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RESEARCH ARTICLE

Biochemical and anatomical aspects of copper deficiency induced by high nitrogen supply in Citrus

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Abstract

Aims Copper (Cu) is essential for enzymatic systems, electron transport during photosynthesis, and lignin synthesis, affecting plant metabolism and growth. Cu deficiency is observed in young tree orchards, possibly induced by interaction with other mineral nutrients, although visual symptoms are not easily characterized. This study evaluated the induction of Cu deficiency in citrus, due to high nitrogen (N) fertilization, based on the assessment of biochemical parameters, gas exchange, and anatomy of plant tissues.

Methods Valencia orange trees were submitted to levels of Cu supply via fertigation in addition to foliar spraying (control without Cu or with 2.4 g plant^{-1} of Cu), and N (medium = 8.6 or high = 25.9 g plant^{-1} of N) for 210 days.

Results Plants well supplied with Cu exhibited higher electron transport rate, net CO_2 assimilation, transpiration, and stomatal conductance, in comparison to the control without Cu. Those grown with high N exhibited greater electron transport rate compared to medium N. Plants without Cu and high N supply exhibited the lowest nitrate reductase activity, whereas plants fertilized with Cu and high N exhibited the greatest increase in the activity of this enzyme, in addition to the highest levels of total free amino acids. Furthermore, plants without Cu and high N exhibited the lowest Cu/Zn-SOD II isoform activity. Loose parenchyma structures of leaves and branches were associated with visual symptoms of Cu deficiency.

Conclusion Plant responses demonstrated how Cu deficiency is induced in citrus trees by excess N, thus highlighting the importance of establishing best nutrient management for sustainable fruit production.

Keywords Micronutrient · Physiological traits · Enzyme activity · Tissue anatomy · Visual symptom · Nutrient interaction

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Introduction

Copper (Cu) is an essential mineral element for plants, playing a role in cellular metabolism, associated with photosynthesis, production of proteins and lignin, and activity of the antioxidant system (Alloway 2013). Therefore, Cu should be adequately supplied by crop fertilization in agricultural systems (Yruela 2009; Shabbir et al. 2020).

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The occurrence of Cu deficiency in citrus plants has been suggested in newly planted and young orchards in soils with low levels of available nutrient, as well as in the production of seedlings in nurseries, in which the use of organic substrates favors the specific adsorption of this metal and limits root absorption (Mattos Jr. et al. 2010, 2020; Hippler et al. 2017).

Visual symptoms of Cu deficiency in orange trees are known by the growth of vigorous, crooked, elbow-shaped branches, which may exhibit gum leakage. In addition, leaves may exhibit excessively developed leaf blades and protruding veins on the adaxial surface (Hippler et al. 2018a). It has also been suggested that these deficiency-like symptoms also occur in association with the application of high doses of nitrogen (N) fertilizers, which promotes vigorous growth and possibly low lignification of plant tissues (Mattos Jr. et al. 2010, 2020).

Thus, the occurrence of Cu deficiency induced by high doses of N limits the development of plants and, consequently, impairs the maximum productive potential of the orchards. Low tissue lignification due to Cu deficiency might be associated with reduced activity of the polyphenol oxidase enzyme, as well as with reduced activity of the antioxidant enzymatic system, which would cause an increase in the concentration of reactive oxygen species (ROS) and intensify the degradation of cell membranes causing damage to cellular metabolism (Azevedo et al. 1998; Hippler et al. 2016, 2018b).

Total Cu content in leaves and its activity in plant metabolism are related to how much of this ion is bound to protein/chaperones (Yruela 2009). Indeed, Cu activity in metabolism is inversely proportional to the number of ions bound to these nitrogenous compounds and the mobility of the micronutrient in the phloem varies depending on either hydrolysis or degradation of such compounds. With the supply of high doses of N, a certain delay in the movement of Cu from old leaves (source) to newer ones (sink) is expected, not only due to the presence of high levels of nitrogen compounds, but also due to a delay in leaf senescence and release of Cu for redistribution in the phloem (Gilbert 1951). On the other hand, Cu interacts directly with N in the plant, and when there is a deficiency of this micronutrient, there is a reduction in the incorporation of N into proteins, since the process of reducing nitrate to ammonium in the N assimilation path requires the participation of Cu (Hippler et al. 2018b).

In this context, there is a need for a better understanding of the interaction between the nutritional status of citrus trees and the occurrence of visual symptoms, which are considered as Cu deficiency, although they are not well defined in terms of changes in metabolism and in the plant anatomy, which occur mainly after the supply of high doses of N. To test the hypothesis that high fertilization with N causes Cu deficiency in citrus due to the increase in vigor, and damages to the plant metabolism, the present study assessed the effect of supplying different levels of N on the induction of Cu deficiency in young sweet orange trees due to high N fertilization, through biochemical and anatomical parameters until the visual manifestation of the symptoms.

Material and methods

Design and performance of the experiment

The study was performed in a greenhouse at the Sylvio Moreira Citrus Research Center (IAC), Cordeirópolis, State of São Paulo, Brazil (22°27′40”S - 47°24′4”W; altitude = 639 m). Valencia orange trees [*Citrus sinensis* (L.) Osbeck] grafted onto Rangpur lime (*C. limonia* Osbeck), 18-month-old, with fully expanded and newly mature leaves were used for the experiment. They were grown in pine-bark substrate, in 12 dm^3 pots. The experiment was designed in a 2 × 2 factorial scheme, combining two levels of Cu applied via fertigation, in addition to foliar sprays (control without Cu supply or 2.4 g $plant^{-1}$ of Cu; Mattos Jr. et al. 2010; Hippler et al. 2018c) and two doses of N (medium = 8.6 or high = 25.9 g N $plant^{-1}$; Dovis et al. 2021), between July and February (210 days), with five replications.

N was applied in the form of calcium nitrate plus ammonium nitrate (keeping the percentage ratio of 70:30 for NO_3^-: NH_4^+). The applications of Cu to the plants were divided and performed at intervals of 30 days, initially via foliar fertilization and subsequently via fertigation. The adjustment of the applied volume and the Cu concentration of the solutions were performed based on the estimates of the volume of water retained by the leaves of the control plants, using a manual sprayer with previous compression

and an empty cone-jet spray tip to produce droplets, with diameter < 200 µm. The volume of applications was sufficient to allow maximum wetting of the leaf surfaces without significant run-off.

During the entire course of the experiment, adequate nutritional status of the plants was maintained through fertilization with other nutrients, also applied via fertigation, in the total amounts per plant of: 0.32 g of P, 0.39 g of K, 0.97 g of Ca, 0.15 of Mg, 0.14 g of S, 31.3 mg of B, 43.5 mg of Fe, 28.3 mg of Mn, 5.2 mg of Mo and 39.1 mg of Zn, as adapted from Dovis et al. (2021). Plants were also manually irrigated in sufficient amount to maintain approximately 70% (w/w) of the field capacity in the substrate. Phytosanitary treatments, when required, excluded Cu applications.

The plants were assessed in a non-destructive manner for physiological parameters (gas exchange and chlorophyll *a* fluorescence) 210 days after the imposition of fertilization with different levels of Cu and N. New leaves and/or branches were collected for further enzymatic analysis, quantification of free amino acids, and anatomical analysis.

Subsequently, the plants were harvested destructively to determine the production of dry mass (DM) of leaves, branches, and roots, and the total leaf area, through the leaf area integrator (mod. LI-3100, Li-Cor, Lincoln, NE, USA). The leaves and branches were clipped, divided into 'old' part (grown before the imposition of treatments) and 'new' part (grown after the imposition of treatments). The total leaf area of plants was measured after destructive harvesting with the aid of the leaf area meter. The material collected was dried in an oven with forced air circulation at 60 °C until constant mass weight and ground to pass through a 200-mesh sieve. The analyses to quantify the levels of nutrients in the plants were performed on samples after wet digestion of plant tissue with sulfuric acid and then steam Kjeldahl distillation for N (AOAC 1999), or nitric acid plus perchloric acid and elemental determination by inductively coupled plasma for Cu (Havlin & Soltanpour 1980).

Gas exchange and chlorophyll *a* fluorescence

Measurements of gas exchange and chlorophyll fluorescence were obtained using an infra-red gas CO_2 analyzer system integrated with a fluorescence chamber (IRGA mod. Li-6800, Li-Cor, Lincoln, NE, USA). These measurements were performed on newly emerged mature leaves of the third flush of growth, between the 3rd and 5th branch positions and exposed to the sun. The photosynthetically active photon flux density (PPFD) was fixed at the beginning of each measurement based on the incident radiation at the time and used for the other repetitions at the same time of day.

The variables related to gas exchange measured were net CO_2 assimilation (P_N, µmol m^{-2} s^{-1}); stomatal conductance (g_s, mol m^{-2} s^{-1}); transpiration (E, mmol m^{-2} s^{-1}); and intercellular concentration of CO_2 (Ci, µmol mol^{-1}). The variables related to chlorophyll *a* fluorescence were minimal fluorescence (Fo) and maximal fluorescence (Fm), measured after dark adaptation (30 min); and fluorescence under dynamic equilibrium (F′) and maximal (Fm′), measured after light adaptation (Schreiber et al. 1998). The measurements of Fm and Fm′ were obtained with a saturation pulse of λ <710 nm, PPFD ~10,000 µmol (photon) m^{-2} s^{-1}, 0.8 s. The Fo parameter was measured using far-red light pulse (λ = 735 nm, PPFD ~50 µmol [photon] m^{-2} s^{-1}, 3.0 s). The maximal variable fluorescence in the dark and after light adaptation, as well as the term Fq' were calculated according to Baker (2008). These variables were used to calculate maximum quantum efficiency (Fv/fm), maximal photosystem II (PSII) efficiency (Fv'/Fm′), operational efficiency of PSII (Fq'/Fm′), apparent electron transport (ETR), and non-photochemical fluorescence quenching (Baker 2008). These measurements were performed at 9:00 a.m. and 1:00 p.m.

In vitro activity of superoxide dismutase

When the plants exhibited two flushes of vegetative growth already developed, approximately five to six months after treatment imposition, the leaves were collected and storage in a freezer at −80 °C, for further analysis. The enzymatic activity of superoxide dismutase (SOD) was determined in leaves stored at −80 °C and macerated in 100 mmol phosphate buffer (pH 7.5) containing 3 mmol DTT, 1 mmol EDTA, and 4% (m/v) PVPP. The suspension was centrifuged at 12,000 x *g* for 35 min at 4 °C. The supernatant was used for biochemical analyses. Protein content was determined by the method proposed by Bradford (1976) using bovine serum albumin (BSA) as a standard. SOD activity was performed in 12% (w/v)

polyacrylamide gel electrophoresis (PAGE). A standard sample of beef liver SOD and 50 µg of protein from protein extracts were applied to each gel (Hippler et al. 2016). After protein separation by electrophoresis, the gels were incubated in a light-free environment for 30 min in a reaction solution containing 50 mmol potassium phosphate buffer (pH 7.8), 1 mmol EDTA, 0.1 mmol NBT, 0.05 mmol riboflavin, and 0.3% (w/w) TEMED. After incubation, the reaction solution was removed and the gels were exposed to light for a few minutes for photo-oxidation, with the bands corresponding to SOD activity remaining without photo-oxidation. The photo-oxidation of the gel was interrupted by inserting the gels into 7% acetic acid (m/v) for 15 min. To determine the isoforms, they were then classified as Cu/Zn-SOD, Fe-SOD or Mn-SOD. Cu/Zn-SOD is inactivated by KCN and H_2O_2. Fe-SOD is inactivated by H_2O_2, and resistant to KCN. The Mn-SOD form is resistant to both (Azevedo et al. 1998).

In vivo nitrate reductase activity

The in vivo activity of the enzyme nitrate reductase (NRase) was performed according to Dovis et al. (2014), and leaf sampling was performed between 9:00 a.m. and 10:00 a.m.

Two 3-to 6-month-old leaves, exposed to the sun, and located between the 3rd and 8th position from the end of the branch and in the middle third of the canopy, were collected. After cleaning with paper and distilled water, 20 discs were cut per sample and placed in a syringe. The incubation medium was composed of 100 mmol sodium phosphate buffer (pH 7.5) + 1% n-propanol (m/v), being incubated with phosphate buffer +200 mmol KNO_3. The samples were vacuum infiltrated and placed in a 37 °C water bath to incubate for 30 min. NO_3^- reduced to NO_2^- was quantified by adding sulfanilamide diluted in 2.4 N HCl + N-(1-naphthyl)-ethylenediamine dihydrochloride and subsequent reading in a spectrophotometer at 540 nm.

Total free amino acids

Soluble amino acids were extracted according to the method proposed by Bieleski and Turner (1966). Fine powder of biological material (200 mg) was used in 2 mL of MCW (methanol:chloroform:water, in the proportion 12:5:3). The mixture remained overnight in the refrigerator and then centrifuged at 10,000×*g* for 20 min. Subsequently, 500 µL of chloroform and 750 µL of distilled water were added to the supernatant, and then the sample was centrifuged again, and the aqueous phase formed was carefully removed. The collected material was placed in a water bath for one hour at 38 °C and then in a Vacufuge concentrator (Eppendorf, Westburg, NY, USA) for complete concentration of the samples. The samples were resuspended in 100 µL of Milli-Q water, filtered through 0.22 µm Millex, for later determination of total free amino acids according to Yemm and Cocking (1955).

Light microscopy

Leaves and branches of orange trees from the different treatments with Cu and N were collected. From the plants that received high N and did not receive Cu, leaves and branches with symptoms of excessive growth (symptoms not observed in the other treatments) were also collected. The samples were fixed in Karnovsky's solution (Karnovsky 1965 - modified), remaining in a vacuum pump for five minutes and kept in a refrigerator for at least 24 h.

Fixed samples were dehydrated in a series of ethanol concentrations [30, 50, 70, 90, and 100% (three times)], remaining 20 min in each stage. After dehydration, the samples were infiltrated in a 1:1 solution of acrylic resin and 100% ethanol for six hours, and then in pure acrylic resin for 24 h. Once infiltrated, the samples were polymerized with acrylic resin for 72 h at room temperature. They were cut to a thickness of 5 µm in a manual rotating microtome using a type C steel knife. The sections were placed on histological slides, stained with 0.05% toluidine blue and pH 3.2 (Feder and O'Brien 1968 - modified) for five minutes and washed in water for three minutes. The slides were mounted with Entellan® resin and coverslips and then analyzed under a light microscope.

Statistical analysis

A two-way analysis of variance (ANOVA) was used to evaluate effects of Cu and N supply to plants. Differences in the means of one factor were tested separately on each level of the other factor by the Tukey's multiple comparison test at 5% as proposed by Kitsche and Schaarschmidt (2015).

Results

Dry mass production and foliar nutrient concentration

The mean accumulation of root dry mass of plants supplied with +Cu was lower (452 g plant^{-1}) in comparison to the plants fertigated without the metal (-Cu) (565 g plant^{-1}). Although the measures of total shoot dry mass and leaf area did not differ between the different levels of N and Cu, the highest N supply to plants in the +Cu treatment determined the highest shoot to root ratio (S:R = 1.8) observed in the present study (Fig. 1).

The levels of N and Cu in the leaves of plants varied according to the treatments and were higher with the increase in the supply of each one individually, except for root N (Fig. 2). Trees supplied with medium N level exhibited higher leaf N concentration with +Cu (~23 g kg^{-1} of N), whereas the same was the highest despite the varying Cu supply (30 g kg^{-1} of N) (Fig. 2). Furthermore, the highest supply of N was associated with the concentration of Cu in the leaves (30 mg kg^{-1} of Cu), about twice as low as observed in plants grown with medium N (~60 mg kg^{-1} of Cu). These levels of Cu in the leaves, despite the varying N supply, were higher than those in roots (<30 mg kg^{-1} of Cu) (Fig. 2).

Gas exchange and chlorophyll *a* fluorescence

Plants supplied with +Cu exhibited greater assimilation of CO_2 (P_N), transpiration (E), and stomatal conductance (g_s) when compared to those without Cu supply, which differences were 12–47% higher for plants grown with the high N regime (Fig. 3).

Regarding the electron transport rate (ETR), plants submitted to high N exhibited higher mean values of ETR (58 µmol m^{-2} s^{-1}) in comparison to plants supplied with medium N (45 µmol m^{-2} s^{-1}) (Fig. 3). Also, plants fertilized with +Cu exhibited ETR 27% higher than plants without Cu supply (Fig. 3).

Enzyme activity and total amino acid content

The activity of the nitrate reductase (NRase) was highest in the treatment with high N and + Cu supply (Fig. 4). However, as the N supply increased, plants without Cu exhibited the lowest NRase activity, about twice as low as plants with +Cu (Fig. 4).

Plants with medium N had similar total free amino acid concentrations at the two Cu levels (62.5 µmol g^{-1} DM). However, when the N supply was increased, the plants fertilized with +Cu exhibited the highest amino acid content (125 µmol g^{-1} DM) (Fig. 4). Therefore, plants that

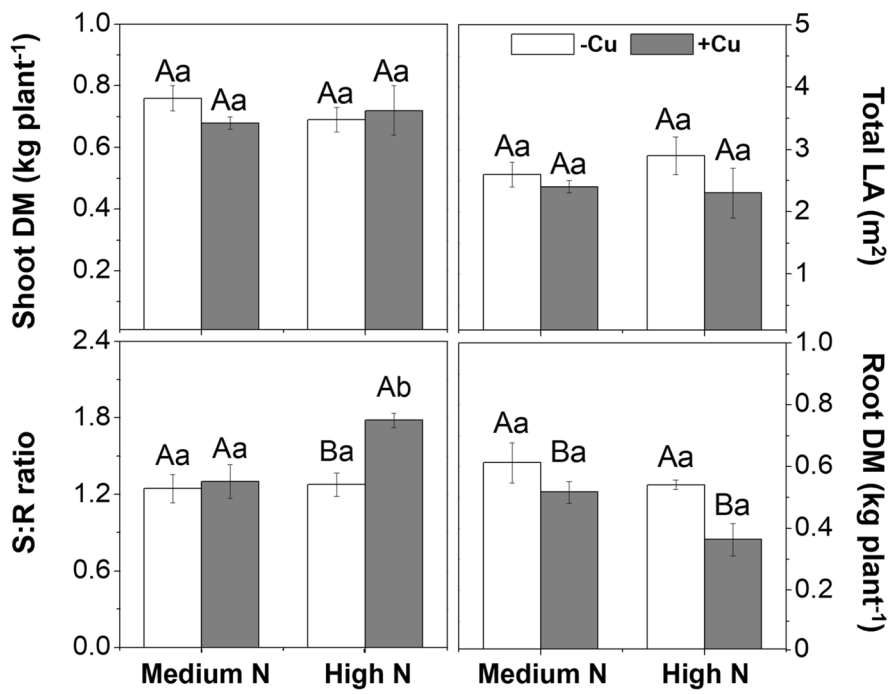

Fig. 1 Dry mass (DM) of shoot and roots, total leaf area (LA), and shoot to root ratio (S:R) of young plants of Valencia sweet orange trees supplied with different nitrogen levels (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper [control without Cu (-Cu) or adequate = 2.4 g plant^{-1} of Cu (+Cu)], after 210 days of treatments. Same lowercase letters between N levels, and capital letters between Cu levels did not differ according to Tukey's test ($p < 0.05$)

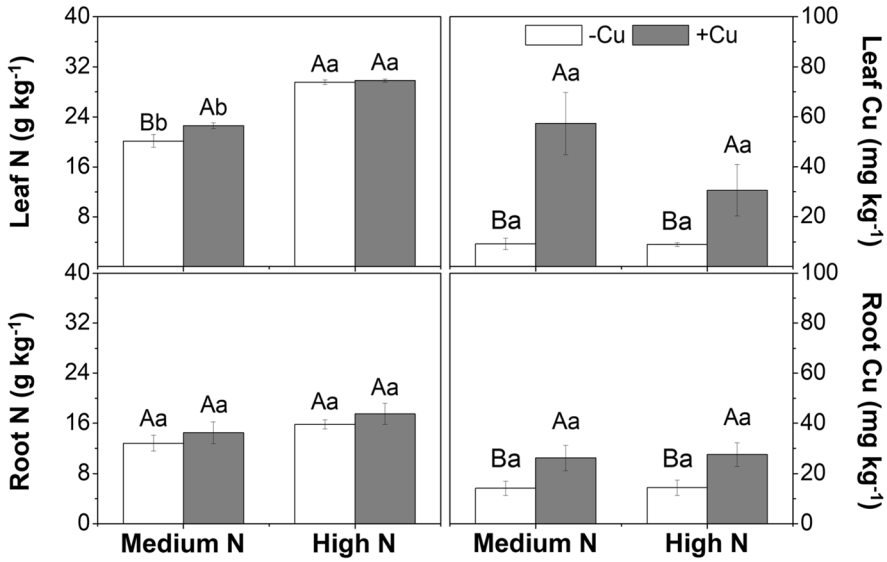

Fig. 2 Nitrogen (N) and copper (Cu) concentrations in leaves and roots of young Valencia sweet orange trees supplied with different levels of nitrogen (medium N = 8.6 or high N = 25.9 g plant^{-1}) and Cu (control without Cu or adequate = 2.4 g plant^{-1} of Cu), after 210 days of treatments. Caption: equal lowercase letters between N levels and capital letters between Cu levels did not differ according to Tukey's test ($p < 0.05$)

exhibited highest NRase activity (Fig. 4) also had highest amounts of amino acids.

The activity of superoxide dismutase (SOD) in the orange tree leaves was reduced mainly in the Cu/Zn-SOD II and III isoforms when the plants were submitted to high N without Cu (Fig. 5).

Visual symptoms of copper deficiency

In the plants of the experiment that did not receive Cu applications, either from the high or medium N treatments, visual symptoms of Cu deficiency were observed. They were characterized by the presence of long and crooked branches, leaves with protruding veins, and irregular leaf blades (Fig. 6).

Light microscopy

The leaves of plants that did not receive Cu in the nutritional management, both with the high dose and the medium dose of N, exhibited altered anatomical structure when compared to plants that were supplied with Cu (Fig. 7). The palisade parenchyma and mesophyll of the plants that did not receive Cu exhibited lax cells with less surface of contact between cell walls of adjacent cells. The same plants had a greater number of intercellular spaces in the spongy

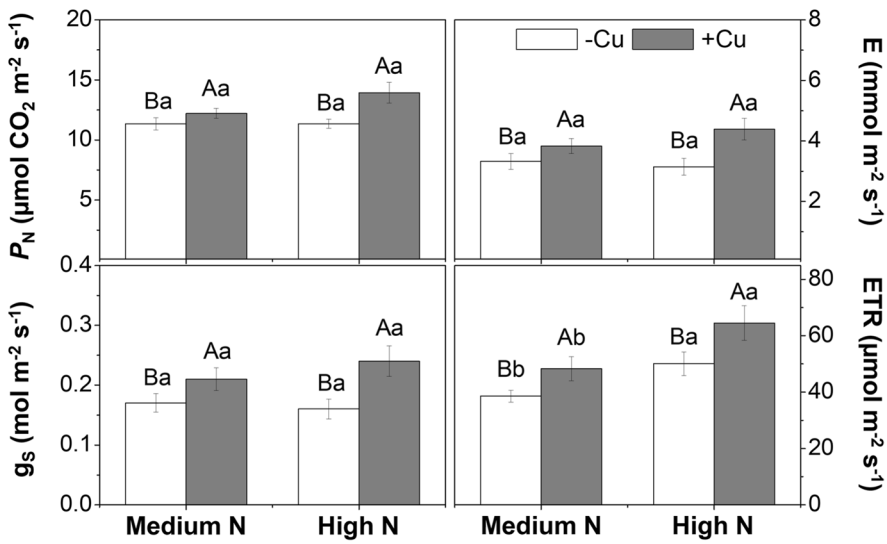

Fig. 3 Net CO_2 assimilation (P_N,), transpiration (E), stomatal conductance (g_s), and electron transport rate (ETR) in leaves of young Valencia orange trees supplied with different nitrogen levels (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper (control without Cu or adequate = 2.4 g plant^{-1} of Cu), 210 days after treatments. Same lowercase letters between N levels, and capital letters between Cu levels did not differ according to Tukey's test ($p < 0.05$)

parenchyma. Such characteristics were prominent with the high dose of N, due to the greater elongation of the cells (forming a thicker mesophyll), together with the less structuring of the cell walls that ensure the adequate development of the tissue, evidenced by the weak staining of the cell walls of this treatment (Fig. 7).

The leaves of the orange trees that received Cu, both with the medium and high doses of N, exhibited palisade parenchyma cells with greater uniformity of shape and size, which allowed the observation of a greater surface of contact between the cell walls of the adjacent cells and, consequently, a compacted tissue. These plants also produced mesophylls with greater number of cells in the spongy parenchyma, which reduced the intercellular spaces and favored the formation of compacted tissue. As the N dose increased in plants that received Cu, the mesophylls were also thicker. However, due to the better structuring of the cell walls (evidenced by the more intense staining of the walls), the tissue was formed with greater compaction (Fig. 7).

As in the leaves, the branches of the orange trees that received Cu (with both doses of N) exhibited a well-organized anatomical structure of cells and tissue (Fig. 8). The branches of the plants that did not receive Cu had fissures in the vascular tissue, which demonstrates the lower compaction and resistance of these tissues. As the N dose increased in plants that were not fertilized with Cu, the branch tissues

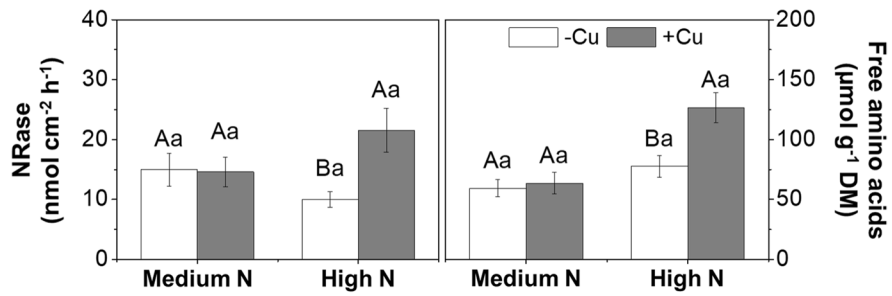

Fig. 4 In vivo activity of the enzyme nitrate reductase (NRase) and total free amino acids in the leaves of young Valencia orange trees supplied with different nitrogen levels (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper (control without Cu or adequate = 2.4 g plant^{-1} of Cu), after 210 days of treatments. Same lowercase letters between N levels, and capital letters between Cu levels did not differ according to Tukey's test ($p < 0.05$)

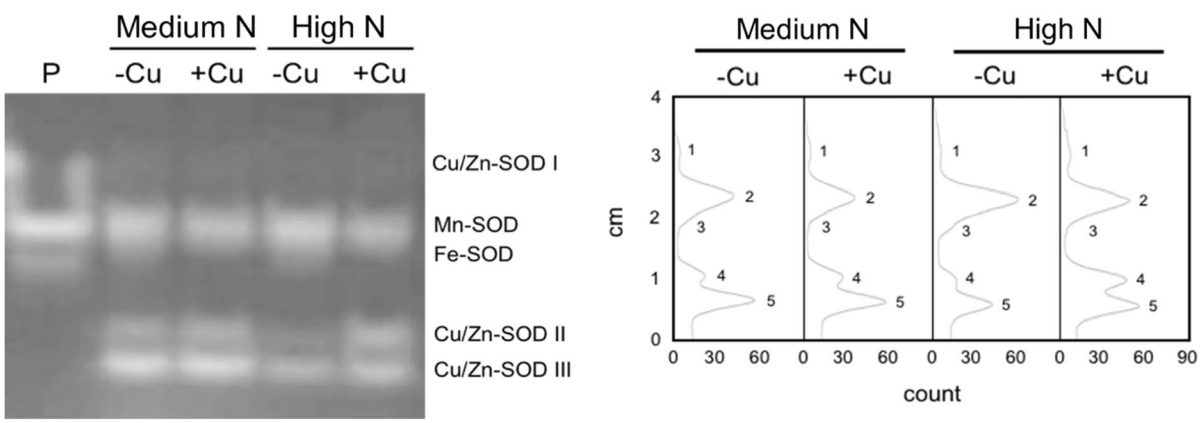

Fig. 5 Polyacrylamide gel electrophoresis (PAGE 12%) activity and densitogram of superoxide dismutase (SOD) activity in leaves of young Valencia orange trees supplied with different nitrogen levels (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper (control without Cu or adequate = 2.4 g plant^{-1} of Cu), after 210 days of treatments. Caption: PAGE: P - bovine SOD standard; Densitometry: 1 - Cu/Zn-SOD I; 2 - Mn-SOD; 3 - Fe-SOD; 4 - Cu/Zn-SOD II; and 5 - Cu/Zn-SOD III

exhibited excessive elongation with the formation of smaller and lax cells (vascular and parenchyma) (Fig. 8).

When Cu was applied, the plants (at both N doses) exhibited branches with compact and resistant tissues, with no cracks in the vascular tissue. In addition, vascular and parenchyma cells were formed with greater diameter and greater intensity in staining, which demonstrated the better structuring of cell walls when Cu was applied. With the increase in the dose of N in plants that received Cu, the branch tissues also exhibited greater elongation; however, due to the better structuring of the cell walls, the tissues formed were compact (Fig. 8).

In symptomatic branches and leaves with excessively vigorous growth (higher dose of N and without Cu), the anatomical characteristics described above were even more prominent than in asymptomatic leaves and branches of the same treatment (Fig. 9). The mesophyll of symptomatic leaves had greater number of intercellular spaces, due to excessive cell elongation and the low uniformity of size and shape of the palisade and spongy parenchyma cells. Such cellular development generated the tissues with less compaction of all the leaves assessed.

Symptomatic branches exhibited the greatest number of cracks in vascular and parenchymatic cells, showing the lowest resistance to the cut performed for the assembly of the blades and, consequently, the slacker tissue structure of all branches assessed. Also, the symptomatic branches exhibited the formation of a layer of phenolic substances (green color), which was observed in non-symptomatic branches only in the same treatment (higher

Fig. 6 Symptoms of copper (Cu) deficiency in young Valencia orange trees without Cu supply, after 210 days of treatments. **A**: protruding veins and irregular leaf blades; **B**: long and crooked branches

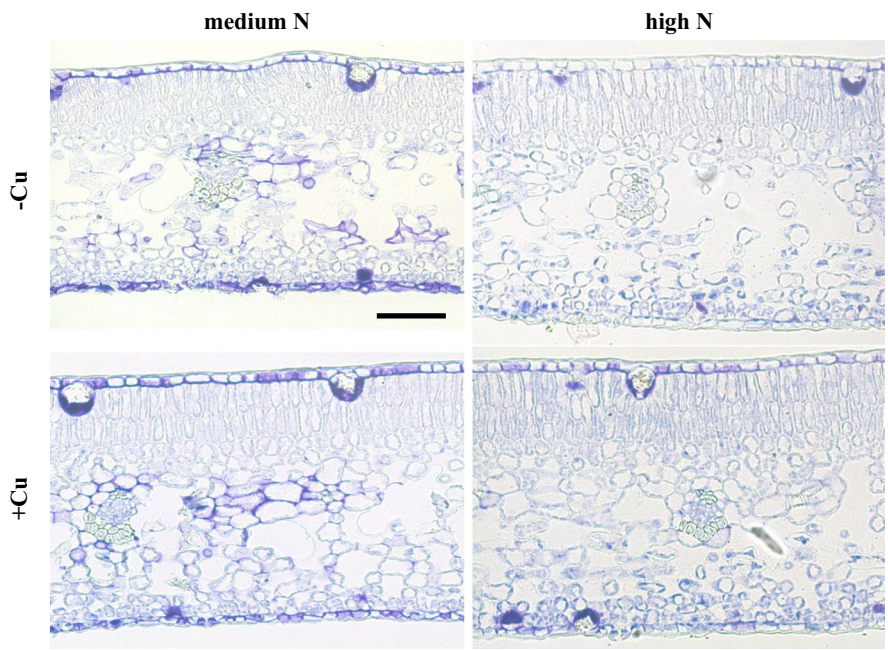

Fig. 7 Light microscopy images of the mesophylls of leaves from young Valencia orange trees supplied with different levels of nitrogen (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper (control without Cu or adequate = 2.4 g plant^{-1} of Cu), after 210 days of treatments. Bar: 20 µm

dose of N and without Cu). The formation of these phenolic layers indicates inadequate tissue development, possibly generated as a plant response to cell lysis.

Discussion

Modern citrus farming focuses on increasing productivity and fruit quality, achieved with the implementation of orchard management practices for the sustainability of the industry. For instance, the planting new orchards, in the Brazilian citrus belt, sought to increase the density of plants in the field, which has probably led to greater efficiency in the use of nutrients provided by fertilization.

Thus, young orchards with vigorous growth have been frequently observed, resulting from the greater use of N by plants. Associated with this excess vigor, we see the occurrence of abnormal growth of branches and leaves, which visual symptoms suggest that they are associated with Cu deficiency induced by excess N.

In this study, the interplay of N and Cu is characterized based on the effects of different levels of nutrient supply on plant nutritional status related to tissue anatomy and plant growth, as well physiological traits, assimilation of nitrate into amino acids and activity of enzymes of the antioxidant system. The understanding of this array of events is important for fine tuning nutrient supply for best management of fertilization in the orchards.

Well-nourished plants, such as those with high N supply, invest least carbon in root production when there is no limitation of resources for plant growth (Gunatilleke et al. 1997; Illenseer and Paulilo 2002). This explains the fact that the S:R ratio was highest for trees supplied with high N and + Cu (Fig. 1). The relevance of such a response demonstrates the ability of trees to invest greater biomass and N partition to grow leaves rather than roots, which translates into improved NUE as demonstrated for lemon compared to orange trees (Dovis et al. 2021).

We assume that the estimated 15–20% increase in leaf N concentration in trees with the most limiting supply of N and + Cu was associated with increased N uptake by roots (Fig. 2). The Cu supply improves the N uptake by roots, by up-regulating the expression levels of both NO_3^- and NH_4^+ transporter genes, while the N supply significantly promotes the root-to-shoot translocation of Cu and distributed more Cu into the shoots and leaves of grain crops (Cui et al. 2022).

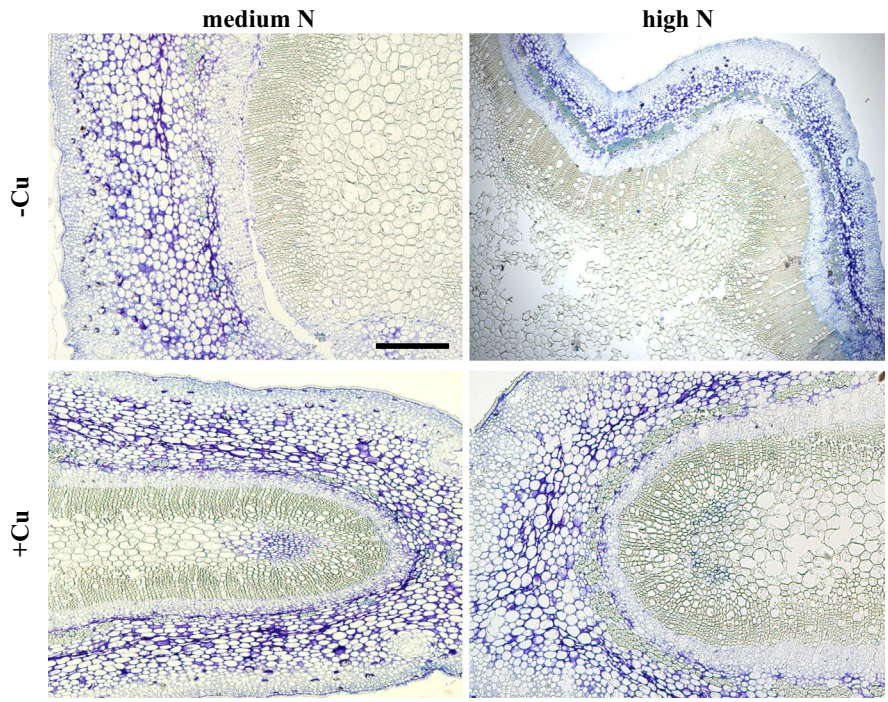

Fig. 8 Light microscopy images of young branches of young Valencia orange trees supplied with different levels of nitrogen (medium N = 8.6 or high N = 25.9 g plant^{-1}) and copper (control without Cu or adequate = 2.4 g plant^{-1} of Cu), after 210 days of treatment. Bar: 20 µm

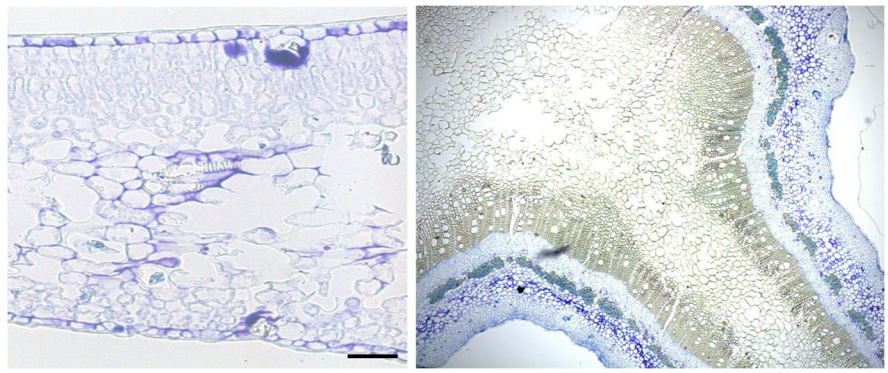

Fig. 9 Light microscopy images of symptomatic leaf mesophyll (left) and young branch (right) with excess and vigor in young Valencia orange trees supplied with high N level (25.9 g $plant^{-1}$) and without Cu. Bar: 20 µm

Furthermore, based on the previous, it would be reasonable to expect greater differences in plant growth for the citrus trees in a longer period, especially for the medium N and Cu supply, tested in this study.

Plants subjected to low availability of nutrients exhibit impaired growth, frequently adapting mechanisms to survive in the environment under stress, for instance, decreasing the S:R ratio under N or phosphorus (P) deficiency (Zambrosi et al. 2013a). Such changes in the architecture of the root system favor the plant ability to exploit the soil and take up nutrients (Cakmak et al. 2023). This may be an effect related to lower root growth when high N was applied, resulting in almost 30% lower root DM compared to medium N (Fig. 1), reducing the ability of tree roots to absorb Cu. Although there were no differences in N contents in plant roots regardless of the treatments tested, those of Cu were higher due to Cu supply (Fig. 2).

The interaction of Cu with other nutrients on the nutritional status of plants was also determined, although in conditions where the Cu concentration was considerably high in the plants. Under excess Cu, adequate supply of phosphorus (P) may reduce the high uptake of this metal by the roots of citrus plants (Zambrosi et al. 2013b). Also, high supply of N or adequate Ca can reduce phytotoxic effects in orange trees (Hippler et al. 2018b).

Greater photosynthesis in trees supplied with +Cu resulted from greater stomatal opening and transpiration as well greater ETR compared to those without Cu (Fig. 3). Moreover, ETR was highest with high N, highlighting the N and Cu interplay on plant growth (Fig. 3). Cu is part of plastocyanin, a photosynthesis-related protein involved in the transfer of electrons from cytochrome f to P700+, which is responsible for the electron transport chain (Peers and Price 2006). Such results also demonstrate the importance of Cu in the activity of NRase, the first enzyme in the process of N assimilation via reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is followed by production of ammonium and, later, incorporation as a protein (Dovis et al. 2014).

Nitrate reductase activity levels gradually increased in roots of *Luffa cylindrica* with increasing copper concentrations (up to 50 µmol in the nutrient solution) compared to a nil control (Zhang et al. 2014). Thus, when there is a deficiency of this micronutrient, there may be a reduction in the production of free amino acids (Fig. 4) and further assimilation into proteins, since this process requires the participation of Cu. On the contrary, nitrate reductase activity is reduced to negligible levels in response to excess Cu exposure.

The primary N metabolism is reduced in leaves, although to a lesser extent than in roots, also in response to inhibition of nitrate uptake induced by the metal (Llorens et al. 2000). Furthermore, NRase consumes large amounts of energy to reduce nitrate to nitrite (Dovis et al. 2014). Since the lack of Cu impairs the electron transport chain between the photosystem, as demonstrated by varying ETR (Fig. 3), it may result in less energy for NRase enzyme activity (Llorens et al. 2000).

In cases of deficiency of this nutrient, there is a reduction in the transport of electrons due to the decrease in the synthesis of this protein (Printz et al. 2016) as suggested by data in Figs. 3 and 4. This dysfunction in electron transport can cause an increase in ROS, which cause the degradation of membranes and proteins in cells (Gill and Tuteja, 2010; Hippler et al. 2016).

Interestingly, the activity of Mn-SOD was increased to compensate for the reduction of the activity of Cu/

Zn-SOD (Fig. 5). Specific changes in one or another dismutase may indicate specific plant response for homeostasis maintenance, since nutritional deficiencies generate an increase in the concentration of ROS, which in turn may affect organelle cell processes (Gratão et al. 2005). In citrus trees, the enzymatic antioxidant system proved to be important in the detoxification of these radicals (Hippler et al. 2016, 2018c).

Fe-SOD and Mn-SOD are much related, although Fe cannot replace the Mn ion in the active center (Soares et al. 2019). Moreover, ferric superoxide dismutase (Fe-SOD) likely compensates Cu/Zn-SODs during the Cu starvation, therefore saving the nutrient for essential functions, as in plastocyanin, when plants undergo Cu deficiency, revealing that Cu and Fe plant pools are interconnected (Carrió-Seguí et al. 2019; Wairich et al. 2022). Then, increased activity of Mn-SOD could represent a similar mechanism contributing to plant homeostasis maintenance.

Nitrogen decreases Cu concentrations in plants, suggesting that Cu deficiency is induced by inhibition of the Cu transport from roots to shoots. With excess N, plant growth is stimulated to such an extent that the absorbed nutrient reach deficient concentrations in stems and leaves. Similarly, in our observation of visual symptoms on leaves (Fig. 6), Chaudhry and Lonerag (1970) described young wheat leaves, supplied with N but not Cu, folded at the ligule and assumed a position almost perpendicular to the stem, with ears facing the floor. Cu deficiency also altered shoot architecture in *Arabidopsis*, which exhibited increased shoot branching, leaves with chlorotic spots, and a longer primary inflorescence (Ishka and Vatamaniuk 2020).

The cell wall is a complex structure made of cellulose fibrils with a higher degree of polymerization and lignin. Cu as other metals influence lignin deposition in a wide variety of plant species, from oxidized phenols (Printz et al. 2016). The lack of structural cell wall components leads to abnormal cells, which affects epidermis and parenchyma normal organization and causes loose growth of stems and leaves that become increased in size and floppy as described in our work. In addition, Cu deficiency also exerts changes related to auxins, affecting plant architecture, whose homeostasis and nutritional signals lack further studies (Ishka and Vatamaniuk 2020).

In fact, the biochemical aspects of the plants assessed in the experiment of the present study demonstrate in part that Cu deficiency is induced in citrus plants by high doses of N, characterized mainly by the decrease in metabolic and enzymatic activity in the plants, with consequent loss of photosynthetic capacity, assimilation of nitrate into amino acids, and tolerance to oxidative stress.

Also, anatomical aspects of loose parenchyma structures of leaves and branches were associated with visual symptoms of Cu deficiency, thus confirming characteristics observed in citrus growing conditions in the field and seedling nurseries, when excess plant vigor occurs. Thus, our data allowed the identification of the nutritional disorder caused by Cu deficiency, subsidizing the definition of fertilization of citrus orchards, avoiding the excessive use of N in new planted areas, as well as promoting the foliar spraying of Cu, to meet the nutritional requirements of trees (Mattos Jr. et al. 2020), aiming at the sustainability of the citrus industry.

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