



Phosphorus availability and rootstock affect copper-induced damage to the root ultra-structure of *Citrus*



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ABSTRACT

The control of several citrus diseases requires continuous applications of fungicides containing copper (Cu) which favor to the accumulation of this metal in the soil. Therefore, the evaluation of how nutrient availability and rootstock interact with Cu toxicity in the citrus trees is required to maintain sustainability of fruit production in Cu-contaminated soils. Valencia orange trees on Sunki mandarin (SM) or Swingle citrumelo (SC) rootstock were grown in nutrient solutions combining adequate Cu ($1.0 \mu\text{mol L}^{-1}$), excess Cu ($50.0 \mu\text{mol L}^{-1}$), deficient phosphorus (P) (0.01 mmol L^{-1}) and sufficient P (0.5 mmol L^{-1}). The excess Cu reduced root and shoot growth, chlorophyll and relative water content in the leaves of the trees compared to those under adequate Cu supply. Furthermore, excess Cu caused severe damage to the root ultra-structure, characterized by the degeneration of the middle lamella and the presence of a thin and sinuous cell wall, as well as, starch accumulation in the plastids, disruption of the mitochondrial membranes and cellular plasmolysis. The damage caused by excess Cu in the cell wall and middle lamella on the root cells of SC was less severe than SM. Sufficient P supply improved the structure of the cell wall and middle lamella of trees subjected to excess Cu in comparison to P-deficient ones. Thus, the occurrence of more preserved cell wall and middle lamella supports the idea that sufficient P availability in the rooting medium and the use of SC rootstock might contribute to increase the ability of young citrus trees to cope with Cu toxicity.

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1. Introduction

The excessive increase in the amount of copper (Cu) in agricultural soils has been associated with the continuous use of fungicides containing this metal (Mirlean et al., 2007; Nóvoa-Munñoz et al., 2007). In the case of citrus groves, successive applications of Cu fungicides occur for the control of foliar and fruit diseases, such as citrus scab (*Elsinoë fawcettii* and *Elsinoë australis*) and citrus canker (*Xanthomonas citri* subsp. *citri*) (Bettiol et al., 1994; Meneguim et al., 2007). Consequently, the accumulation, mobility, and availability of Cu in the soil have remained in proportion to the age of the citrus groves because annual doses of up to 30 kg ha^{-1} of Cu can be used (Fan et al., 2011). In this scenario, the accumulation of Cu in the soil as a result of the continuous use of fungicides becomes a constraint for citrus production due to the elevated phytotoxic potential of this element (Pätsikkä et al., 1998; Kukkola et al., 2000) and to the

slow conversion of active forms of Cu into inactive forms in the soil (Pietrzak and Mcphail, 2004).

Copper toxicity in plants causes oxidative stress and compromises physiological and biochemical processes (Yruela, 2005), resulting in the inhibition of shoot and root growth (Kopittke and Menzies, 2006). An excess of Cu also leads to serious damage to the ultra-structure of the roots (Liu and Kottke, 2004; Panou-Filotheou and Bosabalidis, 2004). Since in Cu-contaminated soils the Cu toxicity primarily occurs in the roots because of the accumulation of the metal in the soil and the direct contact between Cu ions and the root surface, the sustainability of citrus production in these locals relies on strategies that can minimize Cu damage to the ultra-structure and growth of the roots. For instance, considering that P deficiency has a demonstrated influence on the intensity of abiotic stress in plants, including salinity (Qadar, 1998) and drought stress (Garg et al., 2004), appropriate management of P nutrition might be also important to minimize Cu toxicity in citrus. This notion is supported by the fact that reduced leaf area, photosynthesis, and root growth (Zambrosi et al., 2011, 2012a,b) and enhanced oxidative stress (Tewari et al., 2007) have been associated with P deficiency, which, in turn, might contribute to making plants more susceptible

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Table 1
Plant growth, leaf and root tissue concentrations of copper (Cu) and phosphorus (P), leaf relative water content (RWC) and SPAD readings of young citrus trees according to the supply of P and Cu in the nutrient solution.

P supply	Cu supply			F test ^a		
	Adequate Cu	Excess Cu	Average	P	Cu	P*Cu
RDW (g tree ⁻¹)						
Deficient P	17.4 bA	14.1 aA	15.8			
Sufficient P	22.5 aA	13.4 aB	18.0	*	*	*
Average	20.0	13.8				
NSDW (g tree ⁻¹)						
Deficient P	24.0 bA	4.6 aB	14.3			
Sufficient P	28.0 aA	3.9 aB	16.0	*	*	*
Average	26.0	4.3				
TSDW (g tree ⁻¹)						
Deficient P	42.6 bA	20.6 aB	31.6			
Sufficient P	50.8 aA	19.2 aB	35.0	*	*	*
Average	46.7	19.9				
Leaf Cu (mg kg ⁻¹)						
Deficient P	4.8	8.5	6.7			
Sufficient P	4.1	8.5	6.3	NS	*	NS
Average	4.5 B	8.5 A				
Root Cu (mg kg ⁻¹)						
Deficient P	29.1 aB	557.9 aA	293.5			
Sufficient P	18.1 aB	415.9 bA	217.0	*	*	*
Average	23.6	486.9				
Leaf P (g kg ⁻¹)						
Deficient P	1.4 bB	1.9 aA	1.7			
Sufficient P	1.9 aA	1.7 aA	1.8	*	*	*
Average	1.7	1.8				
Root P (g kg ⁻¹)						
Deficient P	1.3 bA	1.3 aA	1.3			
Sufficient P	3.2 aA	1.5 aB	2.4	*	*	*
Average	2.3	1.4				
RWC (%)						
Deficient P	88.6	82.5	85.6			
Sufficient P	91.4	81.9	86.7	NS	*	NS
Average	90.0 A	82.2 B				
SPAD readings						
Deficient P	80.1	52.7	66.4			
Sufficient P	78.9	53.8	66.4	NS	*	NS
Average	79.5 A	53.3 B				

P supply comparison: means (*n* = 3 or 6) followed by different lowercase letters within columns are significantly different by the *F* test (*p* < 0.05). Cu supply comparison: means (*n* = 3 or 6) followed by different uppercase letters across paired columns are significantly different by the *F* test (*p* < 0.05). Deficient P: 0.01 mmol PL⁻¹; sufficient P: 0.5 mmol PL⁻¹; adequate Cu: 1.0 μmol Cu L⁻¹; excess Cu: 50.0 μmol Cu L⁻¹. RDW: root dry weight; NSDW: new shoots DW; TSDW: total shoots DW.

^a *F* test in the ANOVA of P supply (P) vs Cu supply (Cu) for each parameter evaluated. **p* < 0.05 and NS = not significant (*p* > 0.05).

to abiotic stresses. As a result, there is a practical need to evaluate how the variation of P availability in the rooting medium affects the pattern of Cu toxicity in citrus trees since P deficiency is a common constraint to citrus production (Quaggio et al., 1998).

The selection of rootstock is also an important approach for the management of citrus groves based on the fact that rootstocks differ in regard to the adaptation to nutritional stresses (García-Sánchez et al., 2002; Pestana et al., 2005; Zambrosi et al., 2012b). Although, the deleterious effects of excessive Cu on growth and on nutrient uptake by citrus were previously reported (Alva and Chen, 1995; Alva et al., 1999; Mattos Jr. et al., 2010), we still find a lack of detailed information about the mechanism by which the rootstock influences Cu toxicity in the scion. Indeed, the characterization of such responses could help to better define the recommended rootstocks for cultivation in Cu-contaminated soils and more adequate management practices for citrus trees requiring the continuous application of Cu fungicides (i.e. groves under a high disease pressure).

Taking into account that the maintenance of improved cell ultra-structure is important for the tolerance of the plants to metals toxicity (Doncheva et al., 2009; Gzyl et al., 2009; Ali et al., 2013), our research was based on the hypothesis that more conserved root cell ultra-structure of citrus trees could be obtained with sufficient P supply in the rooting medium and with the use of Cu-tolerant rootstocks. Thus, the examination of the ultra-structure would contribute to improve the understanding about the effects of the excess

Cu supply on the roots and also to define some practical strategies (i.e. nutrients management and rootstock selection) to increase the adaptation of citrus on Cu-contaminated sites. Accordingly, we aimed to investigate the interactions of P supply vs rootstock vs Cu supply in the rooting medium on the growth, water and nutritional status, and root ultra-structure of young citrus trees.

2. Materials and methods

The citrus trees were grown for 140 days, from August 2011 to December 2011, in an unshaded greenhouse with average day/night temperatures of 35 °C/23 °C. Uniform, 1 year-old nursery trees of Valencia sweet orange [*Citrus sinensis* (L.) Osbeck] on either Sunki mandarin [*Citrus reshni* hort. ex Tanaka, SM] or Swingle citrumelo [*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf., SC] rootstock were obtained from a commercial nursery, bare-rooted and supported in non-draining pots containing 8 L of ¼ strength nutrient solution (NS) (Zambrosi et al., 2011) without P and Cu for plant establishment. Five days after transplanting, this ¼ strength NS was replaced with full-strength NS [in mmol L⁻¹, 9.6 N (11% as NH₄⁺), 3.0 K, 4.5 Ca, 1.2 Mg, 1.2 S and, in μmol L⁻¹, 41.6 B, 54.0 Fe, 8.2 Mn, 2.5 Zn and 1.0 Mo] with variable Cu and P concentrations (see below). Each pot with NS contained one citrus tree and was equipped with a tube extending to the bottom through which air was continuously bubbled for the NS aeration. The solution pH during the experimental period was monitored and maintained close

Table 2

Plant growth, leaf and root tissue concentrations of copper (Cu) and phosphorus (P), leaf relative water content (RWC) and SPAD readings of young citrus trees according to the rootstock and Cu supply in the nutrient solution.

Rootstock	Cu supply			F test ^a		
	Adequate Cu	Excess Cu	Average	Rootstock (R)	Cu	R*Cu
RDW (g tree ⁻¹)						
SM	18.3	14.8	16.6	NS	*	NS
SC	21.7	12.7	17.2			
Average	20.0 A	13.8 B				
NSDW (g tree ⁻¹)						
SM	23.1 bA	4.4 aB	13.8	*	*	*
SC	29.4 aA	4.2 aB	16.8			
Average	26.3	4.3				
TSDW (g tree ⁻¹)						
SM	41.6 bA	19.8 aB	30.7	*	*	*
SC	51.1 aA	20.0 aB	35.6			
Average	46.4	19.9				
Leaf Cu (mg kg ⁻¹)						
SM	3.4 bB	8.6 aA	6.0	*	*	*
SC	5.5 aB	8.3 aA	6.9			
Average	4.5	8.5				
Root Cu (mg kg ⁻¹)						
SM	25.8	453.7	239.8	NS	*	NS
SC	21.5	520.1	270.8			
Average	23.7 B	486.9 A				
Leaf P (g kg ⁻¹)						
SM	1.7	1.7	1.7	NS	NS	NS
SC	1.7	1.9	1.8			
Average	1.7	1.8				
Root P (g kg ⁻¹)						
SM	2.6	1.6	2.1 a	*	*	NS
SC	2.0	1.1	1.6 b			
Average	2.3 A	1.4 B				
RWC (%)						
SM	90.7	83.3	87.0	NS	*	NS
SC	89.1	81.3	85.2			
Average	89.9 A	82.3 B				
SPAD readings						
SM	78.9 aA	60.7 aB	69.8	*	*	*
SC	80.0 aA	45.9 bB	63.0			
Average	79.5	53.3				

Rootstock comparison: means ($n=3$ or 6) followed by different lowercase letters within columns are significantly different by the F test ($p<0.05$). Cu supply comparison: means ($n=3$ or 6) followed by different uppercase letters across paired columns are significantly different by the F test ($p<0.05$). SM: Sunki mandarin; SC: Swingle citrumelo; adequate Cu: $1.0 \mu\text{mol Cu L}^{-1}$; excess Cu: $50.0 \mu\text{mol Cu L}^{-1}$. RDW: root dry weight; NSDW: new shoots DW; TSDW: total shoots DW.

^a F test in the ANOVA of rootstock (R) vs Cu supply (Cu) for each parameter evaluated. * $p<0.05$ and NS = not significant ($p>0.05$).

to 5.0. The water lost through evapotranspiration was replaced every day, and the hydroponic solutions were replaced every two weeks.

The Valencia sweet orange trees on SM or SC were cultivated with two concentrations of Cu, $1.0 \mu\text{mol L}^{-1}$ (adequate Cu) and $50.0 \mu\text{mol L}^{-1}$ (excess Cu), and with two concentrations of P, 0.01 mmol L^{-1} (deficient P) and 0.5 mmol L^{-1} (sufficient P) in the NS. The combination of rootstocks with Cu and P supply in the NS resulted in 8 treatments, with 3 replicate trees each (24 trees total).

After 135 days of treatments, the plants were harvested and separated into old shoots (trunk, leaves and twigs present before the beginning of the treatments), new shoots (leaves and twigs occurred after the beginning of the treatments), and the entire root system. The tissues were rinsed with deionized water and oven-dried at 60°C until constant dry weight (DW) for the determinations of root DW (RDW), new shoots DW (NSDW) and total shoots DW (TSDW). The entire root system and mature leaves from the new shoots were ground and digested in nitric-perchloric acid for the determination of the Cu and P concentrations (Bataglia et al., 1983).

Immediately before terminating the experiment, the leaf greenness (chlorophyll index) was evaluated on the mature leaves from new shoots using a SPAD-502 (Minolta Corp., Ramsey, NJ, USA). The same leaves were employed to quantify the relative water content (RWC) (Morgan, 1986). To evaluate root ultra-structure,

the samples were collected from mature region of representative roots and prepared according to Rocha et al. (2012) for subsequent analysis by transmission electron microscopy (TEM, model EM900, Zeiss, Germany).

The data of tree growth, Cu and P concentrations in the roots and leaves, RWC and SPAD readings were analyzed using a factorial analysis of variance (ANOVA). If no significant three-way interaction ($p>0.05$) was observed for rootstock vs P supply vs Cu supply, the two-way analysis of variance was performed using averaged values of rootstocks and P supplies to investigate the interactions P supply vs Cu supply and rootstock vs Cu supply, respectively. The effects of the treatments were compared using the F test at $p<0.05$. A linear correlation was used to describe the relationships between selected variables. The TEM images of the root ultra-structure were interpreted according to a comparative evaluation of the treatments.

3. Results

There was no significant three-way interaction ($p>0.05$) for P supply vs rootstock vs Cu supply on the parameters of tree growth, Cu and P concentrations in the roots and leaves, RWC and SPAD readings in the leaves. As a result, the following two-way interactions, P supply vs Cu supply and rootstock vs Cu supply, were

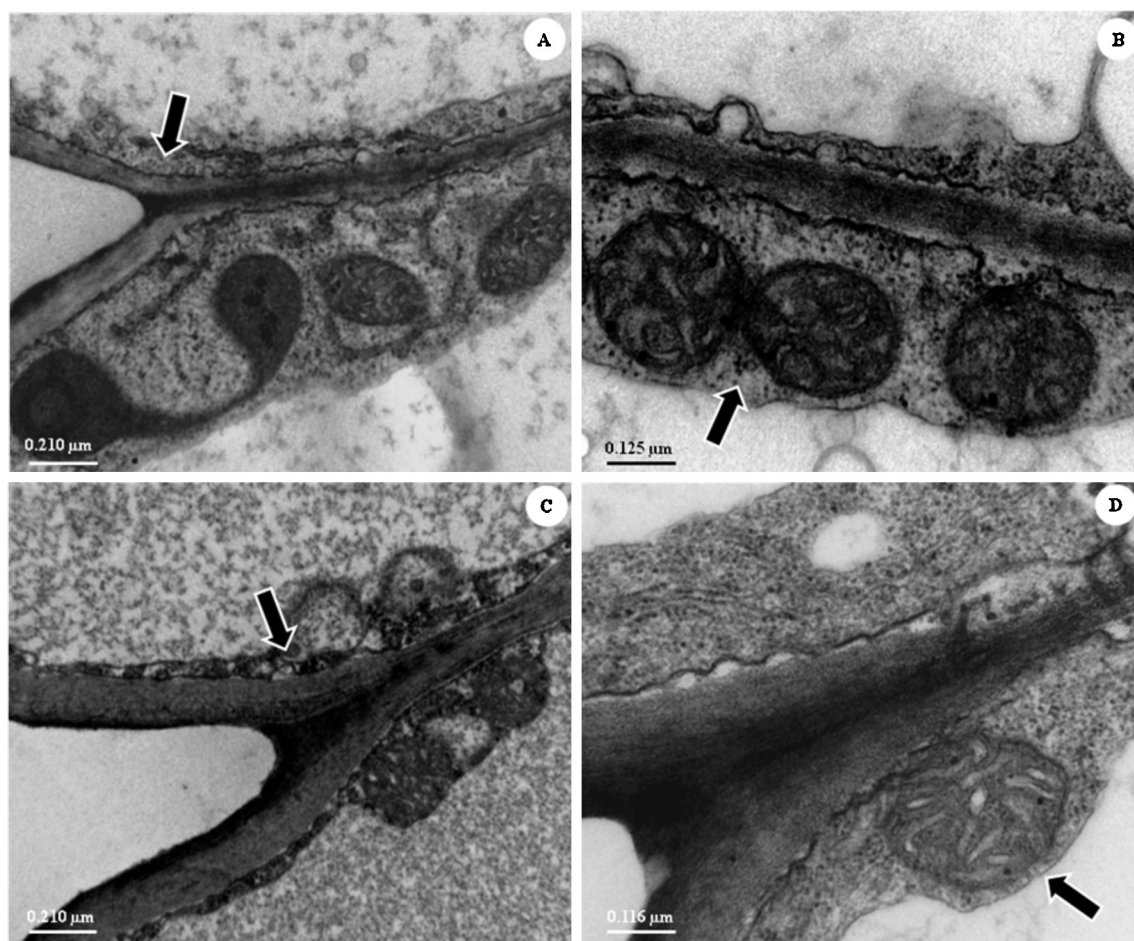


Fig. 1. Transmission electron microscopy of root cells of citrus trees on two rootstocks grown in adequate copper and sufficient phosphorus nutrient solution. (A) Sunki mandarin: cell wall and middle lamella integrity (→); (B) Sunki mandarin: mitochondrial integrity (→); (C) Swingle citrumelo: cell wall and middle lamella integrity (→); (D) Swingle citrumelo: mitochondrial integrity (→).

studied individually to determine the effects of the P supply in the rooting medium (Table 1) and rootstock (Table 2) on the responses of young citrus trees to Cu toxicity.

3.1. Effects of P availability on the responses of young citrus trees to Cu toxicity

The parameters of tree growth showed significant interaction ($p < 0.05$) for P supply vs Cu supply (Table 1). As a result of enhanced DW produced by the trees in the sufficient P, the proportional reduction on growth caused by excess Cu in relation to adequate Cu was greater in sufficient P than in deficient P. For instance, the RDW of the trees in the excess Cu was 40% lower compared to adequate Cu in the sufficient P, whereas no difference was found between Cu supplies in the deficient P (Table 1). The NSDW was reduced by 81–86% in the excess Cu in comparison to adequate Cu, and this decrease in NSDW negatively affected TSDW by 52–62% (Table 1). The P deficiency reduced tree growth by 14–23% in adequate Cu, whereas no effect was detected in the excess Cu (Table 1).

A significant interaction for P supply vs Cu supply was not observed ($p > 0.05$) with regard to the leaf Cu concentration, but did occur ($p < 0.05$) for the root Cu concentration and P concentration in the leaves and roots (Table 1). On the average of the P supplies, the leaf Cu concentration was 89% higher in excess Cu compared to adequate Cu (Table 1). The root Cu concentration was enhanced by 19–23-fold in excess Cu compared to adequate Cu. On the other hand, for the trees grown in the excess Cu, the sufficient P reduced

root Cu concentration in 26% compared to deficient P (Table 1). Furthermore, the Cu concentration was higher in the roots than in the leaves, mainly for the trees in the excess Cu. The P concentration in the leaves and roots of the trees grown in the adequate Cu and deficient P treatment was 26% and 59% lower, respectively, than trees in adequate Cu and sufficient P treatment (Table 1). With regard to RWC and SPAD readings, no significant interaction ($p > 0.05$) was detected for P supply vs Cu supply (Table 1). Therefore, on the average of the P supplies, the excess Cu diminished RWC by 9% and SPAD readings by 33% in comparison to adequate Cu (Table 1).

3.2. Effects of rootstock on the responses of young citrus trees to Cu toxicity

There was significant interaction ($p < 0.05$) for rootstock vs Cu supply on NSDW and TSDW, but the same was not observed for the RDW ($p > 0.05$) (Table 2). On the average of the rootstocks, the RDW was 31% lower in the excess Cu than on the adequate Cu (Table 2). The difference in NSDW and TSDW between trees grown in excess Cu and adequate Cu corresponded to 18.7–25.2 g tree⁻¹ and 21.8–31.1 g tree⁻¹, respectively (Table 2). The trees on SC had 23–27% greater shoot growth than those on SM in the adequate Cu, but there was no variation in DW accumulation in the excess Cu (Table 2).

A significant interaction ($p < 0.05$) for rootstock vs Cu supply occurred for the Cu concentration in the leaves, whereas root Cu concentration was influenced exclusively by Cu supply (Table 2).

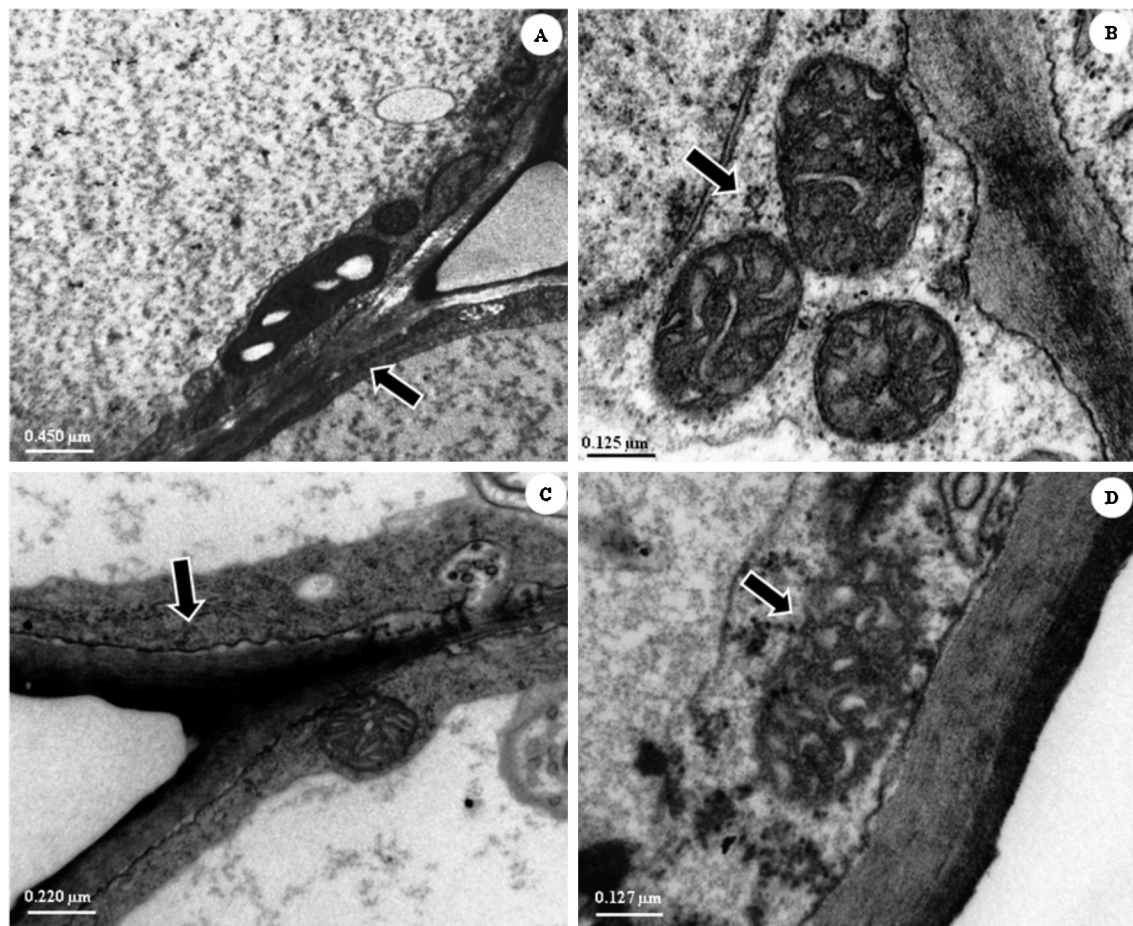


Fig. 2. Transmission electron microscopy of root cells of citrus trees on two rootstocks grown in adequate copper and deficient phosphorus nutrient solution. (A) Sunki mandarin: cell wall and middle lamella integrity (→); (B) Sunki mandarin: mitochondrial integrity (→); (C) Swingle citrumelo: cell wall and middle lamella integrity (→); (D) Swingle citrumelo: disarrangement of the mitochondrial membranes (→).

The trees on the SC had greater leaf Cu concentration than those on SM in the adequate Cu, but there was no difference in the excess Cu (Table 2). On the average of the rootstocks, the excess Cu increased the root Cu concentration by 21-fold in relation to the adequate Cu (Table 2). The leaf P concentration did not vary as a function of the rootstock or Cu supply, but root P concentration was lower for the trees on SC and grown in the excess Cu compared to those on SM and in the adequate Cu, respectively (Table 2). A significant interaction ($p < 0.05$) between rootstock vs Cu supply was found for the SPAD readings but not for RWC ($p > 0.05$) (Table 2). On the average of the rootstocks, the RWC was reduced by 9% in the excess Cu compared to adequate Cu (Table 2). The trees on SM exhibited SPAD readings that were reduced by 23% in the excess Cu compared to adequate Cu, whereas the corresponding reduction was 43% for the trees on SC. Furthermore, Valencia on SM had greater SPAD readings than Valencia on SC in the excess Cu (Table 2).

3.3. Analysis of root ultra-structure

The root cells of the trees on SM and SC grown in adequate Cu and sufficient P treatment maintained the integrity of the cell wall, middle lamella, and mitochondria (Fig. 1A–D). The trees on both rootstocks presented also integrity of the cell wall and middle lamella in the adequate Cu and deficient P treatment (Fig. 2A–D). In this same treatment, the root cells of the Valencia on SM had mitochondria with membranes visible and well formed (Fig. 2B), whereas certain disarrangement in the mitochondrial membranes of the Valencia on SC was observed (Fig. 2D).

In the roots of the Valencia on SM subjected to the excess Cu and deficient P treatment there was degeneration of the middle lamella, the presence of a thin and sinuous cell wall, the occurrence of cellular plasmolysis and the absence of organelles (Fig. 3A and B). Similar sinuosity of the cell wall in the roots of the Valencia on SC was observed, but improved organization of the cell wall and middle lamella as well as the presence of plastids with starch accumulation were visualized (Fig. 3C). In contrast to the observations for Valencia on SM, even with alterations in the membranes, the mitochondria could be identified in the root cells of the Valencia on SC in the excess Cu and deficient P treatment (Fig. 3D).

The roots of the Valencia on SM grown in the excess Cu and sufficient P treatment showed fragmentation of the middle lamella, a thin cell wall displaying sinuosity and the occurrence of cell plasmolysis (Fig. 4A). Furthermore, these trees showed mitochondria with a severe degeneration of the membranes (Fig. 4B). On the other hand, Valencia on SC grown also in the excess Cu and sufficient P treatment had root cells with middle lamella and cell walls and that were better structured (Fig. 4C) in comparison with Valencia on SM (Fig. 4A). In addition, the root cells of the Valencia on SC presented less pronounced degeneration of mitochondrial membranes (Fig. 4D) than Valencia on SM (Fig. 4B). A protective role of sufficient P supply against excess Cu was detected in the root cells of Valencia on SC. In these trees under excess Cu, the sufficient P treatment improved conservation of the cell wall and middle lamella (Fig. 4C and D) in comparison with the excess Cu and deficient P treatment (Fig. 3C and D).

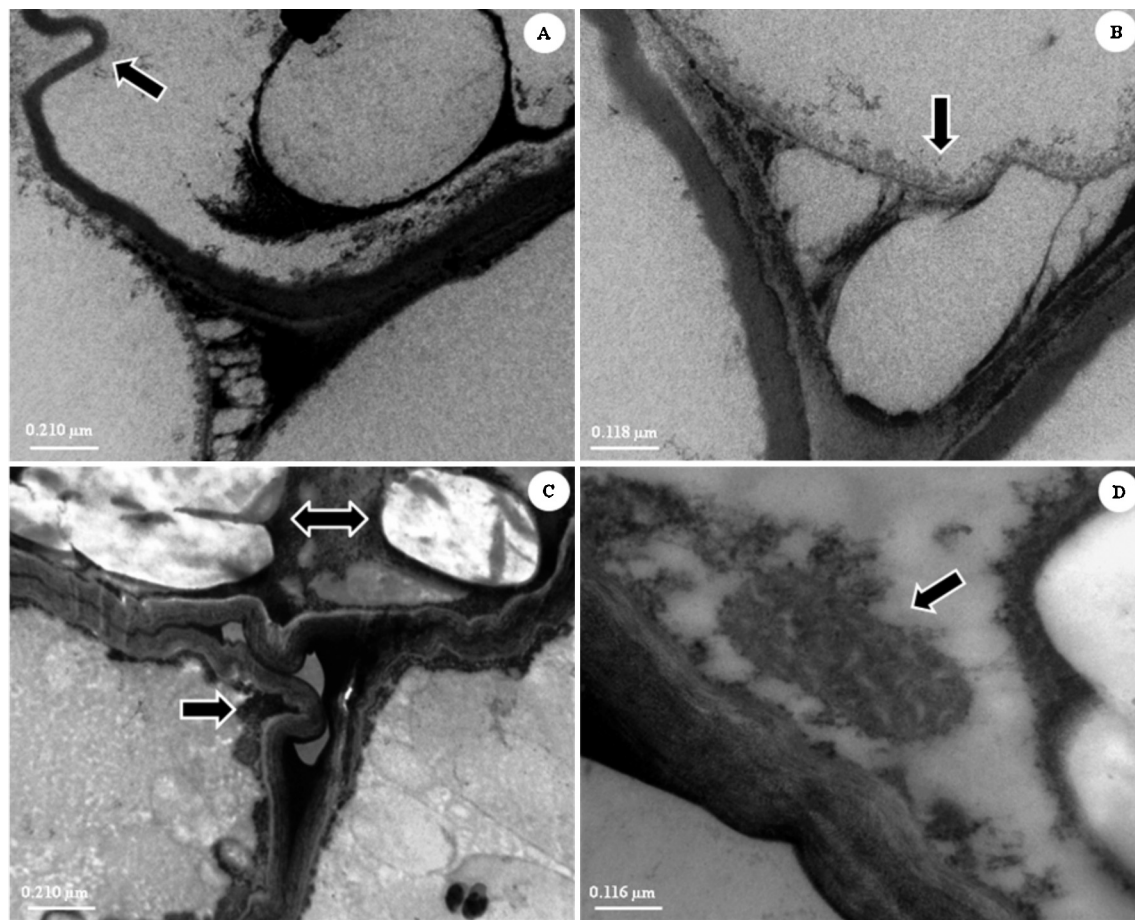


Fig. 3. Transmission electron microscopy of root cells of citrus trees on two rootstocks grown in excess copper and deficient phosphorus nutrient solution. (A) Sunki mandarin: a thin cell wall with sinuosity (→); (B) Sunki mandarin: degeneration of middle lamella (→) and absence of mitochondria; (C) Swingle citrumelo: cell wall with sinuosity (→) and plastids with starch accumulation (↔); (D) Swingle citrumelo: presence of mitochondria but with degeneration of mitochondrial membranes (→).

4. Discussion

The Cu toxicity occurs in adult citrus trees due to continuous applications of Cu fungicides and consequent accumulation of Cu in the soil over the growing seasons, and also in young citrus trees planted in Cu-contaminated sites. In this context, our study was proposed to investigate the latter condition, when the Cu in the soil is already in toxic levels and the trees are more susceptible to Cu toxicity. Taking into account that root Cu concentration is very responsive to Cu availability in the rooting medium and it has been proposed as diagnostic of Cu toxicity (Alva et al., 1995), the root Cu concentration could be used to indicate the level of Cu toxicity present in a specific environment. Therefore, as the root Cu concentration in the trees subjected to excess Cu (average of 486.9 mg kg^{-1} of Cu, Tables 1 and 2) were in the range observed in citrus roots grown in Cu-contaminated soils (Graham et al., 1986; Alva et al., 1995, 2000), the responses observed in our study provided insights into the effects of rootstocks and P availability in the rooting medium on the pattern of Cu toxicity in young citrus trees.

The intensity of injury caused by excess Cu in the root ultra-structure of the trees was influenced by both rootstock and P supply, suggesting differential ability of the citrus to cope with Cu toxicity depending on rootstock and P availability in the rooting medium. For instance, the trees in the excess Cu and sufficient P treatment (mainly Valencia on SC) showed enhanced integrity of the cell wall and middle lamella (Fig. 4C and D) compared to the trees in excess Cu and deficient P treatment (Fig. 3C and D). Since the P supply did not affect the root P concentration of the trees grown in the excess Cu (Table 1), the less preserved cell wall and middle

lamella in the root cells of trees in excess Cu and deficient P treatment compared to those in excess Cu and sufficient P treatment could not be related to an increased oxidative stress observed in P-deficient roots (Juszczuk et al., 2001; Malusà et al., 2002). Therefore, the positive effect of sufficient P supply on root ultra-structure was most likely associated to the influence of this nutrient on reducing the Cu concentration in the roots (Table 1) and the activity of Cu ions in the apoplast and subsequent damage to the root cells (Bueno and Piqueras, 2002; Kopittke et al., 2011). There was also less severe damage in the root cells of Valencia on SC compared to Valencia on SM in the excess Cu combined either with deficient P or sufficient P supply. The trees on SC showed an improved cell wall and middle lamella structures and less pronounced degradation of the mitochondrial membranes (Figs. 3C, D and 4C, D) in comparison to the root cells of trees on SM (Figs. 3A, B and 4A, B). Because there was no variation in the root Cu concentration between the trees on SM and SC in the excess Cu (Table 2), the exclusion mechanism of Cu by the roots (Rouphael et al., 2008) seemed not to be an important component of the Cu toxicity tolerance that could be obtained with the use of these rootstocks. Accordingly, these differences in the root cells damage were probably associated with distinct mechanisms of defense against Cu toxicity of each rootstock, such as antioxidative capacity and Cu complexation in the cells (Devi and Prasad, 1998; Gupta et al., 1999; Cuypers et al., 2002; Lou et al., 2004).

The minor damage caused by excess Cu on the root ultra-structure of trees on SC and also in the trees subjected to sufficient P supply was not translated into improved growth (Tables 1 and 2), confirming the complexity of plant responses to high Cu stress. For instance, the absence of improved root growth under Cu toxicity

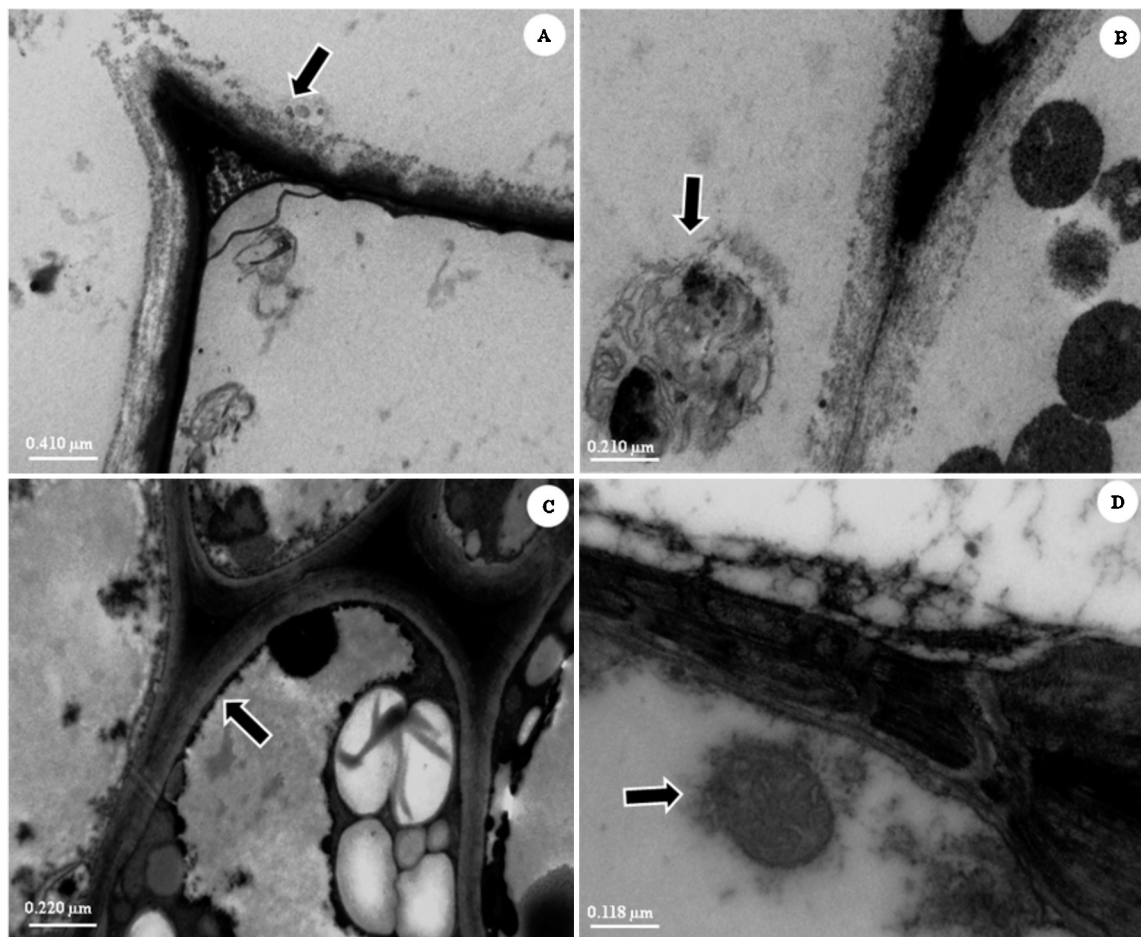


Fig. 4. Transmission electron microscopy of root cells of citrus trees on two rootstocks grown in excess copper and sufficient phosphorus nutrient solution. (A) Sunki mandarin: degeneration of cell wall and middle lamella (\rightarrow); (B) Sunki mandarin: degeneration of mitochondria (\rightarrow); (C) Swingle citrumelo: middle lamella and cell wall integrity without sinuosity (\rightarrow) and starch accumulation in the plastids; (D) Swingle citrumelo: damage to the mitochondrial membranes (\rightarrow).

could be explained by the fact that the minor damages in the root cell wall and middle lamella was not associated with normal rates of cell division and elongation (Ouzounidou et al., 1995) and DNA synthesis of root meristem cells (Doncheva, 1998). Reduced elongation of roots under Cu toxicity has been demonstrated to be also a consequence of the lignification process and the high permeability of the plasmalemma (Arduini et al., 2005). Furthermore, the maintenance of a high leaf area was considered to be critical to sustain growth under Cu toxicity (Vinit-Dunand et al., 2002). Because the SC rootstock and sufficient P did not promote greater leaf growth of the trees in the excess Cu (data not shown), it could be assumed that the absence of enhanced root growth would be due to insufficient carbohydrate supply. However, according to the starch accumulation in the root cell plastids of Valencia on SC subjected to excess Cu with either deficient or sufficient P supply (Figs. 3C and 4C), rather than carbohydrate starvation there was a compromised utilization of carbohydrates for root growth.

The restriction on root growth resulted in compromised new shoot growth in the excess Cu (Tables 1 and 2), as indicated by the positive correlation between RDW and NSDW ($r = 0.75$; $p < 0.0001$; $n = 24$). The inability of sufficient P and SC rootstock in improving chlorophyll content and water status of the trees in the excess Cu (Tables 1 and 2) might have also contributed to the absence of enhanced shoot growth of these trees, since NSDW was positively correlated to SPAD readings ($r = 0.84$; $p < 0.0001$; $n = 24$) and to RWC ($r = 0.80$; $p < 0.0001$; $n = 24$). The lower SPAD readings for Valencia on SC than Valencia on SM (Table 2) were in agreement with more pronounced internodal chlorosis in the former, recognized as

typical symptom of iron (Fe) deficiency induced by high Cu (Kopittke et al., 2009). This finding is also supported by the lower leaf Fe concentration in the trees on SC compared to those on SM (39.0 vs 62.4 mg kg^{-1} of Fe; $p < 0.05$). The lower RWC in the leaves of the trees subjected to excess Cu (Tables 1 and 2) suggested limitations in water uptake and transport to the shoot (Kumar et al., 2008; Nedjimi and Daoud, 2009) and the participation of Cu-induced water stress as a component of Cu toxicity in young citrus trees. Moreover, the root Cu concentration of the trees in excess Cu was not affected by the rootstock, and the decrease provided by the sufficient P in relation to deficient P led to Cu concentration that was still high (415.9 mg kg^{-1}) (Table 1) and capable of inhibiting growth (Alva et al., 2000). Since the root Cu concentration was negatively correlated to NSDW ($r = -0.90$; $p < 0.0001$; $n = 24$), our results did not support the idea that preferential accumulation of Cu in the roots (Tables 1 and 2) could contribute to alleviate the toxicity of Cu in the shoots (Oliva et al., 2010). The Cu accumulation in the roots is a common response to elevated Cu supply (Lin et al., 2003; Wang et al., 2009) and it is a consequence of Cu deposition in the cortical region of the roots (Sahi et al., 2007) and Cu bound on the cell walls (Lou et al., 2004).

5. Conclusions

The treatments imposed in our study represented a condition of new citrus plantings on Cu-contaminated soils. Thus, the success in the occupation of these sites relies on strategies that can ameliorate the detrimental effects of the excess Cu in the roots.

In this scenario, the analysis of root ultra-structure showed that improved tolerance of citrus trees to Cu toxicity could be achieved with the use of SC rootstock and sufficient P supply in the rooting medium. This is supported by the fact that in the excess Cu condition, there was improved integrity of the cell wall and middle lamella in the roots of trees on SC and subjected to sufficient P supply compared to trees on SM and subjected to deficient P supply, respectively. However, the detrimental effects of elevated Cu toxicity on new shoot and root growth prevailed over any benefit that could be provided by this improved root ultra-structure of trees on SC and supplied with sufficient P in the rooting medium. As a result, additional and complementary strategies that could contribute to decrease Cu toxicity and stimulate tree growth need to be investigated and implemented in Cu-contaminated soils in association with the use of SC and sufficient P availability. For instance, our results suggested the need of additional practices to promote greater reduction of Cu availability to the roots and to alleviate the effects of high Cu on decreasing the content of water and chlorophyll in the leaves. Moreover, further studies should be established aiming to better understand the contribution of both improved root ultra-structure and reduced oxidative stress on root growth and also on the adaptation of young citrus trees in Cu-contaminated soils.

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