Evaluation of the Spatiotemporal Dynamics of Oxytetracycline and Its Control Effect Against Citrus Huanglongbing via Trunk Injection

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ABSTRACT

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Citrus huanglongbing (HLB) or greening is a devastating bacterial disease that has destroyed millions of trees and is associated with phloem-residing '*Candidatus Liberibacter asiaticus*' (Las) in Florida. In this study, we evaluated the spatiotemporal dynamics of oxytetracycline in planta and its control effect against HLB via trunk injection. Las-infected 'Hamlin' sweet orange trees on 'Swingle' citrumelo rootstock at the early stage of decline were treated with oxytetracycline hydrochloride (OTC) using trunk injection with varying number of injection ports. Spatiotemporal distribution of OTC and dynamics of Las populations were monitored by high-performance liquid chromatography method and qPCR

assay, respectively. Uniform distribution of OTC throughout tree canopies and root system was achieved 2 days postinjection. High levels of OTC (>850 µg/kg) were maintained in leaf and root for at least 1 month and moderate OTC (>500 µg/kg) persisted for more than 9 months. Reduction of Las populations in root system and leaves of OTC-treated trees were over 95% and 99% (i.e., 1.76 and 2.19 log reduction) between 2 and 28 days postinjection. Conditions of trees receiving OTC treatment were improved, fruit yield was increased, and juice acidity was lowered than water-injected control even though their differences were not statistically significant during the test period. Our study demonstrated that trunk injection of OTC could be used as an effective measure for integrated management of citrus HLB.

Additional keywords: bactericide, antimicrobial, citrus disease control.

Huanglongbing (HLB) or greening is one of the most devastating diseases of citrus in Florida and worldwide (Gottwald et al. 2007). The causal agent is phloem-residing '*Candidatus Liberibacter asiaticus*' (Las), which is yet to be cultured (Bové 2006). Las is transmitted by Asian citrus psyllid (ACP, *Diaphorina citri*) (Halbert and Manjunath 2004). This destructive pathogen affects all citrus cultivars by disrupting flow of nutrients and thereby causing rapid tree decline. HLB weakens root system, increases early fruit abscission and ultimately causes high tree mortality. Since its first discovery in South Florida in 2005, HLB has spread to all major production areas of the state and the economic loss exceeded 3.6 billion dollars (Boina and Bloomquist 2015; Gottwald et al. 2012; Stansly et al. 2014). Currently, more than 80% of citrus trees in Florida have been infected by Las and there is no cure for HLB. Integrated management for HLB control has been ineffective and costly. Genetic and transgenic approaches to modify citrus host (Hajeri et al. 2014; Mirkov and Gonzalez-Ramos 2013) and ACP (Marutani-Hert et al. 2010), which offer great promise for mitigating the HLB problem, are far away from application. Commercial adoption of GMO products takes many years, if not decades, of rigorous field testing and lengthy deregulation process. In the absence of host resistance, to keep existing Las-infected citrus alive and productive requires large reduction of Las populations or long-lasting suppression of bacterial growth. Bactericides such as streptomycin and oxytetracycline (OTC) have such potential and are used extensively in agriculture to control phytopathogenic bacteria on a variety of economically important crops including fruit trees, ornamentals and vegetables (Christiano et al. 2010; Kumar et al. 2005; McCoy 1976; McManus et al. 2002). Recently, under the Emergency Exemptions provisions of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Florida

has declared an HLB crisis that allows use of the following bactericide: streptomycin sulfate (FireWal 50WP, AgroSource, Inc.), oxytetracycline hydrochloride (FireLine 17WP, AgroSource, Inc.), and oxytetracycline calcium complex (Mycoshield, Nufarm Americas, Inc.) in foliar applications to enhance the overall tree health of HLB-diseased trees in Florida citrus groves.

OTC is a member of tetracycline bactericide that inhibits bacterial protein biosynthesis by binding reversibly to the ribosomes (Goodman 1959). More than 10 tons of OTC are applied annually in several states of the United States (McManus et al. 2002). While a small fraction was used for bacterial disease management on vegetables, the majority of OTC is used to control *Xanthomonas arboricola* on peach and nectarine, *Erwinia amylovora* on pear, and in particular streptomycin-resistant isolates of *E. amylovora* on apple (Christiano et al. 2010; McManus et al. 2002). Well-timed applications of OTC are carried out by foliar spray onto tree canopies at rates of 100 to 200 µg/ml during bloom. Once deposited on leaf surface, OTC did not appear to enter leaf tissue (Christiano et al. 2010). Consequently, OTC protects trees by inhibiting bacterial growth on the surface of plant tissues prior to infection. A study of OTC dynamics on peach leaves by Christiano et al. (2010) showed that its residue activity decreased sharply by 77.8, 92.1, and close to 100% within 2, 4, and 7 days after application, indicating high vulnerability for quick degradation in aqueous solution under natural sunlight (Christiano et al. 2010; Maia et al. 2009; Sanderson et al. 2005). Moreover, rainfall washed 60 to 80% of OTC residue off leaves within 5 min (Bernstein 1991; Christiano et al. 2010). These limitations associated with foliar spray of OTC led to reduced efficacy, frequent reapplication, and risky environment contamination. It is possible that OTC will have the same trouble in entering citrus leaves for HLB control (Christiano et al. 2010; McCoy 1976) to suppress phloem-residing Las, thus limiting its control effect when sprayed. This scenario justifies investigation into alternative application methods to improve the efficacy of application, and to reduce the negative effect of OTC on the environment.

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Trunk injection represents a target-precise and environmental friendly method for delivery of chemicals and nutrients directly into plants. Tree injection is used in landscape tree care for treating tree pests and nutrient deficiency (Guillot and Bory 1997; Kielbaso et al. 1978). Due to xylem's quick and high capacity of translocation, it has been demonstrated that trunk injection of therapeutic compounds is a better alternative approach to control phytoplasma, bacteria, fungi, nematodes, and insects on numerous trees species (Aćimović et al. 2015; Aubert and Bove 1980; Buitendag and Bronkhorst 1980; Byrne et al. 2012; Guillot and Bory 1997; Jansson and Rabatin 1997; Keil 1979; McCoy 1976; Percival and Boyle 2005; Tanis et al. 2012; Timmer et al. 1985). In contrast, foliar spray creates excessive drift, has limited reach, and leads to exposure to off-target organisms despite its easiness in application. Similarly, soil drench has a number of limitations such as slower action, microbial degradation, requirement of higher amount of product or repeated application, and negative impact on off-target organisms. McCoy (1976) demonstrated that there was no OTC uptake following foliar spray or soil drench on coconut palm (McCoy 1976).

The long-distance movement of trunk-injected therapeutic compounds within xylem or phloem is affected by numerous factors including compound properties, tree physiology, xylem architecture, and environmental conditions. The mode of translocation (i.e., xylem, phloem, or both) is largely determined by physicochemical properties of the compounds including polarity, molecular weight, and water solubility (Hsu et al. 1988; Kleier 1994). Pesticide formulation had a significant impact on the uptake and transport of the molecules (Mendel 1998; Aćimović et al. 2014). Most of tree species exhibited sectorial sap flow due to specific xylem vessel elements connecting given branches or integrated vessel elements connecting several branches (Byrne et al. 2012; Orians et al. 2004). This led to concerns of uneven distribution of injected compounds throughout tree canopies, potential phytotoxicity due to overdose, and thus variable control effect. For example, sectorial "zigzag" xylem pattern was responsible for sectorial flow distribution of imidacloprid in ash trees (Tanis et al. 2012). Aćimović et al. (2014) found that a minimum of four injection ports were required to achieve uniform spatial distribution of imidacloprid in apple tree canopies.

Fig. 1. Port and sampling schematic of 5-year-old 'Hamlin' sweet orange on 'Swingle' citrumelo rootstock trees that were trunk injected with oxytetracycline hydrochloride (OTC) at 2 g of active ingredient/tree. **A**, A total of 600 ml of OTC solution was injected as follows: (a1) one port (600 ml), (a2) two ports (300 ml for each port), (a3) three ports (200 ml for each port), and (a4) four ports (150 ml for each port). **B**, The first injection port was always located 30 cm below the bottom branch b1 and additional ports were anchored with equal space among them on the trunk. Tissue samples of shoots, flowers, fruits, and leaves were collected from four branches labeled as b1, b2, b3, and b4. **C**, Leaf samples (c1 and c2) and root samples (c3) were also collected from **D**, four quadrants. U, upper level of the canopy; L, lower level of the canopy; NW, northwestern; NE, northeastern; SW, southwestern; SE, southeastern; and R, root.

Trunk injection of bactericide was evaluated for HLB control in Asia and South Africa (Aubert and Bove 1980; Moll and van Vuuren 1977; Vanvuuren et al. 1977; Zhao 1981). The information on trunk injection of bactericide for control of pathogens or pests on citrus remains limited due to the high costs associated with injection technology which was designed primarily for landscape use where high labor costs can be met by the market needs. Those pioneering tests did not provide detailed information about the dynamic distribution of bactericide in planta and antimicrobial effect. Over three decades ago, trunk injection was used to investigate the causes of citrus blight by injecting bactericide and fungicides into blight-affected trees (Lee et al. 1982; Timmer et al. 1985). Several bactericides including OTC have been found highly effective against Las in greenhouse studies (Zhang et al. 2014; Zhang et al. 2012). However, it is not clear whether similar high level activity against Las could be observed on fruit-bearing trees under natural field conditions. Due to partial effectiveness of current control measures, development and use of bactericides is highly needed for the survival of Florida citrus industry. The goal of this study was to evaluate the effectiveness of trunk-injected OTC on saving Las-infected trees under natural field conditions. Our specific objectives were to (i) quantify the spatiotemporal dynamics of OTC concentration throughout citrus tree canopies and root system; (ii) assess the variation in OTC concentration among tree tissues including fruit; (iii) determine the minimum number of injection ports required for a uniform distribution of OTC in trunk-injected trees; and (iv) investigate the effect of trunk-injected OTC on suppression of Las populations, fruit drop, yield, and quality.

MATERIALS AND METHODS

Reagents and standard solutions for high-performance liquid chromatography (HPLC) analysis. HPLC grade oxytetracycline hydrochloride, methanol, and hexane were purchased from Sigma (St. Louis, MO). Analytical reagent grade sodium acetate, calcium chloride, disodium EDTA, citric acid, disodium hydrogen phosphate, sodium hydroxide, and hydrochloric acid were obtained from Thermo Fisher Scientific (Waltham, MA). All solutions were prepared with Milli-Q water. The McIlvaine buffer (pH 4.0) and Na₂EDTA-McIlvaine buffer (pH 4.0 and 8.0) solutions were weekly prepared as previously described (Pena et al. 2007). OTC stock solution (1 mg/ml) was prepared in methanol and stored in a -20°C freezer. Dilution series were prepared daily by diluting stock solution in methanol.

Field site, experiment trees, and trunk injection. Trees of 5-year-old 'Hamlin' sweet orange on 'Swingle' citrumelo rootstock in a central Florida citrus grove were selected for study. The grove is naturally infected by Las with high HLB incidence and severity. The well-drained soil is Astatula fine sand (Typic Quartzipsamment). Trunk diameter of 15 cm above the bud union was 9 cm for all trees in the experiment, which received standard commercial care including irrigation with microsprinklers.

All 15 trees receiving treatments had four primary branches labeled as b1, b2, b3, and b4 (Fig. 1). Each tree received 5 g of Arbor-OTC (Arborjet Inc., MA), equivalent to 2 g of active ingredient (AI) oxytetracycline hydrochloride, dissolved in 600 ml of distilled water. Trunk injection was carried out with tree I.V. MICRO INFUSION (Arborjet Inc.) at the recommended pressure (<50 psi) during a sunny day in February 2015. Holes on the trunk were drilled 30 cm below the first branch to a depth of 2 to 3 cm using 7.14 mm drill bit; a No. 3 Arborplug was set into each hole for proper seal with Arborplug setter and a rubber hammer. The area surrounding drilling site was treated with Ridomil Gold (Novartis) to prevent opportunistic infection by *Phytophthora* spp.

Treatments and experimental design. To assess effect of the number of injection ports on the uniformity of OTC concentration, varying number of injection ports ranging from one to four ports per tree were tested by dividing 600 ml of treatment solution equally among each port. The first port was always located 30 cm directly below the first branch that is closest to the ground and additional

ports were placed on the trunk with even space between any port pair (Fig. 1). Negative control was trees receiving 600 ml of water by trunk injection. A completely randomized design was used and each treatment was replicated with three trees.

Sampling schematic. After trunk injection, each tree was repeatedly sampled at the following six time points: 2, 4, 7, 14, 28, and 270 days postinjection (DPI). A total of 24 samples were collected from each tree at each time point. Six shoots, flowers, and leaves were removed from proximal, middle, and distal portions of four individual branches per tree. A total of four fruit were collected from each tree at 270 DPI. In addition, canopy-oriented sampling scheme was used to collect leaf and root samples. Specifically, the entire canopy was divided vertically into lower half and upper half, and then horizontally by cardinal directions into four quadrants: NE, NW, SE, and SW (Fig. 1D). Therefore, four quadrants in the upper half were designated as UNE, UNW, USE, and USW (Fig. 1c1) and four quadrants in the lower half as LNE, LNW, LSE, and LSW (Fig. 1c2). Six leaves were collected from each quadrant. Four root samples per tree were obtained from soil directly below each quadrant (RNE, RNW, RSE, and RSW) (Fig. 1c3); 5 to 10 g of fine root tissues (2 to 4 cm in length) was taken from 10 to 15 cm depth of soil. All samples were placed in a polyethylene bag on ice and transported back to lab for further analysis.

Sample preparation. Two leaf discs (0.90 cm in diameter) were cut from a leaf using a cork borer. A total of 12 leaf discs were incised from six separate leaves per sample. Root samples were rinsed with tap water to remove sands and were cut into 1 to 3 mm segments. Shoots, flowers, and roots were minced with a scalpel. Either 100 mg (DNA isolation) or 200 mg (HPLC analysis) of minced samples were placed in 2-ml screw cap tubes that contained two 4.5-mm stainless steel beads. All samples were ground in liquid nitrogen using the TissueLyser II (QIAGEN, Valencia, CA). To check the recovery rate of OTC and accuracy of the HPLC method, 200 mg of OTC-free samples (i.e., from water-injected control trees) were spiked with a fortification solution at three levels: 156.3, 625, 1,250 µg/kg. All samples were homogenized using bead beating method with TissueLyser II.

OTC Extraction and clean-up procedure. The polar nature of tetracycline allowed it to be extracted from tissues using pH 4.0 Na₂EDTA-McIlvaine buffer. The HPLC procedure used in this study was based on the method (Maia et al. (2008) for OTC quantification. The modifications to the aforementioned extraction procedure included a tissue grinding step with bead beating method and a centrifugation step at 14,000 rpm. Each ground sample was added with 0.8 ml of 0.10 M Na₂EDTA-McIlvaine buffer (pH 8.0) and the resulting suspension was shaken for 5 min in a vortex mixer at maximum speed and centrifuged for 5 min at 14,000 rpm. The supernatant was subjected to liquid-liquid extraction with equal volume of hexane. The pH value of aqueous phase was adjusted to 4.0 with 1 M citric acid and applied to an octadecyl cartridge (Bond Elut C18 OH 100 mg/ml, Agilent). The cartridge was preconditioned with 1 ml of methanol and 1 ml of Na₂EDTA-McIlvaine buffer (pH 4.0); thereafter, the SPE cartridge was loaded with a sample and washed with 1 ml of McIlvaine buffer (pH 4.0): methanol (vol/vol = 85/15). The OTC was eluted with 1 ml of methanol and air-dried in a vacuum and dissolved in 100 µl of the mobile phase buffer. The final elute was filtered through a 0.45 µm membrane filters Millipore (Agilent).

OTC concentration analysis by HPLC. An Agilent 1260 Infinity Quaternary Liquid Chromatography controlled by Agilent ChemStation (Rev C.01.06) (Agilent Technologies, Palo Alto, CA) was used to determine OTC concentration according to an earlier reported method (Maia et al. 2008). The HPLC system was equipped with G1311C 1260 Quat pump VL, G1329B 1260 ALS, G1316A 1260 TCC, G4212B 1260 DAD, and G1321B FLD Spectra. The column used for separation was an Eclipse plus C18 column (Agilent, 100 mm × 4.6 mm) housed in thermostatted compartment at 22°C. Fluorescence detection is a sensitive and specific tool for tetracycline concentration analysis. The fluorescent signal was produced by

chelating OTC with CaCl₂ and EDTA in mobile phase. The fluorescence detector was operated at 390 nm excitation and 512 nm emission wavelengths. The mobile phase contained buffer (pH 7.3) of 30% methanol and 70% aqueous solution of 0.035 M calcium chloride, 0.025 M disodium ethylenediaminetetraacetate (EDTA) and 0.075 M sodium acetate filtered through a 0.45 µm Omnipore membrane filter (Merck Millipore Ltd., Cork, IRL). The flow rate was 0.5 ml/min and the autosampler was configured to inject 50 µl aliquots of samples. Retention time of OTC was 62 s.

DNA extraction and enumeration of Las. DNA was isolated from macerated tissue using Wizard Genomic DNA purification kit (Promega) according to manufacturer's protocol. The air-dried DNA pellet was dissolved in 100 µl of DNA rehydration solution (supplied by Promega). An aliquot of 4 µl was used as DNA template to determine Las populations using a previously reported TaqMan qPCR protocol (Trivedi et al. 2009; Wang et al. 2006). The primers target the β-operon region of Las with primer probe set CQULA04F-CQULAP10-CQULA04R. All qPCR reactions were performed in triplicate using ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Briefly, a 25 µl of qPCR reaction consisted of 12.5 µl of 2× Quantitect probe PCR master mix (Qiagen, Valencia, CA), 1.25 µl of each primer (10 µM), 0.5 µl of probe (10 µM), 4 µl of DNA template, and 5.5 µl of DNase/RNase free water. The PCR cycling condition was initial activation step at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Cycle threshold (C_t) values were obtained by adjusting threshold to the recommended level of 0.02. All DNA samples were run in triplicate. The copy number of Las genomes in a reaction was calculated using a plasmid (pLBA2) standard curve ranging from 10 to 10⁶ plasmids/µl (Trivedi et al. 2009).

Fruit yield and quality analysis. Number of fruit drop was recorded when fruit was harvested in January 2016 (10 months after bloom). All fruit including decomposing fruit and dry mummies under the tree canopy were counted. Fruit weight was recorded for each tree and an ≈8 kg of composite fruit sample was taken from each tree for quality analysis according to standard methods (Gottwald et al. 2012). Juice content and fruit acidity were expressed as percent juice and percent citric acid. Total soluble solids expressed as fruit brix, a measure of sugar content in fruit, were calculated as weight (grams) of sugar in 100 g of juice. Thereafter, fruit brix and acidity ratio were calculated accordingly. All fruit from the tested trees were destroyed after data collection.

Statistical analysis. SAS software (SAS 9.3, SAS Institute Inc., Cary, NC) was used to perform all statistical analyses. Linear regression analysis (Proc REG) was used to obtain linear equation for OTC standard curve. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3× and 10× δ/s, where δ is the standard deviation of the response and s is the slope of the standard curve. OTC concentration data were first tested for normality and variance homogeneity and thereafter were log transformed to meet the assumption of normality as needed. The temporal distribution were analyzed with models for repeated measurement analysis using a compound symmetry covariance structure (Littell et al. 1998). Analysis of variance (Proc GLM) was used to determine statistical significance of main effects and interactions for the number of injection ports, tissue types, and time (DPI). When main effects were significant, Fisher's protected least significant difference (LSD, *P* < 0.05) were used for mean separation. Las populations in leaves and roots were log concentrations. Unequal variance *t* test was conducted to compare fruit yield and quality performance between OTC treatment and water-injected control.

RESULTS

OTC concentration analysis by HPLC. The standard curve for OTC quantification was established with seven concentrations of internal standard OTC as triplicates (i.e., 78, 156.3, 312.5, 625, 1,250, 2,500, and 5,000 µg/kg) (Fig. 2). The linear quantification range was from 40 to 5000 µg/kg. The intercept, slope, and linearity

(R^2) for the quantification curve were 0.6304, -34.451, and 0.9996, respectively. The LOD and LOQ were 12 and 40 $\mu\text{g/kg}$, respectively. There was relatively small variability in the OTC recovery rate among five citrus tissue types (Table 1), indicating that OTC extraction method worked effectively with all tissue types used in this study. The mean recovery rates at three OTC fortification levels of 156.3, 625, and 1,250 $\mu\text{g/kg}$ ranged from 84.5 to 95.4%, 85.0 to 92.3%, and 86.6 to 95.6%, respectively (Table 1).

Spatial distribution of OTC. The number of injection ports and canopy positions did not significantly influence the leaf OTC concentrations ($F = 3.28$, $P \geq 0.0794$; $F = 0.40$, $P \geq 0.5276$), although small to moderate variations were found in the leaf OTC concentration among four canopy quadrants (Table 2) or branches (Table 3). Higher injections port number, i.e., two, three, or four injection ports, resulted in quicker distribution of OTC across four cardinal quadrants of tree canopies when compared with one injection port at 2 or 4 DPI (Tables 2 and 3). Increasing the number of injection ports led to significantly higher leaf OTC concentrations than a single injection number ($F = 4.75$, $P < 0.0029$), particularly at 2 or 4 DPI (Tables 2 and 3). For example, the leaf OTC concentrations of trees with two, three, and four injection ports were 151, 231, and 138 $\mu\text{g/kg}$ higher than those of one injection port at 7 DPI. However, the number of injection ports did not affect the overall leaf OTC concentrations at 28 and 270 DPI ($F = 0.86$, $P \geq 0.4673$; $F = 1.05$, $P \geq 0.3783$).

Variation in OTC concentration among tree tissues. When compared with two injection ports or more, a single injection port delivered significantly less amount of OTC to leaves, roots, and flowers at 2 DPI (Fig. 3). In general, two injection ports provided similar level of OTC concentrations to three or four ports in all tissue types (Fig. 3). A repeated-measures ANOVA showed that citrus tissue type and time (as DPI) significantly affected OTC concentration in trunk-injected trees ($F = 130.08$, $P < 0.0001$; $F = 24.86$, $P < 0.0001$). While there was no significant difference in OTC concentration between leaf and root at all but 4 DPI, OTC concentrations in leaf and root were significantly higher ($P < 0.05$) than those of shoot and flower; OTC concentration in shoot was significantly higher than that in flower at all but 2 DPI. OTC concentration in fruit was several-fold lower than those of other tissue types.

Persistence of OTC in relation to Las population dynamics in citrus tree. At the injection rate of 2 g of AI/tree, OTC was detected in shoot, flower, leaf, and root tissues within 2 days of trunk injection at concentrations of more than 500 $\mu\text{g/kg}$, indicating rather fast uptake and transport of OTC in vascular system throughout trees. While shoot had little gain in accumulation of OTC after 2 DPI, OTC concentration increased steadily in leaf and root and peaked above 1,000 $\mu\text{g/kg}$ at 14 DPI (Fig. 3). In contrast,

OTC concentration exhibited a decline trend in flower after 2 DPI. After 14 DPI, OTC concentration started to decline gradually in all four types of tissues, but over 500 $\mu\text{g/kg}$ were detected in both root and leaf tissues when sampling was concluded at 9 months postinjection (Fig. 3). At 270 DPI or 1 month prior to harvesting, OTC concentration of 202 $\mu\text{g/kg}$ was detected in fruit (Fig. 3). The Las populations in leaves and roots dropped rapidly as the OTC concentration increased from 0 to 1,200 $\mu\text{g/kg}$ during the first 28 DPI (Fig. 4). In contrast, Las populations in water-injected trees stayed at similar level during the test period. The lowest Las populations occurred at 28 DPI, 2 weeks after the peak OTC concentration, which was 2.19 and 1.76 log reduction in leaf and root, respectively. Las populations in leaf and root at 270 DPI grew to some extent when compared with those at 28 DPI, but still substantially lower than those at 2 DPI (Fig. 4).

HLB symptom, fruit yield, and quality. When OTC was injected at 2 g of AI/tree at the beginning stage of early spring flushes and bloom in mid-February, the OTC concentration in new shoots attained the peak of 733 $\mu\text{g/kg}$ during the first month following injection. Moderate phytotoxic effect ranging from brown discoloration to leaf burning was observed on some newly developed leaves located at the distal portions of branches. In contrast, either young fully expanded leaves or mature leaves did not exhibit any phototoxic effect, except that the foliage showed a slight yellowing when compared with water-injected trees within 3 months postinjection. During summer flushes in mid-June and July, OTC-treated trees no longer showed yellowing and produced many more vigorous new shoots with large expanded leaves when compared with water-injected trees. Although OTC-treated trees had an average of 18.5% fewer fruit dropped and fruit weighed 14.3% higher than water-injected trees, such improvement was not statistically significant (t test: $P \geq 0.4251$, $P \geq 0.3706$) (Table 4). Juice acidity from

TABLE 1. Recovery rate in several tissue samples spiked with different concentrations of oxytetracycline (OTC)

Sample type ^y	Recovery rate (%) ^z		
	156.3 $\mu\text{g/kg}$	625 $\mu\text{g/kg}$	1,250 $\mu\text{g/kg}$
Root	94.4 $\pm$ 10.4	88.4 $\pm$ 7.2	95.6 $\pm$ 0.9
Leaf	84.5 $\pm$ 1.1	87.2 $\pm$ 6.0	86.6 $\pm$ 6.8
Shoot	87.0 $\pm$ 6.3	92.0 $\pm$ 2.6	93.7 $\pm$ 4.2
Flower	95.4 $\pm$ 5.8	92.3 $\pm$ 1.1	95.6 $\pm$ 0.9
Fruit	87.0 $\pm$ 2.7	85.0 $\pm$ 2.0	87.9 $\pm$ 5.5

^y Each sample (200 mg) was spiked with 100 μl of OTC solution at different fortification concentrations of OTC.

^z Recovery rate was expressed as means $\pm$ standard deviation of three replicated samples.

Fig. 2. Standard curve for quantification of oxytetracycline hydrochloride (OTC) in a citrus sample. Each concentration was run as triplicates and the standard curve was included for each high-performance liquid chromatography run.

OTC-treated trees was reduced ($P \leq 0.0971$) when compared with that from water-injected trees (Table 4). There were no significant differences in other fruit quality parameters between OTC-treated and water-injected trees.

DISCUSSION

This is the first study characterizing spatiotemporal dynamics of OTC concentration in several citrus tissue types and demonstrating persistent Las suppression under field conditions. Our results indicated that trunk-injected OTC were readily transported to all citrus tissue types. Our findings of high OTC concentration in leaves and shoots, lower in flower and extremely low in fruit agree with previous results obtained on trunk-injected sweet orange (Aubert and Bove 1980; Lee et al. 1982; Timmer et al. 1985) and coconut palms (Hunt et al. 1974; McCoy 1976). This is the first study to demonstrate high level of OTC concentration in root system. Earlier studies detected either little OTC activity or low activity in some but not all root samples (McCoy 1976; Timmer et al. 1985). This difference may be due to the much lower OTC extraction efficiency and lower sensitivity of OTC bioassay than HPLC procedure used in the current study. The detection of high level of OTC concentration in root system may suggest that OTC transport occur in not only xylem, but also phloem. This is probably due to water exchange between phloem and xylem. It was suggested that in all vascular plants, phloem and xylem tissues are located next to each other, and there is clear evidence that these tissues exchange water (Sevanto 2014). It was shown that OTC was translocated into the fruit of treated coconut palms at extremely low levels (McCoy, 1976). Similarly, OTC was detectable in fruit at 202 $\mu\text{g/kg}$, but this concentration is 42.3% below the tolerance level of 350 $\mu\text{g/kg}$ permitted

by governmental agencies (United States Environmental Protection Agency 2006, 2008). Tetracyclines are administered in humans at doses from 1 to 2 g daily for at least 7 days, which is equivalent to a minimal exposure of 7 to 14 g for a prescribed cycle (<https://www.drugs.com/dosage/tetracycline.html>). Assuming a kilogram of citrus fruit contains 202 μg of OTC, a person would need to consume daily about 4,950 kg of orange for exposure of minimal daily therapeutic dose of tetracycline. United States Environmental Protection Agency (2008) concluded that the potential dietary exposure of humans to OTC used in agriculture would result in no harm compared with its pharmaceutical usage.

Different tree species differ in the xylem architecture and sap flow patterns with some species have relatively constrained (sectored) vascular connections, while others have relatively unconstrained (integrated) vascular connections. Consequently, the transport pathways of trunk-injected therapeutic compounds in vascular systems might vary greatly among tree species. While lateral movement of inorganic solutes occurs by radial diffusion in lateral transport system (Baker and Milburn 1965), the vertical transport systems are not straight sectorial, but instead either helical or “zig zag” sectorial. The majority of tree species were reported to have sectorial xylem architecture (Larson et al. 1994; Orians et al. 2004; Tanis et al. 2012; Zanne et al. 2006). This phenomenon requires multiple injection ports spaced radially around the stem for a uniform distribution of trunk-injected compounds throughout the canopy (Aćimović et al. 2014; Baker and Milburn 1965; Larson et al. 1994; Percival and Boyle 2005). For example, Aćimović et al. (2014) trunk-injected 29-year-old mature apple trees with imidacloprid and found a spiral pattern for vertical uptake (Aćimović et al. 2014) and a minimum of four injection ports are needed for even distribution of imidacloprid. In our experiments,

TABLE 2. Spatial distribution of trunk-injected oxytetracycline (OTC) (2 g of active ingredient/tree) at various days postinjection in canopy quadrants based on cardinal directions in relation to varying number of injection ports

DPI ^y	Number of injection ports ^z															
	1				2				3				4			
	NE	SE	SW	NW	NE	SE	SW	NW	NE	SE	SW	NW	NE	SE	SW	NW
2	60±15	63±20	78±34	71±10	64±28	137±13	136±14	130±6	142±47	129±29	152±15	115±10	115±44	90±31	78±25	86±27
4	48±13	36±20	43±23	43±27	108±27	102±7	86±2	103±2	111±3	105±2	108±10	108±7	123±28	103±55	112±35	114±31
7	108±10	105±12	107±18	92±9	95±27	112±66	74±12	127±23	123±19b	178±57a	168±50a	119±25b	144±35	153±25	158±51	131±28
14	173±27	160±30	150±21	144±33	177±40	135±46	178±65	187±41	118±16	135±29	139±22	133±14	130±7	145±11	109±48	127±43
28	76±6	54±9	58±3	130±36	87±43	89±14	109±1	102±8	112±4	109±9	105±3	103±16	56±4	56±9	63±7	100±20
270	43±3c	62±15b	72±14a	61±4b	64±1	65±6	51±6	74±23	56±11	99±8	64±8	67±5	68±21	73±29	68±7	81±25

^y DPI is abbreviation of days postinjection.

^z All ports were located 30 cm directly below the first branch that is closest to the ground and additional ports were placed on the trunk with even space between any port pair (Fig. 1). The entire tree canopy was divided vertically into lower half and upper half, and then horizontally by cardinal directions into four quadrants NE, NW, SE, and SW (Fig. 1). The main effect of canopy positions (upper versus lower) was not significant ($P \geq 0.5286$) and therefore the OTC concentrations from upper and lower positions of one cardinal direction were averaged to simplify data presentation. Means followed by different letters within a row of injection ports indicate least significant difference (LSD, $P < 0.05$). Mean OTC concentrations (10 $\mu\text{g/kg}$) of each quadrant are followed by standard deviation of the mean.

TABLE 3. Branch-wise distribution of trunk-injected oxytetracycline (OTC) (2 g of active ingredient/tree) at various days postinjection in relation to varying number of injection ports

DPI ^y	The number of injection ports ^z															
	1				2				3				4			
	b1	b2	b3	b4	b1	b2	b3	b4	b1	b2	b3	b4	b1	b2	b3	b4
2	66±23	71±31	84±38	74±25	64±33b	39±5b	161±53a	211±10a	172±59	147±8	217±70	199±39	117±14	71±29	118±37	88±37
4	97±42	99±34	59±16	56±35	104±28	93±26	75±19	89±30	89±25	95±14	91±23	104±33	128±27	110±7	115±53	159±26
7	101±8	100±19	85±29	97±8	89±22	92±26	99±19	122±9	151±29	144±27	160±14	180±41	126±27	160±41	112±34	125±26
14	152±34	145±62	158±38	161±22	109±28	123±32	149±11	157±23	143±27	133±18	133±25	123±31	102±4	90±25	93±13	97±24
28	58±14	60±25	81±18	175±42	144±16	93±3	114±29	186±37	134±14	71±7	97±15	99±5	76±30	81±23	62±30	183±33
270	83±13	58±6	63±4	76±11	62±13	58±9	69±21	62±7	71±17	64±19	61±17	68±10	62±22	78±19	78±9	83±14

^y DPI is abbreviation of days postinjection.

^z All ports were located 30 cm directly below the first branch that is closest to the ground and additional ports were placed on the trunk with even space between any port pair (Fig. 1). Six leaves were collected from proximal, middle, and distal portions of four individual branches on a tree labeled as b1, b2, b3, and b4 (Fig. 1). Means followed by different letters within a row of injection ports indicate least significant difference (LSD, $P < 0.05$). Mean OTC concentrations (10 $\mu\text{g/kg}$) of each quadrant are followed by standard deviation of the mean.

when trees were injected with a single port, similar level of OTC activity was consistently detected in all branches at different orientations in relation to injection ports (Table 3; Fig. 1) or all cardinal quadrants in both lower and upper canopy (Table 2). However, increasing the number of injection ports did lead to quick distribution of OTC at 2 and 4 DPI (Tables 2 and 3). The observed OTC transduction pattern in citrus is supported by results from an earlier study of water and dye transport in 22-year-old grapefruit trees (Vasconcellos and Castle 1994). Their study showed that the majority of dye movement was within 2 cm depth into trunk and moved ≈ 50 cm up and 25 cm down the trunk within 1 to 2 min after injection; in particular, the dye also spread tangentially to all surrounding xylem vessels at the 0 to 3 cm depth. Mendel et al. (2000) applied radio-labeled imidacloprid to the bark of 9-year-old citrus trees and found imidacloprid in parenchyma and long-distance transport in xylem. In addition, coconut palm trees seemed to have similar interconnecting xylem vessels because it also exhibited fast and even distribution of trunk-injected OTC in tree foliage (Hunt et al. 1974; McCoy 1976). A thorough study with radio-labeled OTC is needed to better understand the vascular sectoriality and chemical distribution in xylem and phloem of citrus.

The rapid transport and uniform distribution of OTC throughout entire trees support the use of trunk injection as an alternative method for efficient delivery of therapeutic compounds into citrus trees. Recently, several similar studies demonstrated the utility of trunk injection in control of insects on a few other fruit trees including apple and avocado (Aćimović et al. 2014; Aćimović et al. 2015; Aćimović et al. 2016; Byrne et al. 2012; VanWoerkom et al. 2014). However, the majority of current injection technologies involves drilling holes on tree trunk. This drilling-based injection procedure is labor intensive and causes injuries to tree (Perry et al. 1991). Therefore, the number of injection ports needs to be optimized for two important practical reasons: the first is to minimize the number of injection ports that will reduce economic costs by minimizing drilling efforts and potential tree injuries; the second is to have sufficient number of injection ports to allow a quick and uniform distribution of compounds at concentrations higher than MIC or MBC (minimum inhibition concentration or minimum bactericidal concentration). Our data indicated that two, three, or four injection ports did result in much higher OTC concentrations within 7 DPI than the single injection port. No significant improvement was observed for three and four injection ports than two injection ports (Tables 2 and 3). These results support the use of two injection ports for 5-year-old citrus trees.

Fig. 3. Oxytetracycline hydrochloride (OTC) concentrations in **A**, shoots, **B**, roots, **C**, flowers, **D**, fruit, and **E**, leaves of 5-year-old 'Hamlin' sweet orange on 'Swingle' citrumelo rootstock trees that were trunk injected with OTC at 2 g of active ingredient/tree. Means followed by different letters at each day postinjection indicate significant differences in OTC concentrations among varying number of injection ports (least significant difference, $P < 0.05$).

It remains to be determined the suitable injection port number for mature trees. Needle-based injection systems have been developed to ameliorate the potential tree injury (Montecchio 2013). However, needle-based injection systems are time consuming to inject liquid into the tree because they are a passive system and not using air pressure to inject the liquid into the tree. They rely solely on sap-flow driven vacuum force to allow uptake of AI solution. Needle-based injection systems are reliant on intensive sap flow driven by strong transpiration from the canopy. If it is cold weather the sap will move very slowly, or not move at all, thus the uptake into xylem will be very weak. Current injection technology is primarily designed for use in landscape tree cares and may not be able to scale up to cope with hundreds of thousands of Las-infected trees. However, the great potential of trunk injection in control of diseases or pests call for development of automated trunk injection system with high efficiency.

The long persistence of OTC in foliage and roots correlated with long-lasting suppression of Las populations in Las-infected trees. The half-life of OTC in foliage and roots of citrus was 3 to 4 weeks, which is similar to the 3 weeks reported for coconut palms (Hunt et al. 1974). It has been reported that plants treated by trunk injection of OTC tended to remain in symptom remission long after OTC concentration was below detectable level in plant tissues (Hunt et al. 1974; McCoy 1976). Although the MIC and MBC of OTC against Las bacteria is unknown, the observation of 1.76 and 2.19 log reduction of Las in roots and leaves by over 1,000 µg/kg of OTC concentration in both leaves and roots indicated that this concentration had an antimicrobial effect on Las. Interestingly, Las population in the roots and leaf of OTC-treated trees remained 2 log lower than the negative control at 270 days posttreatment (Fig. 4). Trivedi et al. (2009) reported that a minimal Las concentration is required for the expression of HLB symptoms. The reduced Las population due to OTC suggests that an

Fig. 4. Population dynamics of ‘*Candidatus Liberibacter asiaticus*’ (Las) in **A**, leaves and **B**, roots of 5-year-old ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock trees that were trunk injected with either oxytetracycline hydrochloride (OTC) at 2 g of active ingredient/tree or water as water-injected control. Las populations represent as means and error bars as standard deviation ($n = 3$ for OTC-treated trees and $n = 3$ for water-injected control) on logarithmic scale.

TABLE 4. Yield and quality of ‘Hamlin’ sweet orange fruit harvested from oxytetracycline (OTC)-treated and water-injected control trees

Treatment ^w	Yield (kg/tree)	Number of fruit dropped ^x	Fruit quality ^y			
			Juice (%)	Brix	Acidity	Brix/acidity
OTC-treated	16.2 ± 4.7	95 ± 25	58.0 ± 2.0	9.8 ± 0.9	0.49 ± 0.01	19.5 ± 1.3
Water-injected	14.1 ± 1.5	116 ± 36	58.1 ± 0.9	10.3 ± 1.0	0.52 ± 0.01	20.0 ± 2.1
<i>P</i> values of <i>t</i> test ^z	0.3706	0.4251	0.9476	0.4966	0.0971	0.7560

^w Five-year-old ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock trees were trunk injected with either water as control or OTC at 2 g of active ingredient/tree during early spring flushes.
^x Counts of fruit abscission were enumerated when fruit was harvested in January (11 months postinjection). All fruit including decomposing fruit and dry mummies under the tree canopy were counted. Each value is presented as mean ± standard deviation.
^y An ≈8 kg of composite fruit sample was taken from each tree for quality analysis according to standard methods (Gottwald et al. 2012).
^z Unequal variance *t* test was used to determine whether there was significant difference between OTC-treated and control trees.

annual application of OTC right after each harvest might be enough to suppress Las population and stimulate tree growth. The finding of strong Las suppression extends earlier observations obtained with Las-infected periwinkle and citrus in greenhouse (Zhang et al. 2011, 2012). The finding of relatively high level of OTC in new shoots corroborated with earlier results of preferential accumulation of imidacloprid in shoots (Mendel et al. 2000). The occurrence of high level of OTC in shoots will prevent new flushes from infection by Las. Trivial phytotoxicity was found on new flushes of a young citrus tree. We speculated that the phytotoxicity will diminish in mature citrus trees after the young trees pass the juvenility. Likewise, our results of decreased fruit drop and increased yield corroborates results from earlier field trial with trunk-injected tetracycline in South Africa (Aubert and Bove 1980). The increase in yield due to OTC treatment is not significant, which might indicate that OTC-treated trees need additional time to recover from the physical damages caused by HLB, e.g., phloem blockage and root decline.

In summary, this research has demonstrated that OTC moved rapidly within citrus trees following trunk injection and a uniform spatial distribution of OTC within citrus trees can be obtained via trunk injection. The research also showed that direct injection of OTC into citrus trunk at 2 g/tree allowed accumulation of sufficient amount in citrus tree canopy and roots. Injected OTC provided long-lasting suppression of Las populations in Las-infected trees and prevented further tree decline by promoting symptomless new growth and by reducing the transmission rate of Las by ACP. Our results indicated that trunk injection can be used as an effective measure for integrated management of citrus HLB and reduce the potential negative impact of bactericide on the environment.

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