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Inconsequential effect of nutritional treatments on huanglongbing control, fruit quality, bacterial titer and disease progress☆

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ABSTRACT

The use of an enhanced nutritional programs (ENPs) to minimize the deleterious effects of the vector transmitted bacterial disease, citrus huanglongbing (HLB) caused by *Candidatus* Liberibacter asiaticus (*Las*), has been a topic of considerable discussion and debate since the discovery of HLB in Florida. Most reports of the putative effects of ENPs are either anecdotal or based on non-replicated trials lacking non-treated controls or proper experimental design and analysis with sufficient statistical rigor. Even so, Florida citrus producers use this unproven and non-validated approach for HLB management *in lieu* of conventional integrated control of inoculum which includes rouging symptomatic trees to reduce inoculum and vector control using insecticide. The formulation of the ENPs varies considerably, but usually consists of foliar applications of standard essential micronutrients, salts of phosphite, and in some programs, salicylate salts. Two field trials were conducted on Valencia sweet orange [*Citrus sinensis* (L.) Osbeck] to test efficacy of widely used ENPs. The first trial consisting of a randomized complete block design with 3 blocks and 4 replicate trees/block was conducted from 2008 to 2010. All trees were PCR+ for *Las* at the onset of the trial, but exhibited only mild HLB symptoms. This stage of infection was chosen based on claims that the ENPs maintain the health and productivity of HLB-infected trees, thereby extending the orchard's commercial viability. Combinations of components were compared with a control consisting of a standard fertilization and control program for psyllids. Additional treatments consisted of phosphite with Mn-carbonate, Mn-metalosate, Cu-metalosate, or Zn-metalosate, and injection treatments using soluble copper or silver mixed with a polymer. After two seasons of three applications each, there were no significant differences in bacterial titer dynamics, fruit yield (number of fruit/tree, kg fruit/tree, proportion of fruit dropped), or juice quality (Brix, acid, Brix:acid ratio) between treated trees and non-treated control trees. In a second trial of six commercial citrus blocks containing 40,885 trees wherein enhanced vector control and rouging of diseased trees was practiced, the ENP in three blocks was compared to conventional fertilization in three blocks. In this commercial trial, yields, disease progress, and epidemic dynamics did not differ between the ENP and conventional fertilization treatments. Results of the large commercial trial corroborated the experimental results of the first trial with more diverse micronutrient treatments. Considering both trials together, the ENP did not sustain tree health, yield, or fruit quality of *Las*-infected HLB-symptomatic trees. Moreover, since the nutritional supplements had no effect on *Las* titer, a major concern is that existing ENP strategies have promoted area-wide buildup of inoculum and increased disease spread within and between citrus orchards.
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1. Introduction

Citrus huanglongbing (HLB) is the most destructive disease of citrus known. The disease has resulted in the decline and/or death of millions of trees in citrus producing areas worldwide (Bové, 2006; da Graça, 1991; Gottwald et al., 2007; Gottwald, 2010). Since the 2006 discovery of the disease in the Western Hemisphere, and its vector *Diaphorina citri* (the Asian citrus psyllid) in 2004 (Halbert and Manjunath, 2004; Manjunath et al., 2008), HLB has spread across

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most of the largest citrus growing regions in Florida, U.S.A., and the states of São Paulo, Minas Gerais, and Paraná in Brazil (Teixeira et al., 2008). HLB was most recently confirmed from the Rio Grande citrus area of Texas in January 2012. HLB has also spread throughout the Caribbean and Central America. Its recently recognized presence in Mexico threatens citrus producing areas in California, and Arizona (Bové, 2006; Gottwald et al., 2007; Gottwald, 2010). In South America, the large epidemic centered in the states of São Paulo and Parana, Brazil, threatens the citrus industries of adjacent and nearby countries including Argentina, Uruguay, and Paraguay. In the Western Hemisphere, the disease is caused primarily by the bacterium, *Candidatus* Liberibacter asiaticus (*Las*) and to a small extent by *Ca*. Liberibacter americanus, in the state of São Paulo, Brazil (Bové et al., 2008; Lopes et al., 2005). Both bacteria are vectored by the Asian citrus psyllid. Liberibacter may not have come in contact with citrus until the late 1800s or early 1900s, probably near India or Southeast Asia (Beattie et al., 2008). It also appears that the bacterium may have originated as an insect parasite/endophyte and recently jumped to citrus. Because of the relatively recent contact between host and pathogen, they do not appear to have coevolved, and thus there is no resistance to *Las* within the major commercial citrus cultivars (Folimonova et al., 2009).

In Florida, the commercial citrus industry currently comprises about 222,000 ha (550,000 acres) and over 60 million trees. Since HLB was first detected in South Florida, the pathogen has spread across the entire citrus growing area of the state. An estimated 18% of the 60 million orange trees are currently infected (Irey et al., 2011). This rate of disease increase and spread is unprecedented for an arboreal pathosystem. HLB causes a vascular dysfunction that results in lack of fruit set, premature fruit drop and quality changes of citrus juice extracted from diseased fruit (da Graça, 1991; Gottwald et al., 2007; Bassanezi et al., 2009, 2011).

Internationally accepted control strategies for HLB are an outgrowth of the United Nations Development Program, Food and Agriculture Organization (UNDP, FAO) Southeastern Asian citrus rehabilitation project (Aubert, 1990). The integrated control program in commercial orchards consists of controlling psyllid vectors with insecticides, removal of HLB-symptomatic trees to reduce inoculum, geographical isolation and nursery certification for propagation of pathogen-free budwood sources and nursery trees. Citrus phytosanitary agencies in Florida and Brazil, require that all nursery production of citrus trees be conducted in secure insect-proof screen houses. In addition, recent studies in Brazil and Florida have concluded that area-wide insect control, over large areas consisting of thousands of hectares, can reduce vector populations to the point where the disease can be managed (Bassanezi et al., 2011). However, in all commercial production areas throughout the world (with the exception of a few that are under intense area-wide vector management), disease control is insufficient and HLB continues to spread and increase (Gottwald, 2010). Genetic transformation of the citrus hosts, the bacterial pathogen, and/or the psyllid vector shows great promise for the development of disease-resistant cultivars and/or amelioration of the bacterial pathogen and insect vector. However, genetic solutions will be several years in the future (Duan et al., 2009). Meanwhile the disease continues to increase and spread at a devastating rate (Gottwald, 2010).

This has led commercial citrus producers to seek any possible means to ameliorate the effects of the disease.

Recently, a commercial citrus producer in South Florida has reported that foliar sprays of various nutritional products, including micronutrients, extended the vigor and productivity of HLB-infected trees (Giles, 2011). Although no replicated trials of this nutritional regime have been completed nor has the effect of an enhanced nutritional program (ENP) on health of HLB-infected trees been quantified, a significant proportion of the Florida citrus industry has moved rapidly toward the use of ENPs and away from traditional methods of disease control using tree removal to reduce pathogen inoculum and aggressive vector control (Spann et al., 2011). However, in China the effect of various fertilization methods and some micronutrients on HLB was examined soon after the disease was first discovered but no significant effects were found (Chen, 1937, 1938; 1940, 1941). In addition, a recent review of the breadth of Chinese research on nutrient and micronutrient effects on HLB came to the conclusion that overall the historical Chinese literature does not present consistent evidence to support the nutritional approach as an effective means of maintaining citrus productivity or slowing disease progress in HLB-infected plants (Xia et al., 2011).

The goals of the present study were to determine: 1) the effects of an ENP on within-tree bacterial titer, yield, fruit drop, and fruit quality parameters; 2) the potential for various metallic ion bactericidal compounds to affect the dynamics of *Las* titer; 3) the effect of an ENP on HLB progression and fruit production in commercial plantings.

2. Materials and methods

2.1. Effect of micronutrients on yield and quality

2.1.1. Field plot design

The trial was conducted in a block of 6-yr-old (at the beginning of the study) Valencia sweet orange trees [*Citrus sinensis* (L.) Osbeck] scion grafted on Swingle citrumelo [*Poncirus trifoliata* (L.) Raf. × *Citrus paradisi* Macf.] rootstock in Martin County, Florida. The experimental design was a randomized complete block with 10 treatments, each consisting of 12 trees divided into three blocks of four trees per treatment. Treatment applications were made every 45–60 days when flush was present starting with the spring flush in April 2008. The premise for the use of micronutrient treatments is that HLB-affected orchards can be maintained in a productive state for a number of years; thus prolonging commercial viability. Individual trees were therefore chosen for inclusion in the experiment based upon the presence of the initial symptoms of HLB. An attempt was made to select trees in the same stage of HLB expression; i.e., initial symptoms in only one or two branches of the tree, i.e., less that 5% of the canopy, suggesting that the infection is likely not completely systemic. All trees included in the experiment were confirmed to be *Las*-positive via a real-time quantitative polymerase chain reaction (qPCR) assay (Li et al., 2006). Soon after beginning the experiment, the commercial collaborator determined that the majority of trees in the plantation where *Las*-positive. Therefore, insecticide sprays for control of Asian citrus psyllid were discontinued. For the remainder of the trial, minimal commercial citrus insecticide, fertilizer, and herbicide applications were applied to the entire plantation. However, trees within the trial area were maintained at commercial standards to allow for full expression of treatment effects.

2.1.2. Micronutrient treatments

Individual treatments were applied with a hand gun sprayer until runoff to ensure complete coverage as follows:

1. Non-treated control, i.e., a standard commercial citrus fertilization program consisting of 201.6 kg N/ha in 3 applications of 21–0–21 N–P–K, Griffin Fertilize Co., Frostproof Florida, USA, without micronutrients plus a minimal pest and weed control program. All subsequent treatments received the same cultural management program and the supplements as follows.
2. Potassium (K) phosphite (0–28–26 N–P–K) fertilizer; K-Phite®, Plant Food Systems, Zellwood, Florida, USA) first application at 4.7 L/ha and subsequent applications 2.35 L/ha.
3. K phosphite as in treatment 2 plus wettable powder nutrients, Mg 6.3–9.5 kg/ha, Mn 6.3 kg/ha, Zn 3.17 kg/ha, Mo 58.6 g/ha

mixed in water at 2642 L/ha, (Diamond R, Fort Pierce, Florida, USA) Applied three times yearly on a calendar basis, with the exception of Zn applied 4 times yearly.

4. K phosphite as in treatment 2, wettable powder nutrients as in treatment 3, plus SAVER® (Plant Food Systems, containing a salicylate salt for systemic acquired resistance activity) at 1.17 L/ha.
5. K phosphite as in treatment 2, plus Mn^{++} metalosate (Albion Laboratories, Clearfield, Utah, USA) at 3.5 L/ha plus a citrus surfactant (Diamond R SA, 2002 spray adjuvant, nonylphenol polyethylene glycol ether, alcohol phosphatic inorganic chelating agents, and alkotyl alcohol 47%, Diamond R Fertilizer Co., Fort Pierce, Florida, USA) at 5 L/ha.
6. K phosphite as in treatment 2, plus Mn^{++} carbonate at 0.45 kg/tree plus citrus surfactant, applied at the base of each tree trunk.
7. K phosphite as in treatment 2, plus Cu^{++} metalosate at 0.58 L/ha plus citrus surfactant.
8. K phosphite as in treatment 2, plus Zn^{++} metalosate at 3.5 L/ha plus citrus surfactant.
9. Magna-Bon® (copper sulfate pentahydrate, Magna-Bon, LLC, Okeechobee, Florida, USA) at 60 ppm/5 ml/tree via injection per month.
10. Silver PDS (polymer delivery system; Smart Anti-microbial Solutions, LLC) formulation via injection at 0.005 g of silver per ml of PDS per volume of carrier material ~0.005% silver.

2.1.3. Confirmation of Las infection via PCR

Trees were assayed by detaching two to four leaves with petioles; leaves with mottling symptoms were chosen if available for quantification of *Las*. For each tree, a combined sample of 100–180 mg of mid-rib was excised prior for DNA extraction (Irey et al., 2006). Following extraction, DNA was quantified with a nano-drop spectrophotometer (Thermo Scientific, Nanodrop Products, Wilmington, Delaware, USA) and adjusted to 25 ng/ul with nuclease-free sterile water. The PCR primer sequences used for all analyses were as described by Li (Li et al., 2006; Irey et al., 2006). The quantitative TaqMan PCR method used 16S rDNA-based primer-probe sets specific to *Las*. The primer/probe system employed was labeled with NED/MGB; i.e., uses a TaqMan MGB (minor groove binding) probe, which incorporates a 5′ reporter dye and a 3′ non-fluorescent quencher (Applied Biosystems, Foster City, California, USA). The reporter dye used was NED. This system was optimal for a real-time qPCR instrument with a *FAST* platform (Applied Biosystems, Foster City, California). Invitrogen Express qPCR Master Mix was used with the addition of the primers, probe, nuclease-free water and PVP (polyvinylpyrrolidone). The primers were used at a concentration of 0.3 uM, the probe at 0.15 uM and the PVP (added to bind PCR reaction inhibitors) at 25 mg/ml mix. The total volume of the reaction mixture was 20 ul.

Cycle parameters for qPCR were: 95 C for 20 s, followed by 40 cycles of 95 C for 30 s and 60 C for 30 s. The baseline was automatically determined by the ABI software (Applied Biosystems, Foster City, California). The baseline was set above the fluorescent background signals created by the PCR reaction in the early cycles, before the target amplification if sufficiently above the background signal. The threshold was automatically assigned by the analysis software. This threshold is the numerical value that represents a statistically significant point above the calculated baseline. The point at which the target DNA in each sample well reaches this threshold is the threshold cycle (Ct) value. Lower Ct values indicate higher initial template in the well. If sample DNA contains *Las* DNA, amplicon will be created in the PCR reaction and the amount will be reflected in the Ct value. To test for *Las* infection, a Ct of 32 or less was considered positive for detection. The range between 32 and 36 Ct was considered putative positive, and trees testing in this range normally resulted in lower Ct values in subsequent assays.

2.1.4. Real-time PCR using a reference gene to estimate Las titer

To estimate the change in *Las* bacterial titer over time, a reference gene, dehydrin (a nuclear gene present in sweet orange hybrids), was assayed simultaneously with *Las* 16s rDNA and the ratio of *Las* 16s rDNA to dehydrin titers used to define titer changed between sampling periods. Prior to use, qPCR was performed to verify that the primer/probe system chosen for the dehydrin gene would provide consistent performance and that the amplification over a wide range of starting DNA will result in linear amplification. This was accomplished by taking the PCR product from the amplification of the reference gene, inserting it onto a plasmid, and a standard curve generated with concentrations ranging from 10^{-8} to 10^{-13} g to assure that amplification was linear and reliable. Real-time PCR was performed as described below to obtain Ct values.

PCR master mixes were made for each primer/probe set (*Las* Li primers with NED/MGB probe and citrus dehydrin primers with FAM/BHQ1 TaqMan probe). The sequence for the dehydrin primer system was designed using Primer Express software (Life Technologies, Applied Biosystems, Foster City, California, USA). Invitrogen Express qPCR Master Mix (Invitrogen, Foster City, California, USA) was used on the ABI Real-time instrument with the FAST 7500 platform using a total volume of 20 μl/well. Cycle parameters: 95 C for 20 s followed by 40 cycles of 95 C for 3 s followed by 60 C for 30 s. Each sample was run against each primer in duplicate.

The Ct value from each sample was relevant only to its corresponding dehydrin reference gene Ct value when using a reference gene for normalization of data. Using a nano-drop to quantify the DNA concentrations is not highly accurate; i.e., each sample was approximated to be in a range of the desired concentration. Therefore, each individual sample will provide an accurate Ct count for both the HLB primer and the reference gene primer for that particular concentration for that sample, accounting for some variation in the Ct values for the reference gene (~2 Ct range is acceptable).

Duplicate Ct values for each primer were averaged and a ratio (*Las*:dehydrin) calculated for each sample. The ratio of the reference gene Ct divided by the *Las* Ct was calculated for each sample. Change in titer was calculated after two or more sample collections according to the following equation:

$$\%\ \text{change in Las titer} = \frac{(\text{mean Ct ratio 2nd sampling} - \text{mean Ct ratio 1st sampling})}{(\text{mean Ct ratio 1st sampling})} \times 100$$

2.1.5. Yield and fruit quality parameter measurements

For each tree in the micronutrient treatment trial, total number of fruit, proportion of fruit drop, total yield (kg/tree), and fruit volume (cm^3) were assessed each year. Representative fruit samples (30 fruit per sample) were collected from individual trees. To calculate fruit volume, fruit height (stem scar to blossom scar) was measured once and fruit diameter was measured twice (90° angles at the fruit equator) for each fruit and used to calculate

volume of a spheroid. Peel color was quantified using a Minolta Chroma Meter (CR-400, Konica Minolta, Ramsey, New Jersey, USA) measuring a* and b* values for red and green color, respectively. Total fruit weight was determined via a mass balance. Fruit were juiced by hand using a Sunkist Commercial Juicer J-01 (Sunkist Growers, Inc., Sherman Oaks, California, USA). Percentage juice was calculated as follows: (juice wt/total wt) × 100.

Juice quality was determined following standard methods using a composite sample from 30 fruit chosen arbitrarily from trees within each replicate, without regard to fruit symptoms to ensure the assay represented the mix of symptomatic and asymptomatic fruit present on each tree. Titratable acidity (TA) was determined by titrating 25 ml of juice to pH 8.2 with 0.3125 N NaOH using an autotitrator (Metler Toledo DL50 autotitrator, Columbus, Ohio, USA). Data were expressed as percent citric acid. Total soluble solids (Brix; the measure of sugar content in fruit; i.e., 1 g of sugar/100 g of juice is equivalent to 1° of Brix) was measured with a refractometer (Atago RX-5000a Atago Co. Ltd Bellevue, Washington, USA). Juice color was measured using Greytag MacBeth Color Eye 3100 spectrophotometer (Greytag Macbeth, New Windsor, New York, USA).

2.1.6. Data analysis

Treatment comparisons for total number of fruit, the proportion of fruit drop, total yield and fruit volume were analyzed by general linear modeling (Proc GLM, SAS Systems, Cary, North Carolina, USA). Main effect means of each yield and quality parameter were calculated and a means separation for the main effects and interactions calculated using Tukey's HSD test (tested at $P = 0.05$ and $P = 0.10$). The rate of change of normalized *Las* titer over time among treatments was compared via *t*-test and by linear regression analysis to establish if there were significant differences in titer dynamics among treatments.

2.2. Micronutrient effects on production in multiple commercial plantation blocks

2.2.1. Field plot design

A field trial was initiated in 2008 by a commercial producer to evaluate the effects of the enhanced ENP on HLB incidence and tree productivity. This commercial trial afforded an opportunity to examine the effect of ENP applications compared to a more conventional micronutrient program in large commercial orchards. The ENP treatments were applied in six commercial citrus blocks and data from the trial was collected by the commercial producer and kindly provided to the authors for statistical analysis. Four of the blocks were 8-yr-old Valencia sweet orange on Carrizo citrange (*P. trifoliata* [L.] Raf. × *Citrus sinensis*[L] Osb.) rootstock planted in 2002 and two of the blocks were 8-yr-old Valencia on sour orange (*Citrus aurantium* L.) rootstock (resulting from a ~95% reset with new trees from late 1990s to 2002). Of these, one of the latter and two of the former blocks were treated with the foliar ENP and conventional psyllid sprays whereas, the three remaining blocks received the conventional foliar fertilization and insect control program. Blocks ranged in size from 2362 to 12,439 trees with an average of 6814 trees, per block.

Commercial blocks treated with conventional fertilization program. For those blocks receiving conventional fertilization in 2007–2008, foliar applications with an air-blast sprayer at 1400 L/ha consisted of:

- 14–3–7–0.05 (N–P–K–B) or 14–7–7–0.05 (N–P–K–B) at 65.48 L/ha plus 22.24 kg/ha K-phosphite (K-Phite® 0–28–26; or Nutri-Phite® Magnum 2–40–16; Biagro Western, Visalia, California, USA) applied as a dormant spray.
- 15–2–18–3 (N–P–K–Mg) plus 0.075 Fe EDTA at a rate of 112.08 kg N/ha applied as a dry broadcast fertilizer twice per year in fall and late winter.
- 10–0–10 (N–P–K) applied at a rate of 112.08 kg N/ha plus 0.05 kg Fe EDTA liquid fertilizer applied in 1120 L/ha over 5 injections from spring thru early summer.

After HLB was discovered in 2009, the conventionally treated blocks received supplemental foliar applications at 1400 L/ha:

- K-Phite® or Nutri-Phite® Magnum (Biagro Western, Visalia, California, USA) at 3.51 L/ha.
- Saver® (Plant Food Systems, Zellwood, Florida, USA, contains a systemic acquired resistance SAR compound) at 1.17 L/ha.
- Manganese sulfate at 5.60 kg/ha.
- Zinc oxide at 3.36 kg/ha.
- 3–0–11 (N–P–KNO_3) (two applications post-bloom and summer 1) at 56.12 L/ha.

Commercial blocks treated with an ENP. For those plots treated with micronutrients, treatment began on November 6, 2007, and proceeded yearly as three micronutrient sprays applied on the flush during bloom (March–April), and twice during summer (June and August). Micronutrients replaced those five components (supplemental foliar treatments) used in the conventional program immediately above, and consisted of the following components applied at 2500 L/ha:

- Serenade Max® (AgraQuest, Davis, California, USA) at 2.52 kg/ha.
- Di-Oxy Solv™ (Upstart Products, Inc., Titusville, Florida, USA) at 6.12 L/ha.
- 3–18–18 (D–K–P) at 74.82 L/ha.
- K-Phite® at 4.67 L/ha.
- Saver® at 2.32 L/ha.
- Epsom salt® (heptahydrate epsomite – 99% anhydrous $MgSO_4 \cdot 7H_2O$), at 9.54 kg/ha.
- Tecmangam® (Manganese sulfate, manganese monosulfate and monohydrate manganese sulfate mixture, Industrias Sulfamex S.A. DE C.V., Tampico, Mexico) at 9.54 kg/ha.
- Zinc sulfate at 3.14 kg/ha.
- Sodium molybdate at 0.06 L/ha.
- 13–0–44 (N–P–KNO_3) at 9.54 kg/ha.

2.2.2. Disease and yield assessment

Disease incidence data were recorded during 24 visual assessments from December 2006 through March 2011. Yield was estimated as the number of boxes of fruit per tree. One box is equivalent to approximately 40.8 kg (90 lbs) of fruit (=~275–300 fruit/box). Yield data were collected from each block for the five-year period of 2007–2011. Therefore the 2007–2008 harvest would not have had the advantage of micronutrient treatments, whereas, for the 2009–2011 yearly harvests, treatment effects could be compared between the ENP and conventional fertilization.

2.2.3. Data analysis of the effect of nutrition on yields in multiple commercial plantation blocks

Yield comparisons were made over the duration of the experiment via an analysis of variance to make comparisons between treatments; i.e., ENP vs. conventional program, with the pre-treatment year 2 (year immediately prior to commencement of the experiment) as a covariate for years when treatments were applied (SAS Proc GLM method using LSD to separate means and $p = 0.05$), to account for pre-treatment differences between blocks.

2.2.4. HLB disease increase and temporal dynamics of commercial blocks

To determine if there were differences between the rates of disease increase for trees treated with ENP applications vs. trees treated with conventional fertilization, disease increase data over time were fitted to linear [$t = y$], logistic [$t = \log(y/(1 - y))$], and Weibull [$t = -\ln(-\ln(y))$] models. Rates of disease increase for the superior model were then compared via *t*-test to examine for significant differences. To examine the dynamics of HLB, disease increase (y/t), the rate of disease increase (dy/dt), and the acceleration/deceleration of the epidemic [$(dy/dt)/dt$] were calculated for each of the blocks, where y = disease incidence of the block and t = time in days. Linear regression was used to examine the slopes (b) of the [$(dy/dt)/dt$] data points, where the arithmetic sign of the slope, $+b$ or $-b$ is indicative of acceleration or deceleration of the rate of the epidemics, respectively. Slope comparisons were made by *t*-test, where

$$t = (b_1 - b_2)/(\mathrm{SE}b_1 - \mathrm{SE}b_2),$$

and where b_1 and b_2 are the slopes of the regression lines to be compared and $\mathrm{SE}b_1$ and $\mathrm{SE}b_2$ are the respective standard errors of the slopes.

3. Results

3.1. Effect of micronutrient treatments on yield and quality

Over the two-year duration, all trees within the experiment declined, expressing increasingly prevalent HLB symptoms; i.e., loss of foliage, dead and dying twigs especially in the upper canopy, foliar symptoms of blotchy mottle, zinc deficiency, veinal chlorosis, shortening of internodes, small upright leaves, plus foliar and fruit abscission. There was no apparent visual difference among treatments in expression of disease symptoms.

There were no significant differences among treatments for the average of the total number of fruit per tree, the average weight of fruit per tree, or number of fruits abscised per tree (Table 1). In 2009, yields were low with 60–70% premature fruit drop attributed to HLB. In 2010, yields dropped even further to only 25–30% of the 2009 yields, and there was an additional 40–50% fruit drop of the few remaining fruit. Total yield (kg/tree) was low in 2009, and in 2010, dropped to approximately 50% of the 2009 yield. The number of individual fruits per tree was also low in 2009 and in 2010 declined to approximately 30–50% of the 2009 yield. Both years, fruit remaining on the trees was close to abscising at harvest and often dropped when the branch was disturbed (Table 1). This drop is abnormal for mature fruits on healthy trees that usually remain tightly bound to the peduncle until harvest.

There were no significant differences in fruit quality parameters among treatments during either year of the experiment. Brix ranged from 8.75 to 11.8 and was similar for both years of the study. Fruit acid decreased by approximately 30 percent from 2009 to 2010. Therefore, the Brix:acid ratio for all treatments increased from 2009 to 2010 (Table 1).

The only parameter that varied significantly among treatments was fruit volume. In 2009, fruit size among all treatments was not significantly different from the non-treated control (Table 1). In 2010, variability among treatments was more pronounced than in 2009. Treatments 2, 6, 8, and 10, consisting of foliar phosphite, K phosphite plus Mn-carbonate, K phosphite plus Zn-metalosate, and silver PDS via injection, respectively, had the highest fruit volume, whereas treatments 1 and 3, consisting of non-treated control and K phosphite plus wettable powder nutrients, respectively, had the lowest fruit volume (Table 1).

3.1.1. Effect of micronutrient treatments on Las titer

There was no significant difference in normalized *Las* bacterial titer among the ten micronutrient treatments. Titer fluctuated each year and in general was lowest during the hottest summer months and highest in late fall (Fig. 1A–C). Because no significant differences occurred among treatments, normalized *Las* bacterial titer data were combined across all treatments. The data demonstrated an overall decline in *Las* titer over the time course of the trial; i.e., 811 days (Fig. 1D).

3.2. ENP effect on production of commercial blocks

Because there were higher yields initially in commercial blocks chosen for ENP treatment compared to those blocks receiving the conventional fertilization, a covariate analysis was used to correct for this pre-treatment difference of yields with respect to the year immediately prior to the initiation of the experiment (Table 2). When prior year yield differences were taken into account, there was no significant difference ($P = 0.05$ and $P = 0.1$) between the ENP and conventionally fertilized trees for any of the succeeding 3 years (2009–2011) of yield data collection.

3.2.1. HLB disease increase and temporal dynamics in commercial blocks

For the three models examined, the linear model was superior to the logistic model and equivalent to the Weibull model to describe increase in disease incidence over time (Fig. 2 for model slopes and regression coefficients). Therefore, the linear model, i.e., the most simplistic model, was chosen for treatment comparison (Fig. 2A). The linear model demonstrated that the rates of disease increase, represented by the slope (b) of the regression line, were not significantly different ($b_{\mathrm{ENP}} = 0.000203$, $R^2 = 0.74$ and $b_{\mathrm{conventional}} = 0.000197$, $R^2 = 0.84$, via *t*-test comparison of the slopes at $P = 0.05$ and 0.01 using the combined data from three replicated block for each treatment), between the ENP and conventional fertilization (Fig. 2B). The change in the rate of the epidemic through time was examined by taking the first derivative, dy/dt, and demonstrated two peaks near elapsed days 200 and 1000, which corresponded to larger numbers of new HLB-symptomatic trees detected during July 2007 and August 2009 assessments, respectively (Fig. 2C). Acceleration or deceleration of the HLB epidemic relative to each replication was examined by taking the second derivative, $(dy/dt)/dt$. Rates of acceleration or deceleration were quite small and ranged from $4 \times 10^{-6} \times \mathrm{day}^{-1}$ to $1 \times 10^{-5} \times \mathrm{day}^{-1}$, indicating that there was no significant acceleration or deceleration of the epidemic through time for all blocks (Fig. 2D). Because there were no statistical differences within treatment for ENP or conventional determined by *t*-test comparisons of individual slopes among the replicates, the data from all replicates within treatment were combined. There was no significant difference for rate of acceleration or deceleration between ENP and conventional treatments as determined by *t*-test comparison of the slopes of the combined data.

4. Discussion

4.1. Effect of micronutrient treatments on yield and quality

Currently, these multiyear trials represent the only replicated field experiments that evaluate the use of ENPs to offset the deleterious effects of HLB on citrus tree productivity. In Florida, other studies and commercial producer observations are either non-replicated or insufficiently designed to allow for statistical validation. In the first trial, wherein all trees were *Las*-positive at the onset, ENP treatments did not sustain the production of HLB-affected trees. Similarly, none of the treatments containing metal

Table 1
Yield and quality of Valencia orange fruit harvested from a commercial citrus planting treated with various micronutrient and heavy metal treatments to suppress HLB in 2009 and 2010.

Treatment	Yield												Quality											
	Fruit number				Fruit weight (kg)				Number fruit dropped				Proportion fruit drop				Fruit volume (cm^3)				Fruit brix			
	2009		2010		2009		2010		2009		2010		2009		2010		2009		2010		2009		2010	
1	56.83	a[1]	27.58	a	6.13	a	3.90	a	111.42	a	39.08	a	0.66	a	0.54	a	947.9	a	1182.2	d	11.3	a	11.8	a
2	47.48	a	23.42	a	6.53	a	4.13	a	109.42	a	36.50	a	0.66	a	0.55	a	1089.6	a	1449.2	b,a	10.3	a	11.7	a
3	90.17	a	28.08	a	8.02	a	4.24	a	142.42	a	22.67	a	0.62	a	0.50	a	926.7	a	1198.5	d	10.0	a	11.8	a
4	68.67	a	22.69	a	7.10	a	3.41	a	120.08	a	27.22	a	0.61	a	0.54	a	927.3	a	1224.1	c,d	10.1	a	11.4	a
5	86.58	a	28.67	a	11.00	a	4.18	a	155.42	a	44.42	a	0.65	a	0.54	a	1081.1	a	1311.5	b,c,d	9.5	a	11.0	a
6	75.00	a	26.67	a	8.00	a	3.95	a	157.08	a	38.08	a	0.67	a	0.54	a	920.7	a	1384.8	b,a	9.8	a	11.4	a
7	49.17	a	27.83	a	6.37	a	4.49	a	109.83	a	34.42	a	0.66	a	0.59	a	1171.6	a	1360.9	b,c	10.0	a	11.3	a
8	46.83	a	24.58	a	4.99	a	4.16	a	116.25	a	23.75	a	0.73	a	0.45	a	1028.6	a	1529.1	a	10.2	a	11.3	a
9	61.25	a	43.25	a	7.37	a	6.85	a	125.83	a	32.67	a	0.62	a	0.46	a	1024.7	a	1358.0	b,c	11.0	a	11.4	a
10	91.75	a	32.11	a	10.42	a	5.15	a	168.47	a	36.08	a	0.65	a	0.56	a	1063.6	a	1415.5	b,a	10.6	a	11.4	a

Treatment	Quality									
	Fruit acidity				Fruit brix acidity ratio					
	2009		2010		2009		2010			
1	1.19	a	0.80	a	9.48	a	14.66	a		
2	1.09	a	0.80	a	9.49	a	14.64	a		
3	1.13	a	0.77	a	8.83	a	15.44	a		
4	1.05	a	0.74	a	9.63	a	15.32	a		
5	1.00	a	0.73	a	9.50	a	15.15	a		
6	1.16	a	0.85	a	8.52	a	13.49	a		
7	1.08	a	0.75	a	9.27	a	15.09	a		
8	1.20	a	0.77	a	8.54	a	14.73	a		
9	1.21	a	0.77	a	9.25	a	14.84	a		
10	1.19	a	0.79	a	8.93	a	14.49	a		

Tukey's Studentized Range (HSD) Test. Tested at the $p = 0.10$ and 0.05 level, no difference between p levels was detected.
Treatments were:
1) Control (standard commercial citrus fertilization, pest and weed control program without micronutrient enhancements). All subsequent treatments received the same minimal control plus augmentations as noted.
2) K phosphite first application 4.7 L/ha; subsequent applications 2.35 L/ha.
3) K phosphite as in treatment 2 plus wettable powder nutrients, Mg 6.3–9.5 kg/ha, Mn 6.3 kg/ha, Zn 3.17 kg/ha, Mo 58.6 g/ha mixed in water at 2642 L/ha (500 gal/ac). Applied three times yearly, with the exception of Zn applied 4 times yearly.
4) K phosphite as in treatment 2, wettable powder nutrients as in treatment 3, plus SAVER® (= salicylic acid as an SAR compound) at 1.17 L/ha.
5) K phosphite as in treatment 2, plus Mn^{++} metalosate 3.5 L/ha plus standard citrus surfactant.
6) K phosphite as in treatment 2, plus Mn^{++} carbonate 0.45 kg/tree plus standard citrus surfactant, applied at the base of each tree.
7) K phosphite as in treatment 2, plus Cu^{++} metalosate 0.58 L/ha plus standard citrus surfactant.
8) K phosphite as in treatment 2, plus Zn^{++} metalosate 3.5 L/ha plus standard citrus surfactant.
9) Magna-Bon® Cu injection 60 ppm/5 ml/tree via injection per month.
10) Silver PDS polymer formulation via injection PDS/Silver at 0.005 g of silver per 100 g of carrier material ~0.005% silver.

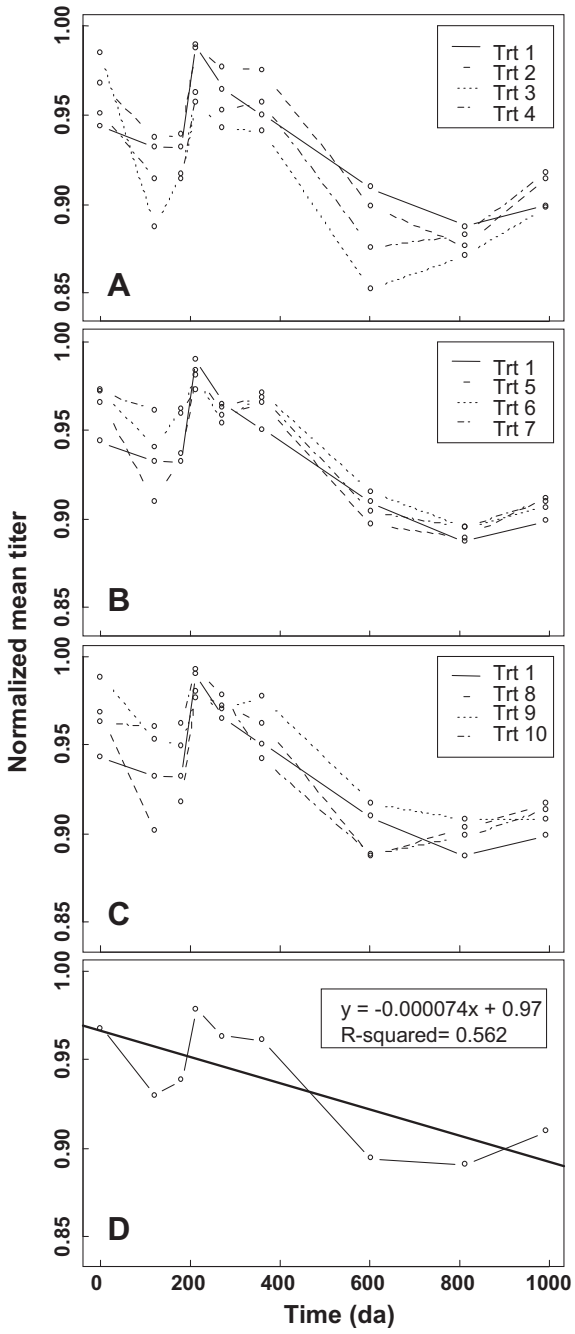

Fig. 1. Dynamics of normalized mean *Las* titer over time for various micronutrient and metal ion treatments to control HLB. To estimate the change in *Las* titer over time, a reference gene, dehydrin, was simultaneously assayed and the ratio of the two used to define titer change between sampling periods. A–C) Treatment 1 (Trt 1) Control = standard commercial citrus fertilization, pest and weed control program without micronutrient enhancements is repeated in each panel to facilitate treatment comparison. Treatments 2–10 (Trt 2–Trt 10) are as indicated in the text. D) Mean normalized titer data combined from all treatments (no significant differences among treatments) demonstrate seasonal fluctuation of titer and general decline in titer through time as the HLB syndrome causes trees to decline.

ion micronutrients reduced *Las* titer as a result of potential bactericidal effects. Micronutrient treatments were also not capable of offsetting losses in yield, fruit weight, and fruit size associated with *Las* infection. Fruit abscission was prevalent among all treatments and continued to become more pronounced as the disease symptoms intensified and became systemic throughout the tree canopy. Although average fruit volume of the 2010 harvest increased slightly compared to the 2009 harvest, average volume of individual fruits was small or smaller than commercially acceptable standards.

Table 2
Commercial block yield [boxes (kg/tree)] comparisons of the enhanced nutritional program (ENP) vs. conventional foliar spray program.

Treatment	Pre year 1	Pre year 2	Post year 1	Post year 2	Post year 3
Conventional	0.70(28.6)	1.36(55.5)	2.13(86.9)[a]	2.29(93.5)	2.59(120.4)
ENP	1.03(53.1)	1.99(81.2)	2.28(93.1)	2.96(120.8)	3.30(134.7)
Difference	0.33(13.5)*	0.63(25.7)**	0.15(6.1)ns	0.67(27.3)ns	0.71(30.0)ns

Three blocks were treated with micronutrient mixture combination and 3 blocks were treated with conventional fertilization. Blocks averaged 6814 trees/block.
Pre years 1 and 2 represent yields prior to application on nutrient treatment; post years 1, 2, and 3 represent yields following nutritional treatment.
PROC GLM with 'Pre year 2' as the covariate to adjust for significant higher yields of nutritional blocks compared to conventional fertilization blocks prior to application of treatments.
Means calculated by LSD.
*, ** = significantly different at $P = 0.10$ and 0.05, respectively.
[a] ns = not significantly different.

Some commercial producers who have applied the ENP consisting of micronutrient combinations have noted that ENP treated trees produce foliage with greater leaf number, size, color, and thickness than conventionally fertilized trees. However, none have documented increases in yield or fruit quality. While, a balanced fertility program of the macronutrients N, P, K, Ca and Mg is well documented to enhance tree vigor, health, and production of citrus (Obreza and Morgan, 2008), evidence for increased production in response to micronutrients is less clear. Results of a single nutrient omission study conducted with newly planted Valencia trees in a previously unfertilized virgin soil in the central ridge of Florida indicated that it takes eight years for trees to become deficient or low enough in Zn and Mn status to affect yield (Koo and Reese, 1971). Recent analyses comparing symptomatic (blotchy mottle) and asymptomatic leaves from *Las*-affected trees and leaves from healthy trees showed that HLB-infected trees, were not actually deficient in Zn, Mn, etc., even when the dry mass of the leaf samples was corrected for the large amounts of starch accumulation caused by HLB (Spann and Schumann, 2009). Sufficiency is measured as the absolute concentration of the nutrient element, which does not necessarily reflect bioavailabilty. Although Zn and Mn deficiency symptoms are commonly seen on HLB-infected trees, whether their micronutrient status is affecting the physiology of the leaves and fruit is unknown. Therefore, foliar fertilizers containing micronutrients may improve the color and abundance of foliage, but not substantially increase fruit yield and quality compared to a conventional fertilization program.

This finding is supported by yields from the commercial blocks studied where enhanced vector control and rouging of diseased trees was practiced. In these blocks there was a lack of yield response to the three years of the ENP compared to the conventional fertilizer and micronutrient spray program. In the commercial blocks, HLB-affected trees were removed within 6 months after their detection. In our replicated block trial, a commercial planting no longer managed by removal of inoculum, citrus trees infected with the *Las* bacterial pathogen did not respond to the micronutrient fertilizer with respect to fruit quality or yield.

Because ENP treatments did not reduce disease progress in the experiments, trees continued to express systemic HLB symptoms. A recent study in Brazil demonstrated a pronounced yield reduction of HLB-symptomatic trees measured as weight of fruit from individual trees, regardless of whether they are early, mid-season, or late-season sweet orange cultivars (Bassanezi and Bassanezi, 2008; Bassanezi et al., 2011). In this Brazilian study, as disease-symptomatic canopy area increased, average yield per tree decreased dramatically, as seen in the trial presented here.

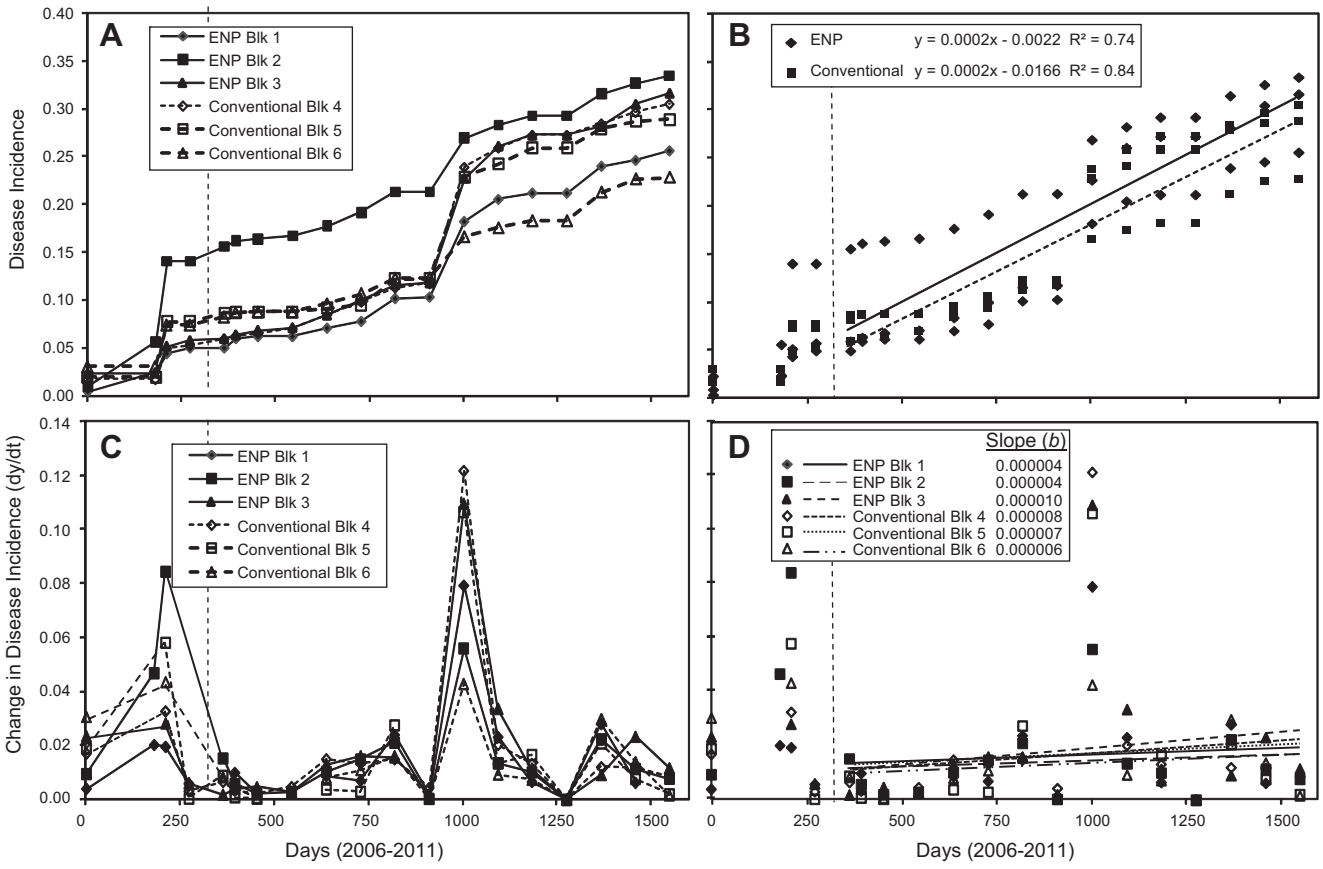

Fig. 2. Temporal dynamics of HLB incidence in six citrus blocks treated with either enhanced nutritional or conventional foliar spray program. Vertical dashed line indicates initiation of nutritional program (ENP) applications in November 2007. A) Increase in HLB disease incidence over time, B) Linearized disease increase of nutritional treatment [solid line] vs. conventional fertilization [dashed line], C) First derivative, d*y*/d*t*, showing the change in the rate of the epidemic through time, D) Second derivative, (d*y*/d*t*)/d*t*, showing the acceleration or deceleration of the HLB epidemic relative to each replication estimated by the slopes (*b*) of the individual regression lines. Note rates of acceleration or deceleration ranged from $4 \times 10^{-6} \times \text{day}^{-1}$ to $1 \times 10^{-5} \times \text{day}^{-1}$, indicating that there was no significant deceleration or acceleration of the epidemic through time for all blocks determined by *t*-test.

HLB has existed in Southeast Asia for a long time and was first inadvertently described in India in 1927 when the authors were reporting the damage due to *D. citri* infestation (Husain and Nath, 1927) and was first noticed by researchers in China in the 1940s (Lin, 1956; Zhao, 1981). Since that time, Chinese researchers have also explored the effects of various nutrient and micronutrient applications to offset the effects of HLB. A series of four studies was conducted in Cheochow, Chaozhou at the Lingnan University Citrus Experiment Station (Chen, 1937, 1938; 1940, 1941). Researchers applied various sprays and soil treatments consisting of zinc sulfate, copper sulfate, boron, calcium sulfate, ferric sulfate, potassium hypo-phosphate, magnesium sulfate, soybean residue, etc. They concluded that no nutritional treatment or treatment combination had a beneficial effect on the health of HLB-affected trees. They did note that yellow shoot symptoms of *Las*-infected trees returned to green within a short period after application of zinc sulfate sprays, but none of the treatments sustained yield or fruit quality. An exhaustive review of the Chinese literature on the many studies nutritional effects on HLB concluded that there was nothing substantive to support a consistent effect of nutrients (Xia et al., 2011).

Cost of the ENP is approximately $1510 per ha, about 3 times the cost of the conventional program at $454 per ha (Muraro, 2009). Claims that the economic lifespan and productivity of HLB-affected citrus plantations can be sustained with ENPs were not substantiated by the present study. Hence, these nutritional programs may increase production costs unnecessarily.

4.2. ENP effect on production and HLB disease progress in commercial blocks

The commercial trial was a robust validation, considering there were 17,195 and 14,590 trees under commercial production located in six large blocks and treated with either ENP or conventional fertilization. Lack of any significant difference in yields of blocks treated with a ENP vs. conventional fertilizers confirms the results of the prior replicated plot trial with an array of micronutrient treatments. Rates of disease increase over time (slope *b* of the regression lines) furthermore revealed that the ENP and conventional programs were nearly identical in disease progress. Examination of the dynamics of the epidemic also revealed that epidemics in ENP or conventional blocks were neither accelerating nor decelerating but were simply progressing at a near linear rate. The relatively uniform and slow rate of disease increase within all blocks is indicative of a commercial HLB management strategy consisting of strong vector population management combined with annual HLB-positive tree removal (inoculum control) and tree replacement.

4.3. Effect of micronutrient treatments on Las titer

An indirect adverse effect of the use of ENP is the maintenance of high bacterial titer in *Las*-infected trees over a prolonged period. Worldwide, management recommendations for management of HLB consist of psyllid vector control and rouging of infected trees to reduce *Las* inoculum. In many cases, commercial citrus producers

who have adopted a nutritional program have not been removing diseased trees. In addition, since many of the trees in orchards treated with ENP are *Las*-positive, some commercial producers have opted to reduce insecticide applications because the trees are already *Las*-infected. The relaxation of vector management and cessation of inoculum reduction has had a devastating effect on the HLB epidemic in Florida, increasing infected tree incidence from 8 to 10% in 2009 to 18% in 2010, which is probably an underestimate (Irey et al., 2011). Thus, commercial producers that have adopted a ENP regime, consisting of only the standard macro- and micro-nutrient elements (and augmented by phosphite and/or salicylate salts) for HLB management, have likely promoted the buildup of inoculum and *Las*-positive vectors. This is supported in that plantations immediately adjacent to those under ENP regimes show a pronounced increase of HLB along the borders of planting undergoing traditional management of HLB consistent with disease edge effects previously reported (Gottwald et al., 2007; Gottwald, 2010). Recent research studies from Brazil report that the HLB incidence of not only adjacent orchards, but plantings within a region have a pronounced impact on well-managed orchards where inoculum reduction and vector management are practiced (Bassanezi et al., 2011).

4.4. Interaction of micronutrient treatments and area-wide control

Recent studies have demonstrated that HLB control can be augmented significantly by regional control strategies; i.e., coordinating insecticide applications for vector control regionally among plantations, often by the use of aerial application (Bassanezi et al., 2011). These regional control strategies suppress vector populations simultaneously over large areas and greatly increase the time that vector populations require to recover after insecticide applications, because local movement between blocks and plantings is suppressed. Simultaneously, all citrus plantations under an area-wide management scenario are encouraged to remove HLB-symptomatic trees to suppress bacterial inoculum. Where this has been practiced in South Florida (Graham, 2011) and São Paulo, Brazil, HLB management has been much more effective than in regions where area-wide management strategies are not employed (Belasque et al., 2010). It is normally recommended that area-wide management should be employed regionally encompassing thousands of hectares of contiguous plantings (Bassanezi et al., 2011). The larger the area under simultaneous management, the greater suppression is achieved. It has been estimated that disease increase can be maintained at the 3–5% per year utilizing area-wide control strategies, whereas, without area-wide control, percent disease increase can be in the double digits (Gottwald and Gilligan, unpublished). Producers and researchers believe that 8% per year disease is economically sustainable rate of production loss (Irey et al., 2011).

4.5. Conclusions

The present study indicates that it may be difficult to integrate ENP with disease control strategies based on area-wide management. This is because with the ENP strategy, diseased trees are not removed but instead an attempt is made with ENP applications to maintain tree health. A major weakness with an ENP approach is that growers tend to cut back on control of vectors, thus leading to a regional increase in disease, inoculum sources, and insect vectors. This outcome is counterproductive to an area-wide management strategy. To be compatible with an effective area-wide control strategy, unless superior bactericides are developed relative to those investigated by the present study, ENPs must be augmented with area-wide vector management, plus rouging of HLB-symptomatic trees. A recent assessment of the Florida citrus industry indicates that many growers are abandoning tree removal in favor of managing with nutritional sprays. This means that all orchard trees, as well as neighboring yard trees, will become infected unless vectors are fully controlled (Timmer et al., 2011). "*We do not believe that citrus industries in Florida and Brazil can survive and be economically viable with 100 percent of the trees affected by HLB*" (Timmer et al., 2011). In addition, due to the high cost of nutritional applications (up to 3 times that of conventional fertilization regimes) and the apparent inability to either directly or indirectly kill the disease organism, this is unlikely to be economically feasible unless a superior ENP strategy is developed that effectively kills the bacteria within infected trees.

The present study indicates that application of many of the standard essential micronutrients, formulated as the standard ionic salts or metalosate ligands, and augmented with putative resistance promoting agents of phosphite and salicylic acid, is not a viable substitute for an effective disease management program, but rather a last resort in a bad situation (Timmer et al., 2011), and facilitates spread of disease. To date, there are no other published controlled experiments in Florida demonstrating that any nutritional application mixture effectively prolongs life and maintains yields of HLB-infected trees (Timmer, 2010). If nutrient management practices are to be used then it is critical that insecticide spray programs be maintained to control arthropod vectors, and that there be more research to better understand the interactions between the HLB associated bacterium and elemental composition of phloem sap.

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