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RESEARCH ARTICLE

Towards soil management with Zn and Mn: estimates of fertilisation efficacy of *Citrus* trees

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Abstract

Nutrient management recommendations for fruit crops lack the understanding of the efficiency of soil fertilisation with manganese (Mn) and zinc (Zn), which could substitute, in part, the traditional foliar applications. Fruit yield of trees in response to Zn and Mn supply via soil may be limited either by sorption reactions with soil colloids or low solubility of fertilisers. We investigated the effects of fertiliser sources and rates of Mn and Zn applied to soils with different sorption capacities on nutrient uptake, biochemical responses and biomass of *Citrus*. Two experiments were carried out with 2-year-old sweet orange trees that received applications of Mn or Zn. The first experiment evaluated the application of Mn fertilisers ($MnCO_3$ and $MnSO_4$) at three levels of the nutrient (0, 0.7 and 3.5 g plant^{-1} of Mn) in two types of soil (18.1% and 64.4% of clay, referred to as sandy loam and clay soils, respectively). The second experiment, likewise, evaluated Zn fertilisers (ZnO and $ZnSO_4$) and nutrient levels (0, 1.0 and 5.0 g plant^{-1} of Zn). Application of Mn and Zn increased nutrient availability in the soils as well as leaf nutrient concentrations in the trees. The lowest rates, 0.7 g plant^{-1} of Mn and 1.0 g plant^{-1} of Zn, both as sulphate, were sufficient to supply these micronutrients to sufficient levels in leaves, flowers and fruits. Metal toxicity to plants occurred with higher doses of both nutrients and to a large extent in the sandy soil. In this case, protein bands lower than 25 kDa were observed as well a decrease on leaf chlorophyll content. In the clay soil, despite increased micronutrient concentrations in the plant, responses were less pronounced because of higher adsorption of metals in the soil. Superoxide dismutase (SOD, EC 1.15.1.1) isoenzyme activity was determined by non-denaturing polyacrylamide gel electrophoresis (PAGE). The Cu/Zn-SOD isoenzymes increased with increased Zn rates, but in contrast, when Mn was applied at the highest rate, the activity of Cu/Zn-SODs decreased. The SOD activity pattern observed indicated increased production of superoxide and consequently an oxidative stress condition at the highest rates of Zn and Mn applied. The results demonstrated that the soil application of Mn and Zn can supply nutrient demands of orange trees, however the low solubility of fertilisers and the high sorption capacity of soils limit fertilisation efficiency. On the contrary, application of sulphate source in sandy soils may cause excess uptake of Mn and Zn and oxidative stress, which impairs the photosynthetic apparatus and consequently tree growth.

Introduction

Manganese (Mn) and zinc (Zn) are limiting nutrients to citrus production under the tropical conditions prevalent in Brazil which is attributed to the inherently low contents in the soil parent material. Furthermore, those are generally found in slightly soluble forms or adsorbed to

colloids, and consequently, their availability in the soil solution to plants is lower (Quaggio *et al.*, 2010).

The low mobility of most micronutrients in the phloem of tree plants hinders the element redistribution from mature tissues to meet the demand of new flushes of shoot growth. Nevertheless, the application of Zn and Mn to fruit crops has usually been conducted with foliar fertilisation during the plant growth cycle (Fageria *et al.*, 2009), justified by the possibility of application and distribution of small amounts of micronutrients in commercial grown areas, and on the contrary, the lack of studies that demonstrate the efficacy of use of those nutrients when applied to the soil.

For micronutrient supply via soil, the sulphates are the most commonly used fertilisers due to their physical properties that allow them to be mixed with other fertilisers (Shaver *et al.*, 2007). The less soluble sources like oxides, carbonates or phosphates when applied to the soil, gradually release the nutrients into the solution, which may reduce absorption rate and consequently the risk of phytotoxicity to plants, and in addition, also reduce adsorption of the metal to the soil (Bradl, 2004; McBeath & McLaughlin, 2014). However, the efficacy of the broadcast application of Mn and Zn to soils with high sorption capacity has not been examined.

Mn and Zn act as co-factors of several enzymes in plants, including superoxide dismutase (SOD), which is responsible for the inactivation of free radicals ($O_2^{\bullet -}$; superoxide), by catalysis to H_2O_2 (Monteiro *et al.*, 2011). Subsequently H_2O_2 is detoxified by enzymes such as catalase, peroxidase and others related to the ascorbate–glutathione cycle in chloroplasts (Cia *et al.*, 2012; Boaretto *et al.*, 2014). Analysis of SOD metalloenzyme was proposed to study the interaction between metals and plants, as well as imbalances among micronutrients in soybean (Leidi *et al.*, 1987), beans (Cakmak & Marschner, 1993) and citrus (Almansa *et al.*, 1989). The enzymatic activity usually responds more quickly to nutritional disorders than plant growth. Therefore, under more severe deficiency of Zn and Mn, reductions in the activity of some enzymes may occur, especially for enzymes such as for SOD, nitrate reductase, carbonic anhydrase and catalase (Broadley *et al.*, 2007).

It is also emphasised that these metals when in excess are harmful to plants, due to the increased level of reactive oxygen species (ROS) in cells (Gratão *et al.*, 2005; Arruda & Azevedo, 2009), and thus causing changes in several physiological processes such as photosynthesis, chlorophyll synthesis and membrane integrity (Lopéz-Millán *et al.*, 2005; Remans *et al.*, 2012). Hence, due to its role on the cell metabolism and metal co-factors, SOD appears to be a useful indicator of the efficacy of Zn and Mn applications and the risk of phytotoxicity at high rates.

Taking into account that the nutrient management recommendations may incorporate strategies for Mn and Zn supply via soil in tropical conditions, this research was based on the hypothesis that fertiliser solubility and soil adsorption capacity, related to soil texture, affects the response of fruit crops to nutrient supply. Therefore, the aim of this study was to evaluate the effect of fertiliser sources and rates of Mn and Zn applied in sandy loam and clay soils on growth and mineral nutrition status of citrus trees.

Materials and methods

Plant material and growth conditions

Two experiments were conducted in a greenhouse with 1–2-year-old trees of Tobias sweet orange [*Citrus sinensis* (L.) Osbeck], which was chosen because of its early flowering, and grown in 20 dm^3 pots containing soil. We used two soils with different texture: a sandy loam soil (18.1% of clay) and a clay one (64.4% of clay), containing respectively, 22 and 37 g dm^{-3} of organic matter, 3.8 and 9.0 mg dm^{-3} of Mn available, and 0.4 and 1.9 mg dm^{-3} of Zn (DTPA-TEA pH 7.3). Soil portions were throughly mixed with dolomitic limestone to reach base saturation of 70% (Quaggio *et al.*, 2010), and then moistened to 60% of field capacity before incubation for about 45 days. Soil pH ($CaCl_2$ 0.01 M L^{-1}) at the end of the experiments ranged for 5.3 and 5.1, for sandy loam and clay soil, respectively.

Orange trees grafted either on Swingle citrumelo [*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.] or Sunki mandarin (*Citrus sunki* Hort. ex Tan.) were transplanted to the sandy loam and to the clay soils, respectively. Before starting the treatments, the plants were grown for 250 days without addition of Mn and Zn to the pots.

Throughout the period of plant growth, soils were irrigated to maintain 70% of field capacity, and the supply of other nutrients was made with application of solutions with the following concentrations: 136.0 mg L^{-1} KH_2PO_4, 365.2 mg L^{-1} KNO_3, 1019.4 mg L^{-1} $Ca(NO_3)_2$, 114.7 mg L^{-1} $CaCl_2$, 492.9 mg L^{-1} $\cdot$ $MgSO_4 \cdot 7H_2O$, 2.9 mg L^{-1} H_3BO_3, 0.08 mg L^{-1} $CuSO_4$, 0.025 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 0.44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ and 1.85 mg L^{-1} $MnSO_4 \cdot H_2O$. Iron was individually applied as Fe-EDDHA (iron chelated with ethylene diamine-N,N′-bis(2-hydroxyphenylacetic acid)) via solution containing 5.0 mg L^{-1} Fe.

Adsorption curves were fitted to describe the adsorption isotherms of Mn and Zn for both soils. Samples with 1.0 g of soil were placed in plastic vials plus 10 mL of 10 mM L^{-1} of $CaCl_2$ solution with Mn or Zn (as $MnNO_3$ or $ZnNO_3$) at concentrations of 0.5, 2.0, 8.0, 32.0, 128.0

and 512.0 mg L^{-1}. The vials were shaken for 48 h in a horizontal shaker at 160 rpm = 2.86 g (25°C), and then centrifuged for 10 min at 905 *g*. After filtration of the supernatant, the concentration of Zn and Mn at the equilibrium solutions were quantified with an ICP-OES spectrophotometer (Perkin-Elmer, mod. 5100 PC, Norwalk, CT, USA). The Langmuir model was used to quantify the metal adsorption capacity of soils (Bradl, 2004) assuming that metal adsorption occurs on homogeneous surface with fixed number of surface adsorption sites. The non-linearized form of the isotherm was fitted, which described the energy of adsorption (K_L) and maximum adsorption capacity (b_L) of Mn and Zn (Fontes *et al.*, 2000; Bradl, 2004).

The first experiment consisted of two sources of Mn applied to the soil ($MnSO_4$ and $MnCO_3$) at: 0 (Control), 0.7 and 3.5 g plant^{-1} of Mn, on both soils. Likewise, the second experiment consisted of two sources of Zn to the soil ($ZnSO_4$ and ZnO) at: 0 (Control), 1.0 and 5.0 g plant^{-1} of Zn, on both types of soil. The fertiliser sources were diluted in 500 mL of dH_2O and applied evenly over the soil surface in a single dose per pot. The $MnCO_3$ and the ZnO sources were a suspension, with 90% of particles with 150–300 and 200–800 nm, respectively. In both trials, the treatments were arranged in a completely randomised design with four replications. The rates for each micronutrient were estimated to meet the nutrient demand of plants during growth or cause excess, considering the solubility of fertilisers.

At 180 days after the beginning of the treatments (application of Mn and Zn), the plants were destructively harvested and separated into trunk, old branches and old leaves (grown before initiation of treatments), new branches and new leaves (grown after treatment applications; 4–6-month-old) and roots to determine the dry mass (DM) of each part by oven drying for 72 h at 65°C. Flowers produced during the experimental period and young fruits (<2.5 cm in diameter) were also dried. Because of the minimal weight of each of these parts, the same was not taken into account on total plant dry matter estimation. The plant materials were then ground to pass a 200-mesh sieve and concentration of Mn and Zn were determined by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA), after digestion of tissue with concentrated $HNO_3 + HClO_4$ (2:1 v/v ratio) (Bataglia *et al.*, 1983). The Mn and Zn concentrations of soil samples were determined after metal extraction with a DTPA-TEA solution at pH 7.3 (Lindsay & Norvell, 1978) and ICP-OES readings. Because of soil analysis was made using a scoop volume measurements (Abreu *et al.*, 1997), the units were given on volume basis (mg dm^{-3}) a standard, convenient and accurate procedure.

Chlorophyll content

At 180 days after application of Mn and Zn, 50 mg of tissue material from new leaves (6-month-old) were collected to measure chlorophyll content as described by Barnes *et al.* (1992), using dimethylsulfoxide (DMSO). Samples were placed in glass vials with 7.0 mL of DMSO and were heated in a water bath at 65°C until tissue depigmentation (approximately 4 h). The absorbance (A) of extracts was read in a spectrophotometer (Femto 600 Plus, São Paulo, Brazil) at 646 and 663 nm. Chlorophyll a (C_a), b (C_b) and total (C_{total}) contents were determined using the equations: $C_a = (14.85{*}A_{663}) - (5.14{*}A_{646})$; $C_b = (25.48{*}A_{646}) - (7.36{*}A_{663})$ and $C_{total} = (7.49{*}A_{663} + 20.34{*}A_{646})$.

Sample preparation for enzyme assay and protein measurement

At 120, 150 and 180 days after application of Mn and Zn, one gram samples were collected from new leaves (4–6-month-old), and then homogenised in 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5), with 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 4% (w/v) polyvinylpolypyrrolidone (PVPP) (Gratão *et al.*, 2008). The suspension was centrifuged at 5 590 *g* for 35 min at 4°C, and the supernatant was used for determination of protein and SOD activity. The total protein content was measured using the method of Bradford (1976), using *bovine serum albumin* (BSA) as a standard.

Sodium dodecyl sulphate (SDS)-PAGE protein profiles were conducted using a discontinuous buffer system (Mini Protean II, Bio-Rad, Hercules, CA, USA) with 5% stacking gel and 12% running gel (w/w) at pH 8.9, prepared according to Laemmli (1970). The volume of the protein extract corresponded to 25 µg of denatured protein in solution. Electrophoresis was performed at 15 mA during 6 h in a cold chamber and the protein bands were stained with 1% (w/w) Coomassie Brilliant Blue G-250 solution. The gel was scanned and the image was analysed with ImageJ (National Institute of Mental Health, Bethesda, MD, USA), estimated by intensity and presence or absence of bands among treatments.

The determination of SOD in 12% non-denaturing polyacrylamide gel electrophoresis (PAGE), was performed as described by Pereira *et al.* (2002). After separation on non-denaturing gel, the gel was incubated in darkness in a solution of 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.1 mM nitro blue tetrazolium (NBT), 0.05 mM riboflavin and 0.3% tetramethylethylenediamine (TEMED) for 30 min. After incubation, the reaction solution was poured off, the gels were rinsed with deionised water and exposed to light

Table 1 Langmuir coefficients, determination coefficients (R^2), binding energy (K_L) and maximum adsorption (b_L) obtained from the fit of the isotherm, referenced to zinc and manganese adsorption in the soils

Soil Type	Model Equations[a]	R^2	K_L ($L\,mg^{-1}$)	b_L ($mg\,kg^{-1}$)
Zn				
Clay	$Y = (0.032 * 1.220C)(1 + 0.032)$	0.97**[b]	0.032	1220
Sandy loam	$Y = (0.035 * 0.790C)(1 + 0.035)$	0.99**	0.035	790
Mn				
Clay	$Y = (0.008 * 1.236C)(1 + 0.008)$	0.74*	0.008	1236
Sandy loam	$Y = (0.010 * 0.620C)(1 + 0.010)$	0.70*	0.010	620

Significant at $^{*}P < 0.05$; $^{**}P < 0.01$.
[a]Original model of Langmuir equation (non-linear).
[b]R^2 denotes data fit to the statistical model.

for a few minutes to photo-oxidation, whereas bands corresponding to SOD activity remained under non photo-oxidation. The photo oxidation was interrupted by the insertion of acetic acid 7% for 15 min. The isoforms were then classified as Cu/Zn-SOD, Fe-SOD or Mn-SOD, of which the Cu/Zn-SOD is inactivated by potassium cyanide (KCN) and H_2O_2, the Fe-SOD by H_2O_2 and is resistant to KCN, and the Mn-SOD is resistant to both reagents (Azevedo *et al.*, 1998).

Statistical analysis

Descriptive statistics was applied and the data were analysed using analysis of variance of the studied factors and their interactions (Source * Rate) considering significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$. Pearson's correlation was used to describe relationships among leaf, flower and young fruit nutrient concentrations.

Results

Soil Mn and Zn adsorption capacity

Micronutrient adsorption characteristics of soils were well described by the Langmuir model, with regression coefficients (R^2) for Zn > 0.97 ($P < 0.05$) and for Mn > 0.70 ($P < 0.01$) (Table 1). The energy of adsorption for Zn and Mn (K_L) was quite similar for the sandy loam and clay soils, even though major difference on the maximum adsorptive capacity (b_L) was observed.

Fertilisers and rates of manganese

Plants that received $MnCO_3$ in the sandy loam soil, regardless of rate, produced more total DM when compared to those that received $MnSO_4$ (Table 2). The latter exhibited lower DM of roots, branches and leaves, including the old leaves after applying the more soluble Mn source, despite the low initial soil concentration of Mn ($3.8\,mg\,dm^{-3}$). Indeed, supply of $MnSO_4$ caused a decrease in shoot DM when compared to the control. In the clay soil, we observed higher DM of new parts of plants (stems = 15 g plant^{-1} and leaves = 34 g plant^{-1}) which received the application of Mn, regardless fertiliser source and rate (Table 2).

The concentration of available Mn increased up to $50\,mg\,dm^{-3}$ in the soil when compared to the Control (Table 3). Accordingly, increases in the concentration of nutrient both in new and in the old leaves of the plants were observed, being greater when the $MnSO_4$ was applied (Table 3). For plants in the sandy loam soil, levels equal to $550\,mg\,kg^{-1}$ of Mn in the old leaves and $951\,mg\,kg^{-1}$ for the newer ones were observed. With the application of $MnCO_3$, Mn was less absorbed by plants suggesting a more gradual release of the nutrient into the soil solution. On the clay soil, only the highest dose of $MnSO_4$ was able to elevate the Mn concentration, with 142 and $84\,mg\,kg^{-1}$ in the old leaves and new ones, respectively compared to either the application of $MnSO_4$ at 0.7 g plant^{-1} or the $MnCO_3$ despite doses in the same soil (Table 3).

When the highest dose of $MnSO_4$ was applied to the sandy loam soil, increased absorption of Mn provided a proportional accumulation of nutrients in flowers and fruits, with 182 and $204\,mg\,kg^{-1}$ of Mn, respectively (data not shown). The other treatments exhibited an average concentration of $22\,mg\,kg^{-1}$ of Mn for both fruits and flowers. Although lower than in the sandy loam soil, the highest dose of $MnSO_4$ applied to the clay soil provided the highest levels of the nutrient in flowers and fruits (23 and $15\,mg\,kg^{-1}$ of Mn), compared to the other treatments (15 and $8\,mg\,kg^{-1}$ of Mn). Furthermore, there was a close correlation between the concentrations of Mn in young leaves and flowers for different treatments and in both soils ($R^2 = 0.95$ and $P < 0.001$).

Fertilisers and rates of zinc

The application of Zn in the sandy loam soil provided greater root DM, averaging 236 g plant^{-1}, while the plants that did not receive Zn (Control) produced only 145 g plant^{-1} (Table 4). In addition, on the sandy loam soil, the new leaves also exhibited higher DM production, 17% higher on average when 1.0 g plant^{-1} of Zn was applied. Among the treatments receiving Zn, there was a decrease in the biomass of new leaves (54 g plant^{-1}) at the highest dose of $ZnSO_4$ (5.0 g plant^{-1} of Zn), probably due to phytotoxicity (foliar Zn > $115\,mg\,kg^{-1}$, Table 5). The clay soil responses on DM production of plants with

Table 2 Dry mass of plant parts of young citrus plants, 180 days after of Mn application in the soil

Treatment (Mn, g plant^{-1})		Sandy Loam Soil (g plant^{-1})					Clay Soil (g plant^{-1})				
		Roots	Old Parts[b]	New Stems	New Leaves	Total	Roots	Old Parts	New Stems	New Leaves	Total
Control	–	213 ± 56[a]	132 ± 27	28 ± 7	65 ± 18	440 ± 104	133 ± 16	93 ± 17	7 ± 3	15 ± 5	248 ± 30
$MnSO_4$	**0.7**	191 ± 31	118 ± 27	32 ± 16	75 ± 18	416 ± 117	139 ± 47	102 ± 20	19 ± 6	43 ± 11	303 ± 79
	3.5	128 ± 41	103 ± 31	17 ± 4	40 ± 16	288 ± 88	143 ± 31	101 ± 15	14 ± 6	31 ± 17	289 ± 57
$MnCO_3$	**0.7**	257 ± 58	146 ± 23	39 ± 5	88 ± 9	530 ± 85	138 ± 32	110 ± 15	15 ± 6	34 ± 12	297 ± 38
	3.5	272 ± 47	143 ± 9	41 ± 21	71 ± 15	527 ± 70	115 ± 24	82 ± 34	12 ± 6	30 ± 17	239 ± 94
Significance (*P*)[c]											
Control vs Rate		0.962	0.683	0.632	0.790	0.993	0.838	0.567	0.033	0.026	0.422
Source		<0.001	0.008	0.016	0.013	<0.001	0.243	0.614	0.170	0.284	0.271
Rate		0.320	0.163	0.265	0.004	0.086	0.347	0.181	0.104	0.205	0.200
Source * Rate		0.112	0.489	0.150	0.227	0.095	0.256	0.281	0.972	0.889	0.355

[a]Standard error of the mean ($n = 4$).
[b]Old parts = branches + old stems + old leaves.
[c]*P*-values refer to a two-way factorial plus control treatment ANOVA.

Table 3 Manganese concentrations in soils and leaves of young citrus trees, 180 days after fertiliser application

Treatment (Mn, g plant^{-1})		Sandy Loam Soil			Clay Soil		
		Soil (mg dm^{-3})	Old Leaves (mg kg^{-1})	New Leaves (mg kg^{-1})	Soil (mg dm^{-3})	Old Leaves (mg kg^{-1})	New Leaves (mg kg^{-1})
Control	–	9 ± 4[a]	40 ± 8	56 ± 18	6 ± 1	45 ± 8	23 ± 5
$MnSO_4$	**0.7**	32 ± 21	104 ± 30	146 ± 33	12 ± 10	54 ± 9	24 ± 4
	3.5	53 ± 11	550 ± 49	951 ± 98	30 ± 14	142 ± 55	84 ± 44
$MnCO_3$	**0.7**	40 ± 14	47 ± 5	81 ± 17	14 ± 9	43 ± 1	23 ± 2
	3.5	64 ± 24	62 ± 19	137 ± 31	27 ± 18	45 ± 8	28 ± 4
Significance (*P*)[b]							
Control vs Rate		0.002	0.007	0.009	0.057	0.324	0.358
Source		0.311	<0.001	0.004	0.909	0.002	0.024
Rate		0.031	<0.001	0.004	0.036	0.008	0.012
Source * Rate		0.877	<0.001	0.009	0.708	0.011	0.028

[a]Standard error of the mean ($n = 4$).
[b]*P*-values refer to a two-way factorial plus control treatment ANOVA.

the application of Zn were limited to an effect of applied sources, in which the ZnO promoted DM production of new stems (mean of 17 g plant^{-1}; Table 4).

The levels of available Zn in soil increased proportionally to the rate of fertiliser applied (Table 5). At the dose of 1.0 g plant^{-1} of Zn, the levels of the micronutrient averaged 30 mg dm^{-3} with application of $ZnSO_4$, and 8 mg dm^{-3} with ZnO despite the soil texture. At 5.0 g plant^{-1} of Zn, besides the effect of fertiliser sources, Zn availability also varied with soil texture. In this case, the largest increase was observed with $ZnSO_4$ applied to the sand, in which the soil level was about 120 mg dm^{-3} of Zn (Table 5).

Concomitantly with the levels found in soils, there was an increase in the Zn concentration in old and new leaves (Table 5). $ZnSO_4$ at the highest dose (5.0 g plant^{-1} of Zn) promoted the highest levels for both parts analysed, either in the sandy loam (80 and 144 mg dm^{-3} for old and new leaves, respectively) or in the clay soil (50 and 115 mg dm^{-3} for old and new leaves, respectively). The concentration of Zn in the leaves correlated with the micronutrient concentration available in the soil ($R^2 = 0.94$; $P < 0.01$; data not shown).

As observed for Mn, Zn concentration in young leaves correlated with that in flowers ($R^2 = 0.95$ and $P < 0.01$). There was an increase in the Zn concentration in the flowers and fruits with $ZnSO_4$ at 5.0 g plant^{-1} of Zn for both soils, with an average of 35 and 20 mg kg^{-1}, respectively, for flowers and fruits (data not shown). The other treatments, including those that did not receive Zn, exhibited average concentrations in the flowers and fruits of 16 and 6 mg kg^{-1}, respectively, for both soils.

Chlorophyll content

The effects of Mn and Zn supply on chlorophyll contents of plant leaves varied with fertiliser sources but did not with rates. Lower levels of chlorophyll were verified for

Table 4 Dry mass of plant parts of young citrus trees, 180 days after of Zn application in the soil

Treatment (Zn, g plant^{-1})		Sandy Loam Soil (g plant^{-1})					Clay Soil (g plant^{-1})				
		Roots	Old Parts[b]	New Stems	New Leaves	Total	Roots	Old Parts	New Stems	New Leaves	Total
Control	–	145 ± 24[a]	129 ± 27	26 ± 8	59 ± 13	359 ± 65	136 ± 43	105 ± 26	15 ± 6	37 ± 19	293 ± 69
$ZnSO_4$	**1.0**	243 ± 51	145 ± 31	33 ± 7	73 ± 29	494 ± 75	116 ± 36	98 ± 10	10 ± 7	25 ± 17	249 ± 60
	5.0	213 ± 39	144 ± 38	29 ± 2	54 ± 4	454 ± 64	103 ± 26	86 ± 9	10 ± 4	24 ± 13	223 ± 46
ZnO	**1.0**	233 ± 96	153 ± 29	29 ± 3	71 ± 15	486 ± 77	127 ± 50	96 ± 23	14 ± 6	28 ± 16	315 ± 62
	5.0	254 ± 31	133 ± 24	36 ± 7	80 ± 7	503 ± 41	147 ± 52	105 ± 12	19 ± 3	44 ± 11	293 ± 43
Significance (*P*)[c]											
Control vs Rate		0.006	0.070	0.090	0.046	0.010	0.538	0.350	0.510	0.457	0.434
Source		0.622	0.670	0.687	0.002	0.642	0.167	0.158	0.003	0.146	0.108
Rate		0.891	0.680	0.651	0.142	0.801	0.877	0.905	0.168	0.325	0.710
Source * Rate		0.415	0.500	0.085	<0.001	0.524	0.396	0.139	0.189	0.270	0.243

[a]Standard error of the mean ($n = 4$).

[b]Old parts = branches + old stems + old leaves.

[c]*P*-values refer to a two-way factorial plus control treatment ANOVA.

Table 5 Zinc concentrations in soils and leaves of young citrus trees 180 days after fertilisers application

Treatment (Zn, g plant^{-1})		Sandy Loam Soil			Clay Soil		
		Soil (mg dm^{-3})	Old Leaves (mg kg^{-1})	New Leaves (mg kg^{-1})	Soil (mg dm^{-3})	Old Leaves (mg kg^{-1})	New Leaves (mg kg^{-1})
Control	–	1 ± <1[a]	9 ± <1	10 ± 2	2 ± <1	11 ± <1	11 ± 2
$ZnSO_4$	**1.0**	32 ± 9	19 ± 5	32 ± 5	29 ± 6	14 ± 2	16 ± 2
	5.0	121 ± 19	80 ± 16	144 ± 17	74 ± 50	50 ± 15	115 ± 36
ZnO	**1.0**	7 ± 2	9 ± 2	12 ± 1	9 ± 6	13 ± 1	16 ± 3
	5.0	84 ± 26	21 ± 5	42 ± 7	45 ± 10	15 ± 1	18 ± 2
Significance (*P*)[b]							
Control vs Rate		0.002	0.007	0.009	0.043	0.344	0.025
Source		0.311	<0.001	0.004	0.032	0.052	0.005
Rate		0.031	<0.001	0.004	0.007	0.047	0.003
Source * Rate		0.877	<0.001	0.009	0.156	0.060	0.004

[a]Standard error of the mean ($n = 4$).

[b]*P*-values refer to a two-way factorial plus control treatment ANOVA.

plants with $MnSO_4$ [C_a (1.5 mg g^{-1} fresh weight (FW)), C_b (1.1 mg g^{-1} FW) and C_{total} (2.6 mg g^{-1} FW)] compared to those with $MnCO_3$ [C_a (1.8 mg g^{-1} FW), C_b (1.5 mg g^{-1} FW) and C_{total} (3.2 mg g^{-1} FW)] in the sandy soil ($P < 0.05$; data not shown), whereas the same was not verified in the clay.

In the second experiment, lower C_b and C_{total} concentrations were also verified for plants that received the more soluble fertiliser ($ZnSO_4$), either in the sandy [C_b (1.3 mg g^{-1} FW) and C_{total} (2.8 mg g^{-1} FW)] or in the clay soil [C_b (1.9 mg g^{-1} FW) and C_{total} (3.8 mg g^{-1} FW)] compared to those with ZnO in both soils, respectively [C_b (1.5 and 2.2 mg g^{-1} FW) and C_{total} (3.2 and 4.3 mg g^{-1} FW)] ($P < 0.05$).

Protein profiles in sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Changes in protein profiles were observed only for plants grown in the sandy soil with respect either to Mn or Zn supply (Fig. 1). With 3.5 g plant^{-1} of Mn, as sulphate, the presence of one band of about 20 kDa was observed.

In the second experiment, with the application of the highest Zn rate (5.0 g plant^{-1}), as sulphate, the presence of one band of 20 kDa was also observed. Furthermore, decreases in the intensity of other bands of about 50 kDa (30%) and <15 kDa (45%) occurred when compared to the other treatments (Fig. 1).

Superoxide dismutase activity

Six isoforms of SOD were identified in the leaves of the orange trees, with three Cu/Zn-SOD (I, V and VI), two Mn-SOD (II and III), and one Fe-SOD (IV) (Fig. 2). A decrease in the activity of the isoforms Cu/Zn-SOD (I and V) was found when the highest dose of $MnSO_4$ was applied to plants grown in the sandy loam soil, during the three periods of evaluation (Fig. 2). The same response was observed for plants grown in the clay soil with the

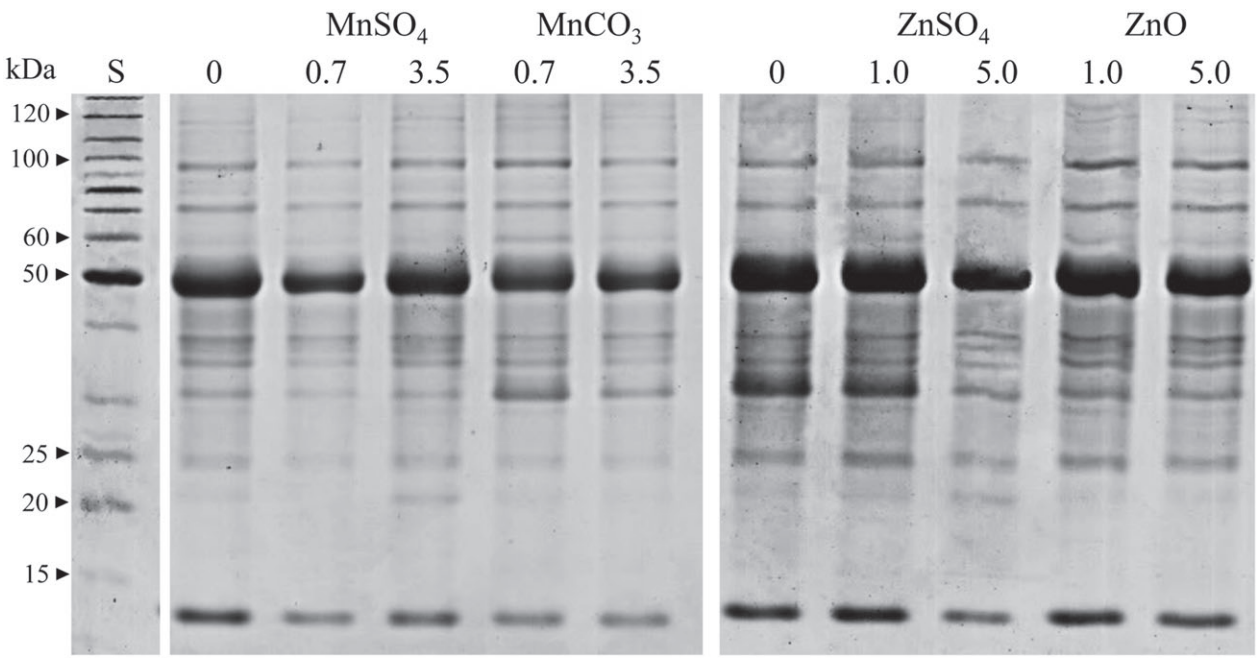

Figure 1 Protein profile in SDS-PAGE to leaves of citrus trees, 180 days after Mn or Zn sources application in sandy loam soil.

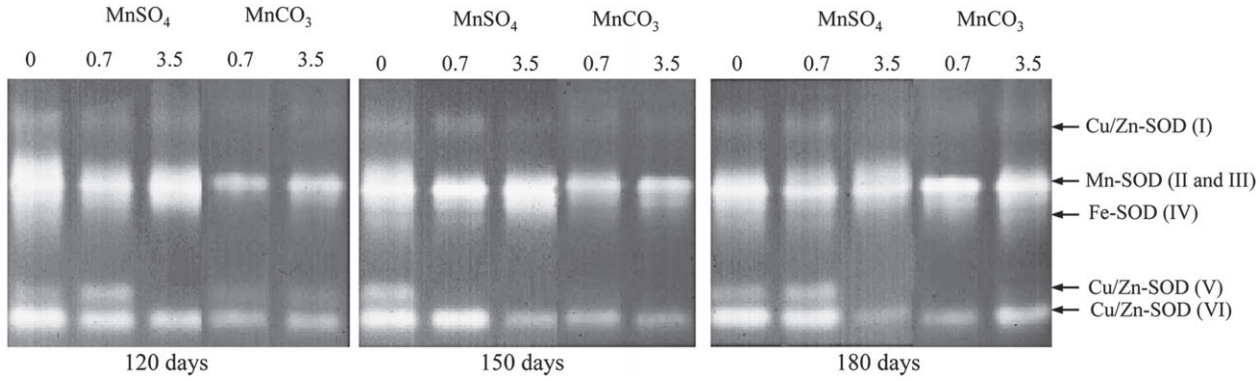

Figure 2 Specific activities of superoxide dismutase (SOD) forms in young leaves of citrus trees grown in sandy loam soil, 120, 150 and 180 days after Mn fertilisers ($MnSO_4$ and $MnCO_3$) application.

highest dose of $MnSO_4$ at 120 days, and when $MnCO_3$ was applied, during the three periods (Fig. 3).

The SOD activity was different with Zn supply, for all periods of enzyme assay. In the sandy loam soil, the highest dose of both Zn sources increased expression of bands corresponding to the Cu/Zn-SOD isoforms (I, V and VI) for all the three periods (Fig. 4). At the 180 day period, both Zn sources also increased expression of the Mn-SOD. In the clay soil application of $ZnSO_4$ decreased the expression of the Mn-SOD (II and III) at 120 days, while, after 150 and 180 days, the highest dose of this same source increased expression of isoform V (Cu/Zn-SOD, Fig. 5). ZnO also affected the Cu/Zn-SOD (isoform VI), whose activity was lower in the first studied period and increased in the last period (Fig. 5). For both soil conditions, besides control plants (with no Zn application), which contained low foliar levels, the activity of the Cu/Zn-SOD only exhibited lower expression of isoform VI in the last period when compared to other treatments that received doses of Zn.

Discussion

The Mn and Zn availability to citrus plants have limited fruit production, mainly when commercial groves are established in highly weathered soils with high sorption

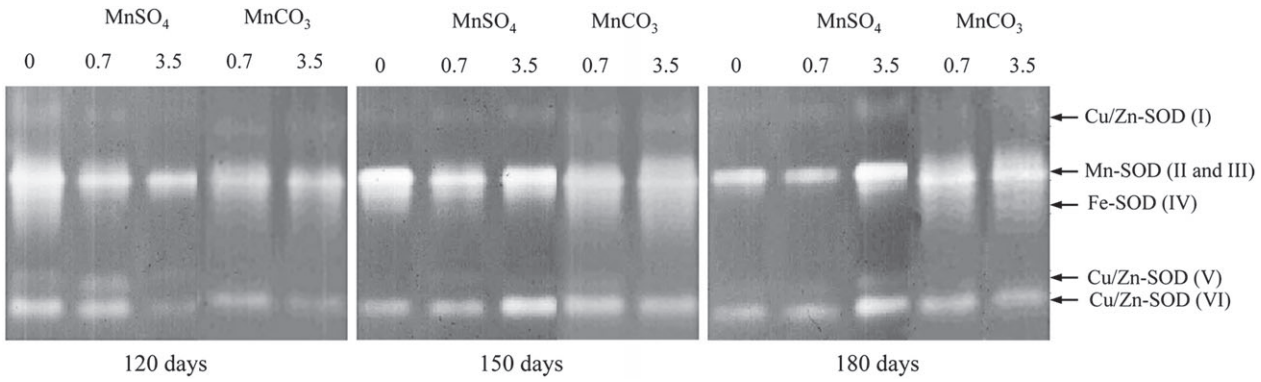

Figure 3 Specific activities of superoxide dismutase (SOD) forms in young leaves of citrus trees grown in clay soil, 120, 150 and 180 days after Mn fertilisers ($MnSO_4$ and $MnCO_3$) application.

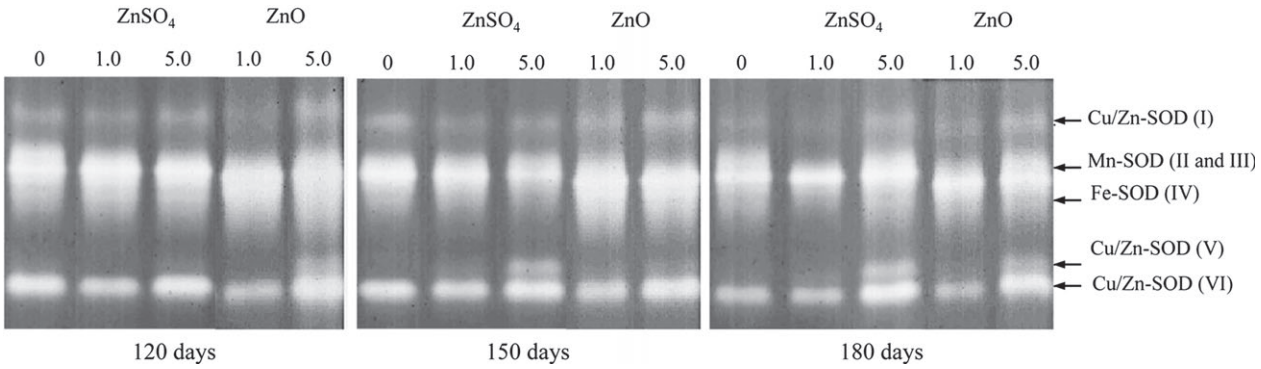

Figure 4 Specific activities of superoxide dismutase (SOD) forms in young leaves of citrus trees grown in sandy loam soil, 120, 150 and 180 days after Zn fertilisers ($ZnSO_4$ and ZnO) application.

capacity for those metals. Zinc adsorption is directly related to the cation exchange capacity (CEC) for the clays and poor related to organic matter content of Oxisols (Abat *et al.*, 2012). Furthermore, the type of clay minerals in soils, i.e., iron oxides, may explain bonding energies inversely related to adsorptive capacity for Zn (Shuman, 1976) as observed for Mn (Table 1). Increasing soil pH by liming affects effective CEC and consequently metal adsorption (Fontes *et al.*, 2000), which is in line with our experimental method before transplanting trees to the pots.

Even though such nutrients are adsorbed onto the soil colloidal matrix, after saturation of adsorption sites, these nutrients are more available for plant uptake (Bradl, 2004). In our study, it was verified that the efficacy of nutrient supply to young citrus plants depends on the interaction among soil sorption and fertiliser solubility and rate. The application of more soluble fertilisers containing Mn and Zn to the soil was able to increase the levels of these elements in the orange trees, especially for growing fluxes developed after fertilisation, namely new leaves, flowers and fruits, which represented the main nutrient sinks in the plants (Grifferty & Barrington, 2000).

In soils, the available concentration of Mn and Zn was high with the highest doses of both fertiliser sources. Furthermore, the adsorption to the colloidal matrix limited the supply of these metals to plants (Table 1). The adsorption capacity (b_L) for Mn in the clay was 1236 mg kg^{-1}, whereas in the sandy loam soil was 620 mg kg^{-1}. Thus, despite the high solubility of the $MnSO_4$, the amount of metal adsorbed after application in the clay soil was high, that the highest dose of this metal did not cause toxicity to plants, as seen in the sandy loam soil. Since fewer roots developed in the clay soil (Tables 2 and 4), the lowest effect of the application of Mn and Zn, in this case, was also likely due to the lowest contact expected between the root–soil solution and consequently nutrient uptake.

Although the plants exhibited lowest concentrations of Mn and Zn when receiving either $MnCO_3$ or ZnO, respectively, compared with sulphate, the less soluble sources promoted higher DM production of roots, leaves and branches in response to fertiliser applications in the

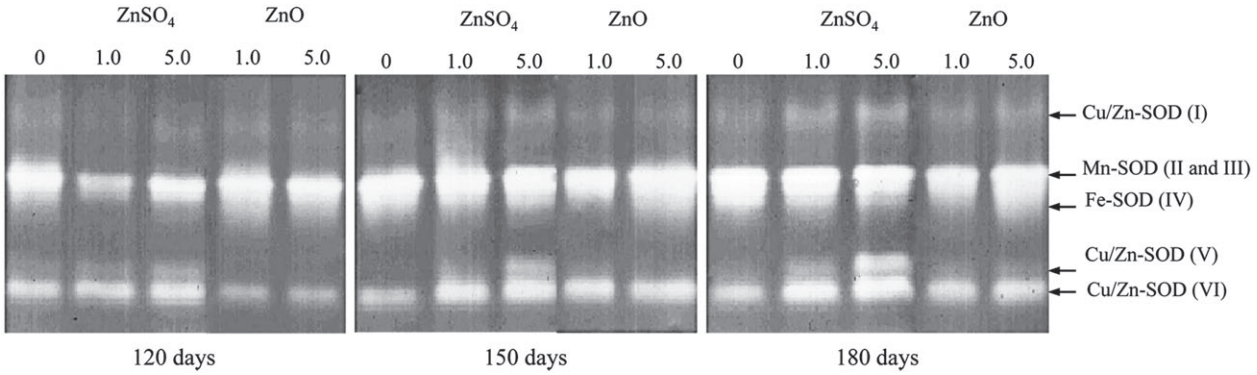

Figure 5 Specific activities of superoxide dismutase (SOD) forms in young leaves of citrus trees grown in clay soil, 120, 150 and 180 days after Zn fertilisers ($ZnSO_4$ and ZnO) application.

sandy loam soil. This occurred because plants grown in the sandy loam soil with the highest rates of Mn and Zn showed visual symptoms of metal toxicity, mainly to Mn, characterised by necrotic spots on leaves. In those plants, the Mn absorbed by roots increased foliar levels up to 550 mg kg^{-1} in old leaves and 951 mg kg^{-1} in new leaves, which are excessive for adult plants (Quaggio *et al.*, 2010).

The excess of metals has been shown to cause reduction of biomass, deformities of leaf surface and increased production of ROS that affect thylakoids, thereby reducing chlorophyll content and consequently the photosynthetic capacity of plants (Millaleo *et al.*, 2010). However, tolerance of plants to excess Mn has been shown to be associated with limited absorption and transport of the element to the shoots and/or tolerance to high levels at the cellular level (Hall, 2002). Although no specific assessment was performed in this study, the Mn concentrations in young leaves of approximately 1000 mg kg^{-1} suggest that citrus does not present efficient mechanisms for alleviating toxicity, on the contrary observed with excess Cu (Alva, 1993) and Zn, which accumulate mostly in the plant roots and woody tissues (Sartori *et al.*, 2008).

The Zn absorption, in spite of having occurred at a smaller magnitude compared to Mn, which for this latter was eightfold higher in leaves, application to the soil was sufficient to elevate the concentrations of Zn within the adequate to high levels in the plant (Quaggio *et al.*, 2010).

In field conditions, other factors may affect the efficacy of Zn fertiliser based on the observation that increases in foliar Zn citrus occurred only 2 years after application to the soil (Quaggio *et al.*, 2003). However, in this later study, lack of irrigation and limited shading of soil surface by canopy of young trees reduced soil moisture, and thus nutrient absorption.

Previous data support the idea that absorption and transport of Zn in citrus is more effective when compared to coffee plants supplied with 100 to 600 g plant^{-1} of Zn, given that foliar plant levels were only 10–35 mg kg^{-1} (Tezotto *et al.*, 2012).

Flowers and fruits, besides of new leaves, proved to be a significant sink for Mn and Zn, particularly at the highest dose of sulphate in the sandy loam soil. This sink effect of young leaves did not limit the transport of nutrients to the flowers as reported for Fe in commercial orchards of orange (Pestana *et al.*, 2005). Malavolta *et al.* (2006) found Mn = 30 and Zn = 26 mg kg^{-1} in flowers of bearing citrus trees, whose concentrations are close to those observed in our experiment when plants were supplied, respectively, with 0.7 g plant^{-1} of Mn and 1.0 g plant^{-1} of Zn. Furthermore, the Zn concentration in flowers and young fruits, when 5.0 g plant^{-1} of Zn as sulphate was applied, were higher than those observed in citrus plants grown in calcareous soils in the Mediterranean region of Spain (flower = 13.2 mg kg^{-1} and fruit = 17.9 mg kg^{-1}; Pestana *et al.*, 2005) and Florida (USA; fruit = 6 mg kg^{-1}; Mattos *et al.*, 2003), where soil adsorption capacity determined low availability of this nutrient for plants.

Elements such as Zn and Mn, although important for plant nutrition, when in excess may cause a number of problems including the establishment of a stressful condition by the generation of ROS (Gratão *et al.*, 2005). Moreover, as far as we are aware, information on antioxidant enzymes are scarce in citrus and the antioxidant stress response is negligible. Edagi *et al.* (2010) investigated the role of ethylene on chilling injury in tangor 'Murcott' and the related responses to cold injuries resistance of fruits, the first study reporting the presence of three SOD isoenzymes isolated from flavedo tissue.

In the present study, we have been able to investigate for the first time SOD activity and their isoforms in citrus leaves in response to Mn and Zn supply. Similarly in a study with *Lupinus angustifolius* (Yu & Rengel, 1999), the activity of Cu/Zn-SOD and Mn-SOD were dependent upon the micronutrient co-factors, but with greater

intensity in cases under severe nutrient deficiency. In our study, there were no visual deficiency symptoms of Zn and Mn on the control plants, only minor expression of Cu/Zn-SOD (VI) at 180 days. Leaf nutrient concentrations of plants that did not receive Zn were low (10 mg kg^{-1}) (Quaggio *et al.*, 2010) for both soil conditions.

Among the isoforms, the activity of Cu/Zn-SOD was more sensitive to different concentrations of Mn and Zn in the orange trees, when compared to Fe-SOD and Mn-SOD isoenzymes, which changed little. The increase in the activity of Cu/Zn-SOD, especially the isoforms V and VI, with the supply of Zn depends on the accumulation of Zn in leaves (Cakmak, 2000), as verified in three varieties of wheat, with the increased activity of Cu/Zn-SOD in plants that grew from deficient to adequate supply of Zn (Hacisalihoglu *et al.*, 2003).

However, with higher doses of $MnSO_4$ and $ZnSO_4$, an increased effect of ROS, specifically the superoxide radical, is expected because of increased nutrient levels in leaves. These excess levels affect the chlorophyll structure by replacing the magnesium ion in the molecule porphyrin ring (Hou *et al.*, 2007). In addition, the associated oxidative stress causes inhibition of uptake and transport of other metal micronutrients in cells, as well impairment of leaf capacity for pigment synthesis by changing protein composition of photosynthetic membranes (Brar & Sekhon, 1976; Hou *et al.*, 2007; Gratão *et al.*, 2005, 2008). These latter are in line with the decrease in chlorophyll content in the new leaves of citrus plants in the sandy soil, in which Mn and Zn concentrations were much higher when sulphate fertilisers were used compared to the less soluble sources (carbonate and oxide, respectively) (Tables 3 and 5). The C_b was more sensitive to the stress condition, as verified in wheat under excess Mn (Macfie & Taylor, 1992). Even though, C_b is considered more resistant than C_a in metal-stress conditions (Ferroni *et al.*, 2004), no specific mechanism has been reported in the literature to explain if excess metals directly impair formation or degradation of photosynthetic pigments.

The condition of Mn or Zn-excess also affected the presence of small proteins (<25 kDa; Fig. 1), which are within the size range of some small heat shock proteins group (sHSPs) (Hall, 2002). There were clear alterations in the intensity of several bands by SDS-PAGE, which confirm that further proteomic analysis using 2D-PAGE will be required for isolation and sequencing of proteins for their identification, and possibly will help gaining further insights into other metabolic routes that may be affected by the conditions to which citrus plants were subjected. For instance, although the literature reports increased expression of sHSPs in plants in response to stress by heavy metals (Neumann *et al.*, 1994), there is no information for citrus trees. So, there is plenty of room for future research with citrus plants on this aspect.

The reduction of the activity of the Cu/Zn-SOD after 180 days when the highest dose $MnSO_4$ was applied on sandy loam soil may be due to excess Mn availability and competition for absorption sites by the roots between Zn and Mn. In rice, the increase in Mn application decreased uptake and redistribution of Zn in plants (Brar & Sekhon, 1976). In our case, Mn-SOD and Fe-SOD isoenzymes expressed constant activity, however not enough, to reduce the effect of ROS by excess Mn in the leaves, as verified by the occurrence of visual symptoms of toxicity in the leaves of plants with 951 mg kg^{-1} of Mn. These symptoms can be associated with the decrease of leaf chlorophyll content (Macfie & Taylor, 1992), the differential expression of some protein bands (Fig. 1) and the Cu/Zn-SOD isoenzyme activity (Figs 2 and 3). The SOD isoenzymes are commonly located into distinct cell compartments. For instance, Mn-SOD is found in the mitochondria, whereas the Cu/Zn-SOD is encountered in the cytoplasm and attached to the thylakoid membrane (Gratão *et al.*, 2005), which was also related to the chlorophyll effects observed in our study.

Specific changes in one or another isoenzyme may indicate specific generation of ROS, which in turn may affect specific organelle cell processes. Such information is important to better understand the stress induced and how it is expressed within the cell. In this work, we concentrated on identifying the SOD isoenzymes and their activity changes in response to the different fertiliser treatments applied, but further work is necessary in order to clearly establish the impact of such response on the cell metabolism. Moreover, it is also important to investigate the generation of ROS, including the H_2O_2, which may have been overproduced in response to the increased Cu/Zn-SOD isoenzymes V and VI activities as a result of the increased Zn supply (Figs 4 and 5). The potential increase in H_2O_2 may lead to the increase of other antioxidant enzymes such as peroxidases which must deal with the excessive production of this ROS. These aspects are currently the focus of an ongoing investigation in our laboratories.

In conclusion, our findings suggest that the soil supply of Mn and Zn increases nutrient availability into the soil solution and nutrient concentrations in the leaves of sweet orange trees, through use of soluble sulphate fertilisers at lower rates, is more prone to supply the nutrient demand of leaves, flowers and fruits. However, higher rates of Mn and Zn in the form of sulphate fertilisers cause plant phytotoxicity, especially when applied to sandy loam soils, compared to Mn-carbonate and Zn-oxide fertilisers. Plant responses to the application of Mn and Zn to clay soils, regardless of the fertiliser source, are less

dramatic because of adsorption of these micronutrients to the soil colloidal matrix. Furthermore, the Cu/Zn-SOD proved to have a major role in the citrus plant metabolism with increased enzyme activity with higher Zn supply and decreased activity with Mn.

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References

Abat M., McLaughlin M.J., Kirby J.K., Stacey S.P. (2012) Adsorption and desorption of copper and zinc in tropical peat soils of Sarawak, Malaysia. *Geoderma*, **175–176**, 58–63.

de Abreu C.A., de Abreu M.F., Soares L.H., de Andrade J.C. (1997) The effect of DTPA extracting conditions on the determination of micronutrients in Brazilian soils. *Communications in Soil Science and Plant Analysis*, **28**, 1–11.

Almansa M.S., Del Rio L.A., Alcaraz C.F., Sevilla F. (1989) Isoenzyme pattern of superoxide dismutase in different varieties of citrus plants. *Physiologia Plantarum*, **76**, 563–568.

Alva A.K. (1993) Copper contamination of sandy soils and effects on young Hamlin orange trees. *Bulletin of Environmental Contamination and Toxicology*, **51**, 857–864.

Arruda M.A.Z., Azevedo R.A. (2009) Metallomics and chemical speciation: towards a better understanding of metal-induced stress in plants. *Annals of Applied Biology*, **155**, 301–307.

Azevedo R.A., Alas R.M., Smith R.J., Lea P.J. (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. *Physiologia Plantarum*, **104**, 280–292.

Barnes J.D., Balaguer L., Manrique E., Elvira S., Davison A.W. (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. *Environmental and Experimental Botany*, **32**, 85–100.

Bataglia O.C., Furlani A.M.C., Teixeira J.P.F., Furlani P.R., Gallo J.R. (1983) *Métodos de análise química de plantas*. Campinas, Brazil: Instituto Agronômico de Campinas(Boletim Técnico 78).

Boaretto L.F., Carvalho G., Borgo L., Creste S., Landell M.G.A., Mazzafera P., Azevedo R.A. (2014) Water stress reveals differential antioxidant responses of tolerant and non-tolerant sugarcane genotypes. *Plant Physiology and Biochemistry*, **74**, 165–175.

Bradford M.F. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.

Bradl H.B. (2004) Adsorption of heavy metal ions on soils and soils constituents. *Journal of Colloid and Interface Science*, **277**, 1–18.

Brar M.S., Sekhon G.S. (1976) Effect of manganese on zinc-65 absorption by rice seedlings and its translocation within the plants. *Plant and Soil*, **44**, 459–462.

Broadley M.R., White P.J., Hammond J.P., Zelko I., Lux A. (2007) Zinc in plants. *New Phytologist*, **173**, 677–702.

Cakmak I. (2000) Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. *New Phytologist*, **146**, 185–205.

Cakmak I., Marschner H. (1993) Effect of zinc nutritional status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves. *Plant and Soil*, **155–156**, 127–130.

Cia M.C., Guimarães A.C.R., Medici L.O., Chabregas S.M., Azevedo R.A. (2012) Antioxidant responses to water deficit by drought-tolerant and -sensitive sugarcane varieties. *Annals of Applied Biology*, **161**, 313–324.

Edagi F.K., Jomori M.L.L., Kluge R.A., Lima G.P.P., Azevedo R.A., Sestari I. (2010) Inhibition of ethylene action delay chilling injury development in tangor 'Murcott'. *Ciência Rural*, **40**, 1530–1536.

Fageria N.K., Barbosa Filho M.P., Moreira A., Guimarães C.M. (2009) Foliar fertilization of crop plants. *Journal of Plant Nutrition*, **32**, 1044–1064.

Ferroni L., Baldisserotto C., Fasulo M.P., Pagnoni A., Pancaldi S. (2004) Adaptive modifications of the photosynthetic apparatus in *Euglena gracilis* Klebs exposed to manganese excess. *Protoplasma*, **224**, 167–177.

Fontes M.P.F., de Matos A.T., da Costa L.M., Neves J.C.L. (2000) Competitive adsorption of zinc, cadmium, copper and lead in three highly-weathered Brazilian soils. *Communications in Soil Science and Plant Analysis*, **31**, 2939–2958.

Gratão P.L., Polle A., Lea P.J., Azevedo R.A. (2005) Making the life of heavy metal-stressed plants a little easier. *Functional Plant Biology*, **32**, 481–494.

Gratão P.L., Monteiro C.C., Antunes A.M., Peres L.E.P., Azevedo R.A. (2008) Acquired tolerance of tomato (*Lycopersicum esculentum* cv. Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321–333.

Grifferty A., Barrington S. (2000) Zinc uptake by young wheat plants under two transpiration regimes. *Journal of Environmental Quality*, **29**, 443–446.

Hacisalihoglu H., Hart J.J., Wang Y., Cakmak I., Kochian L.V. (2003) Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. *Plant Physiology*, **23**, 595–602.

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

Hou W., Chen X., Song G., Wang Q., Chang C.C. (2007) Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemma minor*). *Plant Physiology and Biochemistry*, **45**, 62–69.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Leidi E.O., Gómez M., Del Rio L.A. (1987) Evaluation of biochemical indicators of Fe and Mn nutrition for soybean plants. II. Superoxide dismutases, chlorophyll contents and photosystem II activity. *Journal of Plant Nutrition*, **10**, 261–271.

Lindsay W.L., Norvell W.A. (1978) Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal*, **42**, 421–428.

Lopéz-Millán A.F., Ellis D.R., Grusak M.A. (2005) Effect of zinc and manganese supply on the activities of superoxide dismutase and carbonic anhydrase in *Medicago truncatula* wild type and *raz* mutant plants. *Plant Science*, **168**, 1015–1022.

Macfie S.M., Taylor G.J. (1992) The effects of excess manganese on photosynthetic rate and concentration of chlorophyll in *Triticum aestivum* grown in solution culture. *Physiologia Plantarum*, **85**, 467–475.

Malavolta E., Leão H.C., Oliveira S.C., Lavres J. Jr., Moraes M.F., Cabral C.P., Malavolta M. (2006) Repartição de nutrientes nas flores, folhas e ramos da laranjeira cultivar Natal. *Revista Brasileira de Fruticultura*, **28**, 506–511.

Mattos D. Jr., Quaggio J.A., Cantarella H., Alva A.K. (2003) Nutrient content of biomass components of Hamlin sweet orange trees. *Scientia Agricola*, **60**, 155–160.

McBeath T.M., McLaughlin M.J. (2014) Efficacy of zinc oxides as fertilisers. *Plant and Soil*, **374**, 843–855.

Millaleo R., Reyes-Díaz M., Ivanov A.G., Mora M.L., Alberdi M. (2010) Manganese as essential and toxic element for plants: transport, accumulation and resistance mechanisms. *Journal of Soil Science and Plant Nutrition*, **10**, 476–494.

Monteiro C.C., Carvalho R.F., Gratão P.L., Carvalho G., Tezotto T., Medici L.O., Peres L.E.P., Azevedo R.A. (2011) Biochemical responses of the ethylene-insensitive *Never ripe* tomato mutant subjected to cadmium and sodium stresses. *Environmental and Experimental Botany*, **71**, 306–320.

Neumann D., Lichtenberger O., Günther D., Tschiersch K., Nover L. (1994) Heat-shock proteins induce heavy-metal tolerance in higher plants. *Planta*, **194**, 360–367.

Pereira G.J.G., Molina S.M.G., Lea P.J., Azevedo R.A. (2002) Activity of antioxidant enzymes in response to cadmium in *Crotalaria juncea*. *Plant and Soil*, **239**, 123–132.

Pestana M., Beja P., Correia P.J., Varennes J., Faria E.A. (2005) Relationships between nutrient composition of flowers and fruit quality in orange trees grown in calcareous soil. *Tree Physiology*, **25**, 761–767.

Quaggio J.A., Mattos D. Jr., Cantarella H., Tank A. Jr. (2003) Fertilização com boro e zinco no solo em complementação à aplicação via foliar em laranjeira Pêra. *Pesquisa Agropecuária Brasileira*, **38**, 627–634.

Quaggio J.A., Mattos D. Jr., Boaretto R.M. (2010) Citros. In *Boas práticas para uso eficiente de fertilizantes: culturas*, pp. 373–412. Eds L.I. Prochnow, V. Casarin and S.R. Stipp. Piracicaba, Brazil: IPNI.

Remans T., Opdenakker K., Guisez Y., Carleer R., Schat R., Vangronsveld J., Cuypers A. (2012) Exposure of *Arabidopsis thaliana* to excess Zn reveals a Zn-specific oxidative stress signature. *Environmental and Experimental Botany*, **84**, 61–71.

Sartori R.H., Boaretto A.E., Villanueva F.C.A., Fernandes H.M.G. (2008) Absorção radicular e foliar de ^{65}Zn e sua redistribuição em laranjeiras. *Revista Brasileira de Fruticultura*, **30**, 523–527.

Shaver T.M., Westfall D.G., Ronaghi M. (2007) Zinc fertilizer solubility and its effects on zinc bioavailability over time. *Journal of Plant Nutrition*, **30**, 123–133.

Shuman L.M. (1976) Zinc adsorption isotherms for soil clays with and without iron oxides removed. *Soil Science Society of America Journal*, **40**, 349–352.

Tezotto T., Favarin J.L., Azevedo R.A., Alleoni L.R.F., Mazzafera P. (2012) Coffee is highly tolerant to cadmium, nickel and zinc: plant and soil nutritional status, metal distribution and bean yield. *Field Crops Research*, **125**, 25–34.

Yu Q., Rengel Z. (1999) Micronutrient deficiency influences plant growth and activities of superoxide dismutases in narrow-leafed lupins. *Annals of Botany*, **83**, 175–182.