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Influence of Elevated Manganese Rates on Growth Parameters, Nutrient, and Biomass Accumulation of HLB-affected Trees In Florida

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Enhancing nutrient uptake and tree health play an important role in managing huanglongbing (HLB) affected citrus trees in Florida. A greenhouse experiment was established to evaluate the effect of varying rates of manganese (Mn) on growth and development of 1-year-old HLB-affected sweet orange (*Citrus sinensis*) trees in October 2018 at the University of Florida IFAS (UF/IFAS)Citrus Research and Education Center in Lake Alfred, FL. Fifty percent of the trees were graft-inoculated with the HLB pathogen and the rest of the trees were used as the HLB-free (NHLB), control trees. Four treatments were applied on both sets of the trees in a randomized complete block design with 7 replicates. Data including trunk diameter, tree height and leaf samples were collected, processed and analyzed from trees treated with from four treatments, 0.0 kg·ha^{-1} Mn (Control), 5.6 kg·ha^{-1} Mn (1×, the UF/IFAS recommended rate), 11.2 kg·ha^{-1} Mn (2× UF/IFAS recommended rate), and 22.4 kg·ha^{-1} Mn (4× UF/IFAS recommended rate) on HLB- and NHLB-affected citrus trees. All the other essential nutrients were maintained at current recommendations in all the treatments. Leaf Mn concentrations, tree height and trunk diameter were analyzed by year with repeated measures in SAS-GLIMMIX. Leaf-Mn concentrations were significantly different (P < 0.0001) among Mn rates, and across sampling times in both HLB and NHLB trees in 2019. In both HLB and NHLB-affected trees, the Mn rate of 22.4 kg·ha^{-1} Mn recorded the highest leaf Mn concentration with the least square mean (LSM) of 1131 μg·g^{-1} of dry weight. Tree height and trunk diameter presented significant differences across sampling times in both HLB- and NHLB-affected trees in 2019. The 11.2 kg·ha^{-1} Mn rate increased tree height in HLB-affected trees across sampling-time, while the 22.4 kg·ha^{-1} Mn rate increased the trunk diameter of HLB-affected trees. The results presented in this study show that HLB-affected trees would require higher Mn concentrations than healthy trees (NHLB), for similar physiological functions. The results from this study support higher Mn treatments; specifically, 11.2- and 22.4-kg·ha^{-1} Mn (2× and 4×, respectively) than the UF/IFAS recommendation for better tree growth in HLB-affected trees.

During the past decade, the total citrus production in the United States has declined significantly (Blauer, 2020). Florida, the second-largest citrus producer in the country, has recorded the greatest reduction of more than 70%, from 13.5 million tons in 1998 to about 3.5 million tons in 2019 (Blauer, 2020; USDA, 2019). Despite this reduction, citrus remains the leading tree crop produced in Florida, contributing about $9 billion annually to the state's economy, and employing about 45,000 workers (USDA, 2019). Citrus production decline has been attributed to many causes, primarily huanglongbing (HLB), which was first reported in Florida around 2005. The disease was first found in China in the 19th century and has now spread to most parts of the world thus threatening the citrus industry (Blauer, 2020; Hall and Gottwald, 2011; USDA, 2019).

The HLB is caused by a phloem-limited bacterium called *Candidatus* Liberibacter asiaticus (*CLas*) and spread by an insect vector *Diaphorina citri* (Asian citrus psyllid, ACP) (Gilani et al., 2019; Gottwald et al., 2012; Hijaz et al., 2016; Killiny and Nehela, 2017). The insect-vector pierces through the phloem where it introduces the *CLas*. Once the is in the phloem, the plant blocks the phloem as a defense response and translocation of nutrients occurs (Spreen et al., 2014). One of the effects of the latter is a decline in roots and fibrous root density, which leads to a reduction in nutrient and water uptake, hence, a decrease in citrus yield (Graham et al., 2013; Hamido et al., 2017; Kadyampakeni et al., 2014b, 2014c; Killiny and Nehela, 2017). Since the disease has no cure, the management programs adapted are: intensive chemical control of the ACP, aggressive removal of HLB-affected trees, severe pruning, planting disease-free nursery stock (Hall and Gottwald, 2011; Rouse, 2017) and balanced-nutrition (Vashisth and Kadyampakeni, 2020). But the latter has called for much attention because balanced-nutrition tend to improve tree canopy and yield (Morgan et al., 2016; Nwugo et al., 2013; Rouse et al., 2017; Spann and Schumann, 2009).

Currently, there are no guidelines to determine optimal manganese (Mn) concentration in HLB-affected citrus trees in Florida. These guidelines would enable us to better understand the role Mn plays in the nutrition of HLB-affected trees. The University of Florida's Institute of Food and Agricultural Sciences (UF/IFAS) has updated its citrus nutrition guidelines (Morgan and Kadyampakeni, 2020) to guide farm managers and research

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scientists on balanced citrus nutrition with essential nutrients and their relationship to productivity. However, there is insufficient information on how much more Mn the HLB-affected trees require to maintain balanced levels in tissues of the tree. This study sought to generate information on how much Mn the HLB-affected trees require.

Manganese is an essential plant nutrient present in most plant metabolic processes, particularly in photosynthesis (Millaleo et al., 2010). The form of Mn taken up by plants is Mn^{2+}, a reduced form of Mn. Studies done by most researchers show that Mn is taken up by an active transport system in epidermal root cells, where it is transported by the xylem as Mn^{2+} into the plant system (Gherardi and Rengel, 2004; Marschner, 1995; Millaleo et al., 2010; Pittman, 2005). According to Graham (1979), Mn plays three major roles in the plant system: it assists the movement of electrons in photosynthesis, it affects the reduction of nitrate in nitrogen metabolism, and it acts as a precursor for aromatic amino acids and hormones as in auxins, phenols and lignin (Clarkson, 1988). Insufficient Mn in the plant triggers deficiency symptoms, which are usually observed when plant growth is extremely depressed. However, the productivity of plants is already affected when deficiency symptoms are observed (Schmidt et al., 2016). Zekri and Obreza (2012) reported that Mn deficiency in citrus might significantly reduce yield and fruit quality (Zekri and Obreza, 2012). Deficiency symptoms are observed in newly emerged leaves because Mn is immobile in the phloem, and as a result the remobilization of Mn from older to younger leaves is not possible (Schmidt et al., 2016).

Marschner (1995) mentioned that high levels of Mn could be toxic to biological cells, and intensity of the toxicity will depend on the excess Mn concentration in the plant (Lambers et al., 2015; Marschner, 1995). Finding the level of Mn at which HLB-affected citrus trees may be tolerant is deemed necessary, most importantly, for competition between Mn and other essential nutrients. This is because antagonism between Mn and other mineral elements has been reported; where excess Mn seems to limit the uptake of iron, molybdenum and magnesium, which are equally important for plants growth and development (Rietra et al., 2017).

Even though the mechanism at which HLB-affected trees metabolize Mn is not well understood, elevated levels of Mn has proven to correct deficiency symptoms and reduced *CLas* bacterial titers (Zambon et al., 2019). However, it is still not very clear how much the HLB-affected trees require Mn to maintain optimal concentration. Therefore, the objective of this study was to evaluate the effect of varying rates of manganese (Mn) on the growth and development of 1-year-old HLB-affected *Citrus sinensis* 'Valencia' trees under greenhouse conditions in Florida.

Materials and Methods

Site description and trial establishment. This study was conducted for two years in the greenhouse at the UF/IFAS Citrus Research and Education Center (CREC) in Lake Alfred, FL (Latitude 28°5'37"; Longitude 81°43'30"), to evaluate varying Mn rates on one-year old 'Valencia' (*Citrus sinensis*) trees on *Kuharske citrange* rootstock (*Citrus sinensis* × *Poncirus trifoliata*). The trees were planted in 8.7-L nursery containers. Fifty percent of the trees were graft-inoculated with the HLB-causal pathogen *CLas*. At least 3 leaves were grafted to enhance the probability of *CLas* inoculation. Trees were then left for 3 months for infection establishment. Composite soil samples were collected and analyzed for soil pH and Mehlich-3 extractable nutrients to correct for any deficiency.

Initial measurements of tree height and trunk diameter were taken from each plant before treatments were applied. At the same time, leaves were sampled and analyzed for Leaf-Mn and other essential nutrients. Treatments were then applied once confirmed for *CLas* inoculation using a quantitative polymerase chain reaction analysis (qPCR—Applied Biosystems 7500 Fast Real-Time PCR System; Thermo Fisher Scientific). Water was supplied to each container using a drip irrigation system with pressure-compensating drip emitters at a rate of 2 $L \cdot h^{-1}$ (MaxiJet, Dundee, FL) and trees were provided with all essential nutrients according to the UF/IFAS nutritional guide for citrus production (Morgan and Kadyampakeni, 2020).

Treatment application. Four treatments were applied in total; four treatments including the control for each of the HLB and NHLB-affected trees. Each treatment had a seven single tree replication, arranged in a randomized complete block design (using HLB and NHLB as blocks). The treatments comprised of soil application of varying rates of Mn from manganese (II) sulfate monohydrate ($MnS0_4 \cdot H_2O$, 16% Mn). All treatments were applied and mixed within 5-cm depth of the soil by hand, three times a year. Treatments were as follows:

1. Control: 0.00 $kg \cdot ha^{-1}$ Mn; trees that received this treatment were only supplied with other essential plant nutrients apart from Mn.
2. Standard UF/IFAS recommendation (hereafter denoted 1×): 5.6 Mn; this treatment represents the standard practice for Mn fertilizer application on citrus in Florida.
3. Two times the standard rate (hereafter denoted 2×): 11.2 $kg \cdot ha^{-1}$ Mn; this treatment represents a twice the standard recommendation rate.
4. Four times the standard rate (hereafter denoted 4x): 22.4 $kg \cdot ha^{-1}$ Mn ; this treatment represents four times the standard recommendation rate.

Tree height and trunk diameter. Initial tree height and trunk diameter were recorded for each replicate before treatments were applied. Subsequently, in every three-month interval, a measuring pole height stick (model 807396 by SOKKIA Corp., Olathe, KS) and a digital caliper were used to measure tree height and diameter for each replicate in each treatment. The digital caliper recorded the trunk diameter in the North-South (NS) and East–West (EW) directions of the tree. The results were then averaged and converted into cross-sectional area assuming a circular shape. In terms of tree height and trunk diameter, relative growth was estimated for each replicate by subtracting the first measurements (before treatments were applied) from the subsequent measurements (after treatments were applied) for each measurement period.

Leaf and soil sampling. Leaf samples were collected for each replicate before treatments were applied and afterwards every three months, following treatment application. About 15 fully expanded leaves aged 4–6 months were sampled. Immature-, abnormal-appearing-, and dead-leaves were avoided during every sampling period. The leaves were hand-washed immediately after sampling to remove any surface contamination. Sampled leaves were put into clean paper bags and dried in a ventilated oven at 65 °C for at least 72 h (Morgan and Kadyampakeni, 2020). After drying, leaves were ground with Thomas Type Lab Willey Grinder (PSAW-180, Swedesboro, NJ) with a 20-mesh sieve. Leaf tissue samples were then sent to Waters Agricultural Laboratories, Inc.

Table 1. Effects of manganese (Mn) rates on leaf-Mn concentration, tree height, trunk diameter, and soil Mn for huanglongbing (HLB) and healthy (NHLB) 1-year-old 'Valencia' (*Citrus sinensis*) trees after 12 months of treatment application in 2019.

		Leaf Mn concentration		Tree height		Trunk diameter		Soil Mn	
Tree	Mn Rate	Mean ± SE[x]	DF	Mean ± SE	DF	Mean ± SE	DF	Mean ± SE	DF
	--(kg·ha^{-1} Mn)--	--(µg·g^{-1} dry wt)--		--(cm)--		--(cm)--		--µg·g^{-1} dry wt--	
[z]HLB	0	26 d[w]	48	56 ± 6.96 bc	50.92	0.57 ± 0.053 bc	48	154 ± 442 c	48
	5.6	142 c	48	50 ± 6.96 bc	50.92	0.61 ± 0.053 bc	48	1674 ± 442 bc	48
	11.2	502 b	48	68 ± 6.96 ab	50.92	0.58 ± 0.053 bc	48	2976 ± 442 b	48
	22.4	1131a	48	50 ± 6.96 bc	50.92	0.66 ± 0.053 ab	48	5194 ± 442 a	48
[y]NHLB	0	38 d	48	48 ± 6.96 bc	50.92	0.63 ± 0.053 ab	48	700 ± 442 c	48
	5.6	185 c	48	66 ± 6.96 ab	50.92	0.61 ± 0.053 bc	48	3288 ± 442 ba	48
	11.2	713 b	48	57 ± 6.96 ab	50.92	0.58 ± 0.053 bc	48	2017 ± 442 bc	48
	22.4	1978 a	48	55 ± 6.96 ab	50.92	0.63 ± 0.053 ab	48	3570 ± 442 ba	48

[z]HLB = Huanglongbing affected trees.
[y]NHLB = healthy trees.
[x]SE = standard error of the mean, DF = denominator degrees of freedom.
[w]Different letters indicate statistically significant differences among the studied treatments and same letters indicate no significant differences between them. Statistical significance is set at $P < 0.05$.

(Camilla, GA) to determine elemental concentrations of selected nutrients, including Mn, using the acid digestion method. Soil samples were collected at a depth of 15 cm at the beginning of the study and at the end of the 2-year study period. The samples were then dried in a ventilated oven at 100 °C for at least 24 h. The soil samples were then sent to Waters Agricultural Laboratories, Inc. (Camilla, GA) where they were analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES), for Mehlich III extractable Mn and other nutrients.

Data analysis. Linear mixed model methodology as implemented in SAS PROC GLIMMIX, SAS 9.4 (SAS Institute Inc, 2018), was used to analyzed response data and determine optimal thresholds. The statistical significance level was established as $P < 0.05$ in the experimental design for all measured parameters. Leaf Mn concentrations, relative tree height, and trunk diameter were analyzed by year with repeated measures. An unstructured variance structure was fitted to the repeated measures. A Kenward-Roger type adjusted test was used to account for any bias in cases where the linear mixed model had missing covariates. The mixed model included fixed effects for treatment, blocks, time, and their interactions. A least significant difference (LSD) means separation was used to determine treatment differences for all response data. Leaf surface area, soil Mn concentration, and above-and below-ground biomass were also analyzed using linear mixed models in SAS. An optimal Mn rate was determined on all statistically significant parameters by mixed model polynomial regression in SAS.

Results and Discussion

Effect of Mn treatment on leaf Mn concentration. Leaf Mn concentrations were significantly different among Mn rates and across sampling times in HLB-affected and NHLB trees in 2019 (Fig. 1). The leaf Mn concentrations recorded for both HLB- and NHLB-affected trees that received 2× and 4× the standard rate were above the optimal Mn concentrations for citrus trees (Zekri and Obreza, 2012). It is also important to mention that these values are true only for NHLB-affected trees, as the optimal Mn range values for HLB-affected citrus trees are unknown. Concentration levels for the 2× UF/IFAS rate in HLB-affected trees presented a concave-up shape, suggesting a tendency for further increase with time (Fig. 1). At a similar Mn rate of 11.2 kg·ha^{-1} (2× UF/IFAS), leaf Mn concentrations in the HLB-affected trees tended to be higher relative to concentrations in NHLB-affected trees (Fig. 1). This result agrees with the Zambon et al. (2019) study, where they found that elevated Mn rates restore some biological functions due to increased metabolism experienced by HLB-affected trees. Although the two higher Mn rates (2× and 4×)

Leaf Manganese (Mn) concentration

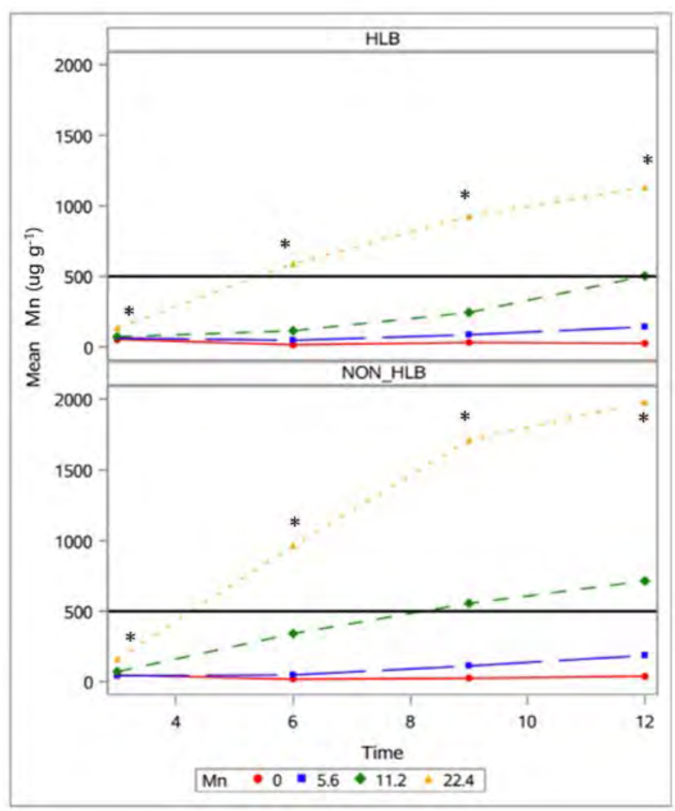

Fig. 1. Leaf manganese (Mn) concentrations across different sampling times (3-, 6-, 9-, and 12-months) after treatments application in 2019, showing the impact of 0.0 kg·ha^{-1} (Control), 5.6 kg·ha^{-1} (University of Florida Institute for Food and Agricultural Sciences recommendation), 11.2 kg·ha^{-1} (2× UF/IFAS rate), and 22.4 kg·ha^{-1} (4× UF/IFAS rate) on HLB-affected and non HLB-affected 1-year-old 'Valencia' (*Citrus sinensis*) trees. Data presented are the least square means and asterisks (*) represents significant differences among treatments ($P < 0.05$).

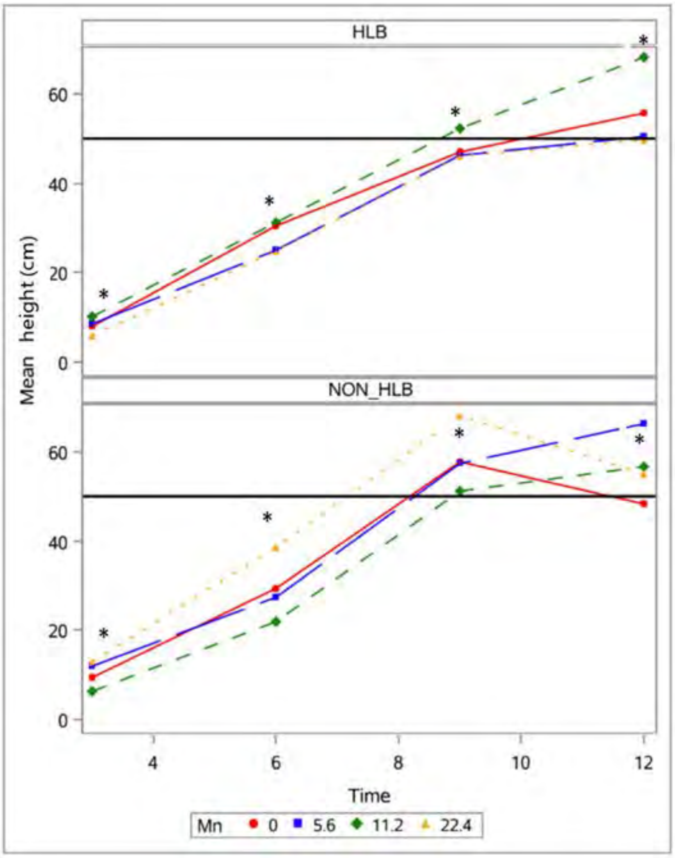

Fig. 2. Effect of Mn treatments 0.0 kg ha^{-1} (Control), 5.6 kg·ha^{-1} (University of Florida Institute for Food and Agricultural Sciences recommendation), 11.2 kg·ha^{-1} and 22.4 kg·ha^{-1} on height of HLB- and non-HLB-affected 1-year-old 'Valencia' (*Citrus sinensis*) trees across different sampling times (3-, 6-, 9-, and 12-months), after treatments application in 2019. Data presented are the least square means and asterisks (*) represents significant differences between sampling time ($P < 0.05$).

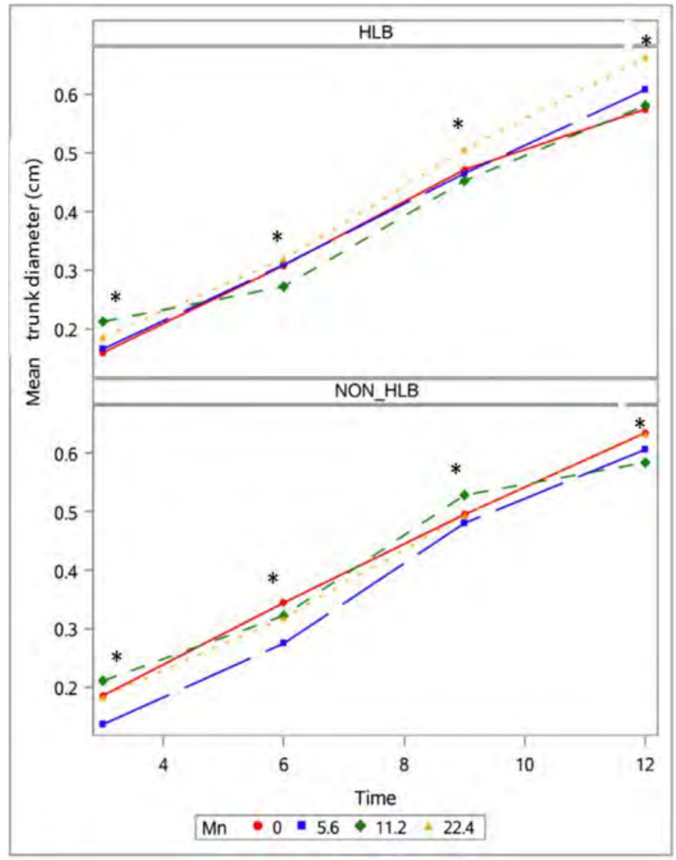

Fig. 3. Effect of Mn treatments 0.0 kg·ha^{-1} (Control), 5.6 kg·ha^{-1} (University of Florida Institute for Food and Agricultural Sciences recommendation), 11.2 kg·ha^{-1} and 22.4 kg·ha^{-1} on trunk diameter of HLB- and non-HLB-affected 1-year-old 'Valencia' (*Citrus sinensis*) trees across different sampling times (3-, 6-, 9-, and 12-months), after treatments application in 2019. Data presented are the least square means and asterisks (*) represents significant differences between sampling time ($P < 0.05$).

used in this study accumulated excess leaf Mn concentrations compared to the standard UF/IFAS recommended rate, there was no visual evidence of toxicity observed in HLB-affected trees.

It is well known that HLB-affected trees have limitations in water and nutrient absorption due to over 70% root loss (Graham et al., 2013; Kadyampakeni et al., 2014a). For this reason, HLB-affected trees may have minimal capacity of acquiring the required Mn through their root system, as is the case of NHLB-affected trees when given the current UF/IFAS recommended rate of 5.6 kg·ha^{-1} Mn (1×). Therefore, the results from this study agree with Zambon et al. (2019), that Mn rate must be increased for HLB-affected citrus trees. An update of Mn rate for HLB-affected trees may be necessary considering that Mn deficiency is detrimental to photosynthesis, and sometimes occurs without visual leaf symptoms (Marschner, 1995; Schmidt et al., 2016).

Effect of Mn treatment on tree growth and trunk diameter. Tree height and trunk diameter were not significantly different among Mn rates (Figs. 2 and 3) for both HLB- and NHLB-affected trees in 2019. However, tree height and trunk diameter showed significant differences across sampling time. The results support the claim that HLB-affected trees show more tolerance to excess Mn when compared to NHLB-affected trees (Fig. 2). For example, as tree height for treatments in NHLB-affected trees peaked in the 9th month, tree height for HLB-affected trees recorded their highest values in the 12th month (Fig. 2). In the Zambon et al. (2019) study, they reported that HLB-affected trees are tolerant to excess Mn levels, which happens to be harmful to *CLas*. Although our study does not present data to support the direct impact of higher Mn levels on *CLas*, our results agree that the HLB-affected trees are tolerant to rates of 2× and 4× the standard UF/IFAS recommended Mn rates (Figs. 2 and 3), as trees show no visual signs toxicity.

The trend of tree height for all treatments in NHLB-affected trees showed quadratic model. However, the trend in HLB-affected trees better fit a linear model suggesting increasing capacity for Mn applications (Fig. 2). This suggests that HLB-affected trees may require more Mn, as they still tend to grow more. Across all sampling-time in HLB-affected trees, tree height was highest at Mn rate of 11.2 kg·ha^{-1} Mn , which is 2× the UF/IFAS recommended rate (Fig. 2). There are mixed results when it comes to research on enhanced nutrition for HLB-affected trees because some researchers found no difference between trials with enhanced Mn treatments and the control plots (Gottwald et al., 2012), others reported a positive impact of balanced Mn and other micro-nutrient treatments (Morgan et al., 2016; Rouse et al., 2017; Zambon et al., 2019). The results from this study support an increase in tree height and trunk diameter with time for HLB-affected trees, when treated with elevated levels of Mn.

Conclusion

The results of this study support higher than the standard Mn treatments (2× and 4×, 11.2- and 22.4-kg·ha^{-1}, respectively) can be beneficial for HLB-affected trees. The rate of 11.2 kg·ha^{-1} Mn increased tree height in HLB-affected trees across sampling time, while the 4× rate of 22.4-kg·ha^{-1} increased the trunk diameter of HLB-affected trees. When trees are affected by HLB, they require higher Mn concentrations than healthy trees (NHLB), for similar physiological functions and possibly, to combat biotic stress. HLB-affected trees that received 22.4-kg Mn·ha^{-1}, which is about 4-times the UF/IFAS recommendation rate presented no visual signs of toxicity.

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