAT activities, with the exception of the first period, whose the highest activity was observed with 2.0 g Cu per plant for both sources (Fig. 9).

Cu sources

significant ($p > 0.05$), * $p < 0.05$, and ** $p < 0.01$. Foliar spray experiment: control versus Cu supply treatments: ns = not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

Discussion

Copper concentration has increased in soils and plants worldwide, due to intensive applications of Cu-based fungicides to control plant diseases (Bakshi et al. 2013; Martins et al. 2014; Silva Jr. et al. 2016). Previously, citrus rootstock varieties were reported to exhibit different antioxidant system responses in leaves when grown with varying Cu concentrations in the root medium (Hippler et al. 2016). In this study, we characterized the enzymatic antioxidative system, as well as homeostatic mechanisms of citrus, associated to the alleviation of Cu stress injuries caused by the metal application either via foliar sprays or via soil.

Because of the low clay content of the soil in the present study (10% of clay), the energy of adsorption of Cu on soil, according to Langmuir's isotherms, was lower ($K_L = 0.089 \text{ L mg}^{-1}$) when compared to other tropical soils with up to 820 g kg^{-1} of clay and 0.468 L kg^{-1} of K_L (Mouta et al. 2008). Furthermore, the soil adsorption capacity for Cu ($b_L = 631.3 \text{ mg kg}^{-1}$) did not limit roots to uptake and

Fig. 5 Sap leakage from the trunk (**a–c**) and twigs (**d, e**) of plants supplied with 8.0 g of Cu via soil, and from twigs (**f**) of plants with 2.0 g of Cu sprayed to the leaves as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Legend: vertical white lines represent: **a** = 1.5 cm; **b** = 0.7 cm; **c** = 4.5 cm; **d** = 0.8 cm; **e, f** = 3.3 cm

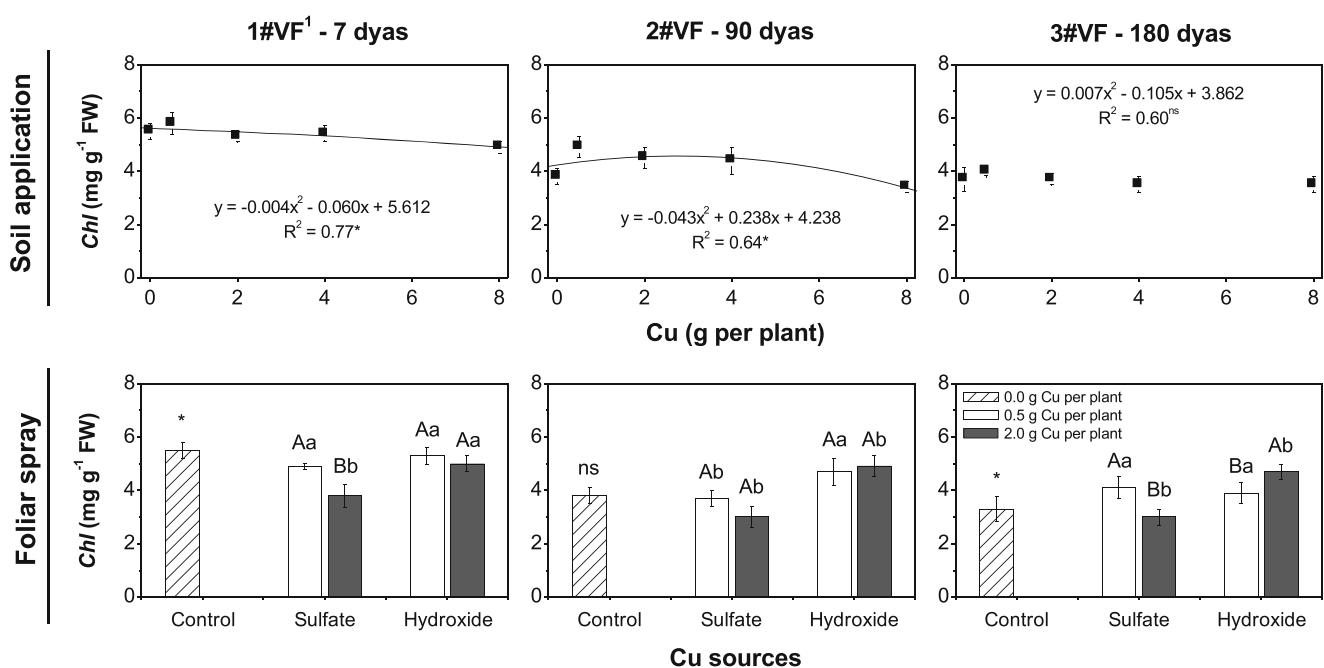
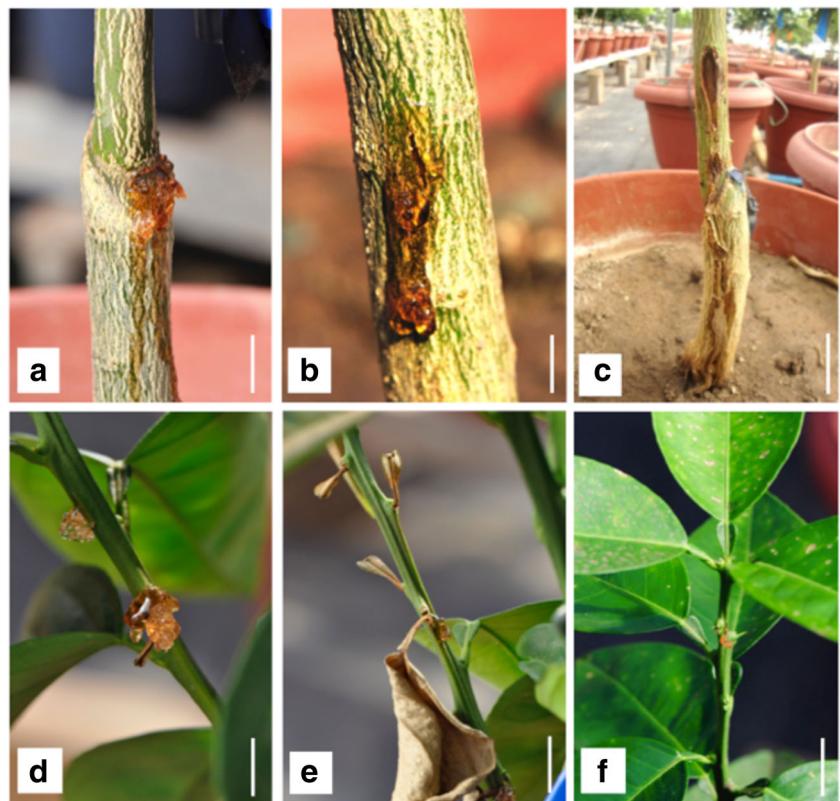


Fig. 6 Concentration of chlorophylls (*Chl*) in leaves of sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO_4 or $\text{Cu}(\text{OH})_2$). Legend: VF¹ = recent and mature vegetative flush; 1#, 2#, and 3# indicates 7, 90, and 180 days after beginning the experiment. Vertical lines represent standard deviation of the mean ($n=5$). Soil application experiment: ns = not significant ($p>0.05$) and $*p<0.05$.

Cu sources

Foliar spray experiment: Control versus Cu supply treatments: ns = not significant ($p>0.05$) and $*p<0.05$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p<0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p<0.05$)

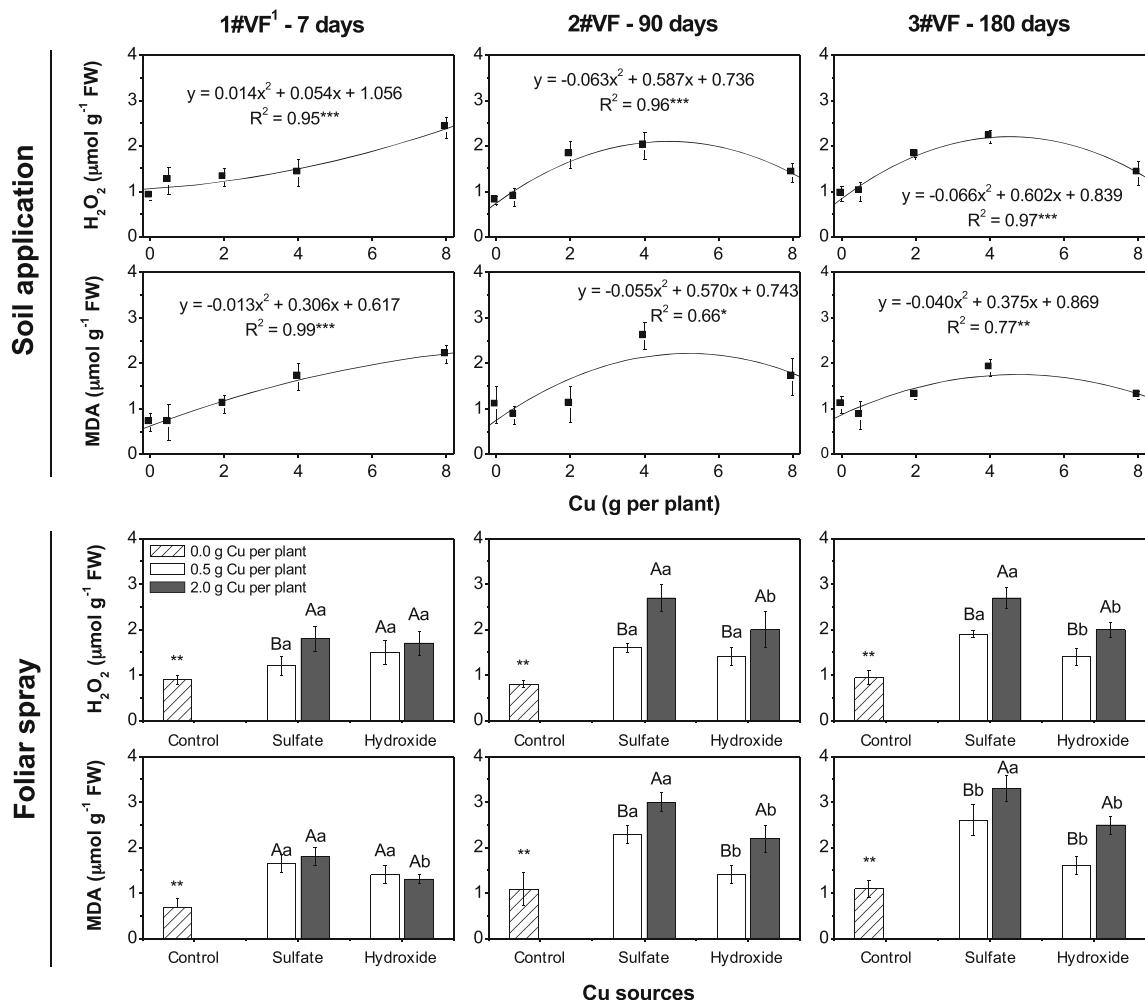


Fig. 7 Hydrogen peroxide (H_2O_2) content and lipid peroxidation (MDA) in leaves of different vegetative flushes (VF) in sweet orange trees 180 days after Cu application via soil or foliar sprays ($CuSO_4$ or $Cu(OH)_2$). Legend: VF = recent and mature vegetative flush; 1#, 2#, and 3# indicates 7, 90, and 180 days after beginning the experiment. Vertical lines represent standard deviation of the mean ($n=5$); Soil

application experiment: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Foliar spray experiment: control versus Cu supply treatments: $**p < 0.01$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

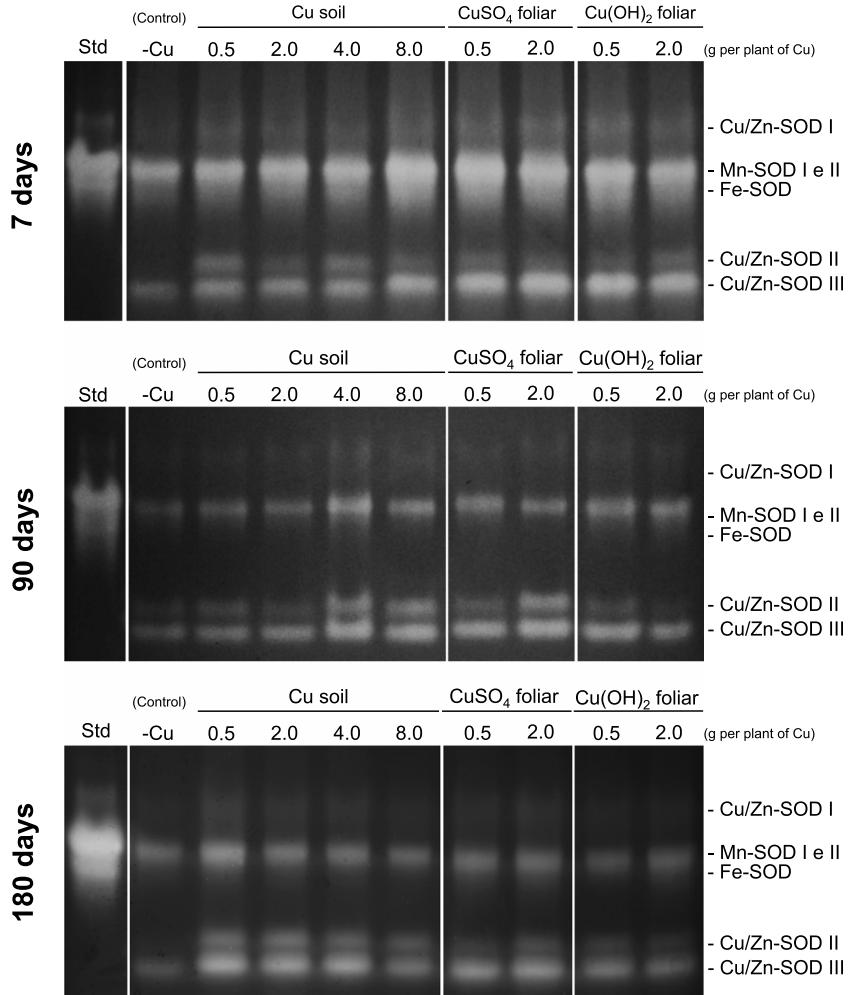
accumulate the nutrient as estimated by leaf concentrations of the metal up to 467 mg kg^{-1} (Fig. 2). However, Cu exhibited low mobility in the trees, mainly from the leaves to the roots when the metal was foliar sprayed. Copper was also accumulated preferentially in the organ that absorbed the metal (leaves or roots, Fig. 2).

High doses of Cu either applied via soil or foliar sprays caused visual symptoms of toxicity, characterized by tissue disruption in the trunk and twigs what resulted in sap leakage (Fig. 5). Copper is considered the most harmful plant metal to membrane and cell wall integrity because of damages caused to proteins (Puig et al. 2007), lipids, and other compounds in membranes (Yruela 2013), as well as build-up of reactive oxygen species (ROS) and consequently, lipid peroxidation (Puig et al. 2007; Andrade et al. 2010). Therefore, observed tissue disruption increased in size and form (Fig. 5), in which would

likely become a potential entry point for pathogens (Behlau et al. 2010, 2017).

On the other hand, sap leakage from the trunk and twigs of trees, 30 days after the highest dose of Cu was applied to the soil (Fig. 2), could reduce plant accumulation of Cu (Table 1) and disturb plant nutritional status by leakage of other nutrients, such as observed for K and Mg (Table 1). However, further studies are necessary to evaluate the severity of damages caused by sap leakage on the citrus nutritional status. Other compounds were also likely leaked from trees that received $8.0 \text{ g per plant Cu}$ via soil, such as phenols and ROS (Kováčik et al. 2009). Even though, such trees exhibited better recovery of the Chl content (Fig. 6), as well as lower contents of H_2O_2 and MDA in the leaves in the second and third vegetative grown flushes, mainly when compared with trees that received $4.0 \text{ g of Cu per plant}$ and did not exhibit visual symptoms of sap leakage (Fig. 7).

Fig. 8 Superoxide dismutase (SOD) activity in leaves of sweet orange trees at 7 days (first vegetative flush), 90 days (second vegetative flush), and 180 days (third vegetative flush) after Cu application via soil or foliar sprays (CuSO_4 or Cu(OH)_2). Legend: Std: bovine SOD standard



Unlikely, plant metabolic recovery was not verified in the second experiment when high levels of foliar-sprayed Cu also caused sap leakage, once it occurred mainly in twigs from the second and third vegetative flushes after 150 days, close to the end of the experiment (Fig. 2d). The sap leakage in plants with Cu foliar sprayed was associated to injuries caused by direct contact of Cu with plant epidermis (Fig. 5), as well as metal accumulation up to 1033 mg kg^{-1} in leaves and 125 mg kg^{-1} in twigs (Fig. 2). Furthermore, sap leakage in the twigs of these trees after leaf sprays with Cu was also characterized by high amounts of nutrients P, Ca, and S (Table 1).

Despite the recovery of trees that received 8.0 g of Cu via soil after sap leakage, these plants exhibited significant damages in root growth, which biomass was approximately 20% lower compared to that of control plants (Fig. 4). Indeed, it is difficult to identify plants injured by high levels of Cu in the soil under field conditions once Cu concentrations in leaves do not clearly substantiate the nutritional disorder they are subjected (Fig. 2), since up to 80% of total plant Cu is likely to accumulate in roots (Hippler et al. 2016, 2018). Similarly, Zn is mainly accumulated in woody parts of *Citrus* when absorbed by roots (Hippler et al. 2015), which could explain

the higher amounts of Zn released in the sap leakage in both experiments (approximately 9.5 mg L^{-1}) compared to other micronutrients; concentrations were in milligrams per liter: 2.5 for Fe, 0.061 for B, and 0.030 for Mn (Table 1).

Few reports characterized visual symptoms of Cu toxicity in woody trees, such as *Citrus*, in which the Fe-chlorosis deficiency symptoms in leaves were described in field conditions (Alva and Chen 1995; Gama et al. 2015) and explained by the interaction between these two nutrients in metal homeostasis in plants (Puig et al. 2007; Waters and Armbrust 2013; Hippler et al. 2016, 2018). Noteworthy, higher levels of Cu taken up either by roots or leaves increased Fe concentration in the root tissue (Fig. 3). This suggests that *Citrus* trees exhibit a regulatory mechanism elicited by a signaling pathway associated to Cu toxicity that limits Fe distribution in the plant, from roots to plant canopy, despite Cu absorption in excess occurs through roots or leaves. Further studies are needed to characterize sensing mechanisms between these two nutrients, mainly in woody trees, which likely involve ferric-chelate reductase enzyme activity, responsible for Fe uptake, as well as some chelator compounds in roots, such as nicotianamine, histidine, and glutathione that limit the

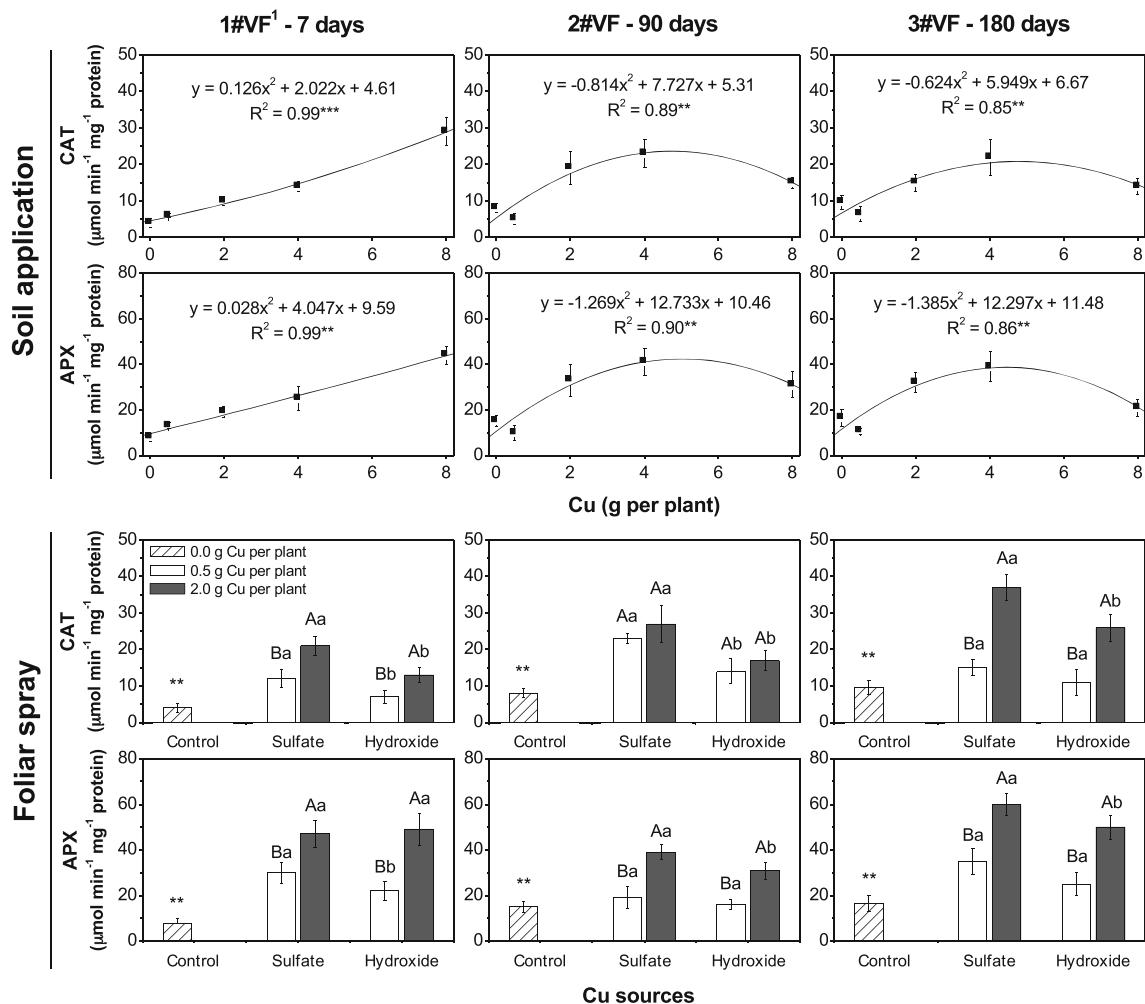


Fig. 9 Catalase (CAT) and ascorbate peroxidase (APX) activity in leaves of different vegetative flushes (VF) in sweet orange trees 180 days after Cu application via soil or foliar sprays (CuSO_4 or Cu(OH)_2). Legend: VF = recent and mature vegetative flush; 1#, 2#, and 3# indicates 7, 90, and 180 days after beginning the experiment. Vertical lines represent standard deviation of the mean ($n = 5$). Soil application experiment: $**p < 0.01$ and

$***p < 0.001$. Foliar spray experiment: control versus Cu-supply treatments: $*p < 0.05$. Cu sources: means followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). Cu levels: means followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$)

mobilization and activity of the metal in the plant (Puig et al. 2007; Deinlein et al. 2012; Waters and Armbrust 2013; López-Clement et al. 2014). Increased expression of genes related to nicotianamine synthesis (*OsNAS1*, *OsNAS2*, and *OsNAS3*) was observed in roots of *Oryza sativa* under Cu excess, which were associated to the reduction of Fe mobilization from roots to sink organs (Andrés-Bordería et al. 2017). Furthermore, *Citrus* groves grown in alkaline soils with low availability of Fe are more susceptible to Cu toxicity, once uptake and accumulation of Cu in this condition increase (Waters and Armbrust 2013).

Plants sprayed with the sulfate source exhibited highest mobility of Cu, supported by the increased translocation of the metal to the roots (Fig. 2). Furthermore, trees with foliar sprays not only exhibited increased Cu concentration in the roots (Fig. 2) but also in the rhizospheric soil (Fig. 1). However, the metal exclusion mechanism by *Citrus* roots

was not likely efficient to eliminate Cu in great amounts to alleviate toxic effects in the trees. Copper exudation by roots through oxalate, malate, or citrate is an important strategy of plants for metal detoxification (Sereno et al. 2007; Lyubanova et al. 2013). On the other hand, due to the low mobility of Cu in *Citrus* (Fig. 2), other strategies might be relevant, such as synthesis of metallothioneins. Metallothioneins are cysteine-rich peptides associated with metal homeostasis and detoxification due to their capacity to bind divalent metals (Sereno et al. 2007). In leaves of *Coffea arabica*, excess Cu upregulated genes encoding metallothioneins what reduced lipid peroxidation levels (Bulgarelli et al. 2016). In *Brassica napus*, Cu excess also caused a reduction in *Chl* content even after the increment of the transcript abundance of genes related to metallothioneins, metalchaperones, and enzymes involved in phytochelatin synthesis (Zlobin et al. 2015).

The reduction of plant canopy and roots biomass by excess Cu correlated with reduced *Chl* content (Figs. 4 and 6) and consequently decreased in plant photosynthetic capacity (Yruela 2013; Syvertsen and Garcia-Sanchez 2014). On the contrary, the application of the hydroxide source to the leaves provided higher Cu concentrations in the plant canopy (Fig. 2), but less damages on *Chl* content (Fig. 6), as well as lower levels of H₂O₂ and MDA compared to those sprayed with sulfate (Fig. 7). Thus, Cu from the hydroxide source was not totally bioavailable to the plant metabolism as confirmed by the lower activity of the Cu/Zn-SOD II in leaves of the second and third vegetative growth flushes (Fig. 8).

The different mobility of the metal in the plant related to the foliar source modified the plant's enzyme antioxidative system responses, in which CAT activity in the leaves was more sensitive when the sulfate source was applied, whereas APX increased only with the increase of the dose applied for both sources (Fig. 9). *Typha latifolia*, grown with different levels of Cu as sulfate or nitrate in the root medium, exhibited differences in APX activity between roots and shoots, which were in accordance to the mobility and partitioning of the metal into the plant parts (Lyubenova et al. 2015).

Reactive oxygen species increased in leaves from all vegetative flushes of analyzed plants, in both experiments, even with the non-increment of Cu concentration in the leaves when ≤ 4.0 g per plant of Cu was applied to the soil (Fig. 7). Despite measurements of H₂O₂ and MDA contents or activities of antioxidant enzymes were not performed in root tissue, an increase of ROS might also be occurring in the trees of the second experiment with high doses of Cu leaf sprayed due to the reduction of root DW (Fig. 4), but low amount of Cu (Fig. 2). In other plant parts, the increase of ROS could occur by signals performed by other compounds, not directly related to metal accumulation in these parts (Tripathy and Oelmüller 2012; Gratão et al. 2015). The signaling network between rootstock and scion has been reported in tomato plants exposed to cadmium (Gratão et al. 2015), which represent an important strategy for metal detoxification by plants. In citrus trees, this signaling process is regulated by rootstock varieties, which affect plant responses under metal stress conditions (Hippler et al. 2016).

The antioxidant enzymatic system is a key factor to alleviate damages caused by ROS (Del Rio 2015; Gratão et al. 2015). The activities of SOD, CAT, and APX were consistent with the alleviation of the oxidative stress levels under excess Cu (Figs. 8 and 9). The activities of the Cu/Zn-SOD II and III isoforms were sensitive to the Cu application, with the lowest activity observed in control plants (Fig. 8). The SOD isoforms are located in different cell compartments (Azevedo et al. 1998; Gratão et al. 2015). Therefore, the regulation of the isoforms activities can be related to the location of the ROS production (Del Rio 2015), or by the availability of the micronutrient co-factor of each enzyme isoform (Hippler et al. 2015), such as verified in this study for Cu/Zn-SOD activity (Fig. 8). Similar to SOD isoform activity responses, total

CAT and APX activities in leaves also increased according to the increment of the Cu doses applied to the soil or via foliar (Fig. 9). These two latter enzymes, responsible to scavenge H₂O₂ in plants, have been more responsive in plants under Cu stress (Hippler et al. 2018). However, the highest activities of CAT and APX were verified in plants with Cu sprayed to the leaves, mainly in those of the second and third vegetative grown flushes (Fig. 9), which agreed with the higher levels of H₂O₂ and lipid peroxidation found in these treatments (Fig. 7).

This work reports visual symptoms of Cu toxicity in *Citrus*, characterized by the rupture of tissues of trunk and twigs and consequent sap leakage. Citrus trees present homeostatic mechanisms to alleviate Cu toxicity, which vary depending upon metal uptake by roots or leaves. The absorption of Cu up to toxic levels by roots is associated to lower injuries in the canopy because of limited metal transport from the roots to the shoots. When Cu is sprayed to the leaves, damages are more severe with sulfate compared to hydroxide metal source, and in this case, Cu is accumulated mainly in the canopy, even though a portion is released in a small portion by roots to the soil. In both metal absorption pathways (leaves or roots), the signaling network and the enzymatic antioxidant system are essential to alleviate injuries and protect plant growth. In fact, understanding tree homeostasis mechanisms is essential for health management of the new challenging groves, where the use of Cu-based pesticides has been increasingly required for diseases control.

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