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Research Article

Efficiency of foliar application of sparingly soluble sources of boron and zinc in citrus

Luiza Oliveira Macedo[1]*, Dirceu Mattos Júnior[1], Rodrigo Jacobassi[1], Franz Walter Rieger Hippler[1], José Antônio Quaggio[2], Rodrigo Marcelli Boaretto[1]

[1]Instituto Agronômico de Campinas/Centro de Citricultura Sylvio Moreira, Rod. Anhanguera, km 158, C.P. 04 – 13490-970 – Cordeirópolis, SP – Brasil.
[2]Instituto Agronômico de Campinas/Centro de Solos e Recursos Ambientais, Av. Barão de Itapura, 1481, C.P. 28 – 13020-902 – Campinas, SP – Brasil.
*Corresponding author <luizaoliveiram@hotmail.com>

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ABSTRACT: This study evaluated leaf supply of zinc (Zn) and boron (B) using either soluble or sparingly soluble fertilizers in young sweet orange trees. Three experiments were set up in a greenhouse to compare two sources and four doses (control, low, adequate and high) of fertilizers as follows: (i) Experiment I (B): boric acid and calcium borate; (ii) Experiment II (Zn): Zn sulfate and Zn oxide; and (iii) Experiment III (B + Zn): boric acid + Zn Sulfate and Zn Borate. The sparingly soluble sources were effective in increasing the Zn and B leaf concentration. Dry matter of the aerial part increased 18 % with B applications in adequate concentration independent of the B fertilizer sources. In contrast, trees did not grow well with applications of adequate concentration of Zn as Zn Sulfate or high Zn concentration as Zn borate. Superoxide dismutase activity in leaves increased with applications of low concentration of Zn as Zn oxide and decreased with high concentration of Zn from either source. Polyphenol oxidase activity increased with application of adequate concentration of B as boric acid and high concentration of B as calcium borate. Furthermore, the upper concentrations of Zn were toxic in orange trees when the source was Zn sulfate. Increases in plant growth without damage to leaf tissue and positive responses of key enzymes of orange trees in a range of nutrient concentration applications demonstrated the practical use of sparingly soluble fertilizers to supplying B and Zn foliarily to plants.
Keywords: micronutrients, microparticles, leaf spraying, fertilizer use

Introduction

The majority of orange groves cultivated in acid to neutral pH soils require frequent supply of B and Zn, otherwise fruit production is limited (Quaggio et al., 2010; Wang et al., 2015; Mattos Jr. et al., 2017). Leaf application has been an effective and preferred management practice employed by growers for micronutrient fertilization in the field (Fageria et al., 2009; Du et al., 2015), which also considers the convenience of distribution of small amounts of products and mixture with defensives (Fernández et al., 2013). Soluble sulfate, chloride and nitrate micronutrient salts represent the main fertilizer sources utilized for leaf spraying in citrus (Quaggio et al., 2010). However, given incidences of leaf "burning" associated with toxic saline symptoms of more concentrated fertilizer solutions (Fernández et al., 2013), the search for new fertilizer sources, such as oxides and carbonates, has been sought by sustainable production systems (Bell and Dell, 2008; Macedo et al., 2017). Efficiency of traditional foliar fertilizers depends on water solubility, while sparingly soluble sources rely upon particle size, in which the smallest particles (0.2 - 20 µm) facilitate the release and further absorption of the nutrient by plant leaves (Du et al., 2015), maintaining a constant nutrient supply for long periods reducing the risk of leaf tissue injury immediately after application (Li et al., 2012).

In addition to the quantification of nutrient concentration in leaves, the analysis of superoxide dismutase activity (SOD) was proposed to study the interaction between metals and plants (Shenker et al., 2004) as Zn is a constituent of Cu / Zn-SOD (Hansch and Mendel, 2009; Hippler et al., 2015a) and may also be indicative of the risk of phytotoxicity in plants supplied with excess micronutrients (Hippler et al., 2015a; Hippler et al., 2018). The activity of polyphenol oxidase (PPO) has been assessed as an indicator of the nutritional status of B since rises in the concentration of phenols in B-deficient tissues may result from restrictions in the biosynthesis of phenolic alcohols (Marschner, 2011).

Considering the need to validate the efficiency of new micronutrient sources, the present research study was undertaken to test the hypothesis that sparingly soluble sources of B and Zn, in microparticles, adequately supply these nutrients to the leaves of orange plants compared to soluble sources traditionally used in orchards.

Materials and Methods

Plant growth conditions and treatments

Three experiments were carried out in a greenhouse on one-year-old sweet orange trees from cv. Pera [*Citrus sinensis* (L.) Osbeck] grafted onto Rangpur lime (*C. limonia* Osbeck). Orange plants were transplanted to 12 L plastic pots, filled with organic substrate (80 % pine bark, 5 % carbonized materials and 15 % vermiculite), and pruned 40 cm above the grafting point to induce new vegetative growth.

During the course of the experiment, both macro- and micronutrients were applied via a nutrient solution modified according to Hippler et al. (2015b). The supply of macronutrients took place over the course of 40 applications, from transplantation up to 180 days after starting treatments, and micronutrient supply with

15 applications from starting treatments up to 180 days after, according to the subsequently described experimental characteristics. Before the beginning of the foliar treatment four plants were destructively harvested and the initial leaf area (LA) and dry matter (DM) canopy measures were 0.38 ± 0.2 m^2 and 74 ± 5.3 g per plant, respectively. Plants chosen for the application of treatments were uniform at that time.

Treatments in each experiment consisted of the application of two sources of fertilizers, one soluble in water and another sparingly soluble in water (0.2-20 µm particle size) as well as four doses of the micronutrient in a 2 × 4 factorial design all together within a completely randomized factorial design replicated four times. Experiment I (B) consisted of foliar application of four doses of B, control (without B), 130, 260 and 520 mg L^{-1} of B (in summary 0, 13, 28 and 55 mg per plant of total B after 180 days) as soluble source boric acid (H_3BO_3) or sparingly soluble source calcium borate [$CaB_3O_4(OH)_3 \cdot H_2O$]. Experiment II (Zn) consisted of foliar application of four doses of Zn, control (without Zn), 200, 600 and 1800 mg L^{-1} of Zn (in summary 0, 23, 65, 200 mg per plant of total Zn after 180 days) as soluble source Zn sulfate ($ZnSO_4.7H_2O$) or sparingly soluble source Zn oxide (ZnO). Experiment III (B and Zn) consisted of foliar application of four doses of B + Zn, control (without B and Zn), 43, 129 and 387 mg L^{-1} of B (in summary 5, 14 and 45 mg per plant of total B after 180 days), and correspondent 200, 600 and 1800 mg L^{-1} of Zn (in summary 23, 65, 200 m g per plant of total Zn) as soluble sources boric acid + Zn sulfate and sparingly soluble source Zn borate ($2ZnO \cdot 3B_2O_3 \cdot 3.5H_2O$). The sources Ca borate, Zn oxide, and Zn borate, used as sparingly soluble sources for foliar treatment, had particle dispersion between 0.37 µm, smaller particle in Zn oxide, and 18.3 µm greater particles in Zn borate. All sources were characterized as microparticulates and contained flowable technology adjuvant for retention of the microparticles in leaves. An adjuvant featuring 200 g L^{-1} of nonyl phenoxy poly (ethyleneoxy) ethanol was mixed at 0.1 ml L^{-1} with the treatment solution of the soluble sources. Doses of nutrients in each experiment were defined based on the adequate concentration levels recommended for citrus orchards by Quaggio et al. (2010), with two additional doses in each experiment as follows - for B (Experiment I) : a half and double of the adequate level; for Zn (Experiment II): a third of and triple the adequate level; and for B and Zn (Experiment III): a third of and triple the adequate level of Zn and doses of B were adjusted based on the adequate dose of Zn. Three treatments were applied - in the first flush of orange vegetation, 160 days after the transplant of trees, and the other two at 60-day intervals, coinciding with the second and third flushes of vegetative growth of trees. At the moment of foliar spraying, a plastic cover was placed on the surface of pots to avoid contamination of the substrate with sprayed solutions. The amounts of solution applied were approximately 25 mL per plant in the first application, 45 mL per plant in the second application and 50 mL per plant in the third application, which were proportional to the LA of plants during every period to guarantee uniform coverage of the leaf surface with a sprayed solution. After the applications, the total amounts of micronutrient retained per plant were estimated for each treatment.

Dry matter production and chemical analysis of plant material

The aerial part of plants was destructively harvested 60 days after the last foliar application in Experiments I (B), II (Zn), and III (B and Zn), and separated into leaves and woody parts (stem + branches). After collection of the plants, before the leaves were dried and processed, LA was measured using the leaf area integrator (LI-COR 3100). The harvested plant material was washed in 5 % diluted detergent and distilled water (Alva and Tucker, 1997) and oven dried at 65 °C to constant weight for quantification of the DM. Leaves and woody parts were ground in a Willey-type mill for determination of plant nutrient concentrations by chemical analysis according to Bataglia et al. (1983).

Enzymatic analysis

Plants in Experiments I (B) and II (Zn) developed new shoots from the main stem after the harvesting of aerial parts in the first part of the experiment, where enzymatic activity analysis was performed on the leaves. During this new period, ten maintenance fertilizations with micro- and macronutrient were applied via a nutrient solution modified according to Hippler et al. (2015b). Completely expanded and mature leaves developed 70 days after harvest, which then received a new foliar application of treatments that was repeated after another 70 days following the first application. The volume of solution applied was approximately 25 mL per plant for both the first and second applications. Amounts of B applied to plants with both fertilizer sources in Experiment I (B) were 0, 6.5, 13, and 26 mg per plant of B. With this, the amounts of Zn applied to plants from both sources in Experiment II (Zn) were 0, 10, 30, and 90 mg of Zn per plant.

Four mature leaves per plant were collected seven days after the second foliar treatment application. The leaves sampled were washed with distilled water to avoid contamination of metals present on the surface, then immediately placed in liquid nitrogen, and subsequently stored in an ultra-freezer at -80 °C until protein extraction.

Extraction of total protein was conducted according to Gomes Jr. et al. (2006). For determination of total protein according to the method adopted by Bradford (1976), 20 µL of the 10-fold diluted aliquot of each sample was added to 1.0 mL of Bradford's solution. Optical density (absorbance) of samples was determined in a spectrophotometer at 595 nm. *Bovine serum albumin* (BSA) was used as a standard.

SOD activity was determined by electrophoresis according to Gomes Jr. et al. (2006) via polyacrylamide gel (polyacrylamide gel electrophoresis; PAGE) at 12 % (m/v). The gels were documented in an Image Scanner. The isoforms of SOD, Cu/Zn-SOD, Fe-SOD, or Mn-SOD were determined by gel electrophoresis according to Hippler et al. (2016).

PPO activity was evaluated following the method adopted by Kar and Mishra (1976). The activity was estimated by measuring absorbance at 420 nm over 1 min. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 min^{-1} to 1 mL of the enzyme and per mg of protein at 25 °C.

Leaves collected after growth of new shoots were washed with 5 % diluted detergent and distilled water (Alva and Tucker, 1997), dried in an oven at 65 °C, and ground in a Willey-type mill to determine the concentration of nutrients (Bataglia et al., 1983).

Statistical analysis

Data collected for greenhouse or laboratory assays were submitted to analysis of variance, and means were analyzed statistically by the F test ($p < 0.05$) using SISVAR (Variance Analysis System, version 5.3). Regression equations were estimated for the parameters that were significant according to the F test for dose or interaction between source and dose ($p < 0.05$).

Results

Plant nutritional status, growth and biomass production

Leaf concentrations of B and Zn increased with doses of soluble and sparingly soluble fertilizers sources sprayed on plants in experiments I (B), II (Zn) and III (B and Zn). In the woody parts, B concentration increased only with calcium borate while Zn concentration rose as a result of the highest doses of either the soluble (Zn sulfate) or sparingly soluble (Zn oxide and Zn borate) fertilizer sources (Figure 1). Furthermore, calcium borate at 520 mg L^{-1} provided the highest B concentration in the leaves (97 mg kg^{-1}), 44 % above the level obtained with boric acid, as well the highest B concentration in the woody parts (12 mg kg^{-1}) of trees at the same dose. No differences were observed in Ca concentration in the leaf owing to the calcium borate sprayed on (data not shown). ZnO had the highest Zn concentration in the leaves (375 g kg^{-1}) and woody parts (55 g kg^{-1}) when the highest dose was applied, though it was 56 % higher in the leaves compared to Zn sulfate with the same dose. In corroboration of Experiments I (B) and II (Zn), the sparingly soluble source (Zn borate) resulted in a higher leaf concentration of Zn (256 g kg^{-1}) at high doses, and, in contrast, the soluble source provided a higher leaf concentration of B (53 g kg^{-1}) with the high dose (387 mg L^{-1}). In the woody parts of the plants of Experiment III, the Zn concentration was the same for both sources with a value of 33 g kg^{-1} at the highest dose (Figure 1).

The DM of canopy and LA in Experiment I increased to the adequate dose and decreased until the highest dose showed no noticeable difference between sources. The maximum LA and DM of leaves and canopy were reached at a dose of 295 mg L^{-1}. The leaf DM and LA were the parameters with the greatest variations, specifically a reduction of 18 % with the deficiency dose and 5 % with the excess (Figure 2).

In Experiment II, orange plants sprayed with ZnO exhibited an increase in the DM of leaves, woody parts, canopy and LA when compared to controls, and this rise occurred with a dose lower than that considered adequate - leaves presenting a concentration of 169 mg kg^{-1} Zn and absent any symptoms of toxicity. These symptoms were even more severe at the high dose, when the leaf concentration reached 375 mg kg^{-1} of Zn. The maximum leaf DM was obtained with a foliar solution containing 428 mg L^{-1} of Zn. The plants that received leaf spraying with Zn sulfate had a reduction in DM throughout the whole canopy at the lowest Zn dose, and a foliar concentration of 60 mg kg^{-1}, the DM remaining constant up through to the high dose, for which the leaf concentration was 164 mg kg^{-1}. With the ZnO source, there was a reduction of 5 % in DM of leaves under deficiency conditions up to 32 % with excess. For Zn sulfate, the decrease in leaf DM from the control treatment at the highest dose was 17 % (Figure 2).

The plants sprayed with doses of B and Zn (Experiment III) experienced an increase in DM of the canopy and LA with adequate doses, with a reduction at the highest dose. When the Zn borate source was used, the plants had maximum DM production of canopy and LA with a dose of 930 mg L^{-1} Zn and 200 mg L^{-1} B. Application of Zn sulfate and boric acid together resulted, on average, in maximum DM production by the plants, at doses of 1500 mg L^{-1} Zn and 290 mg L^{-1} B. The DM of the canopy decreased by 20 % in cases of deficiency for both sources. For the Zn borate source, it decreased 18 % in excess, whereas for the soluble source, there was no decrease in excess (Figure 2). The correlations between the leaf concentration and the production of DM of the canopy in Experiment III (B and Zn) (Figure 3) shows that the limit for toxicity in this experiment was 150 mg kg^{-1} considering that the regression equation exhibited the highest DM for both sources (Zn oxide and Zn sulfate) at this dose (Figure 3).

Symptoms of toxicity owing to salt excess

Orange plants that received foliar spray with the highest dose of Zn sulfate (1800 mg L^{-1} of Zn) in Experiments II and III exhibited symptoms of toxicity because of the high salt content of the soluble fertilizer, characterized by yellowish sores that in the leaf limb, mainly on young leaves (Figure 4C, D, G and H). However, those plants sprayed with the highest dose of Zn, albeit with the sources of Zn oxide and Zn borate did not demonstrate symptoms of toxicity and featured deposition of the microparticulate sparingly soluble fertilizer (Figure 4A, B, E and F).

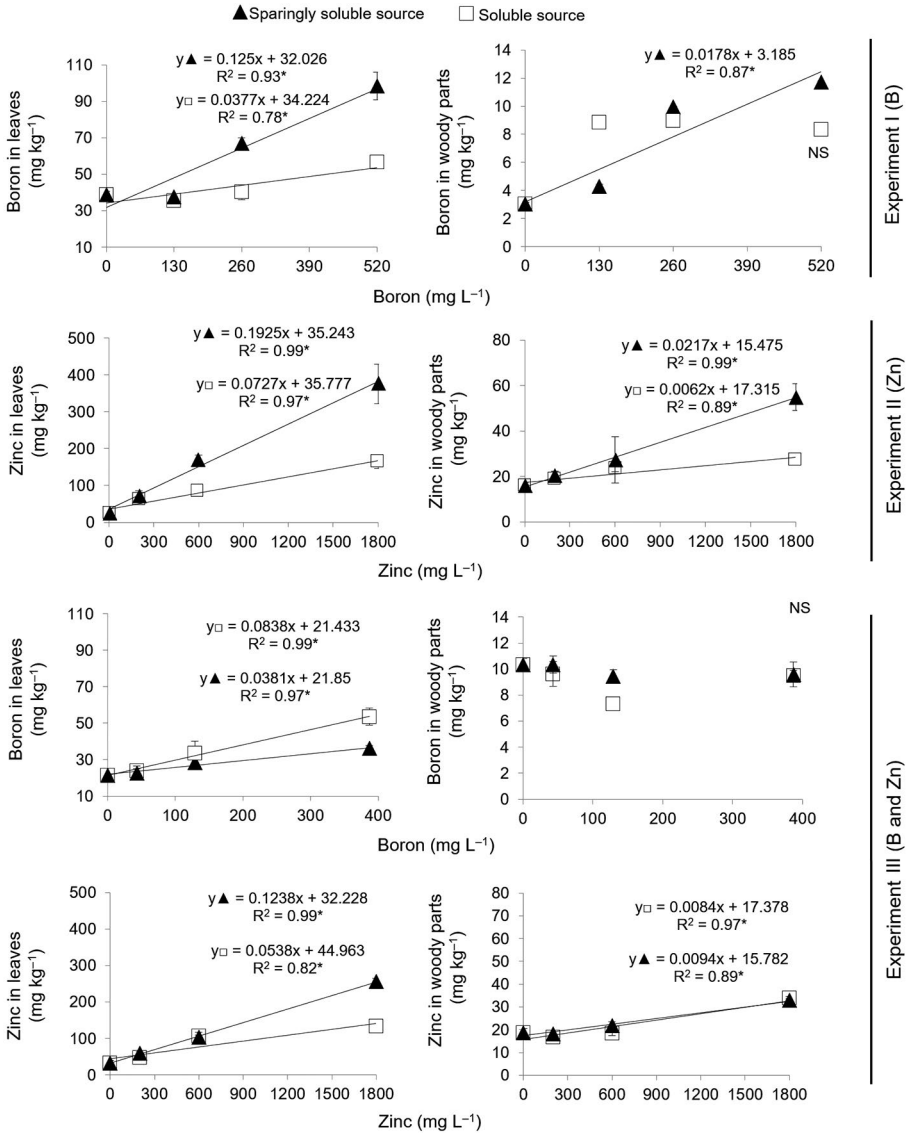

Figure 1 – Zn and B concentrations in the leaves and woody parts of young orange plants sprayed with soluble and sparingly soluble sources of Zn and B at increasing concentrations harvested after 60 days since the last application. Experiment I (B), Experiment II (Zn) and Experiment III (B and Zn). Vertical lines represent the standard error of the mean (n = 4). Legend: *Significant at 1 %; ^{NS}not significant at 5 % according to the F test.

Enzyme activity

Four isoforms of SOD were identified in the leaves of orange plants receiving varying doses of B and Zn: Three Cu/Zn-SOD (I, II and III) and two Mn-SOD (I and II). In Experiment I (B), the control plants, without application of B, showed low Cu/Zn-SOD isoform activity with leaf concentrations of 35 mg kg^{-1} B. When the application of B was carried out with calcium borate, the activity of the Cu/Zn-SOD isoform increased from the lowest dose (130 mg L^{-1} of B) to the highest dose (520 mg L^{-1} of B) with a leaf concentration from 44 mg kg^{-1} up to 70 mg kg^{-1}. The Cu/Zn-SOD isoform with the 130 and 260 mg L^{-1} doses of B, with leaf concentration of 48 mg kg^{-1} and 58 mg kg^{-1}, respectively, when applied in the form of boric acid, presented similar activity to the dose of 520 mg L^{-1} of B in the form of calcium borate. At the highest dose of B as boric acid, when the B foliar concentration was 89 mg kg^{-1}, the activity of the Cu/Zn-SOD isoform experienced a minor decrease compared to the two lower doses. The Mn-SOD isoform's activity was not affected based on the doses and sources of B applied (Figure 5A).

In Experiment II (Zn), the control plants, without application of Zn, when the foliar concentration was 12 mg kg^{-1}, presented low Cu/Zn-SOD (II and III)

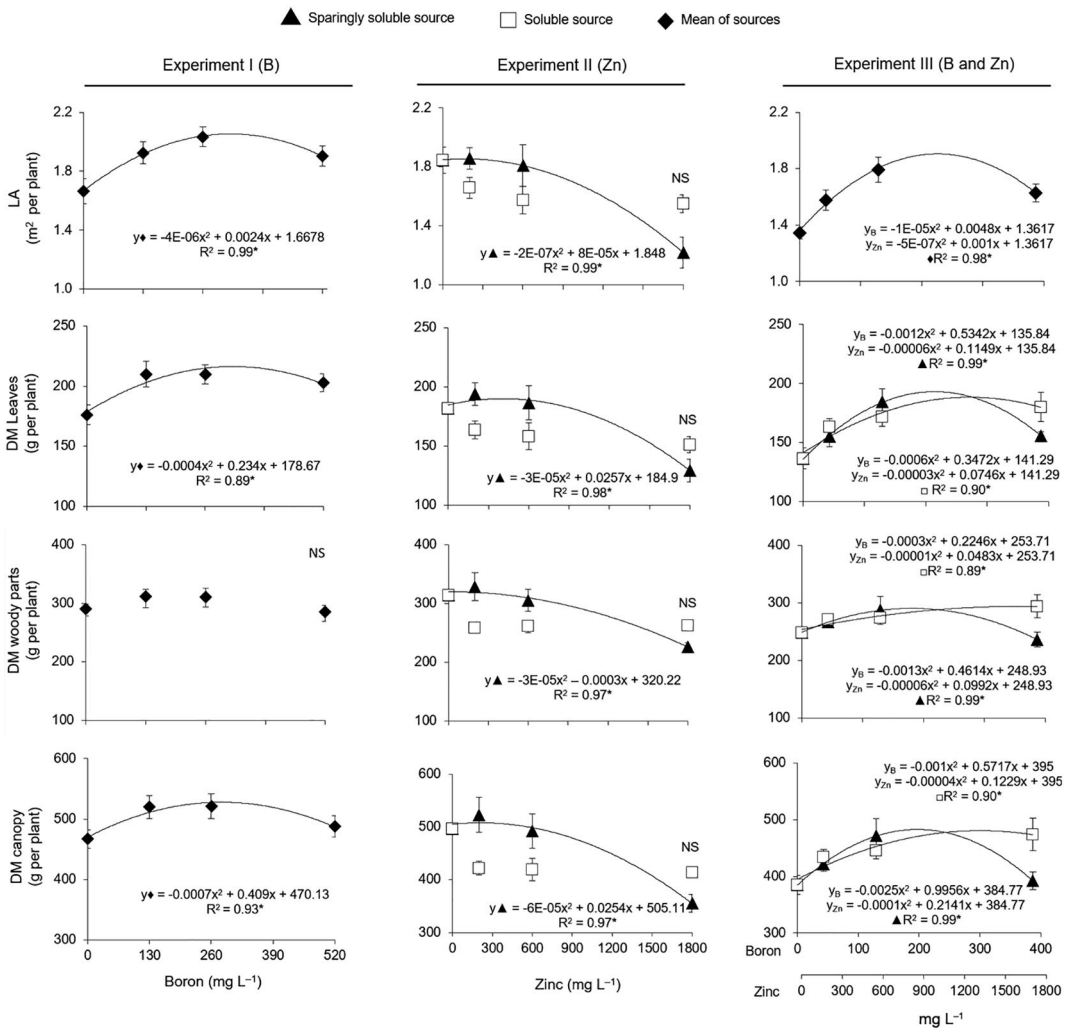

Figure 2 – Leaf area (LA) and dry matter (DM) of leaves, woody parts, and canopy of young orange plants sprayed with soluble and sparingly soluble sources of Zn and B at increasing concentrations harvested after 60 days since the last application. Experiment I (B), Experiment II (Zn) and Experiment III (B and Zn). Vertical lines represent the standard error of the mean (n = 4). Legend: *Significant at 5 %; ^{NS}not significant at 5 % according to the F test.

isoform activity. With the application of ZnO, the Cu/Zn-SOD isoform had a decrease in activity from the low dose to the higher dose, with Zn leaf concentration varying from 29 mg kg^{-1} up to 133.5 mg kg^{-1}. The same pattern was noted with the application of Zn sulfate, where the activity decreased from the smallest Zn leaf concentrations (27 mg kg^{-1}) up to the highest (114 mg kg^{-1}). The activity of the Cu/Zn-SOD isoform was greater in the leaves of the plants that received Zn sulfate spraying at all doses when compared to the activity in the leaves that received Zn oxide. The Mn-SOD isoform exhibited higher activity at doses of 600 and 1800 mg L^{-1} Zn (Figure 5B).

The PPO activity in orange leaves that received the soluble source, boric acid increased 38 % in control plants, without application of B, up to a dose of 334 mg L^{-1} of B, with a subsequent decrease (12 %) at the highest dose of B. When sprayed with the calcium borate source, PPO activity rose 29 % with the control dose up to the highest dose of B. The highest activity of PPO with the application of boric acid was 11 % more than the highest activity achieved with the application of calcium borate (Figure 6).

Discussion

Our study evaluated the use of B and Zn applied foliarly to young orange trees as sparingly soluble fertilizer sources in comparison with soluble fertilizers commonly recommended for orchard nutrient management. Effects of nutrient supply to plants were measured via plant tissue chemical analyses, DM yield, LA, and the activity of selected enzymes on leaf tissue. The positive effects of leaf application of B and Zn on

nutritional status, retention of leaves, yield and fruit quality of citrus trees have been observed previously (Dawood et al., 2001; Eman et al., 2007; Razzaq et al., 2013), fostering the use of new fertilizer sources in commercial orchards (Bell and Dell, 2008).

The sparingly soluble sources (Zn oxide, calcium borate and Zn borate) tested in the experiments were effective in increasing the Zn and B leaf concentration of orange trees according to the increase in nutrient doses (Figure 1). We were able to verify that the sparingly soluble sources provided the highest leaf concentration of B in Experiment I (97 mg kg^{-1}) as well as Zn in Experiments II (375 mg kg^{-1}) and III (256 g kg^{-1}), suggesting that although less soluble than the sulfate and boric acid, when used as microparticles, these fertilizers promoted effective absorption through the nutrients applied to the leaves after dissolution of the particles (Westfall et al., 1999; Du et al., 2015).

The amount of B and Zn absorbed in each experiment was correlated with the DM of the aerial parts of plants. Although the concentration of B in leaves sprayed with calcium borate was higher than that which received boric acid (Figure 1), the LA and DM of both the leaves and the canopy did not vary with the sources tested (Figure 2). Plants exhibited maximum DM and LA with 295 mg L^{-1} of B in Experiment I (Figure 2), which was in accordance with the response of plants in the field when sprayed with the recommended doses of 200 to 300 mg L^{-1} of B, considered adequate for citrus (Quaggio et al., 2010) and usually applied as boric acid. The lack of difference in DM production of plants resulted from different fertilizer sources, demonstrating that foliar application of B, when using a microparticulated sparingly soluble source, supplies the required amounts of nutrients necessary for normal physiological plant processes compared with the use of a soluble source.

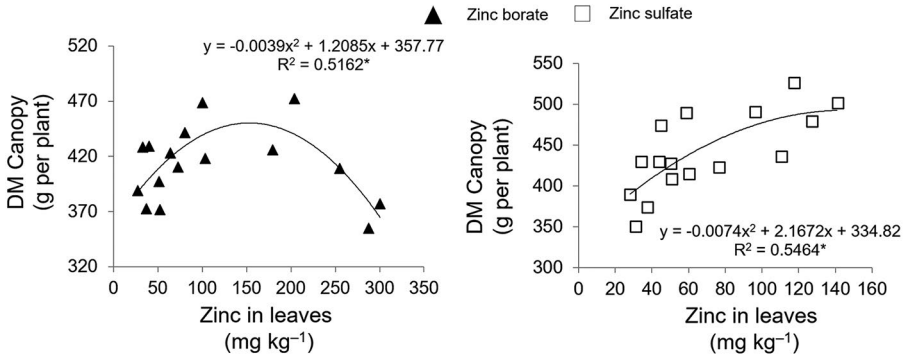

Figure 3 – Correlation between Zn concentration in the leaves and DM mass of the canopy of plants sprayed with Zn borate and Zn sulfate together with boric acid with young orange plants sprayed three times at varying Zn and B concentrations harvested after 60 days since the last application. Legend: *Significant at 5 %.

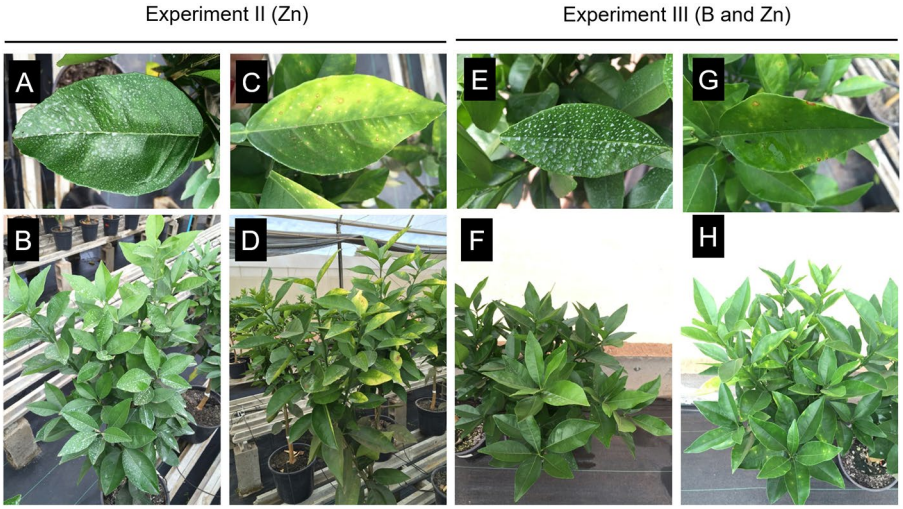

Figure 4 – Young leaf of orange applied with Zn oxide (A), upper view of young orange plant applied with Zn oxide (B), young leaf of orange applied with Zn sulfate (C), upper view of young orange plant applied with Zn sulfate (D), young leaf of orange applied with Zn borate (E), upper view of young orange plant applied with Zn borate (F), young leaf of orange applied with Zn sulfate and boric acid (G), and upper view of young orange plant applied with Zn sulfate and boric acid (H).

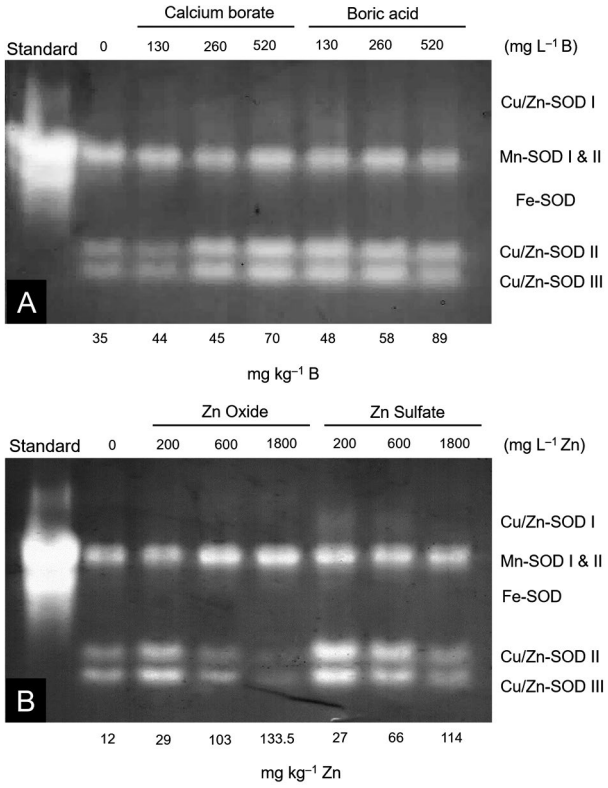

Figure 5 – SOD activity in PAGE (12 %) in leaves of young orange plants sprayed twice with varying concentrations and sources of B (A) and Zn (B) harvested after seven days since the last application. Legend: more abundant white bands represent greater activity of the enzyme.

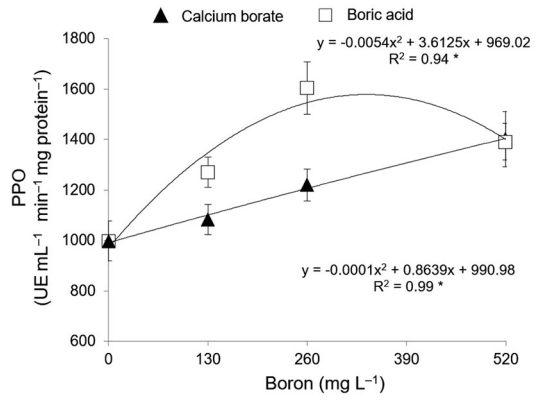

Figure 6 – PPO activity in leaves of young orange plants sprayed twice with varying B concentrations harvested after seven days since the last application. Vertical lines represent the standard error of the mean (n = 3). Legend: *Significant at 5 % according to the F test.

High doses of B applied as calcium borate and boric acid did not result in high concentrations of foliar B as has been found in other works with doses of B above 300 mg L^{-1} (Quaggio et al., 2010; Ullah et al., 2012; Khan et al., 2015). A low concentration of B in the leaves (40 mg kg^{-1} of B) was observed when the lowest dose in this study was applied and, compared to the maximum DM reached with 295 mg L^{-1} of B, reflected a decrease in DM (18 %), characterized as a nutritional deficiency by Mesquita et al. (2016). At the highest dose of B tested, no significant nutrient toxicity was observed, represented by just a 5 % decrease in the DM of the aerial part. This is likely explained by the medium concentration of B (97 mg kg^{-1}) seen in the leaves. Excess B might reduce up to 50 % DM of citrus trees, associated with a decreased net assimilation rate of CO_2 and leaf transpiration when leaf B concentration > 300 mg kg^{-1} (Quaggio et al., 2003; Mesquita et al., 2016; Simón-Grao et al., 2018), a limit considered toxic to citrus seedlings based on Mattos Jr. et al. (1995).

Research results on citrus trees that received adequate doses of Zn sulfate described leaf concentrations in the order of 100 mg kg^{-1} Zn, levels that did not induce plant toxicity (Tariq et al., 2007; Khan et al., 2015). However, concentrations > 100 mg kg^{-1} caused toxicity in citrus (Obreza and Morgan, 2008; Hippler et al., 2015a). We found in Experiment III, with the application of Zn borate and Zn sulfate, that150 mg kg^{-1} of Zn in leaves is the threshold limit for toxicity (Figure 3). Plants supplied with Zn borate exhibited DM reduction from 150 mg kg^{-1} of Zn up to 300 mg kg^{-1} doses of Zn, while the plants supplied with Zn sulfate presented no reduction in DM because they failed to reach 150 mg kg^{-1} of Zn in the leaves (Figure 3).

In Experiment II, ZnO led to high concentrations of Zn (169 mg kg^{-1} Zn) in leaves with adequate doses of the nutrient (Figure 1). A severe reduction in DM production of plants (32 %) was observed when the highest doses of Zn were administered, which correlated with leaf concentrations of approximately 375 mg kg^{-1} of Zn (Figure 2). Similarly, a decrease in DM production of plants (18 %) was observed with application of the highest dose of Zn borate, which correlated with a foliar concentration of 256 mg kg^{-1} Zn (Figures 1 and 2). The reduction in canopy DM at the lowest Zn doses, when the application was administered with Zn sulfate in Experiment II (Zn) (Figure 2), likely occurred because of the amount of salt present in the spraying solution prepared with soluble sources, which caused injury after rapid leaf nutrient absorption, especially during the first application when tissues of plants were young, generating an initial delay in growth (Du et al., 2015).

During the second period of evaluation of the experiments (after harvesting of the aerial part and subsequent growth of new shoots from the main stem), the application of Zn sulfate caused plant injuries characterized by tissue burn of new orange leaves (Figure 4C, D, G and H), probably as a consequence of a cell rupture resulting from differences in osmotic pressure across the cell wall when a solution of fertilizers with high salt content was applied to the leaf surface (Clapp, 2009). This symptom was not observed in plants sprayed

with either ZnO or Zn borate as with the use of sparingly soluble fertilizers, particles adhering to the leaf surface serve as a solid phase to restore micronutrient contents to the plant for a longer time, establishing lower absorption immediately after sprays compared to soluble sources (Peryea, 2006; Li et al., 2012; Du et al., 2015).

A research tool that can assist in assessing the absorption and efficiency of applied nutrients is biochemical tissue testing, which compares the activity of an enzyme in a problem sample with a standard sample. In general, three species of SOD in plants are known and their activity is dependent upon a metal cofactor - Mn-SOD, Fe-SOD, and Cu/Zn-SOD (Shenker et al., 2004). In this study, we investigated the relationship between Zn supply and Cu/Zn-SOD enzyme activity and differences noted between soluble and sparingly soluble fertilizer sources and doses (Figure 5B). The lower activity of the Cu/Zn-SOD II and III isoforms were found in the control treatment when the Zn concentration in the leaves was < 12 mg kg^{-1} Zn, a deficient concentration in citrus leaves (Quaggio et al., 2010), owing to the necessity of Zn as a co-factor for enzyme activation (Hippler et al., 2015a, b). At foliar doses of 200 and 600 mg L^{-1} of Zn applied as Zn sulfate, the Cu/Zn-SOD II and III isoforms were more active compared to the same doses of ZnO application (Figure 5B), and this could be expected because of the high initial absorption that occurs with the application of soluble salt sources, especially in young plant tissues. High nutrient absorption causes an imbalance in plant metabolism, impairing the photosynthetic apparatus, generating reactive oxygen species (ROS). Consequently, the activation of SOD enzymes is necessary for the inactivation of these free radicals (Hou et al., 2007; Hippler et al., 2015a, b).

When the applications of Zn oxide and Zn sulfate provided a concentration of Zn in the leaves > 100 mg kg^{-1} (Figure 5B), considered excessive for citrus, especially for young plants (Obreza and Morgan, 2008; Hippler et al., 2015a), decreases in the activity of the Cu/Zn-SOD II and III isoforms were observed. High levels of metals, such as Zn, can increase the generation of ROS as excess metal levels affect the structure of chlorophyll (Hou et al., 2007) and cause the inhibition of the uptake and transport of other metallic micronutrients in the cells as well as the impairment of the foliar capacity for the synthesis of pigments through the alteration of the protein composition of photosynthetic membranes (Hou et al., 2007; Gratão et al., 2005; 2008). Decreases in the Cu/Zn-SOD II and III isoform activities in this study (Figure 5B), when the Zn content was excessive, corroborated the proposal that SOD activity in leaves increases under moderate and low stress, but above a certain level of stress, inactivation and degradation of SOD prevails (Casano et al., 1997; Panda and Patra, 2000).

The reduction in Cu/Zn-SOD II and III isoform activities at the two higher doses of ZnO may have been induced partially by the contribution of the competition between Zn and Cu. With a high foliar concentration of Zn, the transport of the Cu to the membrane of thylakoids, where isoforms II and III of Cu/Zn-SOD are found, can be compromised, and without Cu, activation of the enzyme does not take place as it is dependent on both Zn and Cu (Figure 5B). This competition would also have consequences for the activity of Mn-SOD, which at the same doses, elevates activity as a way of fostering the inactivation of Cu/Zn-SOD (Gratão et al., 2005).

As the Cu/Zn-SOD enzyme was used to investigate the nutritional status of Zn in citrus, the biochemical test of the PPO enzyme was employed to evaluate the supply of B in this work. It is known that B has the ability to complex with free phenolic compounds and that increases in phenol concentration in B-deficient tissues may lead to the activation of PPO, which processes the hydroxylation of monophenols in diphenols and the oxidation of these diphenols to o-quinones. However, it has been shown that the activity of the enzyme is also expressed by increasing the concentration of B in the leaves to adequate concentrations (Ruiz et al., 1998). PPO activity rises with the B concentration in the leaf to values of 58 mg kg^{-1} and 70 mg kg^{-1} for boric acid and calcium borate, respectively, and decreases with B concentrations of 89 mg kg^{-1} with the application of calcium borate (Figure 6).

Furthermore, it is worth noting that SOD activity in plants that received the application of B, in the form of calcium borate, was in line with the activity of PPO at the same doses applied. Thus, activity rises as the concentration of B is elevated (Figure 5A). This fact can be explained by the generation of active quinones during the phenol transformation process by PPO. These active quinones can receive electrons from the light-saturated photosynthetic process, and when in contact with oxygen, the transfer of these electrons and the formation of superoxide occurs, stimulating the activation of the SOD enzyme during antioxidant processes being carried out by the plant (Marschner, 2011; Simón-Grao et al., 2018). For boric acid, the enzyme is already saturated at the lowest dose, which can be a consequence of the rapid absorption of the soluble source, which, as explained, also leads to the generation of ROS.

In this study, we investigated the activity of SOD and PPO enzymes and their correlation with leaf B and Zn concentration in orange plants with the application of soluble and sparingly soluble sources of these micronutrients. This activity represents biochemical evidence that demonstrates the effect of micronutrient supply from different sources, in addition to that already verified by nutrient concentration and the correlation with the DM of the plants. Further discussion of the generation of ROS, which was not the initial focus of our study, in terms, for example, of quantification of stress products, may be important to explaining effects related to the activities of the enzymes, and could serve as themes for future studies.

Conclusion

Our study demonstrated that the foliar supply of B and Zn to orange plants increases nutrient concentrations in leaves and promotes plant growth, as measured by DM accumulation and LA, similarly with applications of sparingly soluble fertilizer sources (Ca borate, Zn oxide and Zn borate) and soluble sources (Zn sulfate and boric acid). These responses were consistently correlated with enzymatic activity of the antioxidant system, supporting the foliar application of sparingly soluble sources in the nutritional management of citrus orchards.

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Authors' Contributions

Conceptualization: Boaretto, R.M.; Mattos Jr., D.; Quaggio, J.A.; Macedo, L.O.; **Data acquisition**: Macedo, L.O.; Jacobassi, R.; Hippler, F.W.R.; **Data analysis**: Macedo, L.O.; Jacobassi, R.; Boaretto, R.M.; Mattos Jr., D.; Quaggio, J.A. **Design of methodology**: Macedo, L.O.; Mattos Jr., D.; Boaretto, R.M.; **Software development**: Macedo, L.O.; Hippler, F.W.R. **Writing and editing**: Macedo, L.O.; Boaretto, R.M.; Mattos Jr., D.

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