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Interaction of Huanglongbing and Foliar Applications of Copper on Growth and Nutrient Acquisition of *Citrus sinensis* cv. Valencia

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***Abstract*. The following study was conducted in 2016 and 2017 to determine the impact of frequent foliar copper (Cu) applications on Huanglongbing (HLB)-affected *Citrus sinensis* cv. Valencia orange. The experiment was conducted in a psyllid-free greenhouse with HLB-positive and non-HLB control trees grown in an Immokalee fine sand soil. The trees were well-maintained to promote health. Cu was applied to the foliage at 0x, 0.5x, 1x, and 2x the commercially recommended rates, which were 0, 46, 92, and 184 mM, respectively, with applications made 3x in both 2016 and 2017. The impact of HLB and Cu treatments on leaf and root Cu concentrations, vegetative growth, *Candidatus* Liberibacter asiaticus (*C*Lasiaticus) genome copy number, and acquisition of other essential nutrients were determined. HLB caused the roots to acidify the soil more than non-HLB controls, which promoted Cu availability and promoted greater Cu concentrations in leaves and roots. HLB and Cu application treatments suppressed leaf area and total root length observable in rhizotron tubes such that, by the end of the experiment, leaf, stem, root, and whole-plant dry weights were reduced. HLB reduced foliar concentrations of calcium (Ca), magnesium (Mg), manganese (Mn), zinc (Zn) and possibly iron (Fe), but HLB did not affect root concentrations of these same essential nutrients. Cu application treatments did not affect leaf or root concentrations of other essential nutrients except foliar concentration of Fe, which may have been suppressed. Foliar applications of Cu are used to suppress *Xanthomonas citri* ssp. *citri* (*Xcc*) the causal agent of citrus canker, and the frequency of its use may need to be reconsidered in commercial groves.**

HLB is the most serious disease of citrus where it has become endemic, including Florida (Bové, 2006; da Graça et al., 2016; Gottwald, 2010). HLB in Florida is associated with the phloem-limited bacteria *C*Lasiaticus, which has a massive impact on the plant's physiology, growth, development, and productivity, causing whole-plant decline (Ebel, 2017). There is currently no technology that can suppress significantly and economically the bacteria in commercial citrus groves. The bacteria are vectored by the Asian citrus psyllid (*Diaphorina citri* Kuwayama), and the inability to suppress the vector's population adequately, coupled with its ability to increase exponentially, has led to infection of more than 80% of trees across the state (Gottwald, 2010). Once inoculated, the causal agent is apparently capable of reproducing and circulating throughout the tree such that it is permanently affected by HLB (Ebel, 2017). The severe decline observed in commercial groves is a function of repeated inoculation of the bacteria by the psyllid vector and secondary stressors. The impact of HLB on tree growth and development as well as the tree's increased sensitivity to secondary stressors is forcing commercial grove managers in Florida to reevaluate the entire spectrum of horticultural management practices with the twin goals of suppressing the psyllid vector population and minimizing all secondary stressors.

One potential secondary stressor of HLB-affected citrus trees may arise from the frequent applications of Cu to suppress citrus canker caused by *Xcc*. Citrus canker was found in Florida in 1996 and has since become endemic throughout the state (Bouffard, 2006). Canker is another very serious disease of citrus that can cause defoliation, stem dieback, and fruit abscission (Gottwald and Graham, 2000). Foliar applications of Cu are a popular method for suppressing *Xcc* as a result of their low cost. Because the life cycle of *Xcc* is 7 to 21 d (Brunings and Gabriel, 2003; Ebel and Kumar, 2012), Cu applications as frequent as every 3 weeks have been used to suppress canker in severely affected groves. The overuse of Cu promotes high concentrations of Cu in soil that can reduce yields (Bakshi et al., 2013; Behlau et al., 2010; Fan et al., 2011). It is not known how high-frequency Cu applications on HLB-affected trees will impact tree growth, development, and productivity.

The following study was conducted to determine how frequent Cu applications may impact *Citrus sinensis* cv. Valencia affected by HLB. Because it is impossible to conduct studies with trees not infected with *C*Lasiaticus under commercial conditions, this study was conducted under greenhouse conditions where psyllid vectors could be excluded so that non-HLB control trees would remain uninfected. Foliar applications of Cu were conducted in a manner that somewhat simulated commercial grove conditions, although the trees used here were not infected with *Xcc*. The interaction of HLB and Cu treatments on Cu concentrations, vegetative growth, *C*Lasiaticus genome copy number, and nutrient acquisition were evaluated.

Materials and Methods

Plant culture and HLB treatments. The experiment was conducted in a psyllid-free greenhouse at the University of Florida, Southwest Florida Research and Education Center near Immokalee, FL (lat. 26.42° N, long. 81.42° W) from 2016 to 2017. The plants used were 7-year old *Citrus sinensis* (L.) Osbeck cv. Valencia on Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) rootstock. One-year-old trees about 1 m high were obtained from a commercial nursery in 2009, planted in 10-L pots, and double budded with buds highly infected with *C*Lasiaticus, as reported previously (Handique et al., 2012). Before the experiment was initiated, the trees were well maintained in a psyllid-free greenhouse with daily irrigation, fertilization applications according to commercial recommendations, and suppression of insect pests as needed. In April 2016, 24 trees affected by HLB and 24 trees unaffected by HLB (control trees) were transplanted into 110-L pots using Immokalee fine sand soil (sandy, siliceous, hyperthermic Arenic Alaquods), with the roots separated to encourage their exploration into the new soil. The trees were allowed to become established for 3 months before Cu treatments were initiated. The trees were ≈1.5 m in height when the experiment was initiated. Fruit were removed at the start of the experiment and after fruit set in 2017.

The HLB-affected trees were tested on 15 Feb. 2016 using real time–polymerase chain reaction according to Li et al. (2006). The cycle time (Ct) of HLB-affected trees averaged 24.9, which indicates presence of the bacterial genome because the values were less than the threshold of 32. The trees exhibited mild HLB symptoms, including earlier shoot growth and bloom, veinal chlorosis, interveinal chlorosis, whole-leaf chlorosis, retarded leaf and shoot growth, dull cuticle, and slightly reduced growth. Trees were segregated based on leaf area such that the average leaf area per tree was similar for HLB-affected and non-HLB control trees and across Cu treatments to remove bias in tree

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size across all treatments when the experiment was initiated. Total leaf area was determined by counting total leaves per plant, measuring leaf area of 10 randomly selected leaves per tree using a portable leaf area meter (LI-3000A; LI-COR, Lincoln, NE), and multiplying the two quantities.

The trees were irrigated daily (until water dripped from the bottom of the pots) using microjet sprinklers that wetted most of the soil surface. The trees were fertilized with 20–2–20 NPK with 35 g/pot of 0.74% sulfur, 1.1% Mg, 0.1% Fe, 0.05% Mn, 0.05% Zn, 0.025% Cu, and 0.025% boron (B) (Peat-Lite Low Phos Special; Peters Professional, Allentown, PA) every 2 to 5 weeks from July 2016 through Aug. 2017. The trees were also given foliar applications of 0.3% phosphorus (P), 1.2% Ca, 3.6% Mg, Fe, 5.0% Mn, 1.1% Zn, 0.003% Cu, and 2.7% B micronutrients (special formulation, Peters Professional) at 0.8 g/tree on 19 Oct. 2016 and 2.1 g/tree on 1 Nov. 2016. Soil pH was measured in May 2016 and Jan. 2017 by collecting soil samples from 0 to 15 cm and 15 to 30 cm from the soil surface and then pooling the samples for each tree. A subsample of 20 cm^3 soil was placed in a 90-mL cup to which 40 mL water was added. The water–soil solution was stirred, allowed to equilibrate for 30 min, and the pH was measured (model AR15; Fisher Scientific, Hampton, NH).

Cu treatments. Cu treatments were 0x, 0.5x, 1.0x, and 2.0x the commercially recommended rate applied to the foliage of trees on 19 July, 11 Aug., and 30 Aug. in 2016; and 2 May, 6 June, and 26 July in 2017. The rates corresponded to 0, 46, 92, and 184 mM, respectively, using $Cu(OH)_2$, with the recommended rates as given on the product label (Kocide 2000; I.E. DuPont Canada Co., Mississauga, Ontario) with 2.5 L solution applied per tree. Four days after every treatment date, the foliage of each tree was rinsed with 2 L water (to simulate rainfall) to remove residual Cu from the foliage and move it onto the soil surface. This procedure somewhat mimic commercial conditions that exist during the rainy season in Florida, although under natural conditions precipitation is usually more frequent and of longer duration than that used in this study.

Vegetative growth. Measurement of total leaf area as described earlier was determined every 2 to 4 months.

Total observable root length was determined using clear rhizotron access tubes that were 52 cm long with a 64-mm inner tube diameter. The tube was plugged at the bottom. The tubes were inserted into a hole augured vertically (90°) into the soil and 15 cm from the trunk. The tube spanned the distance from the bottom of the pot to the soil surface. Images were taken using a digital camera (model CI-600 In-Situ Root Imager; CID-Bioscience, Camas, WA) that allowed collection of 360° images with a dimension of 21.59 cm × 19.56 cm at 600 DPI. Roots were identified and analyzed using digital imaging software (Root Snap CI-690, version 1.3.2.25; CID-Bioscience). Before sampling on each sampling date, the scanner was calibrated according to the manufacturer's instructions.

In Dec. 2017, the trees were sampled destructively with leaves, stems, and roots separated; dried at 60 °C to a constant weight; and dry weights were determined.

Foliar, root, and soil nutrient analysis. Leaf and root samples were collected using the procedures of Obreza and Morgan (2008); nutrient concentration was determined using standard analytical methods (Hanlon et al., 1997; Jones and Case, 1990; Plank, 1992). Leaf samples of 10 recently mature, fully expanded leaves were collected randomly from each tree and washed with laboratory detergent and 0.2 M HCl to remove residues. Root samples were collected by removing three 15-cm deep-soil cores using a 1.3-cm auger and rinsing the roots with tap and then distilled water. The process produced about 0.5 g roots after drying. Leaf and root samples were dried for 72 h at 60 °C. When the tissues reached a constant weight, they were ground in a mill (model 5K907K; Dayton Electric Mfg., Co., Niles, IL) until all tissue passed through a 60-mesh sieve and was mixed thoroughly. Tissue nitrogen (N) concentration (measured as a percentage) was determined using an NA2500 C/N Analyzer (Thermoquest CE Instruments LTD, Wigan, UK). Other essential nutrient concentrations were determined using a dry ash combustion digestion method (Anderson and Henderson, 1988). A 1.5-g sample of dried ground leaf material was weighed and dry-ashed at 500 °C for 16 h (Hanlon et al., 1997). The ash was equilibrated with 15 mL 0.5 M HCl at room temperature for 0.5 h. The solution was decanted into 15-mL plastic disposable tubes and placed in a refrigerator at ≤4 °C (Plank, 1992) until analyses by inductively coupled plasma atomic emission spectrometry (ICP-AES) (OPTIMA 7000DV; Perkin-Elmer, Billerica, MA) according to Munter et al. (1984). Tissue nutrient concentrations were compared with recommended levels for Florida citrus (Obreza and Morgan, 2008; Obreza et al., 1999).

Soil nutrient analysis was conducted by removing 2.5 g of dry soil and placing it into 50-mL centrifuge tubes with 25 mL of Mehlich-3 extracting solution (Mehlich, 1984). Tubes were covered and shaken for 5 min then filtered through Whatman no. 42 filter paper. Soil essential nutrient concentrations were determined by ICP-AES.

Experimental design and statistical analysis. This study was conducted as a two HLB treatment (HLB and control)-by-four Cu application treatment (0x, 0.5x, 1.0x, and 2.0x, which correspond to 0, 46, 92, and 184 mM, respectively) factorial, completely randomized design. There were six replications per treatment, although some data were collected on only three reps per tree where noted. All data were analyzed using the Statistical Analysis System (SAS for Windows, ver. 9.4; SAS Institute Inc., Cary, NC). Data analyzed over time (months) were analyzed using Proc Mixed with time (month) included as a repeated measure. Proc GLM was used for analyses in which repeated measures were not a factor or when there were too many likelihood evaluations for the Proc Mixed model. Regression equations were determined using Proc REG.

Results and Discussion

Cu in leaves, soil, and roots. There was a significant HLB × Cu treatment × months after Cu treatment interaction (P > F < 0.01) for Cu concentration of leaves (Table 1). Increasing the concentration of Cu led to greater foliar Cu concentrations for both HLB and control trees (Fig. 1A). Foliar Cu declined when Cu applications were ended (e.g., from Oct. 2016 to Apr. 2017), most likely via extrusion that occurs when symplastic Cu concentrations reach excessive levels (Marschner, 1995). The HLB × Cu treatment interaction was also significant

Table 1. Results (*P* > F) of the Proc Mixed (with MAFT) and Proc GLM (without MAFT) models on *in planta* Cu concentrations, vegetative growth, and soil pH of Huanglongbing (HLB)-affected 'Valencia' trees treated with multiple foliar applications of Cu and with dependent variables measured over time.

Model variables and interactions	Cu (mg/kg dry wt)			Leaf area (m^2/plant)	Root length (cm)	Dry wt at end of expt. (kg dry wt)				Soil pH
	Leaf	Soil	Root			Leaf	Stem	Root	Plant	
					P > F					
HLB	0.75	0.25	0.41	0.06	<0.01	**<0.01**	<0.01	**<0.01**	**<0.01**	0.96
Cu	<0.01	**<0.01**	<0.01	<0.01	<0.01	**<0.01**	0.05	**<0.01**	**<0.01**	0.27
HLB × Cu	**<0.01**	0.27	**0.14**	**<0.01**	0.30	0.88	**0.04**	0.55	0.38	0.52
MAFT	<0.01	<0.01	<0.01	<0.01	0.10	—	—	—	—	<0.01
HLB × MAFT	0.90	—	0.70	<0.01	**0.02**	—	—	—	—	**<0.01**
Cu × MAFT	<0.01	—	**<0.01**	<0.01	**0.02**	—	—	—	—	0.81
HLB × Cu × MAFT	**<0.01**	—	**0.20**	**0.03**	0.32	—	—	—	—	0.20

Bold values are referenced in the text.

MAFT = months after foliar treatment with Cu (n = 6).

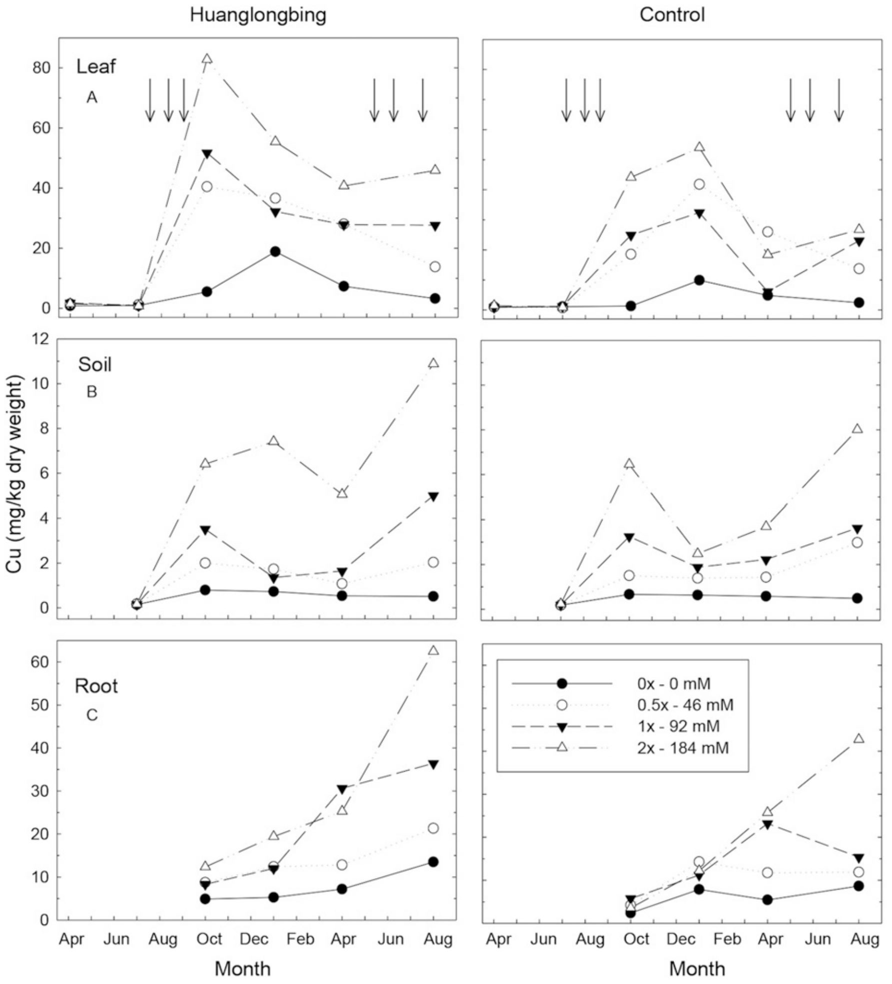

Fig. 1. Interaction of Huanglongbing and foliar applications of copper (Cu) at 0x, 0.5x, 1x, and 2x the recommended rates on Cu concentrations of mature leaves (**A**), soil (**B**), and roots (**C**) of *Citrus sinensis* cv. Valencia (n = 6). Vertical arrows indicate dates of Cu foliar treatments. Symbols and line designations for Cu treatments are located in the legend in the lower right graph.

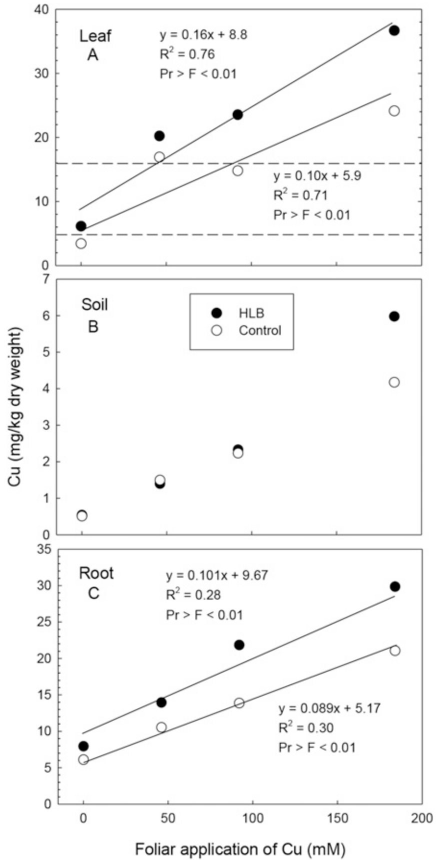

Fig. 2. Interaction of Huanglongbing (HLB) and foliar applications of copper (Cu) on leaf (**A**), soil (**B**), and root (**C**) Cu concentrations of *Citrus sinensis* cv. Valencia (n = 6). The area within the horizontal dashed lines represents the range of foliar Cu concentration considered sufficient (5–16 mg/kg dry weight) (Obreza and Morgan, 2008). Symbols for HLB treatments are in the legend in the middle graph. Data used in the regression analyses were averaged across all sampling dates after the first Cu treatment was applied for each replication (tree); however, only overall means are shown in the graph.

($P > F < 0.01$), with greater Cu rates allowing for greater accumulation of Cu in HLB-affected leaves than in control leaves, indicating a synergistic response (Fig. 2A). The foliar applications of Cu increased leaf Cu concentrations above the upper limit considered sufficient for citrus (16 mg/kg dry weight) at lower rates of Cu applied for the HLB-affected trees than the non-HLB controls. The impact of HLB on Cu uptake has not been defined clearly in the literature, with some studies indicating suppression (Nwugo et al., 2013a, 2013b; Spann and Schumann, 2009), some indicating no effect (Handique et al., 2012; Masaoka et al., 2011; Tian et al., 2014), and, in a recent study, an increase (Hamido et al., 2017), although the Cu foliar analysis in that study was not published (Morgan, personal communication).

Rinsing leaves with water after Cu applications moved excess Cu to the soil, with soil concentrations as high as 11 mg/kg dry weight (Fig. 1B). Soil Cu concentration decreased between foliar applications from Oct. 2016 to April 2017 most likely through uptake by roots and leaching. There were too many likelihood evaluations for soil Cu concentrations to be analyzed using a Proc Mixed model analysis, so a Proc GLM analysis was conducted instead, with data blocked by months after foliar treatment with Cu (MAFT) (Table 1). There was no HLB effect on soil Cu, but soil Cu increased with greater Cu application rates ($P > F < 0.01$), as shown in Fig. 2B.

It would be expected that greater Cu in soil would promote uptake of Cu by roots, and this did occur, as indicated by the significant Cu treatment × MAFT interaction (Table 1: $P > F < 0.01$). Whether there was a preferential uptake of Cu by HLB-affected roots is less clear than it was for leaves (Fig. 1C). The HLB × Cu treatment × MAFT interaction in Cu concentration of roots was only significant at the $P > F = 0.20$ level, and the HLB × Cu treatment interaction was only significant at $P > F = 0.14$ (Table 1), both of which do not suggest an effect of HLB on root Cu concentration. However, when root Cu concentrations were regressed against foliar application concentrations, there was a trend for HLB-affected roots to have greater Cu concentrations, with $P > F < 0.01$ for both regressions (Fig. 2C). The greater acquisition and/or retention of root Cu with increasing Cu application rates indicate at least an additive and possibly a synergistic response for HLB-affected roots. The Cu concentration of roots at the 1x rate was 18.9 and 13.4 mg Cu/kg dry weight for the HLB-affected and non-HLB roots, respectively, which represented a 42% increase caused by HLB. The low R^2 values for both regressions (0.28 and 0.30) indicate high plant-to-plant variation. Unlike leaves, which excreted excess Cu, roots accumulated Cu throughout the study, and an absolute limit—assuming one exists—was never reached, as indicated by no asymptote having occurred (Fig. 1C).

Impact of foliar Cu treatments on CLasiaticus genome copy number. To determine the impact of Cu treatments on the *C*Lasiaticus genome copy number of HLB-affected plants, Ct values taken Oct. 2016, which was after the first three Cu treatments were applied, were compared with those

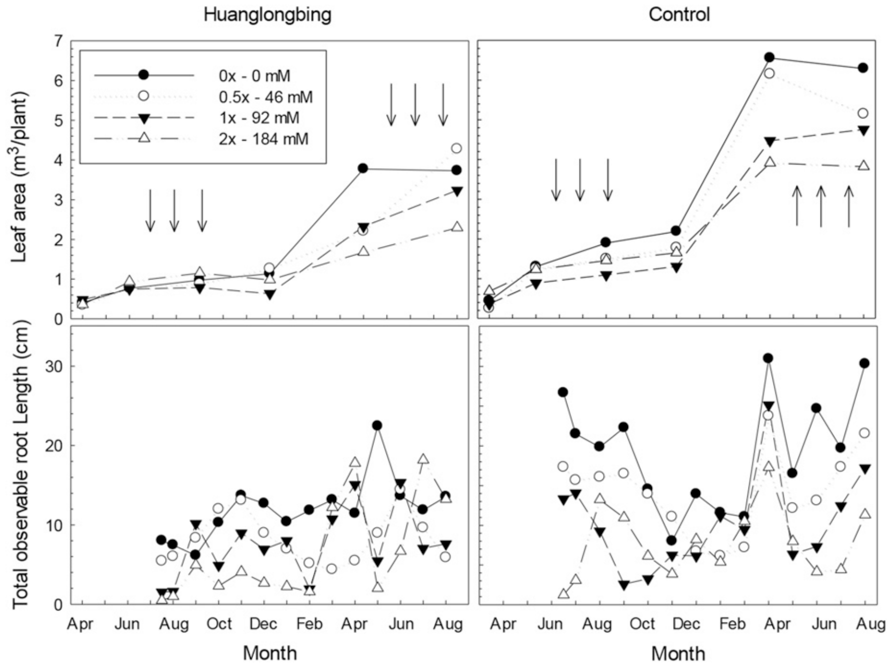

Fig. 3. Change in leaf area and total root length visible in the rhizotron tubes over time of *Citrus sinensis* cv. Valencia affected by Huanglongbing and foliar applications of copper (Cu) at 0x, 0.5x, 1x, and 2x the recommended rates (n = 3). Vertical arrows indicate dates of Cu foliar treatments. Symbols and lines for Cu treatments are in the legend in the upper left graph.

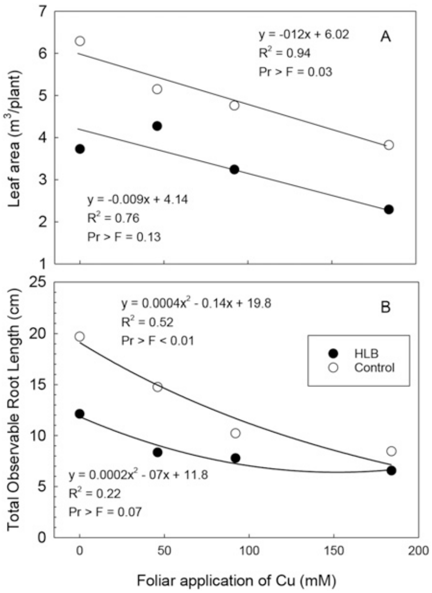

Fig. 4. Leaf area in Aug. 2017 (**A**) and total root length visible (**B**) in the rhizotron tubes and averaged across all sampling dates of *Citrus sinensis* cv. Valencia as affected by Huanglongbing (HLB) and foliar applications of copper (Cu) (n = 3). Symbols for HLB treatments are in the legend in the lower graph. Data used in the regression analyses were averaged across all sampling dates after the first Cu treatment was applied for each replication (tree); however, only overall means are shown in the graph.

taken Feb. 2016, before the experiment was initiated, by determining the Cu treatment × MAFT interaction (analysis not shown). This analysis tested the change in Ct values to determine whether Cu affected proliferation of the bacterial genome copy number. The Cu treatment × MAFT interaction was not significant (P > F = 0.96), indicating that the change in genome copy number was unaffected by Cu treatment. The average Ct values for HLB-affected plants were 24.9 in Feb. 2016 and 29.4 in Oct. 2016.

*C*Lasiaticus and *Xcc* are both Gram-negative bacteria, and it is reasonable to consider that Cu would inhibit both pathogens. However, *Xcc* is limited to the apoplast. It is deposited on the exterior of leaves during rain events and it uses its flagella to move through stomatal apertures and into substomatal chambers (Gottwald and Graham, 1992; Koizumi and Kuhara, 1982; Stall et al., 1982), where it attaches to cell walls to begin the pathogenesis process (Brunings and Gabriel, 2003). When Cu is applied to the foliage, it moves via diffusion into the leaf through the apoplast, where it comes into direct contact with the bacteria. *C*Lasiaticus, on the other hand, resides in the symplast of phloem sieve tubes. The symplast limits Cu concentrations to within a narrow range by reducing uptake and/or pumping excess into the apoplast, where it diffuses to the leaf surface and is removed by precipitation (Marschner, 1995). Most of the Cu applied to foliage does not come into direct contact with *C*Lasiaticus, and thus it is not surprising that the foliar applications of Cu in the current study did not affect the *C*Lasiaticus genome copy numbers as measured by the Ct values of the leaves.

Vegetative growth. Although the HLB-affected and control trees began the study with similar leaf area, there was a significant HLB × Cu × MAFT interaction (Table 1; P > F = 0.03), with HLB and greater Cu treatments suppressing growth of the canopy's leaf area (Fig. 3). Although the HLB × Cu treatment interaction when averaged across all sampling dates was also significant (P > F < 0.01), the interaction was not strong at the end of the experiment (Fig. 4A). Total leaf area in Aug. 2017 declined across Cu treatments for both HLB-affected and control plants, with only a slight difference in slopes. Leaf area averaged 32% less for HLB-affected leaves than controls across all Cu treatments. These data indicate that the impact of HLB and Cu treatments on leaf area were additive.

Total observable root length demonstrated significant HLB × MAFT (P > F = 0.02) and Cu × MAFT (P > F = 0.02) interactions (Table 1). The greater variation in total observable root length across time (Fig. 3) is typical of root growth of plants, which for citrus varies in part based on stage of growth flushes (Bevington and Castle, 1985) and application of essential nutrients (Marschner, 1995). To simplify understanding the impact of HLB and Cu treatment on root growth, data were averaged across all dates for each tree and subjected to polynomial analysis, with quadratic regressions giving better fits (greater R^2 values) than linear regressions (Fig. 4B). Total root length of the HLB-affected roots was 40% less than the controls for the 0x Cu treatment; however, increasing concentrations of applied Cu depressed total observable root length more so for the controls than the HLB-affected plants, such that they were similar at the greatest Cu treatment.

By the end of the experiment, total leaf, stem, root, and whole-plant dry weights were reduced by both HLB and Cu treatments. The HLB × Cu treatment interactions were not significantly different for total leaf (P > F = 0.88), root (P > F = 0.55), and whole-plant (P > F = 0.38) dry weights; however, the HLB and Cu treatment main effect means were all significant at P > F < 0.01 (Table 1). For stem dry weights, there was a significant HLB × Cu treatment interaction (P > F = 0.04). All dry weights were suppressed by HLB and Cu treatments, including the 0.5x Cu treatment (Fig. 5). The HLB and Cu treatment suppression of growth was additive for leaf, root, and total plant dry weight as a result of the lack of significant HLB × Cu treatment interactions and the similarity in slopes.

Soil pH. Soil pH did not differ between HLB (pH = 6.71) and controls (pH = 6.67) before the first Cu treatments were applied. The HLB × MAFT interaction was significant (Table 1; P > F < 0.01) so that by Jan. 2017, soil pH of soil containing HLB-affected trees was 5.96, whereas the soil pH of the controls was 6.36, which represents a 0.40 difference. Cu treatments did not impact soil pH. Low soil pH increases Cu availability, which in turn promotes uptake by plant roots (Harter, 1983; Sims, 1986; Smith, 1994)— a fact on which Cu application recommendations of

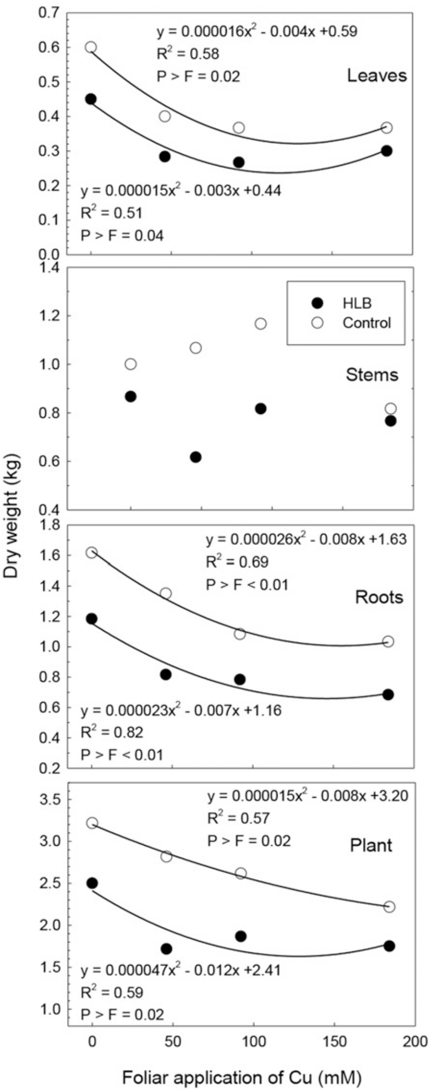

Fig. 5. Interaction of Huanglongbing (HLB) and foliar applications of copper (Cu) on leaf, stem, root and whole-plant dry weights of *Citrus sinensis* cv. Valencia at the end of the experiment (n = 3). Symbols for HLB treatments are in the legend in the second graph. Data used in the regression analyses were averaged across all sampling dates after the first Cu treatment was applied for each replication (tree); however, only overall means are shown in the graph.

Table 2. Results of the Proc Mixed model ($P > F$) on leaf concentrations of essential nutrients of Huanglongbing (HLB)-affected 'Valencia' trees treated with multiple foliar applications of copper (Cu) and with dependent variables measured over time.

Model variables and interactions	Macronutrients and boron				Divalent cations				
	N	K	P	B	Ca	Mg	Fe	Mn	Zn
				$P > F$					
Model									
HLB	0.75	0.41	0.59	0.30	**0.02**	**0.04**	**0.18**	**<0.01**	**<0.01**
Cu	0.42	0.49	0.44	0.75	0.19	0.65	**0.16**	0.15	0.36
HLB × Cu	0.39	0.66	0.57	0.37	0.87	0.93	0.15	0.35	0.25
MAFT	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Main effect means		(%)		(mg/kg)	(%)			(mg/kg)	
HLB treatment									
Control	3.3	2.6	0.14	215	2.2 a	0.29 a	97	147 a	65 a
HLB	3.4	2.6	0.14	204	2.0 b	0.26 b	83	99 b	50 b
Cu treatment									
0×	3.3	2.6	0.15	213	2.2	0.28	103	141	77
0.5×	3.3	2.7	0.14	202	2.2	0.27	98	103	78
1×	3.4	2.6	0.14	216	2.0	0.29	85	126	77
2×	3.4	2.5	0.13	206	2.1	0.27	74	122	66
Recommended levels	2.5–2.7	0.7–1.1	0.12–0.16	36–100	3.0–4.9	0.3–0.49	60–120	25–100	25–100

From Obreza and Morgan (2008).

Bold values are referenced in the text. Letters within columns indicate significant differences among main effect means when the $P > F \leq 0.05$.

N = nitrogen; K = potassium; P = phosphorus; B = boron; Ca = calcium; Mg = magnesium; Fe = iron; Mn = manganese; Zn = zinc; MAFT = months after foliar treatment with Cu (n = 6).

citrus in Florida are based (Obreza and Morgan, 2008). Thus, the greater acidification of soil by HLB-affected roots increased Cu availability, which promoted uptake and greater Cu concentrations in leaves and roots compared with the non-HLB controls.

Essential nutrients: Macronutrients N, P, and K, and B. The inconsistent timing in fertilization of essential nutrients other than Cu makes analyzing foliar and root concentrations as a repeated measure inappropriate. Therefore, the data were analyzed using the GLM procedure with the data blocked by time (MAFT) to remove variation caused by measurements for the different sampling dates. There was no effect of HLB or Cu on N, P, K, and B on foliar (Table 2) or root (analyses not shown) concentrations. None of the foliar concentrations of these essential nutrients were deficient.

Divalent cations Ca, Mg, Fe, Mn, and Zn. HLB has been shown to affect the acquisition and distribution of essential nutrients, with suppression in foliar concentrations of Ca, Mg, Fe, Mn, and Zn being the most often reported (Aubert, 1979; Handique et al., 2012; Koen and Langenegger, 1970; Masaoka et al., 2011; Nwugo et al., 2013a, 2013b; Pustika et al., 2008; Spann and Schumann, 2009; Tian et al., 2014). Foliar concentrations of most of these nutrients were suppressed by HLB ($P > F < 0.05$) in the current study (Table 2), although the analysis for Fe was not strong ($P > F = 0.18$). HLB did not affect concentrations of any divalent cations in the roots (analyses not shown), indicating that although acquisition by roots appeared not to be affected by HLB, their transport to the shoots was reduced.

Acquisition of essential nutrients by plants is a function of several factors including, in part, the 1) relative external concentrations of essential nutrients that have similar valences, 2) closeness of their ionic radii, and 3) soil chemistry, which affects hydration and ionic interactions with the surface of soil particles and other ions and polar molecules (Marschner, 1995). The average soil concentrations of Ca, Mg, Fe, Mn, and Zn across all sampling dates were 478, 59, 111, 17, and 7 mg/kg dry weight, respectively, whereas Cu was as much as 60 mg/kg dry weight in the soil for the greatest Cu treatment. The nonhydrated forms of the divalent cations Mg, Fe, Mn, Zn, and Cu have ionic radii that are within 10% of each other (Shannon, 1976; Wells, 1984), and therefore it would be expected that they would tend to compete for uptake by plant roots (Marschner, 1995). Competition for uptake among divalent cations has been shown in citrus (Martínez-Cuenca et al., 2013; Reuther et al., 1952; Smith and Specht, 1953; Srivastava and Singh, 2005); however, a survey of the literature for citrus revealed that the only divalent cation with an uptake affected by high Cu in soil is Fe (Smith and Specht, 1953). In the current study, the high soil Cu may have suppressed uptake of Fe for HLB and non-HLB control trees ($P > F = 0.16$), where foliar Fe concentrations decreased numerically from 103 to 74 mg Fe/kg dry weight with increasing Cu applied. A more intensive study on the interaction in uptake by the various divalent cations for HLB-affected roots should be conducted to determine the conditions that promote deficiencies in commercial groves.

Conclusions

HLB-affected roots acidified the soil more than non-HLB controls, which increased Cu availability and promoted uptake and greater Cu concentrations in roots and leaves. Excessive levels of Cu were reached in HLB-affected plants at lower foliar application treatments of Cu, which contributed to suppressed growth. As has been shown in other studies, HLB suppressed foliar concentrations of the divalent cations Ca, Mg, Mn, Zn, and possibly Fe. Cu did not affect uptake and foliar or root concentrations of most other essential nutrients except possibly Fe.

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