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Effects of Iron Rates on Growth and Development of Young Huanglongbing-affected Citrus Trees in Florida

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Abstract. **Essential nutrients for citrus [‘Bingo’ (*Citrus reticulata*, Blanco)] production are important for different functions, including photosynthesis, resistance to disease, and productivity. During the past 15 to 20 years, citrus production in Florida has significantly declined as a result of the devastating citrus greening disease also called huanglongbing (HLB). Therefore, a greenhouse study was conducted for 2 years, starting in 2018, at the University of Florida/Institute of Food and Agricultural Sciences Citrus Research and Education Center in Florida to evaluate the effect of varying rates of iron on the growth and development of 2-year-old HLB-affected ‘Bingo’ (*Citrus reticulata,* Blanco) trees on Kuharske citrange rootstock. Four treatments were used in a randomized complete block (HLB status) design with seven single tree replicates for each treatment. The treatments applied were 0.0 (control), 5.6 (standard fertilization, lx), 11.2 (2x), and 22.4 (4x) kg·ha^{-1} iron on HLB-affected and healthy (non-HLB) citrus trees. Data including trunk diameter, tree height, and leaf samples were collected, processed, and analyzed at 3-month intervals for 2 years. At the end of the second year, trees were destructively sampled and processed as above-ground and below-ground biomass. Tree heights were different among iron rates of HLB-affected trees ($P < 0.001$); however, they were similar for non-HLB trees for both years. Higher average trunk diameters ($P < 0.001$) were observed for HLB-affected trees that received the 2x rate compared with the 1x rate and the control. In 2019, non-HLB trees showed 13% to 40% higher iron concentrations in leaves than HLB-affected trees. However, leaf iron concentrations were comparable for HLB-affected and non-HLB trees in 2020. Above-ground biomass for HLB-affected trees had between 33% and 44% more biomass ($P < 0.01$) than below-ground biomass for the corresponding iron fertilization. Iron accumulation correlated positively with all studied nutrients in the above-ground parts for both HLB-affected and non-HLB trees. A 95% confidence interval at which total biomass was nearly maximum corresponded to an iron rate of 9.6 to 11.8 kg·ha^{-1}, which was close to the 2x rate. Therefore, soil iron application using the aforementioned rates may be appropriate for better growth and development of young HLB-affected trees.**

Iron (Fe) deficiency may decrease chlorophyll concentrations of leaves, thus leading to the inability of plants to assimilate CO_2 and use light adequately and affecting productivity. As with chlorophyll, everal other components of the electron transport system involved in photosynthesis are decreased because of Fe deficiency (Marschner et al., 1996; Pushnik and Miller, 1989). Furthermore, Fe is directly involved in the production of reactive oxygen species (ROS) through its role in the Fenton reaction (Bolwell and Wojtaszek, 1997). It is important to understand the role of Fe because its prolonged deficiency can be catastrophic to citrus tree development.

This study was conducted to provide a more comprehensive understanding of how much Fe needs to be applied to HLB-affected [‘Bingo’ (*Citrus reticulata,* Blanco)] trees in Florida, considering its role in photosynthesis and biological defense of the citrus tree. There has been little to no research of the response of citrus mandarins to improved Fe nutrition in endemic HLB conditions. As a micronutrient, Fe is required by most plants in small quantities and is well-known for its use in metabolic processes such as deoxyribonucleic acid (DNA) synthesis, photosynthesis, and respiration (Rout and Sahoo, 2015). It is also a constituent of many electron carriers and enzymes important to plant metabolism (Hell and Stephan, 2003). The presence of Fe in Fe-containing heme proteins makes its levels in plants critical in the electron transfer chain including cytochromes (Hochmuth, 2017).

Plant absorbs Fe by an active process; therefore, energy is provided by the plant to take in Fe (Hochmuth, 2017). The process is dependent on the ability of the plant to reduce Fe^{3+} to Fe^{2+} and remove it from the complex or chelating compound (Hochmuth, 2017). With HLB, one concern is that affected trees may not be able to exert enough energy to absorb required Fe because of the loss of more than 40% of the root system caused by bacterial infection (Graham et al., 2013). Therefore, it has become necessary to maintain adequate amounts of Fe in the root zones of trees.

Furthermore, Fe toxicity can be a serious problem for the growth and development of citrus. Even though this condition is mostly observed in waterlogged soils, it could occur with heavy rainfall and excessive irrigation. Price and Hendry (1991) reported that iron-catalyzed formation of oxygen free radicals in the chloroplasts can cause Fe toxicity under dryland conditions. Because of the complexity of Fe, its recommendation must be based on research through experimentation.

Previous studies linked HLB with tree nutrient deficiencies such as micronutrient deficiencies involving Fe (Atta et al., 2020; Gottwald et al., 2012; Hamido et al., 2019), which have occurred while the trees received standard fertilizer recommendations. This suggests that the level of Fe in HLB-affected trees may not be sufficient considering the standard recommendation of healthy non-HLB trees. Because no cure has been found for HLB, there is an urgent need to investigate required Fe rates for HLB-affected trees to avoid citrus production losses related to Fe deficiency. This may be important for the HLB-affected trees because the appearance of Fe deficiency symptoms suggests a negative impact on citrus growth and production.

During this study, we used a mandarin variety called Bingo that was released in 2015. Since its introduction, citrus growers have shown interest in Bingo because it is an early maturing variety (October/November), has a deep orange color, is easy to peel, is seedless, and has an excellent flavor (Chaires, 2016; Gmitter, 2019). To the best of our knowledge, this is the first time a variable rate nutrition study of this citrus variety has been performed. It may provide a better understanding of which Fe level is appropriate to avoid Fe deficiency in young HLB-affected citrus trees.

The objective of this study was to evaluate the effects of varying rates of Fe on the growth and development of 2-year-old HLB-affected [graft-inoculated with the pathogen that causes HLB *Candidatus* Liberibacter asiaticus (C*Las*) 'Bingo' (*Citrus reticulata,* Blanco)] trees on Kuharske citrange rootstock (*Citrus sinensis* × *Poncirus trifoliata*) under greenhouse conditions.

Materials and Methods

This study was conducted for 2 years in a greenhouse located at the University of Florida/Institute of Food and Agricultural Sciences Citrus Research and Education Center (lat. $28^{\circ}5'37''$ N, long. $81^{\circ}43'30''$ W). The purpose of this study was to evaluate the effects of increasing Fe rates on 2-year-old HLB-affected and non-HLB 'Bingo' mandarin (*Citrus reticulata,* Blanco) trees on Kuharske citrange rootstock [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata*].

The experimental unit was a 2-year-old citrus tree in an 8.7-L pot filled with one part of perlite to four parts of soil by volume. There were seven experimental units for each treatment; therefore, there were 28 experimental units for each HLB-affected and non-HLB tree. The soil used for this study was a Candler fine sand consisting of 96% fine sand and classified as hyperthermic, uncoated, Typic Quartzipsamment with less than 2% organic matter. This soil is typically found in the central Florida ridge orange groves and is excessively well-drained. The topsoil was collected and sieved with a 2-mm mesh to remove unwanted materials and obtain uniform growth medium. A composite soil was collected and dried at 100 °C; then, it was sent to Waters Agricultural Laboratories, Inc. (Camilla, GA) to determine elemental concentrations of selected nutrients, including Fe.

Half (50%), corresponding to 28 trees (seven for each treatment), was graft-inoculated with the HLB-causal pathogen *CLas* before treatment application, and the remaining half was used as HLB-free (healthy trees) controls, similar to a study by Kwakye et al. (2022). Initial measurements of tree heights, trunk diameters, and leaf Fe concentrations were performed after trees were confirmed positive for *CLas* inoculation using a quantitative polymerase chain reaction analysis (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) as described by Vashisth and Livingston (2019) Water was supplied to each pot by drip irrigation with pressure-compensating drip emitters at a rate of 2 $L\cdot h^{-1}$ (MaxiJet, Dundee, FL). Trees were provided with all essential nutrients, except for Fe, according to the University of Florida/Institute of Food and Agricultural Sciences nutritional guide for citrus production (Morgan and Kadyampakeni, 2020).

Treatment application. In the greenhouse, trees were separated into two groups based on the HLB status: HLB-affected and non-HLB-affected (healthy) trees. In each group, there were 28 experimental units with seven replicate pots for each of four Fe rates applied as ferrous sulfate heptahydrate ($FeSO_4.7H_2O$, 20% water-soluble Fe). The treatment description is presented in Table 1. The fertilizers were applied and mixed by hand into the upper 5 cm of soil four times per year. As indicated, all other essential nutrients were applied uniformly to all experimental units (pots) 1 week before Fe application using a similar application method.

Tree height, trunk diameter and leaf Fe concentration. The initial tree heights and trunk diameters were measured for each experimental unit before treatment application. Subsequently, a height-measuring stick (model 807396; SOKKIA Corporation, Olathe, KS) and a digital caliper were used to measure tree heights and diameters, respectively, every 3 months at the same location on the trunk until the end of the study. The digital caliper recorded the trunk diameter in the north–south (NS) and east–west (EW) directions of the tree. Tree height growth and trunk diameter growth were estimated by subtracting the initial application measurement before treatment from subsequent measurements.

Leaf samples were collected from each experimental unit before a treatment was applied, and then every 3 months afterward. Ten fully expanded 4- to 6-month-old leaves were sampled; immature, abnormal-appearing, and dead leaves were avoided during leaf collection. Leaves were hand-washed with deionized water immediately after sampling to remove surface contamination. Leaves were then placed into paper bags and dried in a ventilated oven at 65 °C for 72 h (Morgan and Kadyampakeni, 2020). After drying, leaves were ground with a Wiley Mill (PSAW-180; Thomas Scientific, Swedesboro, NJ) equipped with a 20-mesh sieve. Then, leaf tissue samples were sent to Waters Agricultural Laboratories, Inc. (Camilla, GA) to determine elemental concentrations of selected nutrients, including Fe, using inductively coupled plasma atomic emission spectroscopy (PerkinElmer, Akron, OH).

Table 1. Treatment structure description of the evaluation of varying rates of iron (Fe) on huanglongbing (HLB)-affected Bingo (*Citrus reticulata*) trees in Florida.

Rate[z] (kg·ha^{-1} Fe)	HLB status[y]		Replication
	HLB	Non-HLB	
0.0	Control	Control	7
5.6	1x	1x	7
11.2	2x	2x	7
22.4	4x	4x	7

[z] Rates used are as follows: control = 0.0 kg·ha^{-1} Fe; 1x = 5.6 kg·ha^{-1} Fe; 2x = 11.2 kg·ha^{-1} Fe; and 4x = 22.4 kg·ha^{-1} Fe. 1x represents the standard recommendation by the University of Florida/Institute of Food and Agricultural Sciences.

[y] HLB trees inoculated with Candidatus *Liberibacter asiaticus* (CLas). Non-HLB trees were not inoculated with CLas.

Soil Fe relationship with other essential nutrients. To collect the soil samples, a clean, dry soil probe (Unplated, 7/8" × 21"; Regular Soil Probe; AMS Inc., Jackson, MS) was vertically inserted into the soil within the pot at 15 cm away from the tree trunk to a depth of 15 cm from the surface of the soil. Then, the soil core was removed and placed into a paper bag. The latter was performed for each experimental unit at the beginning and end of the study. The paper bags with samples were dried in a ventilated oven at 100 °C for 24 h. Then, the soil samples were sent to Waters Agricultural Laboratories (Camilla, GA) to determine elemental concentrations of selected nutrients, including Fe, using inductively coupled plasma atomic emission spectroscopy (PerkinElmer). Soil Fe data were correlated with soil phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), boron (B), zinc (Zn), copper (Cu), and manganese (Mn) to identify the relationship between them to explain what happens to the micronutrients when increasing soil Fe.

CLas DNA concentration in leaf tissue. Symptomatic fully expanded leaves with attached petiole were collected during Oct. 2018 and May 2020. The midrib was separated from the leaf, cut into 5-mm-long pieces, and immediately stored in 2 mL centrifuge tubes at −20 °C until DNA extraction. Then, DNA extraction was performed with DNeasy Plant Kits (Qiagen Valencia, CA) by following the manufacturer's step-by-step instruction. Samples were analyzed by quantitative polymerase chain reaction (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) to retrieve the cycle threshold (Ct) values. A polymerase chain reaction in real time was used to quantify the amount of target DNA present in the sample (Ananthakrishnan et al., 2013). For the primers and probe, a working stock solution of 100 uL of 20 uM was made by diluting a 100-uM solution, which is usually purchased from a manufacturer. Then, a method described by Ananthakrishnan et al. (2013) was followed to set-up and run the plate to obtain results.

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Tree dry biomass. At the end of the second year, three out of seven trees were randomly selected from each treatment and separated into roots, trunk, branches, and leaves. Roots were further subdivided into roots with diameter less than 1 mm (fine roots), roots with diameters between 1 and 3 mm (medium roots), and roots with diameters more than 3 mm (large roots). Branches were divided into twigs (diameter <3 mm) and true branches (diameter >3 mm). Fresh mass and dry mass were determined and followed by a nutrient analysis. Fresh plant material was dried to a constant weight in a ventilated oven at 65 °C between 72 h for leaves and 12 d for trunks. The nutrient analysis was used to calculate the amount of Fe (mg) that accumulated in each plant part.

Fe accumulation in tree parts and other nutrients. Accumulation of Fe in each tissue part was calculated by multiplying the dry weight of the tree part by its mean Fe concentration. The information generated from this calculation was used in a regression analysis of dry weight Fe accumulation in HLB and non-HLB trees as a function of the Fe application rate.

*Data analysis.*Linear mixed model methodology with repeated measures as implemented in SAS PROC MIXED (SAS/STAT 15.1; SAS Institute, Cary, NC) (SAS Institute Inc, 2018) was used to analyze all response data. A second-order polynomial was used to model tree height, trunk diameter, and leaf Fe concentration, along with the Fe rate and time (3 month period) according to Eq. [1]. The Fe rate was nested within the time × HLB status combination.

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon \qquad [1]$$

In Eq. [1], y is the response variable being measured, β_0 is the intercept, the coefficients β_1 and β_2 are the linear effect parameters and quadratic effect parameters, respectively, and x represents the model predictors (HLB status, Fe rate, and year), and ε is the error term for predicting y.

The repeated measures aspect was modeled through an R-side model using the REPEATED statement during the aforementioned procedure, either the unstructured or a first-order autoregressive structure with heterogeneous variances provided the best fit based on Akaike's information criterion corrected for the small sample size. Visual inspection of residuals (Kozak and Piepho, 2018) indicated no violations of the underlying assumptions. Means within an Fe × HLB status × time × response combination followed by the same letter are not significantly different at $\alpha = 0.05$. Linear and nonlinear regression analyses were performed to relate Fe accumulation in various tree parts as a function of the Fe application. Pearson's correlation coefficient (r) was used to compare the relationship between soil and tissue Fe accumulation with other essential nutrients.

Results

Tree heights, trunk diameters, and leaf Fe concentrations. For both years, tree heights were different among Fe rates used for HLB-affected trees ($P < 0.001$); however, no significant difference was observed between the Fe rates for non-HLB trees (Table 2). For HLB-affected trees, the 1x and 4x rates showed higher averages of height relative to the 2x rate and the control in 2019 (Table 2). However, in 2020, the 2x rate increased height ($P < 0.01$) relative to the 1x rate and the control. We observed higher average trunk diameters ($P < 0.001$) for HLB-affected trees that received the 2x rate compared with the 1x rate and the control (Table 2). Generally, for both years, we observed a linear increase in the trunk diameter from the control to the 2x rate; thereafter, the diameter decreased until the 4x rate used for HLB-affected trees. However, for non-HLB trees, treatments with increasing Fe rates showed smaller trunk diameters compared with the control during both years.

Regarding leaf Fe concentrations, a linear response was observed with increasing Fe rates used for HLB-affected and non-HLB trees. In 2019, non-HLB trees had higher leaf Fe concentrations than HLB-affected trees, with 13% more for the lower rates and up to 40% more for the higher rate. However, leaf Fe concentrations were comparable among HLB-affected and non-HLB trees in 2020 (Table 2). For the year 2020, we observed no significant difference in the leaf Fe concentration between the standard rate and 2x rate, and trees that received the 4x rate generally had higher Fe contents irrespective of the HLB status. Leaf Fe concentrations for the corresponding Fe rate were compared between HLB and non-HLB trees. We observed that non-HLB trees that were subjected to 5.6 and 22.4 kg·ha^{-1} Fe accumulated 33% greater leaf Fe than HLB-affected trees subjected to the corresponding Fe rates (Table 3). This indicated that when the same Fe rate is used, healthy trees accumulate greater Fe concentrations in the leaf tissue than HLB-affected trees.

Soil Fe relationship with other essential nutrients. Soil Fe concentrations correlated negatively with P, K, Mg, B, Zn, Cu, and Mn and positively with Ca in HLB treatments (Table 4). We observed similar trends for the pots that contained non-HLB-affected trees. Among all the elements that were studied, K, Mg, and Ca comparatively had a stronger correlation with Fe in HLB-affected trees. However, the positive correlation between Fe and Zn was strongest ($P = 0.0074$) for non-HLB-affected trees (Table 4).

Tree dry weight biomass. There was an interaction between the Fe rate and HLB status ($P < 0.001$). This means that the HLB status alone or in combination with the Fe rate did not affect the changes observed in the total dry weight biomass. Above-ground biomass of HLB-affected trees had between 33% and 44% more biomass ($P < 0.01$) than the below-ground biomass with the corresponding Fe fertilization (Table 5). Similarly, we observed that the above-ground biomass was between 34% and 44% greater for non-HLB-affected trees relative to the below-ground biomass ($P < 0.01$). For both HLB-affected and non-HLB trees, varying Fe rates only affected the dry weight biomass of large roots (Table 5). Large roots

Table 2. Least squares iron (Fe) rate × huanglongbing (HLB) × year means for tree heights, trunk diameters, and leaf Fe concentrations as a function of soil Fe application on HLB and non-HLB 2-year-old 'Bingo' (*Citrus reticulata,* Blanco) in 2019 and 2020. Response variables were fitted with a second-order polynomial regression of time nested within Fe rate × HLB combinations. Means ± SE presented are for 12 months after the initial application in each year. Total sample (N = 28) for each category. Means within the Fe × HLB status × time × response combination followed by the same letter are not significantly different at $\alpha = 0.05$.

HLB status	Fe rate (kg·ha^{-1} Fe)	Ht (cm)	Trunk diam (cm)	Leaf Fe (ppm)
HLB			2019	
	0 (control)	35.1 ± 5.41 b	0.48 ± 0.038 b	44.7 ± 3.18 d
	5.6 (1x)	62.2 ± 4.55 a	0.48 ± 0.037 b	62.3 ± 3.24 c
	11.2 (2x)	42.9 ± 5.04 b	0.62 ± 0.039 a	81.5 ± 2.94 b
	22.4 (4x)	62.4 ± 5.04 a	0.56 ± 0.038 ab	94.9 ± 3.17 a
Non-HLB				
	0 (control)	50.9 ± 5.13 a	0.57 ± 0.040 a	51.5 ± 3.79 d
	5.6 (1x)	41.4 ± 4.89 a	0.55 ± 0.040 a	91.2 ± 3.48 c
	11.2 (2x)	41.3 ± 4.59 a	0.56 ± 0.038 a	102.0 ± 3.52 b
	22.4 (4x)	48.9 ± 5.31 a	0.62 ± 0.037 a	157.4 ± 3.85 a
HLB			2020	
	0 (control)	53.2 ± 4.61 b	0.62 ± 0.036 b	29.2 ± 3.68 c
	5.6 (1x)	53.5 ± 6.07 b	0.67 ± 0.039 ab	53.4 ± 3.64 b
	11.2 (2x)	77.7 ± 4.79 a	0.75 ± 0.039 a	62.2 ± 3.51 b
	22.4 (4x)	65.0 ± 4.78 ab	0.63 ± 0.038 b	75.3 ± 3.63 a
Non-HLB				
	0 (control)	73.9 ± 4.80 a	0.76 ± 0.039 a	35.8 ± 2.88 c
	5.6 (1x)	63.2 ± 5.09 a	0.64 ± 0.040 b	54.2 ± 3.28 b
	11.2 (2x)	67.4 ± 5.33 a	0.71 ± 0.039 ab	59.5 ± 2.92 b
	22.4 (4x)	62.9 ± 4.56 a	0.74 ± 0.041 ab	71.0 ± 2.92 a
Sources of variation				
HLB status|Fe rate|year		<0.001	<0.001	<0.001
Time (HLB status × Fe rate × year)		<0.001	<0.001	<0.003
Time × Time (HLB status × Fe rate × year)		<0.001	<0.001	0.487

Table 3. Least squares iron (Fe) rate × HLB status means for leaf Fe concentrations as a function of soil Fe applications on huanglongbing (HLB) and non-HLB 2-year-old 'Bingo' (*Citrus reticulata,* Blanco). Means ± SE presented are for 24 months after the initial application. Total sample (N = 28) for each category. Means within an Fe × HLB status combination followed by the same letter are not significantly different at α = 0.05.

HLB status	Fe rate ($kg \cdot ha^{-1}$ Fe)	Leaf Fe (ppm)	
HLB	0 (control)	47.1 ± 6.93	D
	5.6 (1x)	60.1 ± 3.70	Cd
	11.2 (2x)	77.4 ± 0.66	Bc
	22.4 (4x)	86.6 ± 2.94	B
Non-HLB	0 (control)	55.0 ± 4.96	D
	5.6 (1x)	80.4 ± 1.39	B
	11.2 (2x)	80.1 ± 1.33	B
	22.4 (4x)	111.9 ± 9.27	A
Source of variation			
Fe rate		<0.0001	
HLB status		<0.0001	
HLB status × Fe rate		0.2264	

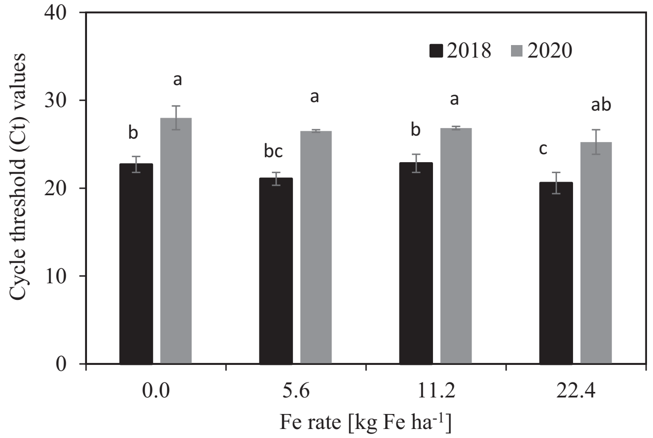

Fig. 1. Concentration of *Candidatus* Liberibacter asiaticus (*C*Las) DNA in leaf tissue taken from symptomatic, fully expanded huanglongbing (HLB)-affected leaves for Oct. 2018 [before iron (Fe) rates were applied] and May 2020 (end of study) as a function of four Fe application rates on HLB-affected 2-year-old 'Bingo' (*Citrus reticulata*, Blanco) in the greenhouse. The Fe rates were 0.0 (control), 5.6 (standard recommendation, 1x, by University of Florida/Institute of Food and Agricultural Sciences), 11.2 (2x), and 22.4 (4x) $kg \cdot ha^{-1}$ Fe. Data presented are the least square means and SEM. Means within the HLB status × Fe followed by the same letter are not significantly different at α = 0.05. Total sample (N = 28) for each category.

(>3 mm in diameter) accounted for 50% of all below-ground biomass for all treatments. The HLB status accounted for changes observed in dry weight biomass accumulation for twigs, small roots (<3 mm in diameter), and large roots.

Iron accumulation in tree parts and other nutrients. Because all essential nutrients in their correct amounts are necessary for citrus growth and development, we studied the correlation between the increasing Fe concentration and other macronutrients and micronutrients. We observed the effect that Fe accumulation had on other essential nutrients in the various tree parts of HLB-affected and non-HLB-affected trees. Iron accumulation correlated positively with all studied nutrients in the above-ground parts for both HLB-affected and non-HLB-affected trees. These correlations were significant for the macronutrients and micronutrients in branches of both HLB-affected and non-HLB-affected trees. In general, the positive correlations generated for Fe with other micronutrients (for example, with Mn) in branches tended to be stronger within HLB-affected trees ($P < 0.001$) relative to the non-HLB trees (Table 6). Iron accumulation in small roots (<1 mm in diameter) had a relatively strong ($P < 0.05$) positive correlation with all macronutrients in HLB-affected trees, suggesting synergism in the uptake of macronutrients in the presence of Fe. However, Fe correlated strongly ($P < 0.05$) only with Zn and Mn, but not with B and Cu, in small roots of non-HLB trees. The relationship between Fe and other nutrients in above-ground biomass was generally positive and stronger ($P < 0.05$) compared with that in below-ground biomass for both HLB-affected and non-HLB trees.

Table 4. Pearson's correlation coefficient (*r*) comparing soil iron (Fe) with phosphorus, potassium, magnesium, calcium, boron, zinc, copper, and manganese as a function of soil Fe application for huanglongbing (HLB)-affected and non-HLB-affected (NHLB) 2-year-old 'Bingo' (*Citrus reticulata,* Blanco) trees at the end of the study. Total sample (N = 28) for each category.

	HLB		NHLB	
Parameter	*r*	*P* value	*r*	*P* value
Phosphorus	−0.64	0.0249	−0.63	0.0295
Potassium	−0.72	0.0083	−0.55	0.0620
Magnesium	−0.76	0.0041	−0.56	0.0594
Calcium	0.77	0.0032	0.39	0.2070
Boron	−0.15	0.6340	0.23	0.4693
Zinc	−0.54	0.0712	−0.73	0.0074
Copper	−0.10	0.7470	−0.40	0.2022
Manganese	−0.16	0.6130	−0.26	0.4215

CLas DNA concentration in leaf tissue. In 2018, the Ct values ranged from 20 to 25. We observed an increase in the 2020 measurements, which ranged from 25 to 30 (Fig. 1). The Fe rates used during this study had no impact on the changes in Ct values that we observed (Fig. 1). This suggests that the Fe rate had no effect on the Ct value.

Optimum iron rate estimation. We calculated the optimum Fe rate for young HLB-affected trees using the total dry weight biomass as a function of variable Fe fertilization. The maximum total biomass calculated was 310 g, which corresponded with an Fe rate of 10.8 $kg \cdot ha^{-1}$ (Fig. 2). A 95% confidence interval at which the total biomass was nearly maximum corresponded with an Fe rate of 9.6 to 11.8 $kg \cdot ha^{-1}$ (Fig. 2). This confidence interval was much narrower than what we observed for non-HLB trees, suggesting the higher accuracy and greater chance of obtaining an observation within that interval.

Discussion

The evaluation of growth parameters in response to Fe fertilization is critical to citrus production, especially for HLB-affected trees. During our study, we observed changes in the tree height, trunk diameter, and leaf Fe concentration as a function of the variable Fe fertilization (Table 2). During the early stage of the experiment, the standard (1x) and 4x rates provided at least 31% greater height than the control and the 2x rate for HLB-affected trees. However, trees fertilized with the 2x rate had 31% greater height than the control and the standard rate during the second year (Table 2). The 2x rate generally resulted in steady and all-around growth by increasing the tree height and trunk diameter. This indicates that early growth by the trees fertilized with 1x may not guarantee sustained growth. The leaf Fe concentration may have affected the growth pattern for height observed for HLB-affected trees because the Fe concentration decreased by 25% for all trees. This decrease caused a disadvantage for the Fe accumulation in trees that received the control treatment and 1x rates. Data of the trunk diameter favored the 2x rate for both years for HLB-affected trees. This suggests that for HLB-affected trees, the 2x Fe rate performed better in terms of tree height and trunk diameter at the end of the second year of this study and might be used as the appropriate Fe rate for promoting tree vigor and performance during early years. A study of the effect of Fe nutrition on the growth of citrus revealed that trees that received Fe treatments had increased trunk circumferences and overall yield (El-Kassas, 1984). Iron deficiency correction has been proven to improve the growth and development of citrus trees, thus leading to better overall tree productivity (Morgan and Kadyampakeni, 2020; Rout and Sahoo, 2015). Rangel de Silva et al. (2020) studied the effects of the micronutrients supply of HLB-infected trees and observed that the HLB bacteria (*C*Las) affects the dynamics of nutrient concentrations in leaves and mentioned that the presence of *C*Las impairs biomass production.

Iron is the fourth most abundant nutrient in the earth crust, but mostly in forms that are

Table 5. Dry matter accumulation and allocation for huanglongbing (HLB) and non-HLB-affected (non-HLB) 2-year-old ‘Bingo’ (*Citrus reticulata,* Blanco) trees as a function of iron (Fe) application. Means ± SE presented are for 24 months of Fe accumulation at 3 months after the final application. Total sample (N = 12) for each category. Means within the HLB status × Fe followed by the same letter are not significantly different at α = 0.05.

	Dry matter (g/plant)			Above ground (%)				Below ground (%)		
Fe (kg·ha^{-1})	Total	Above ground	Below ground	Trunk	Branch	Twigs	Leaves	Small	Medium	Large
HLB										
0 (control)	275 ± 12.2 bc	177 ± 9.7 ab	99 ± 9.2 a	17.1 ± 1.69 a	25.4 ± 1.27 a	43.5 ± 2.16 ab	13.6 ± 0.88 a	10.9 ±± 1.83 ab	32.3 ± 2.07 a	56.6 ± 2.04 a
5.6 (1x)	307 ± 2.5 a	185 ± 2.1 a	123 ± 3.1 a	21.7 ± 1.86 a	26.9 ± 1.30 a	37.9 ± 2.11 b	13.9 ± 0.89 a	13.6 ±± 2.03 ab	31.8 ± 2.06 a	53.9 ± 2.05 a
11.2 (2x)	310 ± 7.2 a	199 ± 6.7 a	112 ± 8.7 a	20.7 ± 1.83 a	27.5 ± 1.31 a	37.9 ± 2.12 b	14.2 ± 0.90 a	13.4 ±± 2.01 a	34.1 ± 2.10 a	52.9 ± 2.05 a
22.4 (4x)	270 ± 8.5 c	162 ± 5.0 b	109 ± 10.2 a	15.8 ± 1.64 a	24.4 ± 1.25 a	46.8 ± 2.18 ab	12.7 ± 0.87 a	12.9 ± 1.98 b	26.5 ± 1.95 a	61.0 ± 2.01 a
Non-HLB										
0 (control)	338 ± 5.0 a	209 ± 5.3 a	128 ± 6.5 a	17.4 ± 1.71 a	26.4 ± 1.29 a	40.0 ± 2.14 a	16.2 ± 0.97 a	12.9 ± 1.98 a	31.4 ± 2.05 ab	55.5 ± 2.04 ab
5.6 (1x)	306 ± 1.5 b	191 ± 0.4 b	115 ± 2.5 ab	18.6 ± 1.75 a	26.0 ± 1.28 a	38.8 ± 2.12 a	16.7 ± 0.98 a	14.9 ± 2.11 a	38.0 ± 2.15 ab	46.9 ± 2.05 ab
11.2 (2x)	294 ± 0.3 c	178 ± 2.0 c	116 ± 2.2 ab	17.2 ± 1.70 a	27.1 ± 1.30 a	39.1 ± 2.13 a	16.8 ± 0.98 a	19.8 ± 2.37 a	35.2 ± 2.11 a	45.4 ± 2.05 b
22.4 (4x)	310 ± 5.6 b	199 ± 7.1 ab	111 ± 0.7 b	20.3 ± 1.81 a	26.9 ± 1.30 a	39.4 ± 2.13 a	13.5 ± 0.90 a	14.2 ±± 2.06 a	32.6 ± 2.07 b	53.4 ± 2.05 a
Source of variation										
HLB status	<0.001	0.001	0.007	0.183	0.784	0.004	0.533	0.047	0.091	<0.001
Fe	0.155	0.168	0.304	2.440	0.407	2.750	0.597	0.060	0.224	0.002
Status × Fe	0.000	0.001	0.009	1.750	0.121	0.401	0.569	0.245	0.664	0.356

Table 6. Pearson’s correlation coefficient (*r*) comparing plant tissue iron (Fe) accumulation with N P, K, Mg, Ca, S B, Zn, Mn, and Cu as a function of soil Fe application for huanglongbing (HLB) and non-HLB-affected (non-HLB) 2-year-old ‘Bingo’ (*Citrus reticulata,* Blanco) trees. Total sample (N = 12) for each category.

Part	N	P	K	Mg	Ca	S	B	Zn	Mn	Cu
	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - *r* - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - -
					HLB					
Above ground										
Leaves	0.56$^{\mathrm{NS}}$	0.51$^{\mathrm{NS}}$	0.15$^{\mathrm{NS}}$	0.51$^{\mathrm{NS}}$	0.52$^{\mathrm{NS}}$	0.74**	0.57$^{\mathrm{NS}}$	0.46$^{\mathrm{NS}}$	0.58*	0.49$^{\mathrm{NS}}$
Twigs	0.58*	0.42$^{\mathrm{NS}}$	0.36$^{\mathrm{NS}}$	0.72**	0.68*	0.76**	0.70**	0.79**	0.65*	0.58*
Branch	0.83***	0.85***	0.88***	0.92***	0.93***	0.90***	0.87***	0.86***	0.90***	0.72**
Trunk	0.72*	0.38$^{\mathrm{NS}}$	0.8**	0.84**	0.88***	0.82**	0.86**	0.69*	−0.32$^{\mathrm{NS}}$	0.56$^{\mathrm{NS}}$
Below ground										
Root (<1 mm)	0.65*	0.71**	0.62*	0.68*	0.50	0.82**	0.74*	0.80**	0.80**	0.60*
Root (1–3 mm)	0.73**	0.49$^{\mathrm{NS}}$	0.47$^{\mathrm{NS}}$	0.56	0.58*	0.63*	0.67*	0.91***	0.82**	0.55$^{\mathrm{NS}}$
Root (>3 mm)	0.26$^{\mathrm{NS}}$	0.36$^{\mathrm{NS}}$	0.39$^{\mathrm{NS}}$	0.41$^{\mathrm{NS}}$	0.3$^{\mathrm{NS}}$	0.52$^{\mathrm{NS}}$	0.42$^{\mathrm{NS}}$	0.45$^{\mathrm{NS}}$	0.64*	0.25$^{\mathrm{NS}}$
					Non-HLB					
Above ground										
Leaves	0.45$^{\mathrm{NS}}$	0.13$^{\mathrm{NS}}$	0.22$^{\mathrm{NS}}$	0.09$^{\mathrm{NS}}$	0.20$^{\mathrm{NS}}$	0.87***	0.86***	0.69**	0.66*	0.15$^{\mathrm{NS}}$
Twigs	0.58*	0.29$^{\mathrm{NS}}$	0.21$^{\mathrm{NS}}$	0.34$^{\mathrm{NS}}$	0.39$^{\mathrm{NS}}$	0.48$^{\mathrm{NS}}$	0.42$^{\mathrm{NS}}$	−0.01$^{\mathrm{NS}}$	0.46$^{\mathrm{NS}}$	0.41$^{\mathrm{NS}}$
Branch	0.75**	0.67*	0.74**	0.82***	0.85***	0.92***	0.84***	0.83***	0.60*	0.79**
Trunk	0.48$^{\mathrm{NS}}$	0.66*	0.63*	0.40$^{\mathrm{NS}}$	0.35$^{\mathrm{NS}}$	0.82**	0.17$^{\mathrm{NS}}$	0.46$^{\mathrm{NS}}$	0.37$^{\mathrm{NS}}$	0.00$^{\mathrm{NS}}$
Below ground										
Root (<1 mm)	0.37$^{\mathrm{NS}}$	0.25$^{\mathrm{NS}}$	0.24$^{\mathrm{NS}}$	0.36$^{\mathrm{NS}}$	0.45$^{\mathrm{NS}}$	0.43$^{\mathrm{NS}}$	0.43$^{\mathrm{NS}}$	0.60*	0.68*	0.2$^{\mathrm{NS}}$
Root (1–3 mm)	0.37$^{\mathrm{NS}}$	0.18$^{\mathrm{NS}}$	0.13$^{\mathrm{NS}}$	0.34$^{\mathrm{NS}}$	0.32$^{\mathrm{NS}}$	0.45$^{\mathrm{NS}}$	0.15$^{\mathrm{NS}}$	0.98***	0.85***	0.39$^{\mathrm{NS}}$
Root (>3 mm)	0.19$^{\mathrm{NS}}$	0.14$^{\mathrm{NS}}$	0.08$^{\mathrm{NS}}$	0.23$^{\mathrm{NS}}$	0.31$^{\mathrm{NS}}$	0.3$^{\mathrm{NS}}$	0.26$^{\mathrm{NS}}$	0.35$^{\mathrm{NS}}$	0.72*	0.18$^{\mathrm{NS}}$

NS, *, **, ***Nonsignificant or significant at $P < 0.05$, 0.01, or 0.001, respectively.

unavailable for plants absorption (Havlin et al., 2016; Rout and Sahoo, 2015; Zuo and Zhang, 2011). During our study, we observed that soil Fe correlated negatively with other soil nutrients except for Ca (Table 4). This suggests that all essential nutrients except Ca seemed to be absorbed better than Fe by the plant because of nutrient competition. Interestingly, we observed a significant ($P = 0.003$) positive correlation between Fe with Ca (Table 4). It is well-documented that calcareous soils (pH, 7.4–8.5) may limit the availability of Fe for plant uptake (Ferreira et al., 2019; Loeppert, 2008; Mengel, 1994). However, during our study, the soil had a pH range of 5.7 to 6.7, suggesting moderate acidity. This pH range favors Fe over Ca, which may suggest why Fe was positively correlated with Ca. Soils around citrus growing areas in Florida have relatively low organic matter content (<2% organic matter); therefore, it is necessary to apply the required Fe rate for better growth and development. Havlin et al. (2016) emphasized that Fe has antagonistic relationships with Cu, Mn, and Zn, and these relationships are usually observed with sensitive crops, including citrus (Fredeen et al., 1990; Havlin et al., 2016; Morgan and Kadyampakeni, 2020).

We observed that the 2x and the standard rate provided the greatest total dry weight biomass relative to the control and 4x rate. At the end of the second year, which corresponded with when the biomass was sampled, leaf Fe concentrations ranged from 35.8 ppm (control) to 71 ppm (4x rate) (Table 2). The trees that received the control treatment and standard rate had concentrations within the low range for citrus production (Morgan and Kadyampakeni, 2020). This suggests that HLB-affected trees that received the 2x rate had the capacity to grow and develop without any limitation on Fe (Tables 2 and 4). This indicates why those trees had a greater average total biomass and aboveground dry weight biomass ($P < 0.05$).

With citrus production, parameters such as biomass, tree height, and trunk diameter have been used to measure the growth response to fertilizer rates by various researchers (Kadyampakeni et al., 2016; McDonald et al., 1996; Morgan et al., 2006; Whitney et al., 1991). In our quest to find an optimum Fe rate for young HLB-affected citrus, we examined the correlation between Fe and other nutrients in various tree parts. In most HLB-affected tree parts, Fe correlated positively with B, Zn, Mn, and Cu. This means that the presence of Fe did not limit the uptake of the aforementioned nutrients in HLB-affected trees. This result is very interesting because we never found any report of the relationship between Fe and micronutrients in HLB-affected trees. Most authors have reported an antagonistic relationship between Fe and Cu and between Zn and Mn in the plant tissue (Havlin et al., 2016; Rai et al., 2021; Rout and Sahoo, 2015). The reason for this contradiction could be HLB. Our study was conducted under controlled

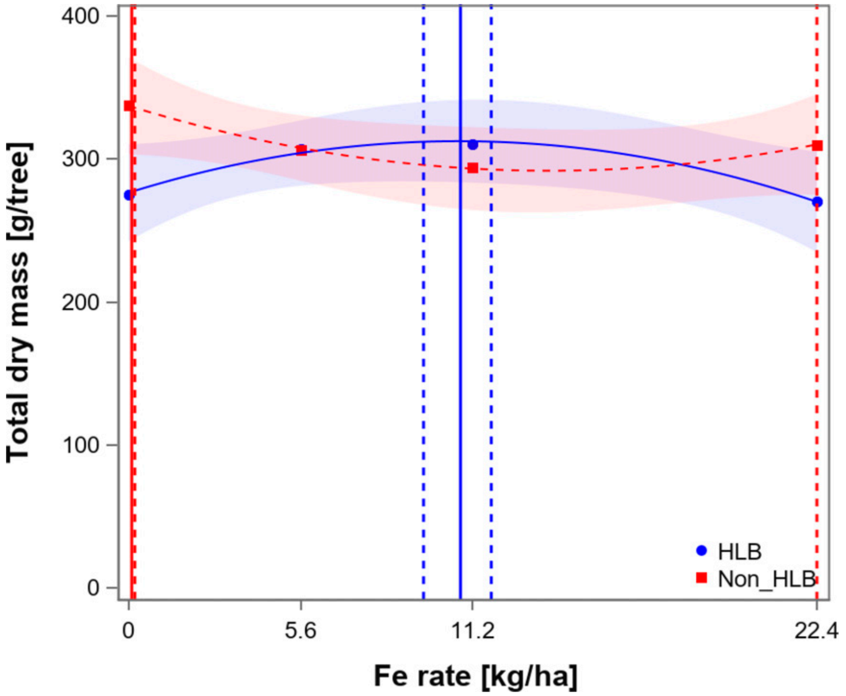

Fig. 2. Maximum dry weight biomass (g/tree) in response to iron (Fe) rates for huanglongbing (HLB)-affected 2-year-old 'Bingo' (*Citrus reticulata*, Blanco) trees. Vertical solid line represents the Fe rate at which the maximal response was achieved. Vertical dotted lines represent the lower and upper 95% confidence interval (CI). Rates were 0.0 (control), 5.6 (standard recommendation, 1x, by University of Florida/Institute of Food and Agricultural Sciences), 11.2 (2x), and 22.4 (4x) kg·ha^{-1} Fe. Total sample (N = 12) for each category.

conditions, and we only applied other essential nutrients as needed. However, when the levels of Cu are high because of the application of pesticides that contain Cu, growers may need to limit the application of extra Cu in the form of fertilizer and apply the required levels of Fe to increase the plant Fe uptake.

Because Fe is strongly involved in chlorophyll synthesis and maintenance of the chloroplast structure (Rout and Sahoo, 2015), adequate amounts should be supplied for optimal plant metabolism. Citrus, like other tree crops, require optimum levels of Fe for plant hormone synthesis (such as ethylene and abscisic acid), photosynthesis, and chlorophyll synthesis, among other critical functions (Kim and Rees, 1992), because Fe has a role as a cofactor for many enzymes required for synthesis.

The rate of Fe application is critical because Fe could easily move from the deficiency state to toxic levels under favorable conditions (Havlin et al., 2016). During our study, the HLB-affected trees that received the 4x rate had smaller trunk diameters and less biomass than trees that received the 2x rate (Table 5). Therefore, the Fe application should be less than 4x the standard recommendation (1x) for better growth and development of young HLB-affected trees. An Fe rate of 9.6 to 11.8 kg·ha^{-1} was calculated for young HLB-affected citrus ('Bingo') trees in Florida (Fig. 2). Soil-applied Fe in this range may be appropriate to enhance the growth and development and result in greater biomass accumulation in young HLB-affected trees.

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