Environmental and Experimental Botany 130 (2016) 42–52

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

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

Franz W.R. Hippler[a,*], Danilo O. Cipriano[a], Rodrigo M. Boaretto[a], José A. Quaggio[b], Salete A. Gaziola[c], Ricardo A. Azevedo[c], 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] Centro de Solos e Recursos Ambientais, Instituto Agronômico, Av. Barão de Itapura, 1481, CP 28, CEP 13020-902, Campinas, SP, Brazil
[c] Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, CP 09, Universidade de São Paulo, CEP 13418-900, Piracicaba, SP, Brazil

ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 9 May 2016
Accepted 10 May 2016
Available online 11 May 2016

Keywords:
Cupric fungicides
Metal homeostasis
Nutritional status
Oxidative stress
Rootstock signalling
Sweet orange

ABSTRACT

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

1. Introduction

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

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

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

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

* Corresponding authors.
E-mail addresses: franz@centrodecitricultura.br, fwhippler@hotmail.com (F.W.R. Hippler), ddm@centrodecitricultura.br (D. Mattos-Jr.).

http://dx.doi.org/10.1016/j.envexpbot.2016.05.007
0098-8472/

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

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

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

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

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

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

2. Materials and methods

2.1. Plant material and growth conditions

Young sweet orange trees cv. Pera [*Citrus sinensis* (L.) Osbeck], grafted onto interstocked Swingle citrumelo [SW; *C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.] or Rangpur lime (RL; *C. limonia* Osbeck) were grown in pots with 11 L of nutrient solution (NS) in a greenhouse. The experiment was set up in a completely randomized, 2×3 factorial design, with two rootstock varieties (SW and RL) and three Cu concentrations in the NS (0.015, 0.60 and 24.0 μM; as $CuSO_4 \cdot 5H_2O$), with four replications.

Six-year-old trees were cultivated in 4-L plastic bags filled with an organic substrate (80% pine bark, 5% carbonized materials and 15% vermiculite) fertilized with macro- and micronutrients, except Cu, for 120 days before transplant to the NS. Plants were adapted to the new growing media for one week at 25% of the final NS concentration and then for two weeks at 50% of the final NS concentration. Plants were then maintained at the following concentrations, in mM: 12 N (80% N—NO_3), 3.4 K, 0.4 P, 4.0 Ca, 25 Mg and 20 S, and the following, in μM: 41.6 B, 48.0 Fe, 8.2 Mn, 3.5 Zn and 1.3 Mo (modified from Zambrosi et al., 2013). Following the first flush of vegetative growth of plants (approximately 30 days after transplant), treatments were started by adding Cu at various concentrations to the NS, which was aerated continuously; the volumes of the containers were kept constant by addition of deionized water when necessary and renewed at intervals of approximately 15 days. The pH of NS was adjusted to 5.0-5.5 with 1 M KOH or 1 M H_2SO_4.

At 45 and 90 days after the start of Cu treatments, the indirect index of chlorophyll (formed after the start of the Cu concentration treatments applied) was determined in young leaves using a portable meter mod, SPAD-502 (Konica Minolta Holding Inc., Tokyo, Japan). The levels of total protein, hydrogen peroxide (H_2O_2) and lipid peroxidation and the activities of the following enzymes: nitrate reductase (NRase; EC 1.6.6.1), superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POX; EC 1.11.1.7), were determined.

Furthermore, at the end of the 90-day period, plants were destructively harvested and separated into trunks, old branches and old leaves (grown before the initiation of treatments), new branches and new leaves (grown after treatment applications) and roots. Total leaf area was measured using the Leaf Area Integrator LI-3100 (LI-COR, Lincoln, NE, USA). Plant parts were washed in 0.01% detergent solution (v/v) to remove external contamination of the epidermis (Hippler et al., 2014) and the dry mass (DM) was determined by oven drying at 60–65 °C for 72 h. The plant material was ground to pass through a 200-mesh sieve and the concentrations of Cu and other nutrients were determined according to Bataglia et al. (1983) by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA). The nitrate (N—NO_3^-) and ammonium levels (N—NH_4^+) in plant parts were determined by steam distillation (Tedesco et al., 1995). The Cu partition (% of Cu) in each plant part was obtained from the product of DM and Cu concentration.

2.2. Nitrate reductase activity

Nitrate reductase (NRase) activity was determined by the *in vivo* method in new leaves as described by Dovis et al. (2014). The assay consists of incubation of 200 mg fresh weight (FW) of leaves in 100 mM of sodium phosphate buffer solution (pH 7.5) with 200 mM of KNO_3 and 1% *n*-propanol (w/v). The samples were vacuum filtered and kept in the dark at 40 °C for 30 min. The NO_2^- was quantified by absorbance at 540 nm, with the addition of 1% sulfanilamide solution in 2.4 N HCl + 0.02% N-[1-naphthyl] ethylenediamine dihydrochloride (NED; w/v). The result was expressed in μmol NO_2^- mg^{-1} FW h^{-1}.

2.3. Hydrogen peroxide and lipid peroxidation

The measurements of H_2O_2 and lipid peroxidation were performed from the same extraction, in which 500 mg of fresh leaves were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 5590 × *g* for 15 min at 4 °C (Alexieva et al., 2001). The supernatant was mixed with 100 mM of potassium phosphate buffer (pH 7.0) containing 1.0 M potassium iodide (1:1:4) and incubated in ice for 1 h in the dark, followed by 20 min at room temperature and the absorbance measured at 390 nm. The blank consisted of 0.1% TCA in the absence of leaf extract. The amount of H_2O_2 was calculated using a standard curve generated