ELSEVIER

Peroxidase activity and total phenol content in citrus cuttings treated with different copper sources

Tatiana P.A. Merlin [a], Giuseppina P.P. Lima [b,*], Sarita Leonel [a], Fabio Vianello [c]

[a] *Departament of Vegetal Production, Horticulture, Faculdade de Ciências Agronômicas (FCA), Universidade Estadual Paulista - UNESP, Botucatu, SP, Brazil*
[b] *Department of Chemistry and Biochemistry, I.B., CP 510, CEP 18618-000, Universidade Estadual Paulista - UNESP, Botucatu, SP, Brazil*
[c] *Department of Comparative Biomedicine and Food Science, University of Padova, Padova, Italy*

Abstract

The proper citrus cutting choice is considered of basic importance for a healthy and productive citriculture. Substrates composed of organic materials are widely used for the production of citrus seedlings but this can lead to plant copper deficiency, mainly caused by substrate high chelating properties. Copper is an essential heavy metal and is involved in many different plant physiological processes. This experiment was carried out in order to evaluate the behavior of 'Rangpur lime' lemon rootstock (*Citrus limonia* Osbeck) grafted with cv. 'Valencia' (*Citrus sinensis* L. Osbeck), when treated with different copper formulations, as cupric oxychloride (50% Cu — Novartis Biociências™), cuprous oxide (32.8% Cu — Yara Vita™), chelated copper (5% Cu — Stoller™) and copper sulphate (25% Cu — Microsal™) analyzing the activity of peroxidase (POD) and the total content of phenols. The different copper formulations did not show significant differences about plant height, diameter, leaf and root dry weight. The comparison among copper treatments shows that cuprous oxide promoted the antioxidant system (POD activity and phenolic content) while chelated copper was not effective.
© 2012 SAAB. Published by Elsevier B.V. All rights reserved.

Keywords: Citrus limonia Osbeck; *Citrus sinensis*; Oxidative stress

1. Introduction

Brazil is considered the major producer of orange in the world. According to Fundecitrus, in 2009 in Sao Paulo State 517 citrus nurseries were screened with a production of about 8.6 million rootstocks and 17.6 million of citrus seedlings (Amaro and Baptistalla, 2010).

The citrus seedlings quality is one of the production factor influencing the success of commercial crops, since plants only reveal their maximum productivity potential six or eight years after planting (Zanetti et al., 2004).

Copper deficiency is common in plants that grow in rich organic matter soils, where this element form insoluble complexes, unavailable to the plants (Turvey, 1984). In citrus, the deficiency symptoms show dark-green leaves in vigorous branches, exaggerated size, exanthema, and the veins may protrude. It can cause dieback (Malavolta, 2006; Rodriguez and Gallo, 1960).

At supra-optimal concentrations, copper disturbs photosynthetic and mitochondrial electron transport, nitrogen assimilation, cell wall metabolism and many other plant functions (Maksymiec, 1997). Moreover, it affects different parameters of plant metabolism as dry mass accumulation (Ali et al., 2002; Zheng et al., 2004), chlorophyll (Liu et al., 2004; Lou et al., 2004), water content (Burzynski and Klobus, 2004) and the balance in macro and micronutrient levels (Ali et al., 2002; Bernal et al., 2007). Its toxicity depends not only on the metal concentration and exposure duration, but on the developmental stage and physiological state of plants, as well (Ahsan et al., 2007).

Heavy metals, including Cu^{2+}, induce secondary oxidative stress by catalyzing the formation of harmful reactive oxygen

---

* Corresponding author. Tel.: +55 1438800596; fax: +55 1438116255.
E-mail address: gpplima@ibb.unesp.br (G.P.P. Lima).

species (ROS) (Hall, 2002; Schutzendubel and Polle, 2002), which consist in enhanced generation of free radicals (hydroxyl radical, $OH^{\cdot}$, phenoxy radical, $RO^{\cdot}$ and peroxyl radicals, $ROO^{\cdot}$) and other ROS (superoxide radical anion $O^{-}{}_{2}$, singlet oxygen $^{1}O_{2}$, hydrogen peroxide $H_{2}O_{2}$) (Posmyk et al., 2009).

Several substances act as protection system in plants against these reactive oxygen species, including peroxidases, catalase, superoxide dismutase and phenolic compounds. The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donating hydrogen atoms or electrons, or chelating metal cations (Amarowicz et al., 2004). On the other hand, peroxidases are considered to protect cells from heavy metal stress (Karataglis et al., 1991) and are used as stress markers in metal poisoning situations (Chaoui et al., 2004).

The aim of the present study was the assessment of the effect of different copper source additions on the growth of citrus seedlings. Further aim of the present study was to investigate the induction of POD activity and phenolic compounds in citrus seedlings when treated with different copper sources.

2. Material and methods

2.1. Plant material

The experiment was carried out in a commercial citrus nursery located in Botucatu, Sao Paulo State, Brazil, 22°54′13.8″ S and 48°27′32.8″ W, 786 m altitude. The average annual temperature was 21 °C, and accordingly to Köppen the region climate is humid subtropical (cwa), temperate/mesothermal. The experiment started in July 2006, with the sowing of 'Rangpur lime' seeds (*Citrus limonia* Osbeck) in coconut fiber substrate, donated by the Citriculture Center "Sylvio Moreira", Cordeiropolis — SP. This variety has been chosen because it represents the major choice of Brazilian citrus growers (65%) (Amaro and Baptistalla, 2010), due to the superior horticultural characteristics such as earliness to bearing and high fruit yield (Pompeu Junior, 1991). In March, plants were grafted with cv. 'Valencia' (*Citrus sinensis* L. Osbeck).

2.2. Copper treatments

Copper treatments were applied in January, February, March and April, 2007. Control plants did not receive any kind of treatment. The second treatment was treated with cupric oxychloride (50% Cu — Novartis Biociencias™) provided two times per week by foliar spray. The third treatment was treated with cuprous oxide (32.8% Cu — Yara Vita™), two times per week by foliar spray, as well. The fourth treatment with chelated copper (5% Cu — Stoller™) was used, applied two times per week, *via* fertirrigation, the fifth treatment was carried out with copper sulphate (25% Cu — Microsal™), once a week, with irrigation water, to avoid phytotoxicity, since according to previous experiments, the damage occurred when copper sulphate was applied two times per week. Every week all the seedlings received insect control pulverization, as recommended for citrus.

2.3. Copper determination

Plant material (leaves) were thoroughly washed with distilled water and desiccated, in a paper bag, at 60–65 °C for 48 h. Samples were then finely ground for chemical analyses. Copper content was determined by atomic absorption according to Malavolta et al. (1997).

2.4. Plant growth measurement

Growth parameters were evaluated as plant height (cm), rootstock diameter (mm) and dry weight of leaves and roots (g). Plants were measured monthly, starting in February (1st), and ending in May (4th), before treatment application. Plants were randomly collected and were taken out of the plot, measured, frozen in liquid nitrogen and kept in deep freezer until biochemical analysis.

2.5. POD activity determination

For POD assay, leaves were ground in liquid nitrogen and the finely ground material (0.3 g) was dispersed in cold 0.2 M potassium phosphate buffer, pH 6.7, and centrifuged at 12,000 ×g for 10 min at 4 °C. Peroxidase activity was determined according to by Lima et al. (1999) on 250 μL of the supernatant, by addition of 10 μmol of hydrogen peroxide, 35 μL of phenol and 2 μmol of aminoantipyrine. The reaction was carried out during 5 min in water bath at 27 °C and the reaction was stopped with 2 mL of ethanol. The reaction occurrence was measured at 505 nm and the results are expressed in μmol of $H_{2}O_{2}$ reduced $minute^{-1}$ g^{-1} fresh material.

2.6. Total phenol content

The amount of total phenolics in leaf extracts was determined according to the Folin–Ciocalteu method (Singleton and Rossi, 1965). Dry material (50 g) was introduced into test tubes and 2.5 mL of Folin–Ciocalteu's reagent and 2.0 mL of sodium carbonate (20% w/w) were added. The tubes were mixed and allowed to stand for 45 min. The total phenolics content were measured at 765 nm and were expressed as mg gallic acid equivalent g^{-1} dry material (GAE).

2.7. Statistical analysis

The experiment was conducted with five treatments and three replicates. The analysis of variance (ANOVA) was carried out on experimental data and in the case of significance; means were compared by t test (LSD) at 5% probability by SISVAR Software.

3. Results

3.1. Copper levels

Copper content in sample leaves was determined as described in methods and reported in Fig. 1. From the data reported in Fig. 1, it can be noted that the treatment with cuprous oxide lead
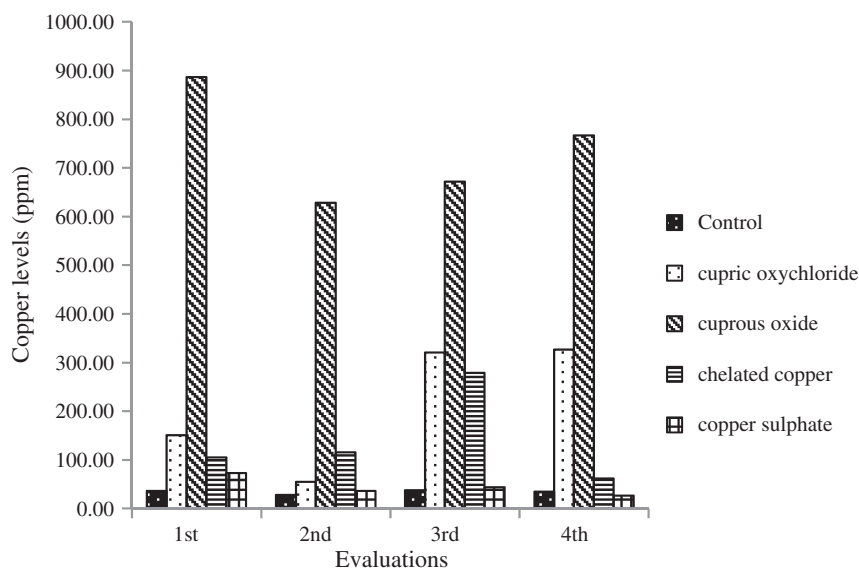
Fig. 1. Copper average content on dry leaves in citrus seedlings determined during the evaluation period on dry leaves.

to an excessive copper accumulation, accordingly with Teofilo Sobrinho et al. (1994), in all the determinations, while the treatment with cupric oxychloride produced high copper levels in 1st, 3rd and 4th evaluations.

3.2. Plant growth measurement

Average plant height (cm) (Table 1), showed significant differences among treatments only in the 2nd evaluation, where the cupric oxychloride lead to the highest height, 64.08 cm, while, the control had 58.50 cm. In the 3rd evaluation, plant height resulted lower than the previous, because samples came from the measurements of the aerial part of the grafted variety.

The average rootstock diameter (mm) (Table 1) was generally unaffected by treatments and increased during plant development, as expected, until the 2nd evaluation. In the 3rd evaluation differences in treatments were observed, treatment with copper sulphate, cupric oxychloride and copper sulphate induced increase in the rootstock diameter. Normally, the ideal rootstock diameter for grafting is around 0.50–0.60 mm, and taking into account

samples in the grafting season, all treatments, included the control reached the ideal rootstock diameter for grafting.

The leaves were from Rangpur lime in the 1st and 2nd evaluations and in the 3rd and 4th evaluations were from cv 'Valencia', after grafting (Table 2). It can be observed that in the fourth evaluation a significant difference among treatments on dry leaf weight, meanwhile significant differences on dry root weight among treatments were shown in the third evaluation.

The dry leaf weight showed, when compared with controls (4th evaluation), a decrease of 6.68% in cupric oxychloride treatment, 5.07% in cuprous oxide treatment, 7.82% in chelated copper treatment and 12.90% in copper sulphate treatment. As regards the dry roots weight, the chelated copper treatment increased by 6.17%, but the other treatments decreased by 2.28% in cupric oxychloride treatment, 4.89% in cuprous oxide treatment and 16.07% in copper sulphate treatment.

3.3. POD activity

Table 3 shows the effect of different copper sources in citrus seedlings leaves. Cuprous oxide treatment gave significantly

Table 1

Plant height average (cm) and rootstock diameter average (mm) in citrus seedlings determined during the evaluations.

Treatments	Evaluations							
	Plant height (cm)				Rootstock diameter (mm)			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Control	30.08[aA]	58.50[abC]	46.33[aB]	51.58[abBC]	0.35[aA]	0.45[aB]	0.62[aC]	0.91[aD]
Cupric oxychloride	27.66[aA]	64.08[bC]	48.66[aB]	48.58[aB]	0.35[aA]	050[aB]	0.67[abC]	0.89[aD]
Cuprous oxide	27.33[aA]	62.50[abC]	46.75[aB]	51.50[aB]	0.34[aA]	0.51[aB]	0.62[aC]	0.93[aD]
Chelated copper	30.33[aA]	61.41[abC]	47.16[aB]	47.58[aB]	0.35[aA]	0.47[aB]	0.68[abC]	0.90[aD]
Copper sulphate	23.25[aA]	54.83[aB]	49.00[aB]	51.79[aB]	0.32[aA]	0.50[aB]	0.71[bC]	0.94[aD]
CV*	11.90				7.68			
LSD**	9.13				0.076			

Values followed by a common letter in the column and capital letter in the line are not different at the $P \leq 0.05$ level of significance.*Coefficient of variance. **Least significant difference.

Table 2
Dry leaf weight average and dry root weight average (g) in citrus seedlings measured during the evaluations.

Treatments	Evaluations							
	Dry leaf weight (g)				Dry root weight (g)			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Control	5.71[aA]	7.14[aA]	17.74[aB]	32.42[bC]	2.82[aA]	5.80[aA]	17.33[aB]	31.73[aC]
Cupric oxychloride	5.85[aA]	9.03[aA]	17.04[aB]	26.88[abC]	4.01[aA]	7.24[aA]	17.78[abB]	27.33[aC]
Cuprous oxide	6.70[aA]	7.84[aA]	17.87[aB]	27.40[abC]	3.30[aA]	6.44[aA]	17.03[abB]	28.09[aC]
Chelated copper	5.96[aA]	10.16[aA]	17.71[aB]	24.25[aB]	4.11[aA]	6.10[aA]	23.30[bB]	27.73[aB]
Copper sulphate	5.91[aA]	7.10[aA]	16.34[aB]	25.53[abC]	3.00[aA]	4.98[aA]	14.74[aB]	25.69[aC]
CV*	30.09				33.67			
LSD**	3.66				3.87			

Values followed by a common letter in the column and capital letter in the line are not different at the P ≤ 0.05 level of significance.*Coefficient of variance. **Least significant difference.

different results in 1st, 2nd and 4th evaluations (3.42, 7.33 and 3.18 µmol of H_2O_2 min^{-1} FM^{-1}, respectively), where the means had a peak in the 2nd evaluation and decreased in the following. The same behavior can be observed on plants treated with chelated copper, which produced the lowest peroxidase activity (2.01 in the 1st evaluation, 2.11 in the 3rd evaluation and 1.67 µmol H_2O_2 min^{-1} FM^{-1} in the 4th evaluation), with respect to other treatments and to controls, as well.

3.4. Total phenol content

The total phenol content is presented in Table 4 for the different copper treatments in citrus seedlings leaves. During the evaluations period (February–May/2007) significant differences were observed among treatments in the 1st, 2nd and 3rd evaluation. As observed for POD activity, the total phenol content presented the highest values in the treatment with cuprous oxide (34.84, 36.58 and 37.45 mg GAE g DM^{-1}). Cupric oxychloride treatment showed 34.79 mg GAE g DM^{-1} as phenol content, higher than other treatments in the 4th evaluation. Meanwhile, control showed the lowest results, when compared with other treatments in the 1st, 2nd and 3rd (22.89, 26.95 and 27.74 mg GAE g DM^{-1}).

4. Discussion

A common problem in Brazilian plant nurseries is represented by copper deficiency in citrus seedlings. However, foliar treatments lead to progressive copper accumulation in the leaf cuticle and was considered excessive according to Teofilo Sobrinho et al. (1994). In this experiment, the average Cu content found in the leaves were about 6 times higher using cupric oxychloride, 22 times higher for cupric oxide, 4 times higher for chelated copper (Fig. 1), with respect to controls. High Cu levels were also observed by Ferreira (2007) in leaves of olives trees treated with copper oxychloride, copper hydroxide and Bordeaux mixture. Therefore, the secondary aim of the experiment was to observe the effect of excessive levels of Cu in citrus seedlings and how the plants manage to control the stress.

Copper is an essential micronutrient for plant growth, metabolism and enzyme activity; but if in excess, it is also a proven inhibitor of various physiological functions. Cu excess can interfere with enzyme function and can decrease protein synthesis and carbohydrate, nucleic acid and lipid metabolism (Marschner, 1986).

Among the treatments used in the present work, significant differences were observed only in the 3rd evaluation for dry root weight, and in the 4th evaluation for dry leaf weight (Table 2). It appears that treated plants presented a lower average values when compared to controls, indicating a possible toxic effect of the copper sources.

As Cu can affect membrane integrity, this could lead to a reduction of root water content, affecting pressure potential and

Table 3
POD average activity (µmol H_2O_2 min^{-1} FM^{-1}) in citrus seedlings leaves determined during the four evaluations.

Treatments	Evaluations			
	1st	2nd	3rd	4th
Control	3.46[bB]	3.09[aB]	3.54[bB]	1.69[aA]
Cupric oxychloride	3.28[abB]	5.67[bC]	2.21[aAB]	1.92[abA]
Cuprous oxide	3.42[bA]	7.33[cB]	2.33[abA]	3.18[bA]
Chelated copper	2.01[aA]	4.52[bB]	2.11[aA]	1.67[aA]
Copper sulphate	3.50[bB]	7.91[cC]	2.25[aAB]	2.09[abA]
CV*	23.25			
LSD**	0.64			

Values followed by a common letter in the column and capital letter in line are not different at the P ≤ 0.05 level of significance.*Coefficient of variance. **Least significant difference.

Table 4
Total phenol average content (mg GAE g DM^{-1}) in citrus seedlings during the evaluations.

Treatments	Evaluations			
	1st	2nd	3rd	4th
Control	22.89[aA]	26.95[aAB]	27.74[aAB]	33.14[aB]
Cupric oxychloride	32.74[bcA]	35.07[bcA]	32.55[bA]	34.79[aA]
Cuprous oxide	34.84[cA]	36.58[cA]	37.45[bA]	32.63[aA]
Chelated copper	26.68[abA]	27.57[abA]	32.42[abA]	28.87[aA]
Copper sulphate	28.30[abcA]	34.59[bcA]	30.66[abA]	31.97[aA]
CV*	14.71			
LSD**	3.82			

Values followed by a common letter in the column and capital letter in line are not different at the P ≤ 0.05 level of significance.*Coefficient of variance. **Least significant difference.

growth (Jouili and Ferjani, 2003). The same copper effect was observed in *Matricaria chamomilla*, which showed a significant and visible growth depression in leaf rosettes and roots (Kovácik et al., 2008). Mourato et al. (2009) noticed a root growth reduction at the maximum copper doses applied in *Lupinus luteus* and Chaoui et al. (2004) in *Pisum sativum* L. According to Jiang et al. (2001) and Wojcik and Tukiendorf (2003) this is a commonly described effect of toxic concentrations of copper. Meanwhile, despite the toxic copper concentrations found in citrus leaves, there was no evidence of toxicity; this fact can be attributed to the protection offered by the plant antioxidant system.

Copper, at high concentrations, causes oxidative stress due to overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can be cytotoxic and can damage important cell compounds (Kovácik and Backor, 2007; Maksymiec and Krupa, 2006; Posmyk et al., 2009). The generation of ROS is considered to be the primary event under a variety of stress conditions. Consequences of ROS formation depend on the intensity of the stress and on the physicochemical conditions in the cell (i.e. antioxidant status, redox state and pH) (Posmyk et al., 2009).

This can be seen in plants treated with cuprous oxide, since they had the highest copper content (Fig. 1). The high level of copper caused an oxidative stress and resulted in a fast and high production of antioxidant components, such as POD and phenols. The increase of POD activity can also be observed with copper sulphate treatment. The same effect was observed on *L. luteus* by Mourato et al. (2009) and on *Len culinaris* Medic. by Janas et al. (2010) where a high Cu amount was accumulated in the roots, inducing oxidative stress and excessive ROS production, resulting in the rapid deterioration of membrane lipids. Reports on copper sulphate effects in increasing POD activity were described by Fang and Kao (2000) in rice, and this effect was attributed to the generation of lipid hydroperoxides, that are toxic for cells and should be scavenged by POD as soon as possible. Thus, free radical generation is likely to be involved in induction of POD activity by Cu^{2+}. It can be also noted that the increase in POD activity depended on the different copper treatment (Table 3).

POD activity showed a peak in the 2nd evaluation (except in the control) probably due to the initiation of plant lignification. Baccouch et al. (1998), Weckx and Clijsters (1996) and Chen et al. (2002) observed an increase in POD isoenzymes is in part responsible for lignin synthesis in radish (*Raphanussativus*) roots and may remove the excess of hydrogen peroxide caused by Cu, thus serving as a detoxifying system during Cu treatment. According to Lin et al. (2005), Cu strongly enhanced POD activity in soybean roots, which was correlated with root growth inhibition and increased lignin content.

Lignin is a highly branched polymer of phenylpropanoid compounds, generally formed from three distinct phenylpropanoid alcohols, including coniferyl, coumaryl, and sinapyl alcohols. The polymerization of these lignin precursors is catalyzed by POD in the presence of H_2O_2 (Mäder and Füssl, 1982) and by laccases in the presence of O_2 (Sterjiades et al., 1993). Or it can be due to the increase in POD content, which also oxidizes IAA in response to the presence of copper ions. This could decrease the endogenous

presence of auxins in roots of metal-treated plants and thereby reduce their growth (Chaoui et al., 2004). This can be observed as the reduction of dry mass of shoots and roots in the last evaluation for all treatments, when compared to control (Table 2).

Thus, high copper concentrations promoted an elevated ROS production, initializing the antioxidants systems to fight against these reactive species and to balance the redox plant status. We noted that copper, in the sulphate or oxide formulation induces oxidative damage in plants, promoting an increase in peroxidase content. In this case, this enzyme could be an indicator of the copper use in citrus seedlings production.

It had been reported that the antioxidant activity of plant materials is well correlated with the content of phenolic compounds (Skerget et al., 2005). The biological and antioxidant activities of phenolic compounds (PhC) can be, at least in part, attributed to their high tendency to chelate heavy metals, in particular, the hydroxyl and carbonyl groups of PhC can strongly bind Cu and Fe. This chelating behavior makes PhC strong candidates in preventing metal-catalyzed free radical formation (Lopes et al., 1999).

In the second evaluation, for all copper treatments, total phenol content increased with respect to controls, being increased also in the third evaluation with cuprous oxide and chelated copper treated plants (Table 4). However, in the 4th evaluation, with the exception of control plants, cited treatments did not promote the same behavior. An explanation of the increase of peroxidase activity in the 2nd evaluation can be due to the concomitant increase of phenol content, as enzyme substrate, indicating that plants are subjected to a lignification process. This is in accord with stem diameter data.

These observations agree with reports of Rice-Evans et al. (1996), proposing that phenolic antioxidants appear more powerful than peroxidase, and even suggested that peroxidases can use them as substrates, along with reduced ascorbate, as an efficient H_2O_2 scavenging system in plant vacuoles (Zancani and Nagy, 2000). In this case, phenols acted as reducing agents, scavenging ROS and chelating copper, thus reducing the metal toxicity in cells (Gordon and Roedig-Penmam, 1998), even in the presence of low copper doses, where plants had no toxicity symptoms and adequate growth.

The response of phenolic compounds to Cu^{2+} can vary among plant species and in different tissues, as well as with metal concentration (Ali et al., 2006; Caldwell, 2002; Gordon and Roedig-Penmam, 1998). Various plants species react differently to excess Cu^{2+}, but differences in plant responses seem to depend not only on copper concentration but also on the capability of plants to increase their antioxidant protection system against heavy metal stress.

The experiment carried out by testing different sources of copper is in accordance with reported literature about the increase of peroxidase activity and phenol content relate to growth and lignification process as well as plant performance against metal induced free radical damage. The resulting plant behavior leads to an asymptomatic high copper content and normal growth.

It can be concluded that the different copper treatments do not interfere significantly with the growth and development of

Rangpur 'lime' seedlings, despite being at toxic levels. The comparison among copper treatments shows that cuprous oxide promoted the antioxidant system (POD activity and phenolic content) while chelated copper was not effective.

Acknowledgment

References

Amarowicz, R., et al., 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry 84, 551–562.

Ahsan, N., et al., 2007. Excess copper induced physiological and proteomic changes in germination rice seeds. Chemosphere 67, 1182–1193.

Ali, N.A., et al., 2002. Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. Plant and Soil 239, 103–111.

Ali, M.B., et al., 2006. Phenolics metabolism and lignin biosynthesis in root suspension cultures of *Panax ginseng* in response to copper stress. Plant Science 171, 147–154.

Amaro, A.A., Baptistalla, C.S.L., 2010. Citrus nurseries — an economic view. Discussion Texts 23, 13.

Baccouch, S., et al., 1998. Nickel-induced oxidative damage and antioxidant responses in *Zea mays* shoots. Plant Physiology and Biochemistry 36, 689–694.

Bernal, M., et al., 2007. Foliar and root Cu supply affect differently Fe-and Zn-uptake and photosynthetic activity in soybean plants. Environmental and Experimental Botany 60, 145–150.

Burzynski, M., Klobus, G., 2004. Changes of photosynthetic parameters in cucumber leaves under Cu, Cd and Pb stress. Photosynthetica 42, 505–510.

Caldwell, C.R., 2002. Effect of elevated copper on phenolic compounds of spinach leaf tissues. Journal of Plant Nutrition 5, 1225–1237.

Chaoui, A., et al., 2004. Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. Journal of Plant Physiology 161, 1225–1234.

Chen, E.L., et al., 2002. Effect of copper on peroxidase activity and ligninin content in *Raphanus sativus*. Plant Physiology and Biochemistry 40, 439–444.

Fang, W.C., Kao, C.H., 2000. Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc. Plant Science 158, 71–76.

Ferreira, I.C.F.R., 2007. Antioxidant activity and phenolic contents of *Olea europaea* L. leaves sprayed with different copper formulations. Food Chemistry 103, 188–195.

Gordon, M.H., Roedig-Penmam, A., 1998. Antioxidant activity of quercetin and myricetin in liposomes. Chemistry and Physics of Lipids 97, 79–85.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany 53, 1–11.

Janas, K.M., et al., 2010. The impact of copper ions on growth, lipid peroxidation, and phenolic compound accumulation and localization in lentil (*Lens culinaris* Medic.) seedlings. Journal of Plant Physiology 167, 270–276.

Jiang, W., et al., 2001. Effects of copper on root growth, cell division, and nucleolus of *Zea mays*. Biologia Plantarum 44, 105–109.

Jouili, H., Ferjani, E.E., 2003. Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess. Comptes Rendus Biologies 326, 639–644.

Karataglis, S., et al., 1991. Effects of heavy metals on isoperoxidases of wheat. Biologia Plantarum 33, 3–9.

Kovácik, J., Backor, M., 2007. Phenylalanine ammonia-lyase and phenolic compounds in chamomile tolerance to cadmium and copper excess. Water, Air, and Soil Pollution 185, 185–193.

Kovácik, J., et al., 2008. Phenolic compounds composition and physiological attributes of *Matricaria chamomilla* grown in copper excess. Environmental and Experimental Botany 62, 145–152.

Lima, G.P.P., et al., 1999. Polyamines and peroxidase activity in bean (*Phaseolus vulgaris* l.) grown under saline stress. Scientia Agricola 56, 21–26.

Lin, C.C., et al., 2005. Rapid effect of copper on lignin biosynthesis in soybean roots. Plant Science 168, 855–861.

Liu, J., et al., 2004. Bioaccumulation and ecophysiological responses to copper in two populations of *Rumexdentatus* L. from Cu contaminated and non-contaminated sites. Environmental and Experimental Botany 52, 43–51.

Lopes, G.K.B., et al., 1999. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Biochimica et Biophysica Acta 1472, 142–152.

Lou, L.Q., et al., 2004. The copper tolerance mechanisms of *Elsholtzia haichowensis*, a plant from copper-enriched soils. Environmental and Experimental Botany 51, 111–120.

Mäder, M., Füssl, R., 1982. Role of peroxidase in lignification of tobacco cells: regulation by phenolic compounds. Plant Physiology 70, 1132–1134.

Maksymiec, W., 1997. Effect of copper on cellular processes in higher plants. Photosynthetica 34, 321–342.

Maksymiec, W., Krupa, Z., 2006. The effects of short-term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. Environmental and Experimental Botany 57, 187–194.

Malavolta, E., 2006. Funções dos macro e micronutrintes – cobre. In: Malavolta, E. (Ed.), Manual de nutricao mineral de plantas. Agronomica Ceres, São Paulo, pp. 336–347.

Malavolta, E., et al., 1997. Avaliação do estado nutricional das plantas: princípios e aplicações, 2nd ed. POTAFOS, Piracicaba. 319 pp.

Marschner, H., 1986. Mineral nutrition in higher plants. Academic Press Harbcourt B. and Company Publishers, London.

Mourato, M.P., et al., 2009. Physiological responses of *Lupinus luteus* to different copper concentrations. Biologia Plantarum 53, 105–111.

Pompeu Junior, J., 1991. Porta-enxertos. In: Rodrigues, O., et al. (Ed.), Citricultura brasileira. Fundação Cargil, Campinas, pp. 265–268.

Posmyk, M.M., et al., 2009. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicology and Environmental Safety 72, 596–602.

Rice-Evans, C.A., et al., 1996. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology & Medicine 20, 933–956.

Rodriguez, O., Gallo, J.R., 1960. Copper deficiency. Bragantia 26, 133–137.

Schutzendubel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal induced oxidative stress and protection by mycorrhization. Journal of Experimental Botany 53, 1351–1365.

Singleton, V.L., Rossi Jr., J.A., 1965. Colorimetric of total phenolics with phosphomolybdic–phosphotungstic acid reagents. American Journal of Enology and Viticulture 16, 144–158.

Skerget, M., et al., 2005. Phenols, proanthocyanidinis, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry 89, 191–198.

Sterjiades, R., et al., 1993. Extracellular laccase and peroxidase from sycamore maple (*Acer pseudoplatanus*) cell suspension cultures. Planta 190, 75–87.

Teofilo Sobrinho, J., et al., 1994. Recomendações de adubação e calagem para citros no Estado de Sao Paulo. Revista Laranja 27.

Turvey, N.D., 1984. Copper deficiency in *Pinusradiata* plant planted in a podzolin Victoria, Australia. Plant and Soil 77, 73–86.

Weckx, J., Clijsters, H., 1996. Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amount of copper. Physiologia Plantarum 96, 506–512.

Wojcik, M., Tukiendorf, A., 2003. Responses of wild type of *Arabidopsis thaliana* to copper stress. Biologia Plantarum 46, 79–84.

Zancani, M., Nagy, G., 2000. Phenol-dependent H_2O_2 breakdown by soybean root plasma membrane-bound peroxidase is regulated by ascorbate and thiols. Journal of Plant Physiology 156, 295–299.

Zanetti, M., et al., 2004. Effect of pyroligneous acid in the spraying solutions on foliar nutrients content of 'Rangpur' lime. Revista Brasileira de Fruticultura 26, 529–533.

Zheng, Y., et al., 2004. Response to copper toxicity for three ornamental crops in solution culture. HortScience 39, 1116–1120.

Edited by A Andreucci