



# Following the effects of micronutrient supply in HLB-infected trees: plant responses and '*Candidatus Liberibacter asiaticus*' acquisition by the Asian citrus psyllid

Jefferson Rangel da Silva<sup>1</sup> · Fausto Veiga de Alvarenga<sup>1</sup> · Rodrigo Marcelli Boaretto<sup>1</sup> · João Roberto Spotti Lopes<sup>1</sup> · José Antônio Quaggio<sup>2</sup> · Helvecio Della Coletta Filho<sup>1</sup> · Dirceu Mattos Jr<sup>1</sup>

Received: 10 November 2019 / Accepted: 11 May 2020

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## Abstract

Despite efforts of research to establish best nutrient management practices in HLB-affected citrus orchards, there are still doubts about the contribution of such strategies to minimizing losses caused by the disease in the citrus industry. We evaluated the effects of micronutrient (Zn, Mn, and/or Cu) supply and '*Candidatus Liberibacter asiaticus*' (CLas) infection on physiological and growth traits of sweet orange trees (*Citrus sinensis* (L.) Osbeck) to understand if enhanced micronutrient supply would improve the growth of plants infected with CLas and reduce bacterial infection, as well as the acquisition of CLas by adults and nymphs of *Diaphorina citri*, correlated with nutritional status of trees. Plants were either grafted with buds obtained from micrografted plants (healthy, −) or buds infected with CLas (diseased plants, +). Infected plants were exposed to one of the five nutrient treatments, applied to leaf and root: (i) nilZnMnCu+: not fertilized with Cu, Mn, and Zn; (ii) Zn+: fertilized with zinc sulfate ( $ZnSO_4 \cdot H_2O$ ); (iii) Mn+: fertilized with manganese sulfate ( $MnSO_4 \cdot H_2O$ ); (iv) Cu+: fertilized with copper hydroxide [ $Cu(OH)_2$ ]; and (v) ZnMnCu+: fertilized with all three micronutrients. Likewise, healthy plants were exposed to one of the two treatments as above: (i) nilZnMnCu− or (v) ZnMnCu−. We found that CLas impairs plant biomass production regardless of nutrient treatments, especially root growth, and increases specific leaf dry weight as disease progresses because of starch accumulation. Moreover, individual supply with Zn, Mn, or Cu can mitigate such deleterious effects of HLB on starch metabolism. HLB also changes nutrient concentrations in both leaves and sap extract, regardless of nutrient treatments, although treatments do not reduce the CLas titer in plants as determined by RT-qPCR. On the contrary, micronutrients applied in combination (ZnMnCu+) can reduce the acquisition of CLas by adults and especially nymphs of *D. citri*, likely reducing the disease infection in citrus orchards.

**Keywords** Huanglongbing · Greening · Citrus · Zinc · Manganese · Copper · Starch content · Bacteria acquisition

## Abbreviations

(+)	Plants infected with ' <i>Ca. Liberibacter asiaticus</i> '	(−)	Plants not infected with ' <i>Ca. Liberibacter asiaticus</i> '
CLas	' <i>Ca. Liberibacter asiaticus</i> '	' <i>Ca. Liberibacter asiaticus</i> '	
$C_T$	Cycle threshold values		
Cu+	Infected plants fertilized with copper hydroxide		
DAINT	Days after initiating nutrient treatments		
HLB	Huanglongbing		
LA	Leaf area		
LDW	Leaf dry weight		
Mn+	Infected plants fertilized with $MnSO_4 \cdot H_2O$		
nilZnMnCu+	Infected plants not fertilized with Cu, Mn, and Zn		

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s40858-020-00370-9>) contains supplementary material, which is available to authorized users.

✉ Dirceu Mattos, Jr  
ddm@ccsm.br

<sup>1</sup> Centro de Citricultura Sylvio Moreira, Instituto Agronômico (IAC), Cordeirópolis, SP 13490-970, Brazil

<sup>2</sup> Centro de Solos e Recursos Ambientais, Instituto Agronômico (IAC), Campinas, SP 13020-902, Brazil

nilZnMnCu(–)	Non-infected plants not fertilized with Cu, Mn, and Zn
PCR	Polymerase chain reaction
qPCR	Real-time quantitative PCR
RDW	Root dry weight
ROS	Reactive oxygen species
SLW	Specific leaf weight
SOD	Superoxide dismutase
TotalDW	Total plant dry weight
ZnMnCu+	Infected plants fertilized with ZnSO <sub>4</sub> .H <sub>2</sub> O, MnSO <sub>4</sub> .H <sub>2</sub> O, and copper hydroxide
ZnMnCu(–)-non	Infected plants fertilized with ZnSO <sub>4</sub> .H <sub>2</sub> O, MnSO <sub>4</sub> .H <sub>2</sub> O, and copper hydroxide
Zn+	Infected plants fertilized with (ZnSO <sub>4</sub> .H <sub>2</sub> O)

## Introduction

Citrus fruits are nearly the most cultivated fruit in the world and are grown on every continent. Estimates demonstrate that there is approximately 7.5 million ha of citrus growing in the world, with an annual production of more than 120 million tons of fresh fruit (FAOSTAT 2017). However, the incidence of several diseases in the orchards (Bové and Ayres 2007; Molin et al. 2012; Singerman and Useche 2015; Stover et al. 2016) have increased production costs (Gottwald and Graham 2014; Fundecitrus 2017; Singerman 2019) and challenged the sustainability of citrus cultivation.

In this context, citrus greening or huanglongbing (HLB) is the most devastating citrus disease worldwide (Bové 2006). This disease is mostly caused by '*Candidatus Liberibacter asiaticus*' (CLas) (Bové 2006), a phloem-inhabiting bacterium that impairs the translocation of photoassimilates from shoot to root due to callose and P-protein depositions within phloem vessels, which impairs plant growth (Folimonova et al. 2009). Some typical symptoms of HLB are characterized by leaf mottling associated with accumulation of starch within the chloroplasts, which results in blockage of starch hydrolysis into maltose molecules (Stettler et al. 2009; Fan et al. 2010; Mikkelsen et al. 2005).

Furthermore, CLas is naturally transmitted by the Asian citrus psyllid (ACP) *Diaphorina citri* (Bové 2006). Fast plant-to-plant disease dissemination within and between citrus orchards, long incubation periods, presence of multiple latent infections, and intermittent transmission rates by *D. citri* make it difficult to eradicate and control the HLB (Gottwald 2010). Thereby, it is imperative to search for management practices to reduce dissemination and, consequently, losses caused by HLB.

A proposed strategy for mitigating the HLB effects is the supply of micronutrients as a complement to fertilizations usually recommended to citrus trees (Spann et al. 2011). Indeed, plants infected with HLB exhibit nutritional imbalances resulting from damages caused to the root system, which affect absorption, assimilation, transport, and utilization of water and nutrients by plants (Johnson et al. 2014). Moreover, pathogens sequestrate nutrients either at the rhizosphere or within the infected tissue, causing nutrient deficiency (Dordas 2008; Spann and Schumann 2009). Thereby, enhanced micronutrient fertilizations could be used to improve plant growth with HLB (Dordas 2008). In addition, micronutrients can trigger a systemic acquired resistance in plants, so would work as elicitors to reduce damages caused by diseases (Spann et al. 2010; Gottwald et al. 2012) and also inhibit pathogens development either by toxic effects or by regulating genes associated to pathogenicity (Dordas 2008; Fones and Preston 2012). Therefore, modifications in sap composition due to micronutrient supply could also represent a new approach to mitigating development and maintenance of CLas in the phloem (Hijaz and Killiny 2014; Killiny 2016; Hijaz et al. 2016). In fact, studies have shown that Zn can reduce bacterial infection in plants (Fones et al. 2010). Manganese is a key component for non-structural carbohydrate formation, for N metabolism, and for phenols and phytoalexin production (Datnoff et al. 2007). Finally, Cu has been used since the nineteenth century to reduce phytosanitary issues caused by microorganisms (Russell 2005). Taking all those together, it is possible that increasing micronutrient concentrations in plants through both foliar and soil fertilizations could reduce HLB damaging effects in citrus trees. Even though, the impact of different micronutrient fertilizations on CLas acquisition by adult and nymph specimens of *D. citri* is still unknown.

We highlight that HLB pathogenesis is not totally understood, no effective strategies to eliminate such a disease is available, and no resistant citrus cultivars have been identified (Hao et al. 2016). Thus, we undertook an investigation to answer the following questions: (i) Would growth of plants infected with CLas be affected by micronutrient supply? (ii) Would nutrient concentrations in leaves and sap extract of plants infected by CLas vary with different micronutrient supply? (iii) Could micronutrient supply mitigate the presence of CLas in citrus trees? (iv) Could the acquisition of CLas by adults and nymphs of *Diaphorina citri* in infected trees be possibly affected by micronutrients?

## Material and methods

### Plant material and growth conditions

This study was conducted in a greenhouse covered with transparent plastic and closed on the sides with an anti-aphid

screen. Sweet orange plants cv. Pera (*Citrus sinensis* (L.) Osbeck), 12 months old and grafted onto Rangpur lime (*C. limonia* (L.) Osbeck), were initially grown in 1.27-L plastic bags. Before transplanting to 12.0-L pots, the tips of roots (*ca.* 5 cm) of nursery trees were trimmed and immersed into 0.6 g L<sup>-1</sup> of Cu(OH)<sub>2</sub> to prevent fungal diseases and stimulate root growth. A total of 140 plants were then grown in the pots filled with pine bark-based substrate (volumetric water content = 49.1%; C:N ratio = 65.3; pH = 6.6; organic carbon = 346 g kg<sup>-1</sup>; N = 5.3 g kg<sup>-1</sup>; P = 9.7 g kg<sup>-1</sup>; K = 4.4 mg kg<sup>-1</sup>; Ca = 7.0 g kg<sup>-1</sup>; Mg = 5.4 g kg<sup>-1</sup>; S = 0.8 g kg<sup>-1</sup>; B = 49.9 mg kg<sup>-1</sup>; Fe = 3.3 g kg<sup>-1</sup>; Cu = 7.2 mg kg<sup>-1</sup>; Mn = 90.0 mg kg<sup>-1</sup>; Zn = 21.2 mg kg<sup>-1</sup>). Substrate fertilization with macronutrients were conducted during plant growth for 4 months before initiating the micronutrient treatments described below. Overall, 500 mL of nutrient solution containing 0.5 g L<sup>-1</sup> of Ca(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O, 0.6 g L<sup>-1</sup> of Mg(NO<sub>3</sub>)<sub>2</sub>, and 0.1 g L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub> were applied 10 times during such period.

### Plant bacterial infection and micronutrient treatments

At 20 days after transferring plants to the 12-L pots, they were separated into two groups and either (i) bud grafted onto opposite sides of the main stem with two buds obtained from micrografted plants (healthy, −) or (ii) bud grafted onto opposite sides of the main stem with two bud woods infected with CLas (infected plants, +) obtained from CLas source plants maintained in our laboratory. The source plants exhibited typical visual symptoms of HLB and were diagnosed positive for CLas by qPCR (“real-time quantitative PCR”) prior to the grafting. In both groups of plants, buds with lignified tissue presenting *ca.* 4-cm length were used.

At 90 days after inoculation (when leaves of the 2nd vegetative flush of growth were fully expanded), 100 infected plants (+) were separated into other groups with 20 individuals, and each group was exposed to one of the five nutrient treatment leaves and roots applied: (i) nilZnMnCu+: not fertilized with Cu, Mn, and Zn; (ii) Zn+: fertilized with zinc sulfate (ZnSO<sub>4</sub>.H<sub>2</sub>O) by both foliar nutrition (750 mg L<sup>-1</sup> of Zn) and substrate fertilization (200 mg of Zn per plant); (iii) Mn+: fertilized with manganese sulfate (MnSO<sub>4</sub>.H<sub>2</sub>O) by both foliar nutrition (500 mg L<sup>-1</sup> of Mn) and substrate fertilization (140 mg of Mn per plant); (iv) Cu+: fertilized with copper hydroxide (Cu(OH)<sub>2</sub>, Kocide® WDG BIOACTIVE, Du Pont, USA) by both foliar nutrition (800 mg L<sup>-1</sup> of Cu) and substrate fertilization (240 mg of Cu per plant); and (v) ZnMnCu+: fertilized with all three micronutrients. Likewise, 40 healthy plants (−) were separated into two groups of 20 individuals and exposed to one of the two nutrient treatments as above: (i) nilZnMnCu− or (v) ZnMnCu−. For both infected

and healthy plants, the fertilizations were conducted every 30 days for 9 months.

At the end of the experiment (270 days after initiating nutrient treatments (DAINT)), each plant of the correspondent treatment received 41 mg of Cu, 27 mg of Mn, and 40 mg of Zn *via* foliar nutrition, as well as 1.3 g of Cu, 0.9 g of Mn, and 1.3 g of Zn *via* substrate fertilization. Throughout the first, second, and third evaluation dates (90, 180, and 270 DAINT, as described below), each plant received 261, 470, and 610 mg of Cu; 160, 287, and 480 mg of Mn; and 230, 410, and 650 mg of Zn, respectively.

After initiating nutrient treatments, all plants were also fertilized with macro- and micronutrients (except Zn, Mn, and Cu) every 30 days as follows: 0.5, 0.3, and 0.7 g of N per plant as ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>), calcium nitrate [Ca(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O, and magnesium nitrate [Mg(NO<sub>3</sub>)<sub>2</sub>], respectively; 0.4 g of Ca as [Ca(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O per plant; 0.6 g of Mg as [Mg(NO<sub>3</sub>)<sub>2</sub>] per plant; 1.2 g of K as phosphate monopotassium (KH<sub>2</sub>PO<sub>4</sub>) per plant; and 0.9 g of P as (KH<sub>2</sub>PO<sub>4</sub>) per plant. In addition, 200 mL of a solution containing 6.66 mg L<sup>-1</sup> of Mg as magnesium sulfate (MgSO<sub>4</sub>), 0.05 mg L<sup>-1</sup> of B as boric acid (H<sub>3</sub>BO<sub>3</sub>), and 0.05 mg L<sup>-1</sup> of Mo as sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O) were applied to the substrate.

### Dry weights and nutrient concentration in the leaves

Of each treatment, three plants were destructively harvested at 90, 180, and 270 DAINT. Shoots (leaves + stems; LDW) and roots (RDW) were dried at 60–65 °C for 72 h and weighed ( $\pm$  0.001 g) for dry mass determination. Leaf area (LA) was measured from 30 randomly sampled leaves using an area meter device (LI-3000C, Li-Cor, Lincoln, NE, USA) at 270 DAINT. Total plant dry weight (TotalDW = LDW + RDW) and specific leaf weight (SLW = leaf dry weight/leaf area) were calculated.

The 3rd, 4th, 5th, and 6th leaves counted from the tip of flushes of growth were sampled, dried as above, and ground to pass through a 200-mesh sieve in a Wiley mill (Thomas Wiley Mini-Mill Cutting Mill, Swedesboro, NJ, USA). Macro- and micronutrient concentrations were measured after nitro-perchloric digestion of ground samples according to Bataglia et al. (1983), by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA). Data obtained for the 90, 180, and 270 DAINT were pooled and statistically analyzed (details provided below).

### Nutrient concentration in the sap extract

Sap extract collected from the youngest vegetative flushes of growth was analyzed according to Cadahía and Lucena (2005), with adaptations (Souza et al. 2012). Leaves were removed from stems, and the latter of those were cleaned with

wet-gauze and distilled water, cut into 20-cm pieces, and placed into plastic pots. Ether (98.0% v/v) was then added to the pots in volume sufficient to cover the plant material. Pots were stored in a refrigerator at 15 °C for 15 days. Thereafter, samples were transferred to a funnel in order to physically separate the sap extract from the ether. Sap was diluted 10 times in deionized water (1:9), and nutrient concentrations (P, Ca, Mg, S, B, Cu, Fe, Mn, and Zn) were determined with ICP-OES (Perkin-Elmer 5100 PC, Norwalk, CT, USA). The Kjeldahl procedure was used to estimate total N concentration, and both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were estimated through steam distillation as described in Cantarella and Trivellin (2001). Sap analysis was performed in three plants per treatment at 90, 180, and 270 DAI NT. Data were then pooled across treatments, and means were calculated.

### Spad values and chlorophyll content

Spad values were measured at 90, 180, and 270 DAI NT on the 3rd and 4th leaves counted from the tip of the youngest flushes of growth (3 plants per treatment), between 08:00 and 10:00 h. There were five Spad values averaged in the same sampled leaf, using the SPAD-502 Chlorophyll Meter (Minolta Co. Ltd., Osaka, Japan). The same leaves were then collected, and the total chlorophyll amount was evaluated according to Barnes et al. (1992). In glass vials, 50 mg of fresh leaf tissue was collected, cut into small strips, and placed with 7.0 mL of dimethyl sulfoxide. The vials were heated in a water bath at 65 °C for ca. 4 h (until tissue depigmentation). The absorbance of extracts was read in spectrophotometer (600 Plus, Femto, São Paulo, Brazil) at 646 and 663 nm, and the determination of  $\text{Chl} = (7.49 \times A_{663}) + (20.34 \times A_{646})$  was performed.

### Starch content

Starch content (measured as in leaves) was measured at 270 DAI NT using the method described by Amaral et al. (2007). We added 10 mg of dried (60–65 °C for 72 h) and ground (20 mesh) samples to an 80% ethanol solution at 80 °C for 20 min to extract soluble sugars and phenols. In order to digest the starch, 0.5 mL of  $\alpha$ -amylase solution (110 U mL<sup>-1</sup>) in MOPS buffer (10 mM, pH 6.5) and 0.5 mL of amyloglucosidase (30 U mL<sup>-1</sup>) in sodium acetate buffer (100 nM, pH 4.5) were used at 75 °C and 50 °C, respectively. Thereafter, 100  $\mu\text{L}$  of perchloric acid 0.8 M was added to precipitate the proteins and stop the reactions. Glucose in leaves was read by adding 50  $\mu\text{L}$  of digested extract to 750  $\mu\text{L}$  of Glucose Reagent PAP Liquiform (CenterLab, Brazil). Samples were submitted to a water bath at 37 °C for 15 min and read with a MultiskanGo microplates reader (Thermo Fisher Scientific Oy, Finland). A standard curve was plotted using six different concentrations of glucose.

### HLB visual symptoms and CLas in the plant by qPCR

The presence of visual symptoms of HLB in plant leaves was monitored every 30 days until the end of the study. Furthermore, cycle threshold values ( $C_T$ ) were determined at 90, 180, and 270 DAI NT as indirect measures of CLas titer in the samples. DNA extraction was performed according to the CTAB method originally described in Murray and Thompson (1980). Briefly, the petiole and midribs of two fully expanded leaves were collected, cut into small pieces, and placed in 2-mL plastic tubes (300 mg each) containing 5-mm beads and 625  $\mu\text{L}$  of buffer 1 (100 mM Tris [pH 8.0], 50 mM EDTA, and 500 mM NaCl). The plant tissue was ground in a homogenizer TissueLyzer II (Qiagen, MD, USA) at 30 Hz for 120 s, and then 725  $\mu\text{L}$  of buffer 2 (5% CTAB, 10% Sarcosyl and 10 mM B-mercaptoethanol) was added to the tube. This was held at 65 °C for 30 min and then centrifuged for 5 min at 3500 rpm. The supernatant was transferred to a new 1.5-mL microtube, followed by extraction with chloroform/isoamyl alcohol 24:1 and precipitation with isopropanol, and the total DNA was suspended in 400  $\mu\text{L}$  of 1/10TE + RNase. DNA concentration was quantified through spectrophotometry at 260/280 OD ratio using the nanodrop system (Thermo Scientific, Wilmington, NC, USA). DNA was standardized to a 100 ng  $\mu\text{L}^{-1}$  concentration.

qPCR was performed with a total volume of 13.5  $\mu\text{L}$  (3  $\mu\text{L}$  of total DNA, 6.75  $\mu\text{L}$  of 2x Maxima® Probe/Rox qPCR Master mix buffer (Fermentas, St. Leon-rot, Germany), 0.4  $\mu\text{mol L}^{-1}$  of each primer, 0.2  $\mu\text{mol L}^{-1}$  of probe (IDT Integrated DNA technology, Leuven, Belgium)), using an ABI7500 (Applied Biosystems, Foster City, CA, USA) at default condition of cycling, totaling 40 cycles. The primers and probes (FAM/Iowa Black FQ—IDT Inc., Coralville, IA) used were based on TS elongation fraction (efTs) of ‘*Ca. Liberibacter asiaticus*’ (Hong et al. 2010). All reactions used internal control primers (GAPDH gene) according to Boava et al. (2015). Results were expressed as the number of PCR cycles, in which reporter emission reaches a value higher than the threshold.  $C_T$  values are inversely proportional to bacteria concentration in the sample, and it has been shown that  $C_T$  values lower than 36 are considered ‘*Ca. Liberibacter asiaticus*’ positive (infected, +) (Ammar et al. 2011; Hilf 2011; Coletta-Filho et al. 2014; Canale et al. 2017). As the negative control, tissues of healthy plants were used, with Milli-Q autoclaved water as the mock sample.

### Bacteria acquisition by *D. citri*

*D. citri* were reared in cages (45 cm × 45 cm × 50 cm) containing *Murraya koenigii* (L.) plants (Nava et al. 2007; Alves et al. 2014). The rearing colony was maintained in a climate-controlled room (25 ± 2 °C, 60 ± 0% RH, and 14-h 75 light/10-h dark photoperiod). The insects were frequently

submitted to qPCR tests to confirm that no specimens in the colony were infected with '*Ca Liberibacter asiaticus*'.

At 270 DAI NT, uninfected *D. citri* (free of '*Ca. Liberibacter asiaticus*') were confined for 7 days on four branches (new shoots, 10–15 m long) per each of the three CLas infected plants of each nutrient treatment. Therefore, 500-mL transparent plastic cups sealed with mesh bags (four per plant) were used to confine both 60 adult specimens of *D. citri* on each plant and 60 nymphs (5th instar) specimens of *D. citri* on each plant. The confinement was performed in a laboratory, in which minimum and maximum temperatures ranged from 18.4 to 19.0 °C and from 19.4 and 22.4 °C, respectively. Seven days after confining the insects on infected plants, all adults and nymphs were separately transferred to healthy sweet orange plants (free of CLas) for 15 days in order to provide the necessary latency period for bacterial development within the insects (Canale et al. 2017). Thereafter, the insects (30 adults and 30 nymphs) were randomly collected, killed by freezing, stored in 2.0-mL Eppendorf tubes containing 70% ethanol, refrigerated in a freezer at –20 °C, and tested individually for the presence of CLas through qPCR.

DNA was extracted from the psyllids individually, according to the method described by Coletta-Filho et al. (2014). Each insect was macerated using the TissueLyser II system (Qiagen, Valencia, CA) at 25 Hz for 60 s in a 2.0-mL microtube containing 100 µL of STE buffer (10 mM Tris-HCl, 1 mM EDTA, and 25 mM NaCl) and two 3-mm beads. After the addition of 15 µL Proteinase K (200 µg mL<sup>-1</sup>), samples were incubated at 56 °C for 30 min. Total DNA was purified using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI), following the manufacturer's instructions. The final pellet was eluted to a final volume of 50 µL with elution buffer (10 mM Tris, 1 mM EDTA, 20 µg mL<sup>-1</sup> RNase) and stored at –20 °C prior to the qPCR tests. The presence of CLas in the psyllid samples was determined by qPCR using the same condition described above. Absolute acquisition efficiency was estimated as the number of qPCR-positive insects divided by the total number of insects tested. Relative acquisition efficiency was estimated as the percentage of qPCR-positive insects.

## Statistical analysis

A completely randomized design was used with seven treatments (nilZnMnCu+, Zn+, Mn+, Cu+, ZnMnCu+, nilZnMnCu–, and ZnMnCu–). Data were subjected to analysis of variance tests and the mean pairwise comparisons made using Fisher's least significant difference (LSD) post hoc tests test at 5% probability (growth related traits, Spad values, chlorophyll, and starch contents, and nutrient concentrations). In addition, orthogonal contrasts at both 5% and 1% probability were used to analyze nutrient concentration data for comparisons between groups of means in order to evaluate both main

and interaction effects (Nogueira 2004). Pearson coefficients ( $r^2$ ) were also used to correlate nutrient concentrations in both leaves and sap extract. Standard error and *P* values of Student's unpaired *t* test were calculated for qPCR data. Statistical analysis was made using the SAS (Statistical Analysis System) software.

## Results

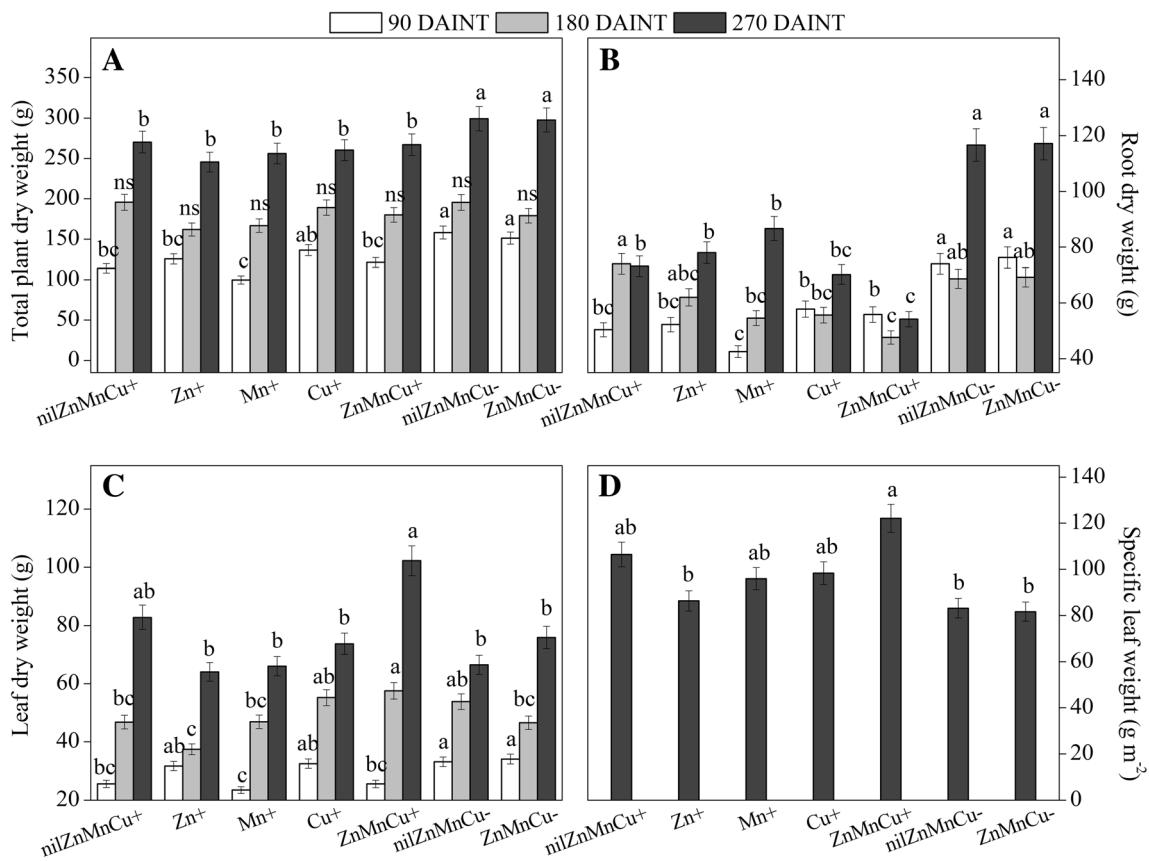
### Dry mass and nutrient concentrations in leaves and sap extract

Healthy trees (nilZnMnCu– and ZnMnCu–) exhibited higher TotalDW than those infected (+) at 90 and 270 DAI NT, while no differences were observed among treatments at 180 DAI NT (Fig. 1a). Correspondently, higher TotalDW of both (–) treatments were associated with higher RDW at 90 and 270 DAI NT, which did not happen to infected plants (+) (Fig. 1b). Overall, (–) plants exhibited RDW values *ca.* 45% higher than (+) plants.

At 90 DAI NT, trees with ZnMnCu– and nilZnMnCu– exhibited higher LDW than ZnMnCu+, Mn+, and nilZnMnCu+ (Fig. 1c). However, such differences decreased over time, so that at 180 DAI NT, LDW of nilZnMnCu– was only higher than Zn+, while no significant differences were observed between Zn+ and ZnMnCu– (Fig. 1c). At 270 DAI NT, Zn+, Mn+, Cu+, and both (–) treatments exhibited lower LDW than ZnMnCu+ (Fig. 1c). No differences in LDW were observed among (–) treatments and Zn+, Mn+, and Cu+ at 270 DAI NT, so that such treatments showed the lowest LDW values (Fig. 1c). Even though no differences were observed among treatments for leaf area (data not shown), both (–) treatments presented SLW significantly lower than ZnMnCu+, whereas Zn+, nilZnMnCu–, and ZnMnCu– exhibited the lowest SLW values, significantly lower than the SLW of ZnMnCu+ (Fig. 1d).

Orthogonal contrasts C<sub>1</sub>, C<sub>2</sub>, and C<sub>4</sub> demonstrated that N, Mg, and S concentrations in leaves differed between (+) and (–) trees (Table 1). Healthy (–) trees exhibited *N* > 30 mg kg<sup>-1</sup>, while infected (+) ones showed *N* < 28 mg kg<sup>-1</sup>. Likewise, (–) plants displayed Mg > 2.2 mg kg<sup>-1</sup> and S > 2.7 mg kg<sup>-1</sup>. Contrasts still did not show differences for Ca, P, and K in leaves (Table 1).

No differences in micronutrients in leaves were observed between (+) and (–) plants, regardless of nutrient treatment, as shown by orthogonal contrast C<sub>1</sub> (Table 2). Likewise, no differences were observed for Fe and B (Table 2). Overall, a LSD test demonstrated higher Mn, Zn, and Cu concentrations in treatments supplied with the respective micronutrients (Table 2). However, Cu+ presented Cu concentrations twice as high as ZnMnCu+, although the same amount of this micronutrient was applied in both treatments (Table 2).



**Fig. 1** Total plant dry weight (**a**), root dry weight (**b**), leaf dry weight (**c**), and specific leaf weight (**d**) of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with *Ca. Liberibacter asiaticus* (+) or healthy (-) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu =

Cu fertilization; ZnMnCu = Zn, Mn, and Cu fertilizations). Each bar represents the mean of either 3 replicates (**a–c**) or 9 replicates (**d**). Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference. DAIT means days after initiating nutrient treatments

Regarding nutrient concentrations in sap extracts, (+) plants had *ca.* 30% less total N (Nt) than (-) plants (orthogonal contrasts C<sub>1</sub> and C<sub>4</sub>, Table 3). Such difference was higher than the same observed for N concentrations in the leaves (6.7%, Table 1). Inorganic N-(NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) was *ca.* 20% of Nt (Table 3). In addition, higher mean values of NO<sub>3</sub><sup>-</sup> were observed in relation to NH<sub>4</sub><sup>+</sup> (Table 3). LSD test demonstrated that NO<sub>3</sub><sup>-</sup> concentration in the sap extract of plants was higher in treatments not fertilized with Cu (nilZnMnCu+, nilZnMnCu-, Mn+, and Zn+, Table 3). Infected trees exhibited lower Mg concentrations in the sap extract than (-) plants (orthogonal contrasts C<sub>1</sub> and C<sub>2</sub>, Table 3). Likewise, S in the sap was lower in (+) plants (orthogonal contrast C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, and C<sub>4</sub>, Table 3). Contrasts still demonstrated that trees growing with the ZnMnCu+ exhibited higher S concentrations than those with Zn+, Mn+, and Cu+ (orthogonal contrast C<sub>3</sub>, Table 3). On the other hand, orthogonal contrasts did not show differences in Ca, K, and P among nutrient treatments (Table 3).

Supply of Zn, Mn, and Cu increased micronutrient concentrations in the sap extract of trees up to sevenfold in relation to

nilZnMnCu trees (Table 4). In addition, positive correlations were observed between nutrient concentrations in the sap extract and in the leaves (Table 5). Generally, there were no differences in either Fe or B concentrations in the sap among treatments (orthogonal contrasts, Table 4). Negative correlations between Cu and both NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were observed in the sap extract (Table 5). Likewise, a negative correlation between Cu in the leaf and NO<sub>3</sub><sup>-</sup> in the sap extract also occurred (Table 5).

### Spad values, chlorophyll content, and starch content

We observed no differences among treatments for both Spad values and chlorophyll contents in this work (data not shown). On the other hand, ZnMnCu+ exhibited higher starch content than all other treatments at 270 DAIT (Fig. 2). nilZnMnCu+ also exhibited higher starch contents than Zn+, Mn+, Cu+, and both (-) treatments (Fig. 2). Moreover, no differences were observed among Zn+, Mn+, Cu+, and both non-infected treatments (nilZnMnCu- and ZnMnCu-) (Fig. 2).

**Table 1** Macronutrient concentrations in leaves of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with ‘*Ca. Liberibacter asiaticus*’ (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and

Cu fertilization). Each treatment mean represents the mean of 9 replicates (3 replicates  $\times$  3 data collections). Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference. \* and \*\* indicate significant difference at 5 and 1%, respectively

Treatment	N g kg <sup>-1</sup>	Ca	Mg	K	P	S
0 nilZnMnCu+	28 <sup>ab</sup>	19 <sup>a</sup>	1.9 <sup>bc</sup>	33 <sup>ns</sup>	1.5 <sup>ns</sup>	2.1 <sup>b</sup>
1 Zn+	28 <sup>ab</sup>	16 <sup>ab</sup>	2.4 <sup>a</sup>	31 <sup>ns</sup>	1.5 <sup>ns</sup>	2.3 <sup>b</sup>
2 Mn+	27 <sup>ab</sup>	16 <sup>ab</sup>	2.1 <sup>abc</sup>	32 <sup>ns</sup>	1.4 <sup>ns</sup>	2.3 <sup>b</sup>
3 Cu+	26 <sup>b</sup>	16 <sup>ab</sup>	1.9 <sup>bc</sup>	30 <sup>ns</sup>	1.5 <sup>ns</sup>	2.3 <sup>b</sup>
4 ZnMnCu+	26 <sup>b</sup>	14 <sup>b</sup>	1.8 <sup>c</sup>	29 <sup>ns</sup>	1.5 <sup>ns</sup>	2.2 <sup>b</sup>
5 nilZnMnCu−	30 <sup>a</sup>	19 <sup>a</sup>	2.4 <sup>a</sup>	33 <sup>ns</sup>	1.6 <sup>ns</sup>	2.7 <sup>ab</sup>
6 ZnMnCu−	30 <sup>a</sup>	17 <sup>ab</sup>	2.2 <sup>ab</sup>	33 <sup>ns</sup>	1.6 <sup>ns</sup>	3.1 <sup>a</sup>
Orthogonal contrasts						
C <sub>1</sub> 5 6 vs 0 1 2 3 4	**	ns	**	ns	ns	**
C <sub>2</sub> 5 6 vs 0 4	**	ns	**	ns	ns	**
C <sub>3</sub> 4 vs 1 2 3	ns	ns	**	ns	ns	ns
C <sub>4</sub> 6 vs 4	**	ns	**	ns	ns	ns
C <sub>5</sub> 0 vs 4	ns	ns	ns	ns	ns	ns

## Visual leaf HLB symptoms and CLas quantification in treated plants

Typical HLB symptoms characterized by blotchy leaves with green and yellow asymmetrical spots were not observed in trees, even though (+) plants exhibited corked

and yellowish central veins (Suppl. Fig. 1). However, bacteria quantifications at 90, 180, and 270 DAINT demonstrated that infections were successful at the starting point of this study once all treatments displayed  $C_T$  values ranging from 19.9 to 28.2 with no effects of nutrient treatments on  $C_T$  values (Table 6).

**Table 2** Micronutrient concentrations in leaves of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with ‘*Ca. Liberibacter asiaticus*’ (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and

Cu fertilizations). Each treatment mean represents the mean of 9 replicates (3 replicates  $\times$  3 data collections). Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference. \* and \*\* indicate significant difference at 5 and 1%, respectively

Treatments mg kg <sup>-1</sup>	Mn	Zn	Cu	Fe	B
0 nilZnMnCu+	19 <sup>b</sup>	10 <sup>b</sup>	1.6 <sup>c</sup>	117 <sup>ns</sup>	50 <sup>a</sup>
1 Zn+	18 <sup>b</sup>	29 <sup>a</sup>	0.9 <sup>c</sup>	91 <sup>ns</sup>	43 <sup>ab</sup>
2 Mn+	57 <sup>a</sup>	9 <sup>b</sup>	0.4 <sup>c</sup>	81 <sup>ns</sup>	46 <sup>ab</sup>
3 Cu+	20 <sup>b</sup>	9 <sup>b</sup>	20 <sup>a</sup>	98 <sup>ns</sup>	45 <sup>ab</sup>
4 ZnMnCu+	48 <sup>a</sup>	31 <sup>a</sup>	10 <sup>b</sup>	81 <sup>ns</sup>	40 <sup>b</sup>
5 nilZnMnCu−	22 <sup>b</sup>	10 <sup>b</sup>	0.8 <sup>c</sup>	37 <sup>ns</sup>	41 <sup>b</sup>
6 ZnMnCu−	50 <sup>a</sup>	33 <sup>a</sup>	10 <sup>b</sup>	100 <sup>ns</sup>	39 <sup>b</sup>
Orthogonal contrasts					
C <sub>1</sub> 5 6 vs 0 1 2 3 4	ns	**	ns	ns	ns
C <sub>2</sub> 5 6 vs 0 4	ns	ns	ns	ns	ns
C <sub>3</sub> 4 vs 1 2 3	**	**	ns	ns	ns
C <sub>4</sub> 6 vs 4	ns	ns	ns	ns	ns
C <sub>5</sub> 0 vs 4	ns	ns	**	ns	ns

**Table 3** Macronutrient concentrations in sap of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with ‘*Ca. Liberibacter asiaticus*’ (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and

Treatment	Nt mg L <sup>-1</sup>	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	Ca	Mg	K	P	S
0 nilZnMnCu+	1400 <sup>ab</sup>	70 <sup>ns</sup>	204 <sup>ab</sup>	452 <sup>ns</sup>	224 <sup>b</sup>	2768 <sup>bc</sup>	354 <sup>ab</sup>	71 <sup>de</sup>
1 Zn+	1100 <sup>ab</sup>	72 <sup>ns</sup>	214 <sup>a</sup>	423 <sup>ns</sup>	234 <sup>b</sup>	2561 <sup>ab</sup>	321 <sup>b</sup>	87 <sup>cde</sup>
2 Mn+	1700 <sup>a</sup>	60 <sup>ns</sup>	223 <sup>a</sup>	568 <sup>ns</sup>	285 <sup>ab</sup>	3151 <sup>a</sup>	423 <sup>a</sup>	99 <sup>bcd</sup>
3 Cu+	700 <sup>b</sup>	58 <sup>ns</sup>	141 <sup>b</sup>	452 <sup>ns</sup>	288 <sup>ab</sup>	2416 <sup>c</sup>	353 <sup>ab</sup>	57 <sup>e</sup>
4 ZnMnCu+	1100 <sup>ab</sup>	55 <sup>ns</sup>	140 <sup>b</sup>	620 <sup>ns</sup>	327 <sup>ab</sup>	2826 <sup>ab</sup>	411 <sup>a</sup>	132 <sup>ab</sup>
5 nilZnMnCu-	1700 <sup>a</sup>	60 <sup>ns</sup>	204 <sup>a</sup>	613 <sup>ns</sup>	380 <sup>a</sup>	2873 <sup>ab</sup>	371 <sup>ab</sup>	117 <sup>bc</sup>
6 ZnMnCu-	1800 <sup>a</sup>	53 <sup>ns</sup>	148 <sup>b</sup>	589 <sup>ns</sup>	328 <sup>ab</sup>	2898 <sup>ab</sup>	366 <sup>ab</sup>	162 <sup>a</sup>
Orthogonal contrast								
C <sub>1</sub> 5 6 vs 0 1 2 3 4	*	ns	ns	ns	*	ns	ns	**
C <sub>2</sub> 5 6 vs 0 4	ns	ns	ns	ns	**	ns	ns	**
C <sub>3</sub> 4 vs 1 2 3	ns	ns	ns	ns	ns	ns	ns	**
C <sub>4</sub> 6 vs 4	*	ns	ns	ns	ns	*	ns	**
C <sub>5</sub> 0 vs 4	ns	ns	*	ns	ns	ns	ns	ns

### Bacteria acquisition by *D. citri*

High or undetermined  $C_T$  values were observed in both adult and nymph specimens confined on ZnMnCu+ branches (Table 7, Supp. Table 1). Only 17% of adults (5 out of 30

Cu fertilizations). Each treatment mean represents the mean of 9 replicates (3 replicates  $\times$  3 data collections). Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference. \* and \*\* indicate significant difference at 5 and 1%, respectively

**Table 4** Micronutrient concentrations in sap of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with ‘*Ca. Liberibacter asiaticus*’ (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and

adults) acquired ‘*Ca. Liberibacter asiaticus*’ after the confinement on ZnMnCu+ branches but with a low bacteria titer in general ( $C_T = 33.65$ ). Also, all nymphs (25 individuals) fed on shoots with this treatment did not acquire CLas (0%; Table 7). On the other hand, 100% of adults and nymphs fed on Mn+-

Cu fertilizations). Each treatment mean represents the mean of 9 replicates (3 data collections  $\times$  3 replicates). Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference. \* and \*\* indicate significant difference at 5 and 1%, respectively

Treatments	Mn mg L <sup>-1</sup>	Zn	Cu	Fe	B
0 nilZnMnCu+	0.7 <sup>b</sup>	1.8 <sup>b</sup>	0.8 <sup>c</sup>	0.7 <sup>abc</sup>	0.7 <sup>abc</sup>
1 Zn+	0.3 <sup>b</sup>	7.2 <sup>a</sup>	0.1 <sup>d</sup>	0.8 <sup>ab</sup>	0.8 <sup>ab</sup>
2 Mn+	2.9 <sup>a</sup>	1.5 <sup>b</sup>	0.1 <sup>d</sup>	0.5 <sup>c</sup>	0.5 <sup>c</sup>
3 Cu+	0.2 <sup>b</sup>	1.3 <sup>b</sup>	1.6 <sup>b</sup>	0.4 <sup>c</sup>	0.4 <sup>c</sup>
4 ZnMnCu+	2.9 <sup>a</sup>	6.7 <sup>a</sup>	2.7 <sup>a</sup>	0.5 <sup>bc</sup>	0.5 <sup>bc</sup>
5 nilZnMnCu-	0.6 <sup>b</sup>	1.5 <sup>b</sup>	0.5 <sup>cd</sup>	0.8 <sup>ab</sup>	0.8 <sup>ab</sup>
6 ZnMnCu-	3.7 <sup>a</sup>	6.9 <sup>a</sup>	2.7 <sup>a</sup>	0.5 <sup>abc</sup>	0.5 <sup>abc</sup>
Orthogonal contrasts					
C <sub>1</sub> 5 6 vs 0 1 2 3 4	**	**	ns	ns	ns
C <sub>2</sub> 5 6 vs 0 4	ns	**	ns	ns	ns
C <sub>3</sub> 4 vs 1 2 3	**	ns	*	ns	ns
C <sub>4</sub> 6 vs 4	ns	ns	ns	ns	ns
C <sub>5</sub> 0 vs 4	**	**	**	ns	*

**Table 5** Pearson correlation matrix ( $r^2$ ) for nutrient concentrations in sap and leaves of 'Pera' orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with '*Ca. Liberibacter asiaticus*' (+) or healthy (-) and exposed to different

nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and Cu fertilizations)

	N <sub>t<sub>sap</sub></sub>	NH <sub>4</sub> <sup>+</sup> <sub>sap</sub>	NO <sub>3</sub> <sup>-</sup> <sub>sap</sub>	P <sub>sap</sub>	Cu <sub>sap</sub>	Mn <sub>sap</sub>	Zn <sub>sap</sub>	N <sub>leaf</sub>	P <sub>leaf</sub>	Cu <sub>leaf</sub>	Mn <sub>leaf</sub>	Zn <sub>leaf</sub>
N <sub>t<sub>sap</sub></sub>	1.00											
Prob. < r	—											
NH <sub>4</sub> <sup>+</sup> <sub>sap</sub>	-0.19	1.00										
Prob. < r	0.6833	—										
NO <sub>3</sub> <sup>-</sup> <sub>sap</sub>	0.40	0.72	1.00									
Prob. < r	0.3751	0.0678	—									
P <sub>sap</sub>	0.35	-0.59	-0.08	1.00								
Prob. < r	0.4479	0.1663	0.8678	—								
Cu <sub>sap</sub>	-0.12	-0.76	-0.93	0.20	1.00							
Prob. < r	0.7962	0.0493	0.0023	0.6602	—							
Mn <sub>sap</sub>	0.53	-0.69	-0.30	0.68	0.55	1.00						
Prob. < r	0.2245	0.0894	0.5062	0.0897	0.1976	—						
Zn <sub>sap</sub>	-0.04	-0.11	-0.32	-0.18	0.47	0.41	1.00					
Prob. < r	0.9361	0.8219	0.4881	0.7015	0.2920	0.3674	—					
N <sub>leaf</sub>	0.73	<0.01	0.27	-0.31	-0.13	0.05	0.07	1.00				
Prob. < r	0.0615	0.9949	0.5539	0.5001	0.7833	0.9149	0.8876	—				
P <sub>leaf</sub>	0.26	-0.29	-0.35	-0.39	0.40	-0.03	0.25	0.72	1.00			
Prob. < r	0.5698	0.5319	0.4447	0.3864	0.3722	0.9409	0.5814	0.0658	—			
Cu <sub>leaf</sub>	-0.60	-0.58	-0.91	-0.03	0.71	0.06	0.07	-0.41	0.14	1.00		
Prob. < r	0.1527	0.1698	0.0045	0.9476	0.073	0.8913	0.8873	0.3566	0.7665	—		
Mn <sub>leaf</sub>	0.47	-0.66	-0.19	0.82	0.39	0.96	0.24	-0.10	-0.26	0.03	1.00	
Prob. < r	0.2818	0.1054	0.6801	0.0235	0.3865	0.0008	0.6033	0.8353	0.5780	0.9478	—	
Zn <sub>leaf</sub>	-0.01	-0.24	-0.43	-0.12	0.58	0.48	0.99	0.09	0.32	0.16	0.30	1.00
Prob. < r	0.9907	0.6095	0.3401	0.7925	0.1707	0.2748	<0.0001	0.8500	0.4771	0.7392	0.5108	—

treated shoots tested positive for CLas, although  $C_T$  values obtained in the insects (nymphs and adults) were higher than in the shoots (Table 7, Supp. Table 1). Insects confined on branches treated with nilZnMnCu+, Zn+, and Cu+ also acquired CLas, exhibiting acquisition efficiency varying from 53 to 73% for the adults, and from 46 to 61% for nymphs (Table 7, Supp. Table 1).

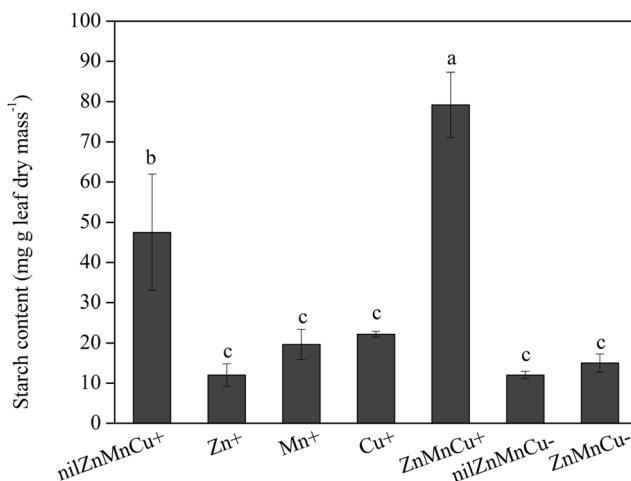
## Discussion

The presence of CLas in all (+) treatments impaired plant biomass production, since lower RDW and, therefore, lower TotalDW were observed, especially at 90 and 270 DAIANT (Fig. 1 a and b). Indeed, as the phloem-inhabiting bacteria, CLas affects the translocation of photoassimilates from shoot to roots, impairs root growth and, consequently, overall plant biomass production (Folimonova et al. 2009). Moreover, CLas also colonizes the root system, contributing to root decline (Graham 2012), as observed in this work (Fig. 1b). Since only mild visual symptoms of HLB (as described in Belasque

et al. (2009)) were observed in shoots (Suppl. Fig. 1), the negative effects of the infection on root growth (Fig. 1b) support the fact that when symptoms of HLB are seen in the shoots, damages to the root system have already developed (Stansly et al. 2013). Nonetheless, no differences were observed among treatments in TotalDW at 180 DAIANT (Fig. 1a), and the RDW of (-) treatments were only higher than ZnMnCu+ at the same evaluation periods (Fig. 1b). Since evaluations at 180 DAIANT occurred after the wintertime (October), it is likely that reduced growth rates caused by low air temperatures (ca. 11–24 °C) throughout the season (Sentelhas 2005) contributed to the lack of significant responses in both TotalDW and RDW (Fig. 1a).

LDW data demonstrated significant effects of both bacterial infection and micronutrient treatments on plants over time. Although (-) plants exhibited higher LDW at both 90 DAIANT, this trend changed 270 DAIANT, so ZnMnCu+ showed higher LDW than Zn+, Mn+, Cu+, and both (-) treatments by the end of the experiment (Fig. 1c). These results correlated with higher starch accumulation within the chloroplasts of plants grown with ZnMnCu+ and in response to the infection with

CLas (Fig. 2). Indeed, one of the main consequences of HLB infection is the accumulation of starch in chloroplasts, likely because of beta-amylases and/or isoamylase enzyme deactivation, which blocks starch hydrolysis into maltose molecules (Mikkelsen et al. 2005; Stettler et al. 2009; Fan et al. 2010). Thereby, starch concentration can be up to 20 times higher in infected leaves, which can increase LDW by 50% (Etxeberria et al. 2009; Spann and Schumann 2009). In this study, starch content was *ca.* 5.8 times higher in ZnMnCu+ than in the (−) treatments (Fig. 2). Thus, as HBL severity progressed over time, starch was accumulated within the chloroplasts of ZnMnCu+ plants (Fig. 2), leading to higher LDW values (Fig. 1c) and, therefore, higher SLW at the end of the experiment (Fig. 1d). nilZnMnCu+ also exhibited higher starch levels than Zn+, Mn+, Cu+, and both (−) treatments (Fig. 2). Since Zn+, Mn+, and Cu+ treatments did not differ in starch content, LDW, and SLW in relation to both non-infected treatments (nilZnMnCu− and ZnMnCu−) at 270 DAIT (Fig. 1 c and d; Fig. 2), we can assume that individual supply with Zn, Mn, and Cu (treatments Zn+, Mn+, and Cu+, respectively) can be important alternatives in order to mitigate the deleterious effects of CLas on starch metabolism in citrus trees. Nonetheless, the negative effects of the interaction among Zn, Mn, and Cu must be better understood since we did not observe differences in chlorophyll content among treatments (data not shown), whereas it has been reported that HLB causes chloroplast disruption due to starch accumulation, as observed in the present work (Fig. 2), which has been



**Fig. 2** Starch content ( $\text{mg glucose} \cdot \text{g leaf dry mass}^{-1}$ ) of 'Pera' orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with '*Ca. Liberibacter asiaticus*' (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and Cu fertilizations) at 270 days after initiating nutrient treatments. Each bar represents the mean of either 3 replicates. Means followed by different letters differ statistically by LSD test at 5% probability. ns indicates no statistical difference

**Table 6** Cycle threshold ( $C_T$ ) values of infected 'Pera' orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with '*Ca. Liberibacter asiaticus*' (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu, Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and Cu fertilizations). Each treatment mean represents the mean of 3 replicates. ns indicates no statistical difference. \* and \*\* indicate significant difference at 5 and 1%, respectively. DAIT means days after initiating nutrient treatments

Treatments	$C_T$ values		
	90 DAIT	180 DAIT	270 DAIT
0 nilZnMnCu+	23.2 <sup>ns</sup>	—	25.1 <sup>ns</sup>
1 Zn+	26.1 <sup>ns</sup>	28.2 <sup>ns</sup>	22.9 <sup>ns</sup>
2 Mn+	24.4 <sup>ns</sup>	20.3 <sup>ns</sup>	22.1 <sup>ns</sup>
3 Cu+	25.7 <sup>ns</sup>	20.2 <sup>ns</sup>	21.2 <sup>ns</sup>
4 ZnMnCu+	22.9 <sup>ns</sup>	19.9 <sup>ns</sup>	19.9 <sup>ns</sup>

associated with phloem dysfunction and/or reduced enzymic hydrolysis of starch (Lemoine et al. 2013; Zeeman et al. 2010).

Negative effects of CLas on root system (Fig. 1b) clearly affected nutrient absorption and/or transport to the shoot (Table 1). Overall, infected plants exhibited lower N, Mg, and S concentrations in leaves (Table 1). Despite such lower N concentration, no significant differences were observed among treatments for Spad values and chlorophyll content (data not shown). Moreover, nutrient treatments (Zn+, Mn+, Cu+, and ZnMnCu+) did not affect N and Mg concentrations in the present work (Table 1). Improved N and Mg concentrations in leaves were expected in plants treated with Zn, Mn, and/or Cu since such micronutrients are important co-factors of superoxide dismutase (SOD), a key enzyme responsible for alleviating oxidative damages to the chlorophylls caused by increased concentrations of reactive oxygen species (ROS) in stressed plants (Gupta et al. 1993; Alscher et al. 2002; Hippler et al. 2018). Since chlorophyll molecules represent a substantial N and Mg investment in plants (Zhang et al. 2008; Sage 2013), less damages to both N and Mg concentrations in leaves of Mn+, Zn+, Cu+, and ZnMnCu+ plants in relation to nilZnMnCu+ were expected. However, this trend was not observed in this work (Table 1). In addition, Cu+ showed Cu concentrations twice as high as ZnMnCu+, although the same amount of this micronutrient was applied in both treatments (Table 2). This response was likely due to the high competition among Mn, Zn, and Cu for the same cell membrane absorption sites in ZnMnCu+ plants (Kumar et al. 2009). Indeed, Hippler et al. (2016) demonstrated that the absorption of micronutrients, such as Zn, Mn, and Cu, can be negatively affected by high concentration of metals in the nutrient solution, which can damage the synthesis and/or activity of

**Table 7**  $C_T$  values (shoot and *D. citri*) and acquisition efficiency of adult and nymph specimens of *D. citri* after confinement on vegetative flushes of ‘Pera’ orange trees (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime rootstock (*C. limonia* Osbeck) either infected with ‘Ca. Liberibacter asiaticus’ (+) or healthy (−) and exposed to different nutrient treatments (nilZnMnCu = plants were not fertilized with Cu,

Mn, and Zn; Zn = Zn fertilization; Mn = Mn fertilization; Cu = Cu fertilization; ZnMnCu = Zn, Mn, and Cu fertilizations). Each  $C_T$  value of shoot represents the mean  $\pm$  SE of 6 replicates. Each  $C_T$  value of *D. citri* represents the mean  $\pm$  SE of 7–15 replicates. Und indicates that the targeted DNA was undetermined through qPCR analysis

Treatment	$C_T$ values—Shoots		$C_T$ values— <i>D. citri</i>		Acquisition efficiency (%)	
	Adult	Nymph	Adult	Nymph	Adult	Nymph
nilZnMnCu+	27.1 $\pm$ 1.1	27.2 $\pm$ 1.3	29.9 $\pm$ 0.6	26.3 $\pm$ 0.8	60	50
Zn+	25.6 $\pm$ 1.2	22.3 $\pm$ 1.2	30.8 $\pm$ 0.6	28.2 $\pm$ 0.8	73	61
Mn+	21.6 $\pm$ 1.3	21.3 $\pm$ 1.1	24.4 $\pm$ 0.4	25.9 $\pm$ 0.3	100	100
Cu+	24.2 $\pm$ 1.0	24.6 $\pm$ 1.6	26.8 $\pm$ 0.7	29.0 $\pm$ 1.1	53	46
ZnMnCu+	27.8 $\pm$ 0.6	27.3 $\pm$ 1.2	33.6 $\pm$ 0.4	und	17	0
ZnMnCu−	und	und	und	und	0	0

specific membrane transporters (Hall and Williams 2003), the activity of H+-ATPases, and the proton pumping capacity of the plasma membrane (Kabala and Burzynski 2012).

Lower N content was also observed in the sap extract of (+) plants (Table 3). The higher concentration of  $\text{NO}_3^-$  in relation to  $\text{NH}_4^+$  observed in (+) plants (Table 3) corroborates with the results obtained in a previous study conducted with healthy adult citrus trees (Souza et al. 2012). In addition,  $\text{NO}_3^-$  concentration in the sap extract was higher in treatments that were not supplied with Cu (nilZnMnCu+, nilZnMnCu−, Mn+ and Zn+, Table 3), and close negative correlations between Cu and both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were observed in the sap (Table 5). We point out that high Cu concentrations impair the assimilation of nitrate by inhibiting specific enzymatic processes, such as nitrate reductase activity (Xiong et al. 2006). Thus, the discrepancy between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations in the sap was rather caused by different absorption rates of such sources of N in (+) plants than by any impairment in the  $\text{NO}_3^-$  reduction process.

Reduced Mg concentration in the sap extract of (+) plants was likely caused by callose and P-protein depositions, which likely restricted the transport of this nutrient within the plant (Folimonova and Achor 2010; Koh et al. 2012). Orthogonal contrast C<sub>6</sub> demonstrated that ZnMnCu+ reduced S concentrations in the sap in relation to ZnMnCu−, supported by the fact that S correlates positively with N concentrations, which was also lower in ZnMnCu+ in relation to ZnMnCu− (orthogonal contrast C<sub>6</sub>, Table 3). Both nutrients are the main constituents of amino acids (Haneklaus et al. 2006). In addition, our work does not support the association between P deficiency and HLB incidence proposed by Zhao et al. (2013), since no differences between (+) and (−) plants were observed for this nutrient in both leaves and sap extract (Tables 1 and 3).

We did not observe the effects of different nutrient treatments tested on bacteria titer, based on  $C_T$  values, although with the tendency of bacteria concentration in plants

increasing from 90 to 270 DAIANT (Table 6). Curiously, Zambon et al. (2019) observed reduced CLas bacterial populations in trees that received higher doses of Mn. However, we need to point out that Mn concentrations in leaves in our study were ca. 33% lower than the values obtained by Zambon et al. (2019). Nonetheless, we also observed that ZnMnCu+ treatment somehow blocked the acquisition of CLas by both nymphs (100%) and adults (83%), with low bacteria titer in the adults that acquired the bacteria (Table 7). Indeed, qPCR analysis only provides a quantification of the target DNA (Hong et al. 2010), so there is no discrimination between viable and unviable cells of CLas. Therefore, it is not surprising that we observed no effects of the applied treatments on  $C_T$  values of leaves (Table 6), whereas we found promising results on  $C_T$  values in *D. citri* specimens (Table 7). Such results show that ZnMnCu+ reduced CLas population, so although the DNA of the bacteria was present at high concentrations in the phloem, it was likely composed of mostly unviable cells. Thereby, the insects acquired reduced concentrations of viable cells from ZnMnCu+ plants, so most adults and specially nymphs of *D. citri* tested negative for HLB after the adequate latency period provided for the development of the bacteria in the insects (Canale et al. 2017). Furthermore, it has been reported that the sap composition is of great importance to CLas growth (Hijaz and Killiny 2014; Killiny 2016; Hijaz et al. 2016). Thus, we suggest that the changes observed in nutrient concentration in the sap extract (such as Cu, Mn, Zn, and S—Tables 3 and 4) in response to ZnMnCu+ treatment could reduce the viability of CLas cells. Considering that infected plants are sources of inoculum for future infections (Belasque et al. 2010), our results are promising, especially because the probability of CLas acquisition by nymphs reared on (+) plants is higher than the probability of acquisition by adults (Pelz-Stelinski et al. 2010; Ammar et al. 2016), and when CLas is acquired by nymphs, the emerging adults will be more infective (Xu et al. 1988; Inoue et al. 2009; Pelz-Stelinski et al.

2010; Grafton-Cardwell et al. 2013; Ammar et al. 2016; Canale et al. 2017). Thus, reduced bacteria acquisition by insects, especially by nymph specimens due to ZnMnCu<sup>+</sup> treatment, can represent an advantage, reducing HLB spread within and between citrus orchards. We point out that such a result was only observed when micronutrients (Mn, Zn, and Cu) were applied in combination (ZnMnCu<sup>+</sup>), since Mn<sup>+</sup>, Zn<sup>+</sup>, and Cu<sup>+</sup> treatments did not reduce CLas acquisition efficiency by vectors, especially for the Mn<sup>+</sup> treatment that reached 100% acquisition (Table 7). However, given the negative effects of HLB on starch metabolism (Fig. 2) of ZnMnCu<sup>+</sup>, as well as the positive effects of individual fertilizations with Zn, Mn, and Cu on starch accumulation in leaves of (+) plants, further studies are required in order to find a balance between plant growth/physiology and CLas effects.

In conclusion, this study demonstrates the following: (i) CLas causes damages to biomass production regardless of nutrient treatments, especially in the root system, supporting the fact that when symptoms of HLB are noticed in the shoot, damages in the root system are severe. Moreover, the effects of HLB on LDW and SLW change as the disease develops due to starch accumulation in the chloroplasts. However, individual fertilizations with Zn, Mn, or Cu can mitigate such deleterious effects of HLB on starch metabolism; (ii) overall, HLB changes nutrient concentrations in both leaves and sap extract of plants, regardless of micronutrient treatments; (iii) although micronutrient treatments do not change  $C_7$  values of plants, (iv) fertilizations with micronutrients applied in combination (Mn, Zn, and Cu—ZnMnCu) can reduce the acquisition of '*Ca. Liberibacter asiaticus*' by adults and, especially, by nymphs of *D. citri*.

**Authors' contribution statement** DMJ, HDCF, JAQ, JRS, and RMB planned, designed, and discussed experimental data; DMJ, HDC, and JRS conducted data analyses, prepared the original manuscript draft, and revised and edited the final version; FVA executed experimental work, conducted data analyses, and discussed experimental data.

**Funding information** São Paulo Research Foundation (FAPESP, grants #2015/13572-8 and #2018/14893-0) and the Fund for Citrus Protection (Fundecitrus) supported the project development. The National Council for Scientific and Technological Development (CNPq) granted D.M.J. fellowship.

## Compliance with ethical standards

**Conflict of interest** The authors declare that there are no conflicts of interest.

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