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Ground Application of Overdoses of Manganese Have a Therapeutic Effect on Sweet Orange Trees Infected with *Candidatus* Liberibacter asiaticus

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Abstract. **There is accumulating evidence that root system collapse is a primary symptom associated with Huanglongbing (HLB)-induced tree decline, especially for commercial sweet orange and grapefruit trees on Swingle and Carrizo rootstocks. Maintaining root health is imperative to keep trees productive in an HLB-endemic environment. Preliminary greenhouse and field studies have shown that HLB-impacted trees had secondary and micronutrient deficiencies that were much greater in the roots than in the leaves, and that treatments containing three-times the recommended dose of manganese (Mn) improved tree health and growth and increased feeder root density in greenhouse trees. These results suggested that trees in an HLB-endemic environment have higher specific micronutrient requirements than those currently recommended. To test this hypothesis, established Vernia sweet orange grafted onto rough lemon rootstock trees were divided into eight supplemental CRF nutrition treatments (including two-times and four-times the recommended doses of Mn and boron) using a randomized complete block design in a commercial grove in St. Cloud, FL. The following supplemental nutrition treatments were used: no extra nutrition (control); Harrell's–St. Helena mix 0.9 kg per tree; Harrell's with 32 g of Florikan polycoated sodium borate (PSB) per tree; Harrell's with 90 g of TigerSul® Mn sulfate (MS) per tree; Harrell's with 32 g of PSB and 90 g of MS per tree; 180 g of MS per tree; 64 g of PSB per tree; and 180 g of MS plus 64 g of PSB per tree applied every 6 months since Fall 2015. Leaf and soil nutritional analyses were performed in Mar. 2017, Sept. 2017, and May 2018; a quantitative polymerase chain reaction was performed for *Candidatus* Liberibacter asiaticus (*C*Las) titer estimation in Nov. 2017. Significantly higher cycle threshold (Ct) values indicating reduced *C*Las bacterial populations were observed in trees that received the higher doses of Mn, especially those receiving four-times the recommended dosage of Mn (180 g Mn). Many trees exhibited Ct values of 32 or more, indicating a nonactive infection. Fruit yields of these trees were also increased. No significant differences in juice characteristics, canopy volume, and trunk section area were found between control plants and plants treated with 180 g Mn. Soil and leaf nutrients B, K, Mn, and Zn were significantly different among treatments at various times during the study. Our results strongly suggest that overdoses of Mn can suppress *C*Las bacterial titers in sweet orange trees on rough lemon rootstock, thus providing a therapeutic effect that can help restore tree health and fruit yields. This response was not observed when Mn and B were combined in the overdose, suggesting an antagonistic effect from B on Mn metabolism. When an overdose of Mn is used, biological functions and tree tolerance lost due to nutritional imbalances caused by HLB might be restored. Further studies are needed to elucidate which metabolic pathways are altered by comparing overdosed and conventionally fertilized HLB-impacted trees and to determine if the observed therapeutic effects can be achieved in trees grafted to other important commercial rootstocks.**

Plant nutrient management is one of the most basic practices used for crop production and environmental sciences. Acquisition of nutrients by feeder roots is crucial for the survival of plants. The application of nutrients such as chemical or organic fertilizers is routine for growers, and it is part of nutrient recycling. To reach maximum growth and overcome stresses, plants develop a relationship with soil microbes (Chen et al., 2014; Jacoby et al., 2017; Navarro et al., 2011). Soil microbes change the ionic status of minerals that plants cannot take-up in molecular form during a process called mineralization (Sattelmacher et al., 1982). The presence of nutrients in ionic form can lead to competition for the same exchange in soil colloid sites by ions of similar sizes, thereby interfering with the absorption, adsorption, and transport of a specific ion and/or a similar chemical–physical structure group (Fageria, 2001).

Citrus are among the most valuable crops worldwide. Since 2005, Florida citrus production has been affected by yellow dragon disease, which is commonly known as citrus greening or Huanglongbing (HLB) (Halbert, 2005; USDA-NASS, 2017). HLB is presumably caused by a fastidious bacterium [*Candidatus* Liberibacter asiaticus (*C*Las)] transmitted by the Asian Citrus Psyllid (*Diaphorina citri*). HLB symptoms include blotchy mottled leaves, starch accumulation in leaves, zinc-like deficiency symptoms, misshapen fruit, low juice quality, high fruit drop rate, decreased root biomass, callose and p-protein deposition in phloem sieve pores, and twig dieback (Bové, 2006; Cimò et al., 2013; Etxeberria et al., 2009; Johnson et al., 2014; Kim et al., 2009). As a result of the presence of *C*Las, nutrient imbalance has been reported, thus aggravating the functional status and physiological status of an already photoassimilate-depleted plant (Nwugo et al., 2013; Spann and Schumann, 2009). Nutrient imbalance causes several drawbacks, especially the deficiency of micronutrients known to be cofactors in enzymatic reactions that protect cell integrity (Hänsch and Mendel, 2009) from excessive reactive oxygen species (ROS) when infected with *C*Las (Pitino et al., 2017).

Several approaches have been reported to improve the health status of HLB-affected plants, such as an overdose of foliar sprays with essentials nutrients (Morgan et al., 2016) and soil acidification, resulting in corresponding increases in root density and yield (Graham, 2016). However, the low mobility of some ions does not address localized nutrient deficiency in roots infected with *C*Las (Johnson et al., 2014). The use of polymer-coated or clay-coated products as an alternative to traditional fertilizer application (fertigation/dry granular fertilizer) has increased recently due to its easy application two times per year without the need for incorporation in the soil, synchrony of the release of nutrients and plant demand, and reduced loss of nutrients in the environment (Obreza et al., 2006).

The objective of this study was to balance the nutritional status and alleviate HLB symptoms of field-established sweet orange trees infected with *C*Las by supplementing a traditional citrus fertilization program with ground-applied, slow-release enhanced nutrition for selected micronutrients, including

Mn and B at double and quadruple the current recommended doses.

Materials and Methods

Greenhouse study. We previously conducted a preliminary greenhouse experiment to investigate the potential of micronutrient overdoses to improve the health of *C*Las-infected trees (Grosser and Barthe, 2015). In the greenhouse, UFR-3 rootstock liners (Nova+Hirado Buntan pummelo × Cleopatra+ Argentine trifoliate orange) were budstick-grafted with HLB-infected Valencia sweet orange. Treatments were established with 10 single-tree replications. Control treatments included either biannual Harrell's® 16–5–10 nursery controlled-release fertilizer (CRF) mix or biweekly liquid fertilizer (Peters). Experimental treatments included biannual treatments of Harrell's CRF mix supplemented with biannual treatments of triple the recommended dose of individual polycoated essential minor elements (Florikan®), TigerSul micronutrients® (sulfate form of Fe, Zn, and Mn embedded in clay prills, and a blend of all three products, as recommended by A. Schumann, personal communication), and double the recommended dose of the individual polycoated essential macronutrients. The experiment was performed for 1 year. Effects on tree health, tree growth, root mass, soil plant analysis development (SPAD), leaf and root nutritional analyses, and leaf and root Liberibacter titers were measured.

Field study. Mature 10-year-old midseason 'Vernia' sweet oranges [*Citrus sinensis* (L.) Osbeck)] grafted onto rough lemon (*Citrus jambhiri* Lush.) were used in this study performed at Lee Groves in St. Cloud, FL. All trees were HLB-scouted at the beginning of the experiment by trained personal, and 100% were classified as HLB-infected based on visual observations. Tree density was 375 trees/ha planted every 4.6 m in rows with 7.6 m between rows on Myakka fine sand. The soil was classified as sandy soil with 96.5% sand, 2.2% clay, and 1.3% silt. Trees were grown with a standard soluble dry fertilizer program (Supplemental Table 1) applied four times per year. Supplemental controlled-released fertilizer treatments (Table 1, Supplemental Table 2) were used twice per year, starting in Fall 2015, and were continued throughout this study. Trees were irrigated with microsprinklers. A traditional sweet orange spray program for pest management (only one or two sprays of pesticide per year) was used, but a full psyllid control program was not (grower's/cooperator's decision). The experimental design was a randomized block with 12 plants per treatment (nutrition) subdivided into six plants per replicate. Nutritional replicates were randomly assigned to each subplot, forming four replications per treatment (Fig. 1). A composite of 10 leaves from each of the trees was randomly sampled and sent for quantitative polymerase chain reaction (qPCR) analysis at Southern Gardens Citrus (Clewiston, FL) for confirmation of *C*Las in Nov. 2017. Plants that showed cycle threshold (Ct) values of 30 or less were considered HLB-positive, those with Ct values of 32 or more were HLB-negative, and results were inconclusive when the value was between 30 and 32. Soil and leaf samples were collected during Mar. 2017, Sept. 2017, and May 2018 for tissue and soil nutrient concentration analyses. Leaf samples were dried at 65 °C for 72 h until dry and then ground with a 20-mesh sieve. The tissue samples were sent to the WaterAg Laboratory (Camila, GA), analyzed using the dry-ashing method, and assessed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) to determine elemental concentrations of selected nutrients. The tissue nutrient concentration was expressed as the percentage of the dry tissue biomass (%). Soil nutrients were extracted using the Mehlich III method and analyzed using the ICP-AES method at the WaterAg Laboratory (Camilla, GA). The nutrient concentration was expressed as nutrient mass per unit of soil mass ($mg \cdot kg^{-1}$). The tree canopy volume and trunk cross-section area were measured during Mar. 2017, Sept. 2017, and May 2018. The trunk cross-sectional area was calculated assuming a circular shape by measuring the average trunk diameter in the east–west and north–south directions and calculating the trunk cross-section area as πr^2, where r is the mean trunk radius. The canopy volume was calculated based on the following formula for the prolate spheroid shape: $(4/3)*(\pi)*(\text{tree height}/2)*(\text{mean canopy radius})^2$ (Obreza and Rouse, 1993). The Brix/acid ratio was calculated in kilograms of solids per box of fruits harvested during Jan. 2018 and was measured at the Citrus Research and Education Center Processing Pilot Plant (Lake Alfred, FL) with a commercial juice extractor (FMC Corp., Philadelphia, PA). A juice color analysis was performed with a GretagMacbeth Color-eye 3100 using Optiview–ProPallete software. The yields of all treatments were collected per replicate plot by Lee Groves personnel for the

Table 1. Slow-release treatments and dosages applied on 10-year-old 'Vernia' trees.

Treatment[z]	Formulation	Amount[y]	Product names
Control	None	0	None
Harrell's	12–3–9	910 g	Harrell's® St. Helena mix
Harrell's + 2× Mn	12–3–9 + 0.08% Mn	910 g + 90 g	Harrell's® St. Helena mix + TigerSul® manganese ($MnSO_4$)
Harrell's + 2× B	12–3–9 + 0.22% B	910 g + 32 g	Harrell's® St. Helena Mix + Florikan® polycoated sodium borate $\{Na_2[B_4O_5(OH)_4]\}$
Harrell's + 2× Mn + 2× B	12–3–9 + 0.08% Mn + 0.22% B	910 g + 90 g + 32 g	Harrell's® St. Helena Mix + TigerSul® manganese + Florikan® polycoated sodium borate
4× Mn	0.16% Mn	180 g	TigerSul® manganese
4× B	0.44% B	64 g	Florikan® polycoated sodium borate
4× Mn + 4× B	0.16% Mn + 0.44% B	180 g + 64 g	TigerSul® manganese + Florikan® polycoated sodium borate

[z]Treatments in addition to a standard citrus nutrition program.
[y]Grams per tree.

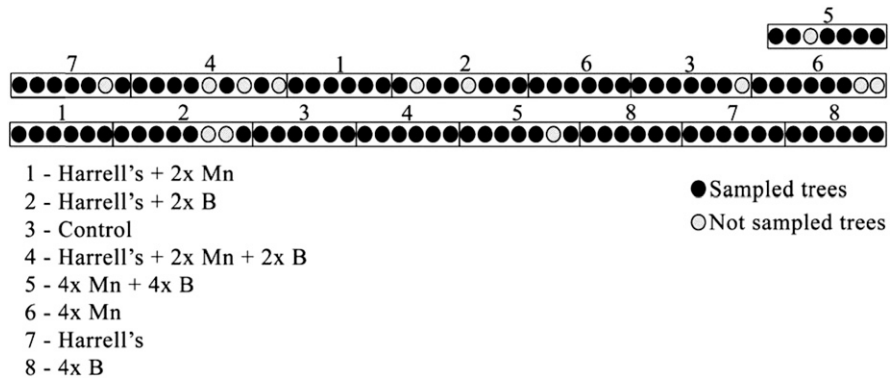

Fig. 1. Diagram of the experimental design and relative tree locations.

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2016, 2017, and 2018 seasons and reported as boxes per acre (40.5 kg/box).

Statistical analysis. R-Studio version 1.1.456 was used for statistical analyses. One-way analysis of variance (ANOVA) and two-way ANOVA were performed using *agricolae* and *lme4*. packages. Tukey's honestly significant difference means separation was performed as a post hoc analysis; when not specifically mentioned, $\alpha \geq 0.05$.

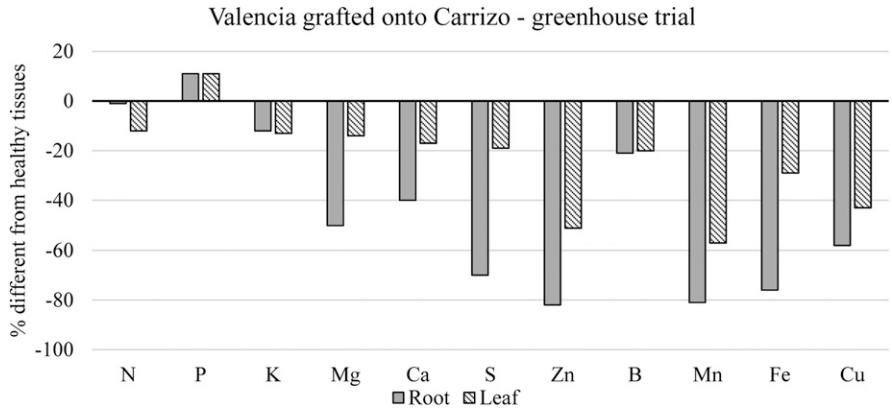

Fig. 2. Nutrient level means for the leaf and root of *C*Las-infected Valencia/Carrizo (Val/Czo) compared with healthy greenhouse trees. Data (average of 10 trees) are presented in % differences based on levels in healthy tissues. *C*Las infections were validated using qPCR.

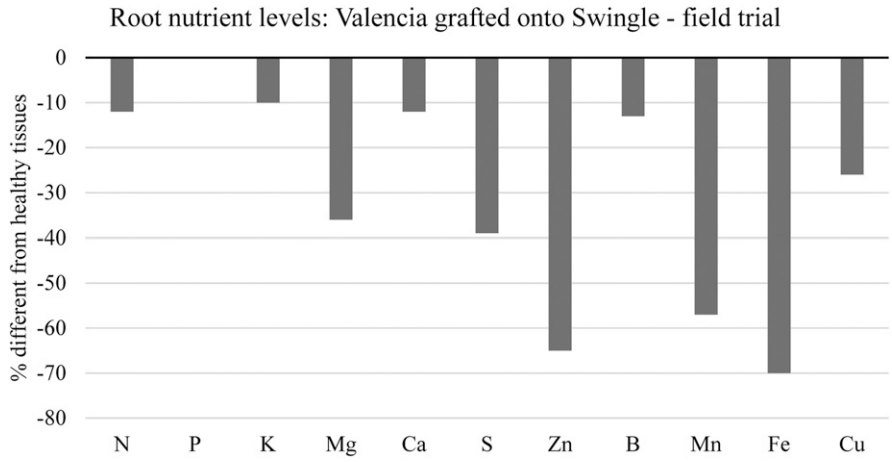

Fig. 3. Root nutrient level means of *C*Las-infected Valencia/Swingle (Val/SW) field trees (6–10 years old) compared with healthy trees. Nutrient contents (averaged from 10 trees) are presented in % differences based on levels in healthy tissues. *C*Las infections were validated using qPCR.

Fig. 4. *C*Las-infected Valencia/UFR-3 greenhouse trees after 1 year: control standard liquid fertilizer (left) and Harrell's CRF plus triple TigerSul® manganese (right).

Results and Discussion

Preliminary data. Preliminary greenhouse and field nutrient analyses were performed before the main experiment reported herein showed that *C*Las-infected trees have higher levels of micronutrient deficiencies in the roots than in the leaves as compared with healthy trees (Figs. 2 and 3). Zinc, Mn, and Fe are the micronutrients most impacted by HLB, especially in the roots of infected trees. Soil pH and micronutrient content do not appear to be responsible for these deficiencies (data not shown).

Several treatments tested during this study significantly improved tree growth and health as compared with the control, especially the triple TigerSul® Mn and triple polycoated sodium borate treatments. Visual examinations (Figs. 4 and 5) and total root length data generated using winRhizo image analysis (Table 2) showed improved feeder root density and health for the two treatments containing the highest levels of Mn.

Canopy volume and trunk cross-section area. Canopy volume and trunk cross-section area means were not significant between treatments. However, the quadruple Mn treatment showed a higher canopy volume compared with the that of the treatment with Harrell's® plus double Mn plus double B in Mar. 2017 (Table 3).

Soil nutrient analysis. The soil nutrient analysis showed differences in the potassium (K) content between months for all treatments; these were possibly caused by plant growth during summer and the beginning of fall. The K soil content during the growing season in Sept. 2017 was statistically significant between treatments because Harrell's treatment resulted in the highest values. Overdoses of B and Mn treatments had the lowest K content in soil in Sept. 2017, possibly because K cations have greater valence (+2) compared with the monovalent K and might have displaced K from exchange sites (Table 4). Another explanation could be that K has a greater ionic radius (280 pm) compared with B and Mn (<100 pm), which might have resulted in greater propensity of the tree roots to use the latter two cations, leading to K losses in the root zone. A similar trend of reduced uptake of Mn and B with high concentrations of K was also observed for rice (Ramadi and Kannan, 1974). A statistical interaction between the treatment and month was observed for Mn in soil (P = 0.000114) (Fig. 6). As expected, the quadruple Mn treatment had the highest value of Mn content in the soil during Sept. 2017 and May 2018. The Mn content for the quadruple B treatment was higher than the treatment with Harrell's® plus double B, suggesting a synergic interaction between Mn and B, because higher Mn contents increased with the presence of four-times the recommended dose of Mn or B in soil. The opposite could be seen for Mn absolute values of Harrell's® plus double Mn and Harrell's plus double Mn plus double B treatments, although this was not

statistically significant. It is possible that lower concentrations of Mn and B (two-times the recommended dose) may interact differently when the soil Mn content is higher. However, the boron content in soil was not significantly different. Except for the treatment including Harrell's plus double Mn, the leaf Mn content remained low (18–24 ppm) or deficient (<18 ppm) with the other treatments during Mar. 2017, but it remained in the optimum range (25–100 ppm) during Sept. 2017 and May 2018. Therefore, it would be ideal to have Mn or micronutrient thresholds for different times of the year because the leaf nutrient content tends to increase during the summer flush (July–September samples), and it tends to decrease when trees remobilize nutrients to fruit and vegetative growth during late fall and early spring, resulting in decreased leaf tissue nutrient concentrations.

Fig. 5. *C*Las-infected Valencia/UFR-3 typical root systems: control standard Harrell's CRF fertilizer (left) and Harrell's CRF plus triple TigerSul® manganese (right).

Citrus roots are one of the first affected sites when infected with *C*Las before any foliar symptoms occur (Johnson et al., 2014); this occurs from 6 months to 3 years after the initial infection (Bové, 2006). Transcriptomes of early infected citrus roots showed stress-related genes were differentially regulated (Zhong et al., 2015) and showed down-regulation of genes related to oxidative stress (Mittler, 2002). Among the genes involved with oxidative stresses, mitochondria superoxidase dismutase is dependent on Mn availability to reduce the ROS pool in cells (Alscher et al., 2002). ROS are reducing agents that are known to extensively damage cells because of either biotic (a pathogen, such as *C*Las) or abiotic stresses (such as drought, wind, cold, heat, and nutrient deficiency). Sufficient Mn in the rhizosphere is critical for scavenging the deadly ROS produced by the presence of *C*Las. To be actively taken-up by the roots, Mn must be in its reduced form (Mn^{+2}) (Gherardi and Rengel, 2003; Marschner, 1995; Pittman, 2005). Reduction of $MnSO_4$ happens by reducing rhizosphere pH, and increasing

Table 2. Effects of nutrient overdoses on *C*Las-infected Valencia/UFR-3 greenhouse trees after 1 year of treatments. Total root length (cm) was determined by winRhizo washed root image analysis.

Treatment	N	Mean[z]	Tukey grouping
Harrell's + 3× TigerSul® Mn	10	2361	A
Harrell's + 3× Tiger®-Arnold's Mix (Mn, Fe, Zn)	9	2270	A
Harrell's + 3× TigerSul®-Arnold's + Biochar	9	1955	AB
Harrell's + 3× TigerSul® Zinc Sulfur	10	1672	AB
Harrell's – control	8	1670	AB
Harrell's + 3× Florikan® sodium borate	10	1554	AB
Harrell's + 3× TigerSul® Fe	7	1419	AB
Liquid fertilizer only – control	6	1349	AB
Harrell's + 3× Florikan® magnesium sulfate	8	1315	AB
Harrell's + 2× Florikan® ammonium sulfate	8	1276	AB
Harrell's + 2× Florikan® urea	8	1173	AB
Harrell's + 3× Florikan® iron sulfate	7	1032	AB
Harrell's + 3× Florikan® super triple phosphate	6	910	AB
Harrell's + 2× Florikan® potash	4	902	AB
Harrell's + biochar	9	559	B

[z]Means separation by Tukey's honestly significant difference test ($P \leq 0.05$).

Table 3. Canopy volume (m^3) and trunk cross-sectional area (cm^2) for Mar. 2017, Sept. 2017, and May 2018.

	Mar. 2017		Sept. 2017		May 2018		Total averaged (2 yr)	
Treatment	Canopy vol.	Trunk cross-section area	Canopy vol.	Trunk cross-section area	Canopy vol.	Trunk cross-section area	Canopy vol.	Trunk cross-section area
4× Mn	27.14 a[z] A[y]	172.1 a A	16 a C	147.0 a A	24.43 ab B	183.17 ab A	33.78	251.13
Harrell's® + 2× Mn	25.84 ab A	149.6 a A	14.68 a C	168.2 a A	26.32 a B	176.07 ab A	33.42	246.93
Harrell's®	25.5 ab A	148.4 a A	18.54 a C	180.3 a A	26.5 a B	160.05 ab A	35.27	244.37
Control	24.94 ab A	154.9 a A	19.69 a B	199.9 a A	24.95 ab AB	191.05 a A	34.79	272.92
Harrell's® + 2× B	23.61 ab A	157.3 a B	23.39 a B	199.9 a A	25.00 ab B	173.4 ab AB	36	265.3
4× Mn + 4× B	20.85 ab A	154.7 a A	18.55 a A	200.2 a A	20.20 ab A	156.92 ab A	29.8	255.91
4× B	20.43 ab A	162.7 a A	21.82 a A	152.9 a A	23.72 ab A	185.77 ab A	32.98	250.68
Harrell's® + 2× Mn + 2× B	17.61 b A	123.2 a A	20.84 a A	179.8 a A	19.8 b A	134.85 b A	29.12	218.92

[z]Means separation within months according to Tukey's honestly significant difference test ($P \leq 0.05$; lowercase).
[y]Means separation between months according to Tukey's honestly significant difference test ($P \leq 0.05$; uppercase).

Table 4. Soil nutrient content for Mar. 2017, Sept. 2017, and May 2018.

		Treatment (F2)							
Month (F1)	Nutrient (mg·kg^{-1})	4× Mn + 4× B	4× B	4× Mn	Harrell's®	Harrell's® + 2× B	Harrell's® + 2× Mn	Harrell's® + 2× Mn + 2× B	Control
Mar. 2017	P	71.6 C[z] a[y]	59.8 B a	63.5 B a	71.4 B a	84.4 B a	67.4 B a	63.4 B a	62.8 B a
Sept. 2017		137.1 A a	138.6 A a	126.4 A a	132.6 A a	155.8 A a	139.0 A a	137.8 A a	138.4 A a
May 2018		108.6 A a	133.9 B a	134.4 A a	129.0 A a	124.0 A a	150.1 A a	120.0 A a	117.8 A a
Total avg		106.5	116.6	114.7	115.5	122.1	126.7	110.3	109.2
Mar. 2017	K	96.0 A a	93.8 A a	68.9 A a	67.0 A a	91.4 A a	80.1 A a	94.1 A a	77.7 A a
Sept. 2017		25.6 A ab	23.3 B b	24.5 B b	42.8 A a	39.3 B ab	31.9 B ab	34.5 B ab	31.4 B ab
May 2018		44.8 A a	44.2 B a	45.4 B a	50.4 A a	43.9 B a	65.2 AB a	45.2 B a	47.8 AB a
Total avg		52.8	51.4	46.1	52.7	54.6	60.6	54.8	51.2
Mar. 2017	Mg	.[x]	.	.	.	.	.	.	.
Sept. 2017		55.8 A a	45.0 A a	34.3 A a	35.6 A a	32.9 A a	23.4 B a	40.9 A a	60.1 A a
May 2018		58.0 A a	68.9 A a	55.5 A a	65.2 A a	55.5 A a	61.2 A a	72.5 A a	60.4 A a
Total avg		56.9	57.0	44.9	50.4	44.2	42.3	56.7	60.3
Mar. 2017	Ca	382.7 A a	415.8 A a	284.7 A a	251.9 A a	306.7 A a	360.2 A a	301.7 A a	311.3 A a
Sept. 2017		452.0 A a	386.4 A a	298.0 A a	286.9 A a	324.9 A a	244.4 A a	350.6 A a	474.6 A a
May 2018		414.0 A a	497.0 A a	372.7 A a	427.6 a	374.6 A a	364.9 A a	463.6 A a	409.9 A a
Total avg		415.7	449.05	332.025	348.5	345.2	333.6	394.875	401.4
Mar. 2017	S	.	.	.	.	.	.	.	.
Sept. 2017		36.9 A a	26.4 A a	74.0 A a	39.0 A a	32.6 B a	31.9 B a	30.6 A a	21.8 A a
May 2018		36.4 A c	42.9 A c	114.9 A a	53.6 A bc	46.6 A bc	92.2 A ab	45.0 A c	37.9 A c
Total avg		36.7	34.7	94.5	46.3	39.6	62.05	37.8	29.9
Mar. 2017	Zn	.	.	.	.	.	.	.	.
Sept. 2017		19.9 A ab	12.1 A b	12.7 A ab	21.6 A a	19.7 A ab	14.8 A ab	20.1 A ab	13.6 A ab
May 2018		14.8 A a	17.7 A a	14.0 A a	20.8 A a	15.1 A a	18.5 A a	18.5 A a	15.1 A a
Total avg		17.35	14.9	13.35	21.2	17.4	16.65	19.3	14.35
Mar. 2017	Fe	.	.	.	.	.	.	.	.
Sept. 2017		173.9 A a	162.4 A a	208.5 A a	172.3 A a	196.1 A a	187.4 A a	167.6 A a	157.4 A a
May 2018		168.0 A ab	143.1 A b	196.7 A a	167.5 A ab	164.2 A ab	178.6 A ab	168.4 A ab	170.5 A ab
Total avg		171.0	152.8	202.6	169.9	180.2	183.0	168.0	164.0
Mar. 2017	Cu	.	.	.	.	.	.	.	.
Sept. 2017		9.9 A a	10.5 A a	7.6 A a	8.2 a	8.3 A a	9.6 A a	8.1 A a	10.3 A a
May 2018		8.4 B a	10.7 A a	10.1 A a	9.7 a	7.3 A a	8.7 A a	7.6 A a	10.8 A a
Total avg		9.15	10.6	8.85	8.95	7.8	9.15	7.85	10.55
Mar. 2017	Mn	44.1	44.1	42.1	35.5	50.8	52.1	67.3	45.6
Sept. 2017		128	78	170.9	72.5	72.9	70 B	92.3	76.8
May 2018		117.2	121.9	185.2	125.5	114.4	162.1	130.9	122.9
Total avg		101.625	103.975	145.85	89.75	88.125	111.575	105.35	92.05
Mar. 2017	B	3.5 A ab	3.7 A ab	3.1 A b	3.4 A ab	3.6 A ab	3.9 A a	3.5 A ab	3.2 A b
Sept. 2017		0.6 B a	0.7 B a	0.2 B a	0.2 C a	0.4 B a	0.2 B a	0.3 C a	0.3 B a
May 2018		0.6 B ab	0.7 B a	0.5 B b	0.5 B ab	0.5 B ab	0.5 B ab	0.6 B ab	0.5 B b
Total avg		1.3	1.5	1.1	1.15	1.25	1.3	1.25	1.1

[z]Means separation between months by Tukey's honestly significant difference test ($P \leq 0.05$; uppercase).
[y]Means separation within months by Tukey's honestly significant difference test ($P \leq 0.05$; lowercase).
[x]Nutrients not measured for Mar. 2017.

microflora conditions in the rhizosphere assist in reducing Mn to an available form (Jacoby et al., 2017).

Higher availability of Mn in the soil profile could have a positive effect on the roots of HLB-infected plants by triggering defense responses against the pathogen because HLB-infected plants have reduced root biomass (Graham et al., 2013). Lower root biomass affects the entire process of water and nutrient uptake, and the plant response to HLB by phloem blocking shortens the distribution of several ions for new growth (Nwugo et al., 2013).

Leaf nutrient analysis. Nitrogen (N), P, K, and Mg concentrations were statistically different between months (Table 5). Calcium (Ca), Mn, Fe, and B concentrations were higher in September, perhaps due to the summer flush during early fall. There was a statistically significant interaction between treatment and month ($P = 2.01e^{-10}$) for boron. Although there was no statistically significant interaction for Mn, its content differed according to treatment over the months.

An accumulation of Mn was noticed for Harrell's® plus double Mn plus double B and for Harrell's® plus double Mn in Sept. 2017; this increase in Mn content was observed in leaves administered twice the recommended dose of B. The quadruple Mn treatment could have led to toxicity and, therefore, reduced uptake compared with the control and even Harrell's® fertilizer treatment groups (Fig. 7). Doses of four-times that recommended for Mn and B also reduced the Mn content in leaves. Double the recommended dose of boron with Harrell's treatment was quasi-linear regarding the accumulation of Mn in leaves over time (Fig. 7). This partially explained the synergic environment for Mn allocation in leaves when in the presence of twice the recommended dose of B in the mix. The opposite was seen in the accumulation of B over time in the leaves. Quadruple the recommended dose of Mn and B showed leaf B concentrations more than 50% of the current Florida guidelines regarding the optimal range for B (200 mg·kg^{-1}) (Fig. 8). Nutrient accumulation from Mar. 2017 to May 2018 and utilization in new meristematic tissues could be the reason why there were high values of B in leaves. Mn could have a synergic effect when used in higher

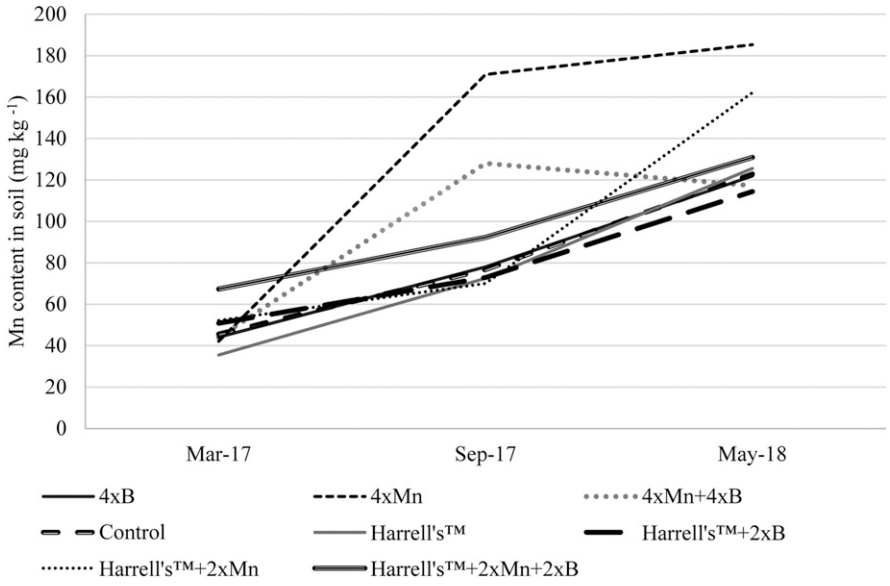

Fig. 6. Interaction plot of months and treatment for manganese content in soil. Statistically significant mean totals are presented. Means separation within months*treatment were determined using Tukey's honestly significant difference test ($P \leq 0.05$).

doses to increase the allocation of B in leaves and when B is present in the fertilizer mix because the quadruple Mn treatment alone had B concentrations within the limits for B content in leaves.

Physiologically, boron has a role in tissue growth and is necessary for vascular tissue repair, which is compromised in *C*Las-infected plants, membrane stability, and metabolism of indole acetic acid, which is a plant hormone responsible for cell multiplication (Blevins, 1998). When B was deficient in squash root apices, Mn-dependent indole-3-acetic acid oxidase (IAAO) activity was higher compared with that of well-supplied B root tips. Higher IAAO activity might be due to the interaction between B with the IAAO cofactors Mn and p-coumaric acid. Lower activity of IAAO was observed in B-sufficient plants. By supplementing Mn in B-sufficient squash root tips, IAAO activity was stimulated, which was an indication that, in presence of B more Mn is needed for enzyme activity (Nguyen et al., 1993).

Compared with other nutrition during the same month, statistically lower concentrations of B were found in March and September with quadruple Mn treatment, indicating an antagonistic effect between B and Mn ions for allocation inside the cell and uses by the plant (Aref, 2012). It is important to notice that quadruple Mn treatment results in the significantly lowest values for B content in leaves for all the months, which is crucial for normal meristematic growth (Blevins and Lukaszewski, 1998).

Juice attributes and yield data. Juice attributes were not significant across all treatments (Table 6). This could be due to an effect of overlapping root zones from neighboring trees with different treatments; therefore, the effect of nutrients on juice quality was negligible (Castle, 1980, 1977). No differences in juice quality were found in a similar nutrition study involving asymptomatic and symptomatic 'Hamlin', 'Midsweet', and 'Valencia' juices analyzed in 2007 (Baldwin et al., 2010).

A breakout of post-bloom fruit drop caused by *Colletotrichum acutatum* during fruiting formation in the 2017 season resulted in decreased yield data across all treatments for that year (Fagan, 1979). Although yield data from three seasons (2016, 2017, and 2018) did not have statistically significant 95% confidence intervals among treatments (Table 7), 'Vernia' trees that received four-times the recommended doses of Mn exhibited lower Ct values compared with the control group (Table 8). During the 2-year trial period, the 12 trees included in the quadruple Mn treatment group produced more than 10 total field boxes compared with the control trees. A field box (40.8 kg/fruit box) of fruit with this quality is currently worth ≈$17 (USDA-NASS 2017). Extrapolating this to a per-acre basis at 150 trees per acre would mean that the quadruple Mn treatment would have the potential to provide more than $2500 extra income per acre as compared with the control during the 2-year trial period.

Soil pH. Nutrient solubility and availability are closely related to soil pH. Acidification can interfere with oxidation/reduction processes on soil colloids. In an experiment involving six vegetable species, pH values ranging from 5.5 to 6.5 were ideal for maximum or near-maximum growth (Islam et al., 1980). Florida's irrigation water is naturally alkaline (pH >7.0) and has high levels of carbonates and bicarbonates of Ca and Mg. Constant applications of alkaline groundwater sources can increase soil pH over time, thus altering the availability/solubility of nutrients, especially micronutrients, and directly affecting plant growth (Albano et al., 2017). To neutralize groundwater alkalinity, Albano et al. (2017) added sulfuric acid to levels of medium and low alkalinity (3 mEq·L^{-1} $CaCO_3$ and 1 mEq·L^{-1} $CaCO_3$, respectively). During our experiment, acidification was achieved by the dissociation of $MnSO_4$ in the soil. The increase in sulfur content in the soil in May 2018 could be observed in all treatments supplied with Mn (Table 4), especially quadruple Mn treatment, by adjusting the pH values to the best range for nutrient availability (Fig. 9). All treatments with sodium borate involved less than the best pH for citrus roots in May because the formation of boric acid was increased by the excess boron accumulated in the soil. When in combination with $MnSO_4$, the pH decreased even more due to the presence of $(SO_4)^{-2}$ in solution.

HLB confirmation. Using qPCR, *C*Las detection was performed in Nov. 2017 (Table 8) for all treatments at the Southern Gardens Laboratory. Plants supplied with control nutrition had the highest Ct values, meaning that more copies of the bacteria were amplified during each cycle (Li et al., 2006; Wang et al., 2006). Plants that received quadruple Mn treatment showed the highest average Ct value, which was significantly different from plants that received the control treatment. Therefore, higher Ct values indicated lower genomic copies of the bacteria, suggesting that an overdose of Mn may limit bacterial growth within trees. Applications of four-times the recommended dose of Mn suggested a decrease of inoculum growth and that Mn could have a role in plant recovery from biotic stress and root damage because it is crucial for enzymatic stress–related processes (Millaleo et al., 2010). High Ct values can be interpreted as a therapeutic effect against *C*Las in sweet oranges when fertilized with quadruple Mn because of the physiological roles played by Mn and B interactions (Tables 4 and 5) in analyzed soil and leaf samples.

Although the accumulation of Mn in soil during Sept. 2017 and May 2018 could be the reason for the therapeutic effect of the nutrient in HLB-affected trees during the full growth season, the accumulation of B in leaves with the quadruple Mn treatment was in the range of optimum levels of the mineral element in citrus, which is now reached with regular foliar sprays.

Root decline happens before any HLB symptom in the aerial part of the trees. Moreover, once infected, the plant quickly proceeds to tissue dieback. By supplying mature plants with ground overdoses of micronutrients, we could reduce the inoculum multiplication compared with nutrient-deprived plants when all the essential mineral nutrients were balanced in the optimum range for a mature citrus plant because absolute yield data can prove the usefulness of quadruple Mn treatment. As mentioned, all trees in this trial were on rough lemon rootstock. Ungrafted rough lemon trees have been

Table 5. Leaf nutrient content for Mar. 2017, Sept. 2017, and May 2018.

Month (F1)	Nutrient (mg·kg^{-1})	Treatment (F2) 4× Mn + 4× B	4× B	4× Mn	Harrell's®	Harrell's® + 2× B	Harrell's® + 2× Mn	Harrell's® + 2× Mn + 2× B	Control
Mar. 2017	N	2.8 A[z] a[y]	2.9 A a	2.9 A a	2.7 A a	2.6 A a	2.8 A a	2.6 A a	2.6 A a
Sept. 2017		2.5 A a	2.4 B a	2.4 B a	2.4 B a	2.3 A a	2.4 A a	2.5 A a	2.4 A a
May 2018		2.5 A a	2.5 B a	2.8 A a	2.6 AB a	2.6 A a	2.6 A a	2.5 A a	2.4 A a
Total avg		2.575	2.575	2.725	2.575	2.525	2.6	2.525	2.45
Mar. 2017	P	0.2 A ab	0.2 A a	0.2 A ab	0.1 A b	0.1 A b	0.2 A ab	0.2 A ab	0.2 A ab
Sept. 2017		0.1 AB a	0.1 B a	0.1 A a	0.1 A a	0.1 A a	0.1 A a	0.1 A a	0.2 A a
May 2018		0.1 B a	0.1 B a	0.1 A a	0.1 A a	0.1 A a	0.1 A a	0.1 A a	0.1 A a
Total avg		0.125	0.125	0.125	0.1	0.1	0.125	0.125	0.15
Mar. 2017	K	1.3 A a	1.5 A a	1.3 A a	1.3 A a	1.1 A a	1.3 A a	1.2 A a	1.3 A a
Sept. 2017		1.4 A a	1.4 A a	1.2 A a	1.1 A a	1.1 A a	1.3 A a	1.3 A a	1.4 A a
May 2018		1.3 A a	1.3 A a	1.4 A a	1.3 A a	1.3 A a	1.3 A a	1.3 A a	1.2 A a
Total avg		1.325	1.375	1.325	1.25	1.2	1.3	1.275	1.275
Mar. 2017	Mg	0.3 B a	0.3 B a	0.3 B a	0.3 B a	0.3 A a	0.3 B a	0.3 B a	0.3 B a
Sept. 2017		0.4 A a	0.4 A a	0.5 A a	0.4 AB a	0.4 A a	0.4 A a	0.4 A a	0.5 A a
May 2018		0.4 A a	0.4 A a	0.4 AB a	0.4 A a	0.4 A a	0.3 A a	0.4 A a	0.4 AB a
Total avg		0.375	0.375	0.4	0.375	0.375	0.325	0.375	0.4
Mar. 2017	Ca	2.6 B a	2.5 B a	2.8 B a	2.8 A a	2.9 B a	2.9 B a	3.1 B a	2.8 C a
Sept. 2017		4.5 A a	3.9 A a	4.2 A a	4.0 A a	4.2 A a	4.1 A a	4.4 A a	4.5 A a
May 2018		4.1 A a	3.8 A a	3.8 A a	3.5 A a	3.7 AB a	3.5 AB a	3.8 AB a	3.8 B a
Total avg		3.825	3.5	3.65	3.45	3.625	3.5	3.775	3.725
Mar. 2017	S	-	-	-	-	-	-	-	-
Sept. 2017		0.6 A ab	0.5 A ab	0.6 A a	0.5 A ab	0.4 B b	0.5 A ab	0.5 A ab	0.5 A ab
May 2018		0.6 A a	0.5 A a	0.6 A a	0.5 A a	0.5 A a	0.6 A a	0.6 A a	0.6 A a
Total avg		0.6	0.5	0.6	0.5	0.45	0.55	0.55	0.55
Mar. 2017	Zn	8.3 B a	6.1 C a	7.8 C a	7.0 C a	5.0 B a	8.6 B a	8.0 B a	3.6 C a
Sept. 2017		13.3 B a	15.0 B a	17.8 B a	15.8 B a	12.7 B a	18.0 B a	17.8 B a	15.3 B a
May 2018		28.1 A a	25.8 A a	27.7 A a	33.6 A a	32.6 A a	31.5 A a	35.0 A a	24.8 A a
Total avg		19.45	18.175	20.25	22.5	20.725	22.4	23.95	17.125
Mar. 2017	Fe	27.2 B a	28.9 B a	44.7 A a	43.4 B a	48.4 B a	49.4 B a	47.5 B a	31.5 B a
Sept. 2017		72.3 A a	67.3 A a	76.5 A a	79. 8 A a	86.8 A a	88.5 A a	85.3 A a	71.3 A a
May 2018		60.0 A a	61.0 A a	84.3 A a	65.7 A a	60.5 AB a	59.4 B a	61.1 B a	51.8 AB a
Total avg		54.875	54.55	72.45	63.65	64.05	64.175	63.75	51.6
Mar. 2017	Cu	-	-	-	-	-	-	-	-
Sept. 2017		19.7 A a	18.3 A a	20 A a	17.8 A a	17.5 A a	18.5 A a	18.8 A a	18.3 A a
May 2018		19.7 A a	19.3 A a	20.3 A a	18.4 A a	19.5 A a	19.2 A a	18 A a	19.1 A a
Total avg		19.7	18.8	20.15	18.1	18.5	18.85	18.4	18.7
Mar. 2017	Mn	6.7 B ab	10.2 B ab	20.1 B ab	10.3 B ab	14 B ab	25.2 B a	23 B ab	5.9 B b
Sept. 2017		34.3 A b	44.5 AB ab	63 A ab	45.8 A ab	51.5 A ab	89.8 A a	91.8 A a	37 A b
May 2018		43.3 A ab	30.7 A b	48.8 AB ab	54.2 A ab	75 A a	77.5 A a	62.2 AB ab	26.5 A b
Total avg		31.9	29.025	45.175	41.125	53.875	67.5	59.8	23.375
Mar. 2017	B	85.3 B a	64.4 B ab	52.4 B b	73.9 B ab	83.1 B ab	82 C ab	83.8 C ab	57.3 C ab
Sept. 2017		331 A a	270 A ab	142 A d	178.5 A cd	228.3 A bc	181.3 A cd	267.5 A ab	161.3 A cd
May 2018		311.9 A a	306.9 A a	115.6 A c	147.8 A bc	214.0 A b	126.0 B c	197.0 B bc	131.6 c B
Total avg		260.03	237.05	106.4	137	184.85	128.825	186.325	120.45

[z]Means separation between months according to Tukey's honestly significant difference test ($P \leq 0.05$; uppercase).
[y]Means separation within months according to Tukey's honestly significant difference test ($P \leq 0.05$; lowercase).

shown to be more tolerant of *C*Las infection, which is attributed to its ability to quickly regenerate new phloem that bypasses phloem compromised by the infection (Fan et al., 2013). Data from our field experiment suggested that enhanced root nutrition may increase the HLB tolerance of sweet orange trees grafted to rough lemon rootstock, making it a viable candidate for future plantings in areas where HLB is endemic. Testing the effects of micronutrient overdoses (especially Mn) on HLB-impacted trees on other important commercial rootstocks is being performed.

To our knowledge, this is the first study to connect ground applications of overdoses of a micronutrient to suppress *C*Las multiplication and to improve the nutritional status and health status of HLB-affected citrus trees. A constant supply of increased levels of secondary nutrients and micronutrients to roots of HLB-impacted trees clearly improved vascular function and, thus, tree health. The data presented herein suggest the possible benefits of providing levels of Mn to HLB-impacted citrus trees that could be toxic to *C*Las but that were not toxic to trees. We are now testing overdoses of polycoated Mn sulfate (6-month release period, Florikan) with various secondary nutrient and micronutrient packages at multiple locations. Further studies regarding the correlation between Mn and B in stress-related genes and nutrient uptake channels in the roots could elucidate the roles played by the two essential micronutrients in the era of HLB and citriculture production.

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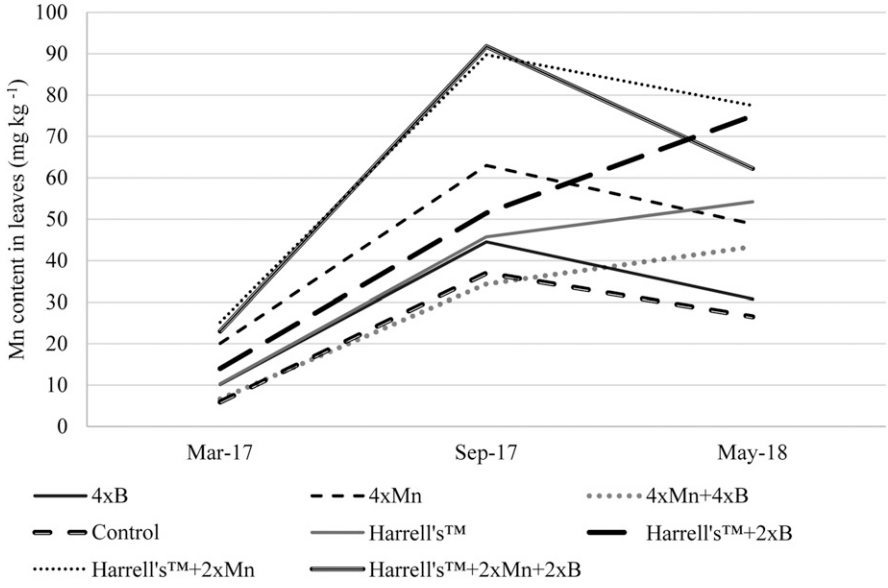

Fig. 7. Manganese content in leaves during Mar. 2017, Sept. 2017, and May 2018. Statistically significant mean totals are presented. Means separation within months were determined using Tukey's honestly significant difference test ($P \leq 0.05$).

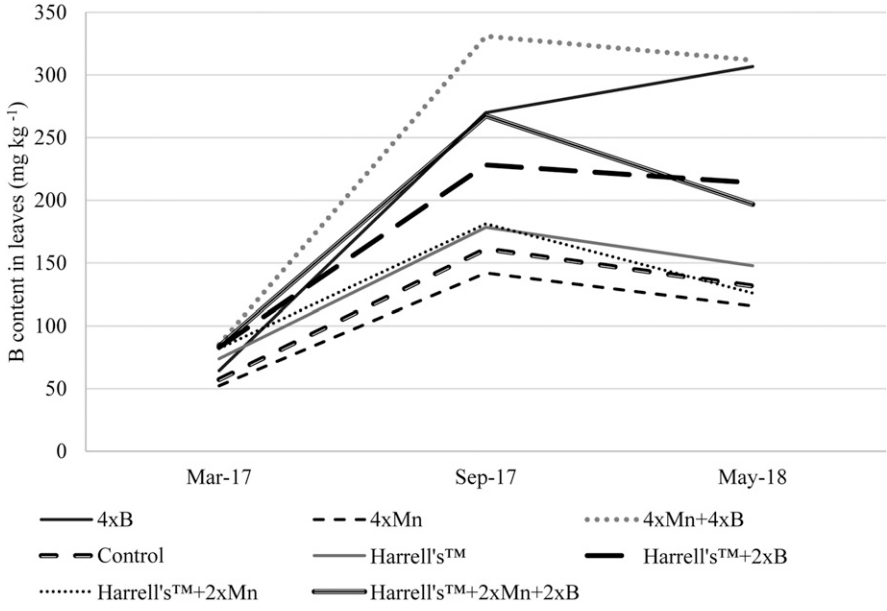

Fig. 8. Boron content in leaves during Mar. 2017, Sept. 2017, and May 2018. Statistically significant mean totals are presented. Means separation within months*treatment were determined using Tukey's honestly significant difference test ($P \leq 0.05$).

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Table 6. Juice attributes for fruits harvested in Jan. 2018.

Treatment	Sample wt (kg)	Juice wt (kg)	Juice per box[z] (kg)	Acidity	Total brix	Ratio	Soluble solutes (kg)	Juice color
Harrell's® + 2× Mn	12.02	7.04	23.95	0.71	12.14	17.10	2.91	38.15
Control	11.93	6.90	23.65	0.71	12.26	17.28	2.90	37.6
4× Mn	11.34	6.53	23.52	0.76	12.51	16.58	2.94	37.95
Harrell's® + 2× Mn + 2× B	11.29	6.62	23.96	0.71	12.05	16.98	2.88	38.05
Harrell's® + 2× B	11.11	6.40	23.56	0.75	12.42	16.56	2.93	37.9
4× B	10.93	6.33	23.64	0.73	12.31	16.87	2.91	38
4× Mn + 4× B	10.89	6.28	23.57	0.74	12.18	16.58	2.87	37.7
Harrell's®	10.80	6.30	23.84	0.70	12.70	18.16	3.03	38.45
P value	0.957	0.924	0.788	0.625	0.80	0.25	0.838	0.535

[z]A field box contains 40.82 kg of fruit.

Table 7. Yield accumulation of 'Vernia' sweet oranges for the 2015, 2016, and 2017 seasons.

Treatment	Yield per yr (boxes[z] per tree) 2016	2017[y]	2018	Cumulative[x]	*P* value Treatment
Control	1.67	0.56	1.71	27.2	NS
Harrell's®	1.5	1.02	1.75	33.2	NS
Harrell's® + 2× Mn	1.5	0.83	1.54	28.4	NS
Harrell's® + 2× B	1.92	0.83	1.71	30.5	NS
Harrell's® + 2× Mn + 2× B	1.5	0.94	1.71	31.8	NS
4× Mn	1.75	0.92	2.21	37.6	NS
4× B	1.58	0.44	1.63	24.8	NS
4× Mn + 4× B	1.5	0.9	1.79	32.3	NS
P value	NS	NS	NS	NS	

[z]A field box contains 40.82 kg of fruit.
[y]2017 yields were significantly reduced by a severe PFD (post-bloom fruit drop disease caused by *Colletotrichum acutatum*) infection.
[x]Cumulative column provides the total number of boxes of fruit per 12 trees receiving each treatment produced during the two seasons after treatments were implemented.
NS indicates not significant.

Table 8. Ct values of *C*Las detection in leaves in Nov. 2017.

Treatment	Ct value[z]
4× Mn	32.7 a
Harrell's® + 2× Mn + 2× B	30.3 ab
Harrell's® + 2× B	29.5 abc
Harrell's®	29.3 abc
4× B	28.1 abc
Harrell's® + 2× Mn	27.6 abc
4× Mn + 4× B	23.8 bc
Control	23.2 c

[z]Means separation by Tukey's honestly significant difference test ($P \leq 0.05$).

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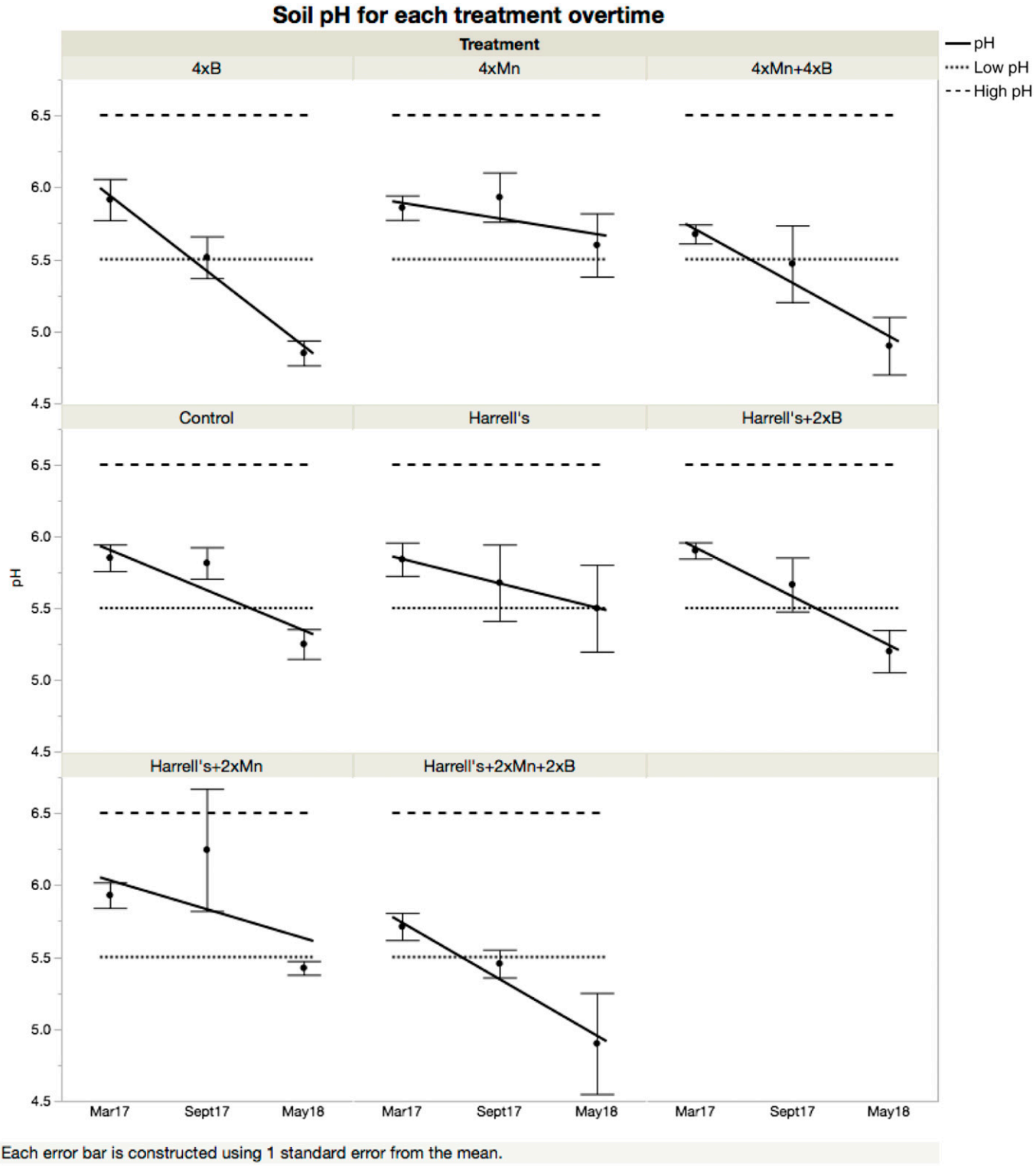

Fig. 9. Mean pH (n = 12) for all treatments during Mar. 2017, Sept. 2017, and May 2018. Horizontal lines correspond to the best pH range for nutrient absorption (high and low: 6.5 and 5.5, respectively).

Supplemental Table 1. Fertilizer and psyllid control spray schedule for the 2016, 2017, and 2018 seasons

Dry soluble fertilizer		
Application date	Chemical composition	Dose (kg·ha^{-1})
Oct. 2015	12–0–15 (0.25 Mn)	415
Feb. 2016	18–0–18	269
May 2016	15–0–15 (1.23 Mn + 0.075 B)	426
Nov. 2016	15–0–17 (0.5 Mn + 0.5 B)	352
Feb. 2017	11–1–11 (1.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	725
May 2017	11–1–11 (1.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	738
Oct. 2017	11–1–11 (1.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	367
Feb. 2018	11–1–11 (2.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	514
June 2018	11–1–11 (2.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	573
Sept. 2018	11–1–11 (2.0 Mn + 0.06 B + 2.5 Mg + 0.15 Fe)	449

Spray (psyllid control), recommended dose	
Application date	Mix composition
Oct. 2015	Mustang + K Nitrate + 435 oil
Apr. 2015	Post bloom spray (Mustang + humectant + 435 oil)
June 2017	Actara + humectant + 435 oil
June 2017	Mustang + humectant + 435 oil
June 2018	Mustang + humectant + 435 oil

Supplemental Table 2. Harrell's® St. Helena Mix formulation (12–3–9)

Nutrient	Percent
Total Nitrogen	12.0000%
Nitrate Nitrogen*	6.8289%
Ammoniacal Nitrogen*	4.3620%
Urea Nitrogen*	0.8100%
Available Phosphate (P_2O_5)*	3.0000%
Soluble Potash (K_2O)*	9.0000%
Calcium (Ca)	4.5270%
Magnesium (Mg)	0.7920%
0.792% Water-Soluble Magnesium (Mg)	
Boron (B)	0.0750%
Iron (Fe)	1.0880%
0.088% Water-soluble iron (Fe)	
0.32% Chelated Iron (Fe)	
Manganese (Mn)	0.9200%
0.065% Water-Soluble Manganese (Mn)	
Molybdenum (Mo)	0.0060%
Zinc (Zn)	0.7130%
0.038% Water-soluble zinc (Zn)	
Derived From: Poly-Mercoated Ammonium Nitrate, Polymer-Coated Calcium Nitrate, Polymer-Coated Monoammonium Phosphate, Polymer-Coated Murlate of Potash, Polymer-Coated Sulfate of Potash, Polymer-Coated Urea, Calcium Borate, Ferric Oxide, Ferrous Sulfate, Iron EDTA, Iron Humate, Iron Sucrate, Manganese Oxide, Polymer-Coated Iron EDTA, Polymer-Coated Magnesium Sulfate, Polymer-Coated Manganese Sulfate, Polymer-Coated Sodium Molybdate, Polymer-Coated Zinc Sulfate, Sodium Borate, Sulfate of Potash-Magnesia, Zinc Oxide	
12% coated slow-release Nitrogen (N), 3% coated slow-release available Phosphate (P_2O_5), and 8.712% coated slow-release Soluble Potash (K_2O)	
Chlorine (Cl), no more than	2.2490%