Control of Citrus Huanglongbing via Trunk Injection of Plant Defense Activators and Antibiotics

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ABSTRACT

Citrus huanglongbing (HLB) or greening is a devastating disease of citrus worldwide and no effective control measure is currently available. Plant defense activators environmentally friendly compounds capable of inducing resistance against many plant pathogens. Earlier studies showed that foliar spray of plant defense inducers could slow down HLB disease progress. In this study, eight plant defense activators and three antibiotics were evaluated in three field trials for their effect to control HLB by trunk injection of young and mature sweet orange trees. Results showed that four trunk injections of several activators, including salicylic acid, oxalic acid, acibenzolar-S-methyl, and potassium phosphate, provided significant control of HLB by suppressing '*Candidatus Liberibacter asiaticus*' titer and disease progress. Trunk injection of penicillin, streptomycin, and oxytetracycline hydrochloride resulted in

excellent control of HLB. In general, antibiotics were more effective in reduction of '*Ca. L. asiaticus*' titer and HLB symptom expressions than plant defense activators. These treatments also resulted in increased yield and better fruit quality. Injection of both salicylic acid and acibenzolar-S-methyl led to significant induction of pathogenesis-related (PR) genes *PR-1* and *PR-2* genes. Meanwhile, injection of either potassium phosphate or oxalic acid resulted in significant induction of *PR-2* or *PR-15* gene expression, respectively. These results suggested that HLB diseased trees remained inducible for systemic acquired resistance under field conditions. In summary, this study presents information regarding controlling HLB via trunk injection of plant defense activators and antibiotics, which helps citrus growers in decision making regarding developing an effective HLB management program.

Citrus huanglongbing (HLB) or greening is one of the most destructive diseases of citrus and causes significant economic losses worldwide (Bove 2006; da Graca and Korsten 2004; Gottwald et al. 2007). Citrus HLB in Florida is associated with '*Candidatus Liberibacter asiaticus*', a phloem-limiting bacterium vectored by Asian citrus psyllid (ACP) *Diaphorina citri* (Halbert and Manjunath 2004). All commercial citrus cultivars are susceptible to HLB and symptom expression on '*Ca. L. asiaticus*'-infected trees varies widely, including yellow shoot, blotchy leaf mottle, vein corking, nutrient deficient foliage, premature fruit drop, small lopsided fruit with off-flavor, twig dieback, and tree death (da Graca and Korsten 2004; Halbert and Manjunath 2004). HLB-diseased trees have an average yield loss of 30 to 100% and often die in 5 to 8 years after diagnosis (Bove 2006). Currently, it has been estimated that almost all mature citrus trees have been infected by '*Ca. L. asiaticus*' in Florida.

Citrus HLB in Florida is currently mitigated by disrupting '*Ca. L. asiaticus*' transmission through ACP control (Boina and Bloomquist 2015). Psyllid control with insecticides combined with foliar nutritional spray has kept ACP populations at low levels, slowed spread of the disease, and maintained yield potential in orchards with high incidence of HLB (Stansly et al. 2014). However, such an HLB management program has not been able to stop HLB from spreading to new plantings (Boina and Bloomquist 2015; Stansly et al. 2014). Several new control tactics such as chemotherapy (Hoffman et al. 2013), chemotherapy (Hu and Wang 2016; Zhang et al. 2014), and transgenic citrus (Mirkov and Gonzalez-Ramos

2013) are in the early stage of development and are likely years away from application in the field. Considering the limited chemical options for controlling bacterial plant diseases in agricultural settings, there are urgent needs to search for better options to expand HLB control arsenal.

Induced resistance, especially systemic acquired resistance (SAR), has been reported to be useful to control many plant diseases (Buzi et al. 2004; Dekkers et al. 2004; Graham and Myers 2011; Justyna and Ewa 2013; Mersha et al. 2012; Obanor et al. 2013; Oren and Yogeve 2002; Perez et al. 2003). The SAR mechanism involves a specific defense signaling pathway that requires the signal molecule salicylic acid (SA) and is associated with accumulation of pathogenesis-related (PR) proteins. SAR may be activated by either the presence of a pathogen or spraying plants with chemical inducers or activators (Aranega-Bou et al. 2014; Vallad and Goodman 2004). Multiple plant defense activators such as acibenzolar-S-methyl (ASM), β -aminobutyric, benzothiadiazole, 2,6-dichloro-isonicotinic acid, (INA) and phosphite have been evaluated extensively for their efficacy on disease control under field conditions (Vallad and Goodman 2004; Walters et al. 2013). Results from the majority of earlier studies showed that SAR inducers provided good but often-incomplete protection against a wide variety of pathogens. Their utility for practical application in disease management has been questioned because of large variability in effectiveness of control. For example, disease reduction by plant defense activators under field conditions could vary from lack of control to 99% and also from field to field (Vallad and Goodman 2004; Walters et al. 2013). Such relatively large variability in their efficacy is thought to be influenced by several factors, including pathogen, plant species, host genotype, plant nutrition, and environmental conditions, as well as application time, frequency, and method. This variability highlights the

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importance of evaluating plant defense activators under local environmental conditions and optimization of application.

Previous research on citrus demonstrated that SAR in citrus was highly inducible and treatment of citrus plants with several plant defense activators resulted in enhanced resistance to both bacterial and fungal diseases. For example, foliar spray of citrus with benzothiadiazole induced resistance to citrus scab, melanose, and *Alternaria* brown spot (Agostini et al. 2003). Soil application of ASM, INA, and imidacloprid increased expression of gene *PR-2* and provided effective control of citrus canker in greenhouse assays (Francis et al. 2009). Furthermore, soil-applied ASM, thiamethoxam, and imidacloprid provided effective protection from canker on foliar flushes of young grapefruit trees under epidemic conditions; however, the efficacy depended on rate, frequency, and timing of application (Graham and Myers 2011). A recent study of citrus revealed that foliar sprays of INA, β -aminobutyric, and benzothiadiazole, individually or in combination, were effective in slowing down progress of HLB disease (Li et al. 2016a). Some of these studies noted that application methods greatly affected activators' effectiveness in disease control, with soil application superior to foliar spray (Dekkers et al. 2004; Francis et al. 2009; Graham and Leite 2004; Graham and Myers 2011). Therefore, optimizing application methods may result in better treatment effects.

Trunk injection is a target-precise method for efficient delivery of compounds into the plant by harnessing the xylem's quick transporting ability. There has been limited research on trunk injection of antibiotics and plant defense activators for better disease control. Currently, the utility of trunk injection of bactericides and plant defense activators in disease management has been demonstrated by several recent field studies. Aćimović et al. (2015) showed that one to two apple tree injection of potassium phosphite and ASM provided good control of blossom and shoot blight (Aćimović et al. 2015). Our previous study demonstrated that oxytetracycline injection provided long-lasting suppression of '*Ca. L. asiaticus*' populations in '*Ca. L. asiaticus*'-infected trees and prevented further tree decline by promoting symptomless new growth (Hu and Wang 2016). As part of an ongoing project to evaluate trunk injection of SAR activators and antibiotics for better control of citrus HLB, we conducted multiyear field trials to ascertain the utility of eight activators and three antibiotics on suppression of '*Ca. L. asiaticus*' titers and HLB disease progress in citrus under natural field conditions.

MATERIALS AND METHODS

Treatments of plant defense activators and antibiotics.

The chemical inducers tested in field trials consisted of SA, oxalic acid (OA), potassium phosphate dibasic (PHI), ASM (Actigard 50 WP; Syngenta), imidacloprid (IMI) (AmTide imidacloprid 2F;

AmTide LLC), L-ascorbic acid sodium salt (AA) (500GR; Acrös Organics), β -aminobutyric acid (BABA) (Sigma-Aldrich), and INA (Astattech Inc.). Antibiotics tested in this study included oxytetracycline hydrochloride (OTC) (Arbor-OTC formulation; Arborjet Inc.), penicillin G sodium salt (PCN) (Sigma-Aldrich), and streptomycin sulfate salt (STM) (Sigma-Aldrich). The concentration and injection volume for each treatment are summarized in Table 1.

Field sites and experimental design. Three field trials were independently conducted in a Mid-Florida Foundation grove located in central Florida from 2014 to 2016. The grove is established on the well-drained soil of Astatula fine sand (Typic Quartzipsamment) and naturally infected by '*Ca. L. asiaticus*', with HLB severity less than 30%. All HLB-affected trees received standard commercial care, including normal irrigation and fertilization as well as conventional pest management practices. A randomized complete block design was used for all three trials. Two weeks prior to the start of each trial, a field survey was carried out to determine the HLB severity of individual trees via visual assessment and '*Ca. L. asiaticus*' titers using a real-time quantitative polymerase chain reaction (qPCR) assay. Trees with a similar stage of HLB symptom expression were selected and divided into blocks (blocking factor) according to the level of '*Ca. L. asiaticus*' titers in an HLB-affected tree. Trials I and II consisted of four blocks of 12 and 9 treatments, randomly assigned to 5-year-old Hamlin sweet orange (*Citrus sinensis* L. Osbeck) on cultivar Swingle citrumelo rootstock. Trial III had 10 blocks of six treatments randomly assigned to 11-year-old Midsweet orange (*C. sinensis* L. Osbeck) on cultivar Carrizo citrange rootstock. To achieve uniform distribution across the tree canopy, higher injection volumes (300 ml) were used in trial III as compared with an injection volume of 200 ml in trials I and II.

Treatment applications via trunk injections. Two injection ports per tree, placed at the opposite side of a tree trunk and positioned at approximately 15 cm above the bud union, were made by drilling 20 to 30 mm into the xylem tissue with a 7.14-mm drill bit. Ports were properly sealed with Arborplug number 3 using Arborplug setter and a rubber hammer (Arborjet Inc.). Treatment liquid in a pressurized bottle was injected into each port on a tree using Arborjet tree I.V. Micro Infusion at the recommended pressure (<50 psi) during a warm and sunny day (Hu and Wang 2016). The area surrounding the drilling site was treated with Ridomil gold (Novartis) to prevent opportunistic infection by *Phytophthora* spp. Trunk injections of different treatments were conducted when new flush was present. In all three trials, four injections were carried out on an approximately 3-month basis. Trial I started in June 2014 and ended in October 2015; trial II started in September 2014 and ended in October 2015; and trial III started in February 2015 to February 2016.

TABLE 1. Concentrations and dosages of plant defense activators and antibiotics used for trunk injection^a

Trial I			Trial II			Trial III		
Treatments	Rate (mg/ml)	Volume (ml/tree)	Treatments	Rate (mg/ml)	Volume (ml/tree)	Treatments	Rate (mg/ml)	Volume (ml/tree)
T1 = WIC	NA	200	T1 = WIC	NA	200	T1 = WIC	NA	300
T3 = SA	4	200	T5 = SA	4	200	T3 = SA	3	300
T4 = ASM	5	200	T8 = ASM	5	200	T5 = ASM	3	300
T2 = OA	5	200	T4 = OA	5	200	T2 = OA	5	300
T5 = PHI	5	200	T6 = PHI	5	200	T4 = PHI	3	300
T6 = IMI	5	200	T7 = IMI	5	200	T6 = OTC+STM	4	300
T7 = PCN	12.5	200	T10 = OTC	6.25	200	...	...	...
T8 = PCN	6.25	200	T3 = AA	5	200	...	...	...
T9 = STM	12.5	200	T2 = BABA	5	200	...	...	...
T10 = STM	6.25	200	T9 = INA	5	200	...	...	...
T11 = OTC	12.5	200	...	...	...	...	...	...
T12 = OTC	6.25	200	...	...	...	...	...	...

^a WIC = water as control, NA = not applicable, SA = salicylic acid, ASM = acibenzolar-S-methyl, OA = oxalic acid, PHI = potassium phosphite, PCN = penicillin G sodium salt, STM = streptomycin, OTC = oxytetracycline hydrochloride, AA = L-ascorbic acid sodium salt, BABA = β -aminobutyric acid, and INA = 2, 6-dichloro-isonicotinic acid.

Disease assessment, fruit yield, and quality analysis. Visual assessments of disease severity were conducted on a 3-month interval using the HLB severity scale described by Gottwald et al. (2007). Each individual tree canopy was divided horizontally and vertically into eight sections and disease severity was scored on a section basis on a scale from 0 to 5, where 0 = none and 1 = ≤20, 2 = 21 to 40, 3 = 41 to 60, 4 = 61 to 80, and 5 = 81 to 100% of limbs affected. Mean HLB score was obtained by averaging these eight scores of an individual tree, which was used to calculate the amount of disease severity, expressed as the standardized area under the disease progress stair (sAUDPS) using Simko and Piepho's formula (Simko and Piepho 2012).

Fruit were harvested and weighed in October 2015 for trials I and II and in February 2016 for trial III. The yield data were recorded for each tree and a composite fruit sample (approximately 8 kg) was randomly sampled from each tree and subjected to juice quality analysis using a standard procedure (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 trees receiving antibiotic treatment were destroyed after data collection.

DNA isolation and 'Ca. L. asiaticus' titer enumerations. Due to the uneven distribution of the 'Ca. L. asiaticus' bacterium throughout the tree canopy, three sets of composite leaf samples (four leaves with mottling symptoms per limb) were collected from three different limbs of a tree canopy at a given time point. The first set of leaf samples was collected approximately 2 weeks prior to start of the first trunk injection. Thereafter, the same set of three limbs were repeatedly sampled 1 week after each treatment application. Leaf samples were placed in a polyethylene bag on ice and transported back to the lab for DNA extraction. Midribs were minced and 100 mg of minced midrib tissue was frozen in liquid nitrogen and ground using the bead-beating method with Qiagen Tissue Lyser II (Retsch, Inc.). DNA was isolated from ground tissue using the Wizard Genomic DNA purification kit (Promega Corp.) according to the manufacturer's protocol. The air-dried DNA pellet was dissolved in 100 µl of DNA rehydration solution. The concentration of isolated DNA was estimated on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to 100 ng/µl. An aliquot of 4 µl was used as DNA template to determine the 'Ca. L. asiaticus' titers using a real-time qPCR method (Wang et al. 2006). All qPCR were performed in triplicate on an ABI PRISM 7500 sequence detection system (Applied Biosystems), with cycling conditions described previously (Hu and Wang 2016). Specifically, a 25-µl qPCR consisted of 12.5 µl of 2× QuantiTect probe PCR master mix (Qiagen), 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

conditions consisted of an 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 the threshold to 0.02, as recommended by the QuantiTect Probe PCR Kits (Qiagen). A C_t value was converted into 'Ca. L. asiaticus' titers as genome copy number using a standard curve described previously (Trivedi et al. 2009). To determine the effect of treatments on 'Ca. L. asiaticus' titers in leaves, treatment effect was expressed as percent change in 'Ca. L. asiaticus' titer = [(mean titer after treatment – mean titer prior to treatment)/mean titer prior to treatment] × 100.

PR gene expression in leaves. Gene expression analyses were conducted for trials II and III. Leaf samples were taken from the same designated trees at five time points: 0, 2, 4, 7, and 14 days after the first trunk injection. At a given sampling time point, three sets of composite leaf samples representing three biological replicates per treatment were collected from three treated trees. A composite leaf sample consisting of eight leaves was randomly collected from an individual tree receiving plant defense activators or water. The leaf samples were wrapped in a prelabeled aluminum foil bag and immediately frozen in liquid nitrogen and stored at –80°C until further analysis. Sixteen leaf discs (0.90 cm in diameter) cut from eight leaves per sample using a cork borer were ground in liquid nitrogen to fine powder. Total RNA was isolated using the RNeasy mini kit for plants (Qiagen), following the manufacturer's instructions. RNA purification was conducted using the DNA-free DNase Treatment and Removal kit (Ambion). The quality and quantity of RNA samples were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The concentration of RNA samples was adjusted to 25 ng/µl and an aliquot of 4 µl of RNA sample (100 ng) was used in the following assay. The gene expression of *PR-1*, *PR-2*, *PR-3*, and *PR-15* was quantified by a quantitative reverse-transcription (qRT)-PCR assay using one-step qScript qRT-PCR Kit (Quanta Bioscience) in an ABI PRISM 7500 sequence detection system. In total, three biological replicates from three treated trees were performed for each treatment. Each biological replicate was run in three separate PCR (three technical replicates). The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase-C (*GAPDH-C*) was used as an endogenous control. All primer sequences used in this study were specific to sweet orange (*C. sinensis*) and their origins and targets are summarized in Table 2.

Relative gene expression was computed using the $2^{-\Delta\Delta C_t}$ method described by Livak and Schmittgen (2001). Briefly, the fold change in the target gene was normalized to plant *GAPDH-C* gene and relative to the expression at time 0 was calculated for each sample using the equation $\Delta\Delta C_t = (C_{t,Target} - C_{t,GAPDH})_{Time\ x} - (C_{t,Target} - C_{t,GAPDH})_{Time\ 0}$, where time x is any day after trunk injection of activators and time 0 is the time point when trunk injection was carried out.

Statistical analysis. The main effects of treatment and time on disease severity and 'Ca. L. asiaticus' titer data in trial III were

TABLE 2. Target genes, their encoding protein, and corresponding primers used in gene expression analysis by quantitative reverse transcription polymerase chain reaction

Target	Function of encoding protein	Primer–probe sequence 5'–3'	Reference
<i>CsPR1</i>	Antifungal and antioomycetic	Forward: AACTCGCCTCAAGACTACCT Reverse: TGCAACTGTGTCGTTCCATA	Dutt et al. (2015) ...
<i>CsPR2</i>	β-1,3-Glucanase	Forward: TTCCACTGCCATCGAAACTG Reverse: TGTAATCTTGTTTAAATGAGCCTCTTG	Francis et al. (2009) ...
<i>CsPR3</i>	Chitinase, antifungal	Forward: TCCGACTACTGTGATCCTAACT Reverse: TCTCCGCACCGTAAGTAATTG	Newly designed ^a ...
<i>CsPR15</i>	Oxalate oxidase	Forward: TGGTTTGAGCAAAGAGGGTAATA Reverse: AGAGTATTGAGGCCAGGAAATG	Newly designed ^b ...
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase-C	Forward: GGAAGGTCAAGATCGGAATCAA Reverse: CGTCCCTCTGCAAGATGACTCT	Francis et al. (2009) ...

^a Primers were designed based on sequence *orange1.lg026315m* obtained by searching the homologous pathogenesis-related (*PR*) gene *PR-3* sequence within the citrus genome.

^b Primers were designed based on sequence *30147.m014037* obtained by searching the homologous *PR* gene sequence within the citrus genome.

analyzed using the MIXED procedure in SAS 9.3, with repeated measures best adjusted to heterogeneous autoregressive variance covariance structure of first order ($\alpha = 0.05$) (SAS Systems). Treatment comparisons for change in '*Ca. L. asiaticus*' titer, sAUDPS, fruit yield, juice quality parameters, and relative gene expression were analyzed by the general linear model procedure in SAS. To determine whether there were significant differences between treatments and the water-injected control, main effect means were calculated and a comparison for the main effects was performed using Dunnett's test (tested at $P < 0.05$). All data in the graphs were presented as mean $\pm$ standard error. Error bars represent standard error of mean ($n = 4$ in trials I and II and $n = 10$ in trial III).

RESULTS

Effect of activators and antibiotic treatments on '*Ca. L. asiaticus*' titer and HLB disease increase. A significant suppression effect of the injected compounds on both '*Ca. L.*

asiaticus' titer and HLB disease development was observed in all three field trials. Although the degrees of '*Ca. L. asiaticus*' titer and disease suppression varied greatly among treatments, the three injected antibiotics and all activators except IMI and AA, in general, significantly reduced '*Ca. L. asiaticus*' titer in treated trees and substantially slowed the HLB disease progression as compared with water-injected control trees (Figs. 1, 2, and 3). Some activators exhibited significant '*Ca. L. asiaticus*'-diminishing effects. For example, whereas water-injected trees had 59.3 and 10.4% increase in '*Ca. L. asiaticus*' titer as well as 110 and 40% increase in disease severity, SA reduced '*Ca. L. asiaticus*' titer by 65.8 and 45.5% and provided 36.6 and 30.0% disease control (reduction in sAUDPS) in 5-year-old young trees in trials I and II, respectively (Fig. 1A and 1B). In trial III, SA initially lowered '*Ca. L. asiaticus*' titer (Fig. 3A) but '*Ca. L. asiaticus*' titer began to increase after reaching the bottom, whereas the combination of oxytetracycline and streptomycin had a long-lasting effect in suppressing '*Ca. L. asiaticus*' populations. SA also significantly reduced disease

Fig. 1. Effect of various treatments to control huanglongbing on '*Candidatus Liberibacter asiaticus*' (Las) titers in leaf samples ($n = 12$, four trees per treatment and three replicated samples per tree) of Hamlin trees in field **A**, trial I and **B**, trial II. '*Ca. L. asiaticus*' titers were estimated with quantitative real-time polymerase chain reaction. Treatment effect was expressed as percent change in '*Ca. L. asiaticus*' titer = [(mean titer after treatment - mean titer prior to treatment)/mean titer prior to treatment] $\times 100$. Data were means and standard errors of four replicates. Columns followed by an asterisk (*) indicates significant reduction ($P < 0.05$; Dunnett's test) in '*Ca. L. asiaticus*' titer 1 week after trunk injection as compared with that prior to treatment. Abbreviations: WIC = water as control, OA = oxalic acid, SA = salicylic acid, ASM = acibenzolar-S-methyl, PHI = potassium phosphite, IMI = imidacloprid, PCN = penicillin G sodium salt, STM = streptomycin, OTC = oxytetracycline hydrochloride, BABA = β -aminobutyric acid, AA = L-ascorbic acid sodium salt, and INA = 2, 6-dichloro-isonicotinic acid.

severity (Fig. 3B) in treated trees as compared with water-injected control trees.

In trial I, the most effective compounds in suppression of '*Ca. L. asiaticus*' titer growth and disease development were PCN and OTC, followed by SA, OA, PHI, STM, and ASM (Figs. 1A and 2A). It is noteworthy that streptomycin at 1.25 g/tree had almost no effect in suppressing '*Ca. L. asiaticus*'. In contrast, little effect was observed with injected IMI. Similarly, in trial II, injected OTC provided the most effective disease control and significant reduction of '*Ca. L. asiaticus*' titer, followed by SA, OA, and ASM (Figs. 1B and 2B). On the other hand, there were no controlling effects of IMI and AA on the bacterial population or disease severity. At a higher HLB disease pressure, as indicated by disease severity of approximately 3.5 in mature trees at the beginning of trial III (Fig. 3B), a combination of OTC and STM at 1.2 g of each killed nearly all '*Ca. L. asiaticus*' in treated trees and revived HLB trees by greatly reducing disease severity as compared with before treatment. All four activators (i.e., SA, ASM, PHI, and OA) significantly reduced '*Ca. L. asiaticus*' titer and disease severity at the end of trial (Fig. 3A and B).

Effect of inducer and antibiotic treatments on yield and quality. In trial I, all three antibiotics significantly increased fruit yield (Fig. 2A), whereas fruit yield increase by all activators were not significant. In trial II, only oxytetracycline significantly increased fruit yield. In contrast, fruit yield increases were not significant in treatments of SA, PHI, ASM, and INA. In trial III with an 11-year-old Midsweet orange tree, the average weight of fruit per tree receiving treatments ranged from 46.7 to 51.7 kg whereas the water-injected control yielded 43.5 kg/tree (Fig. 3C; Table 3). Due to large variations among treated trees, such a yield increase of 7.4 to 18.9% by activator and antibiotic treatments was not statistically different from that of control trees. In general, all fruit quality parameters were within normal ranges and there were no significant differences between treatments and the water-injected control in most of the parameters. However, percent juice content was significantly higher in fruit from trees receiving injection of SA, ASM, and PHI (Table 3). In addition, trees treated with ASM and antibiotics OTC + STM showed a higher brix and brix/acidity ratio, indicating an increased level of sugar content.

Fig. 2. Standardized area under the disease progress stairs (sAUDPS) for citrus huanglongbing and fruit yield in field **A**, trial I and **B**, trial II. Data were means and standard errors of four replicated trees. An asterisk (*) indicates significant difference ($P < 0.05$; Dunnett's test) between the treatment and water-injected control. Abbreviations: WIC = water as control, OA = oxalic acid, SA = salicylic acid, ASM = acibenzolar-S-methyl, PHI = potassium phosphite, IMI = imidacloprid, PCN = penicillin G sodium salt, STM = streptomycin, OTC = oxytetracycline hydrochloride, BABA = β -aminobutyric acid, AA = L-ascorbic acid sodium salt, and INA = 2, 6-dichloro-isonicotinic acid.

PR gene expression in leaves. Different expression levels of *PR* genes were induced when trunks were injected with OA, SA, PHI, and ASM. When compared with the water-injected control, trunk injection of SA into citrus trees resulted in significant induction of *PR-1*, ranging from 2.6- to 3.5-fold change, and *PR-2*,

ranging from 9.6- to 14.5-fold change at 2, 4, and 7 days after injection (Fig. 4A and B). Similarly, ASM injection led to significant induction of *PR-1* of 2.9- to 4.1-fold change and *PR-2* of 13.9- to 15.8-fold change at 2, 4, and 7 days after injection (Fig. 4A and B). PHI injection significantly induced *PR-2* expression at

Fig. 3. Effect of various treatments to control huanglongbing (HLB) of mature Hamlin trees in field trial III on **A**, ‘*Candidatus*’ *Liberibacter asiaticus*’ titers in leaf samples ($n = 10$ trees per treatment, three leaf samples per tree); **B**, temporal dynamics of disease severity; and **C**, standardized area under the disease progress stairs (sAUDPS) for citrus HLB and fruit yield. An asterisk (*) indicates significant difference ($P < 0.05$; Dunnett’s test) between the treatment and water-injected control (WIC) at a given time point. Abbreviations: OA = oxalic acid, SA = salicylic acid, PHI = potassium phosphite, ASM = acibenzolar-S-methyl, OTC = oxytetracycline hydrochloride, and STM = streptomycin.

levels ranging from 5.3- to 11.8-fold change at 2, 4, and 7 days after injection (Fig. 4B). None of the four activators was able to induce *PR-3* gene expression (Fig. 4C). Significant induction of *PR-15* gene expression at levels from 3.4- to 5.5-fold change was observed with OA injection at 2, 4, and 7 days after injection (Fig. 4D). The temporal induction pattern was similar among *PR-1*, *PR-2*, and *PR-15* in all treatments, with a peak expression level at 4 days after injection induction, which quickly diminished at 7 days after injection. At 14 days after injection, induction of *PR* gene expressions in all treatments was reduced to a level similar to that of water-injected control trees.

Trunk injection of activators and antibiotics. Although antibiotic compound OTC needs to be formulated for injection, PCN and STM were highly soluble in water and their aqueous solutions were suitable for trunk injection. Among eight plant defense activators tested in this study, five activators (i.e., AA, BABA, SA, OA, and PHI) were water soluble and their aqueous solutions can be easily delivered into the xylem via trunk injection. However, the uptake and transport of the remaining activators (i.e., ASM, IMI, and INA) was extremely slow through trunk injection due to their low solubility in water. For example, INA must first be dissolved in ethanol and then diluted to low concentrations with water. Therefore, injection-specific formulations for these three activators are required for higher dosages. In both trials I and III, no symptoms of chemical injury, stunting, and other abnormal appearance were observed over the course of 12 months in all trees receiving activators and antibiotics, indicating their lack of phytotoxic effect at concentrations tested. However, INA at rates of 1.0 g/tree (5 mg/ml, 200 ml) caused chemical burns of leaf margins and dieback of young twigs on some branches of a young citrus tree, indicating that a much lower dosage is needed to alleviate their phytotoxic effects.

DISCUSSION

This study evaluated the control effect of trunk injection of plant defense activators and antibiotics against HLB. Harnessing a plant's induced resistance for practical disease control represents a sustainable approach to crop protection. In tree crops, attempts to transform this concept into consistent and adequate protection from pathogen attack has had limited success to date. Several field trials have demonstrated that the degree and magnitude of plant defense activators to activate plant defense weaponry against citrus canker and HLB varied greatly from limited to moderate efficacy under field conditions (Francis et al. 2009; Graham and Leite 2004; Li et al. 2016a). Among many factors responsible for such a varying and partial efficacy, application method of foliar spray and soil drench may be a significant limiting factor that results in poor absorption and translocation of activators in a citrus tree. In addition, plant defense activators such as ASM and SA are highly photodegradable and biodegradable (Silva et al. 2007; Sleiman et al. 2017) as well as vulnerable to other environmental

conditions. Trunk injection of activators and antibiotics may avoid problems of degradation and poor absorption associated with foliar spray and soil drench. Overall, trunk injection of plant defense activators SA, ASM, OA, and PHI showed a positive control effect against HLB, even though it is much less than provided by antibiotics.

Injecting SA, ASM, OA, and PHI into HLB-diseased citrus induced expression of SAR-related *PR* genes *PR-1*, *-2*, *-3*, and *-15*. Similarly, injection of ASM and PHI into apple trees induced expression of *PR-1*, *PR-2*, and *PR-8* genes and resistance against fire blight (Aćimović et al. 2015). It is well known that PR proteins are induced in many plant species in response to pathogens and inducing compounds and exhibit antimicrobial activities in vitro (Walters and Fountaine 2009). For example, PR-1 protein had inhibitory activities against oomycetes and inhibited the differentiation of infection hyphae in leaves of acquired resistant broad bean (Alexander et al. 1993; Rauscher et al. 1999). PR-2 protein exhibits β -1,3-glucanase with hydrolase activity on cell walls of fungi and oomycetes (Mauch et al. 1988). Whether induction of *PR-1* and *PR-2* contributes to resistance against HLB remains unknown. On the other hand, PR-3 protein exhibits chitinolytic activity against the bacterial pathogen of apple fire blight (Maxson-Stein et al. 2002). PR15 protein is oxalate oxidase inducible during seed germination and pathogen infection of many crops (Ferreira et al. 2007). In this study, we found that OA significantly induced expression of the *PR-15* gene. OA is a key pathogenicity factor in several fungal species, including *Sclerotinia* spp., and oxalate oxidase can break down OA into carbon dioxide and hydrogen peroxide (Bernier and Berna 2001). Hydrogen peroxide is a signaling molecule involved in plant defense mechanisms. *PR-15* gene homologs are present in citrus and, thus, OA may be a good inducing agent for high-level expression of *PR-15* gene. It is important to note that some of these plant defense-inducing compounds under certain conditions may have direct antimicrobial activities at high concentrations. For example, SA has antifungal activity against *Penicillium expansum* (Da Rocha Neto et al. 2015). OA has strong antibacterial activity against nine plant-pathogenic bacteria, including *Ralstonia solanacearum* (Kwak et al. 2016). Hexanoic acid, a natural compound activator, inhibited growth of *Botrytis cinerea* (Leyva et al. 2008). Therefore, the question of how plant defense activators lead to '*Ca. L. asiaticus*' suppression in injected trees remains to be investigated.

Trunk injection of plant defense activators and antibiotics effectively prolonged life and maintained the yields of HLB-infected trees. Our field studies indicated that induced resistance by trunk-injected SA, ASM, OA, and PHI had some positive impact on fruit yield and quality. This result is in agreement with results from earlier studies on induced resistance by foliar spray of plant defense inducers in citrus (Li et al. 2016a, 2017). However, it has been speculated that induced resistance may be costly because of metabolic competition between processes involved in plant growth and the synthesis of defense-related compounds. There are few

TABLE 3. Yield and quality of Midsweet orange fruit harvested from field trial III

Treatment ^b	Yield (kg/tree)	Fruit quality ^a			
		Juice (%)	Brix	Acidity	Brix/acidity
T1 = WIC	43.5	53.49	11.51	0.49	23.44
T2 = OA	47.5	55.00	12.24	0.52	23.39
T3 = SA	48.5	58.22*	11.60	0.64	19.12
T4 = PHI	47.3	57.58*	11.66	0.51	22.99
T5 = ASM	46.7	58.39*	12.39*	0.52	25.28*
T6 = OTC+STM	51.7	55.35	13.37*	0.51	26.29*

^a Approximately 8 kg of composite fruit sample was taken from each tree for juice quality analysis according to standard methods (Gottwald et al. 2012). Each value was the mean of 10 replicated trees per treatment. Values followed by an asterisk (*) indicate significant difference between treatment and control ($P < 0.05$, Dunnett's test).

^b WIC = water as control, OA = oxalic acid, SA = salicylic acid, PHI = potassium phosphite, ASM = acibenzolar-S-methyl, OTC = oxytetracycline hydrochloride, and STM = streptomycin; OTC+STM = 1.2 g of OTC + 1.2 g of STM per tree.

reports of such costs of induced resistance on wild plants or crops (Cipollini et al. 2003). Heil et al. (2000) showed that healthy wheat treated with BTH (synonym of ASM) had lower numbers of ears and grains as well as plant biomass, especially when these treated plants were under nitrogen stress. Therefore, we believe that a good

nutritional program is important to avoid stressful conditions when treating HLB-infected citrus with activators, because stressful conditions on HLB-infected trees may inflict yield costs. Additionally, '*Ca. L. asiaticus*' encodes a functional SA hydroxylase that degrades SA, thus affecting its ability to induce plant

Fig. 4. Relative expression of pathogenesis-related (*PR*) genes **A**, *PR-1*; **B**, *PR-2*; **C**, *PR-3*; and **D**, *PR-15* in 11-year-old Midsweet orange (*Citrus sinensis* L. Osbeck) leaves after the first trunk injection of four plant defense activators in trial III. The relative gene expression (postinjection versus prior to injection) was calculated as fold change using the $2^{-\Delta\Delta Ct}$ method. Values are means of three biological replicates and each sample consisted of eight pooled leaves from a treated tree. Error bars represent standard error. Asterisks indicate significant difference ($P < 0.05$, Dunnett's test) between treatments and water-injected control (WIC). Abbreviations: OA = oxalic acid, SA = salicylic acid, PHI = potassium phosphite, and ASM = acibenzolar-S-methyl.

defenses (Li et al. 2017). Trunk injection of SA seems to be able to overcome the SA degradation by the enzyme.

Trunk-injected antibiotics had better performance than injected activators in reducing '*Ca. L. asiaticus*' bacterium and increasing fruit yield in an HLB-infected tree. The antimicrobials induced by plant defense inducers might not reach the same high concentration and same effectiveness as injected antibiotics. In addition, '*Ca. L. asiaticus*' encodes one SA hydroxylase that degrades SA and suppresses plant defenses (Li et al. 2017). Injected OTC, PCN, and STM provided excellent control of citrus HLB severity by substantial reduction of '*Ca. L. asiaticus*' titers in HLB-diseased plants (Figs. 1A and 3A). By combining STM (bactericidal) and OTC (bacteriostatic) into a single treatment, 99% reduction in '*Ca. L. asiaticus*' titer was achieved in field trial III. Such a dramatic inhibitory effect on '*Ca. L. asiaticus*' populations may be due to the synergistic effect between OTC and STM. A synergistic effect of OTC and STM was well documented on several bacterial species, including plant-pathogenic bacteria (Novy et al. 2011; Richardson and Holt 1962; Sabet 1956). It is noteworthy to point out that the qPCR method used in this study underestimated treatment effect on '*Ca. L. asiaticus*' titers because it does not differentiate live bacterial cells from dead ones. From the perspective of epidemiology, the substantial reduction of '*Ca. L. asiaticus*' titers in a '*Ca. L. asiaticus*'-infected tree will significantly lower the likelihood of inoculum spread among neighboring trees in a citrus grove, because '*Ca. L. asiaticus*' acquisition by psyllids positively correlates with bacterial concentration in the host plant (Coletta-Filho et al. 2014). Trunk injection of antibiotics is capable of delivering a large dose to phloem vessels of stem and root tissues where '*Ca. L. asiaticus*' titers reside. Our previous study demonstrated that trunk injection of OTC resulted in quick translocation and uniform distribution of OTC across the entire plant, including root systems, and a low level of residue in fruit (Hu and Wang 2016). Many studies have examined streptomycin residues on apple and pear trees, whereas fewer have addressed residues of oxytetracycline. All earlier studies demonstrated that fruit from trees treated with streptomycin or oxytetracycline for fire blight management does not have residues near the tolerance levels permitted by governmental agencies (Gardan and Manceau 1984; Shaffer and Goodman 1969; Stockwell and Duffy 2012; Stockwell et al. 2008). The amount of antibiotic in positive apple and citrus was far below the minimum inhibitory concentration for gut flora, strongly suggesting that any antibiotics transferred to the gut would have negligible impacts on gut flora.

Given the epidemic status of citrus HLB in Florida, a multifaceted HLB management program is urgently needed for saving HLB-infected trees and prolonging their productivity. Trunk injection of antibiotics or plant defense activators may provide a useful tool for the citrus growers in the battle against citrus HLB in Florida if the residue level in juice due to injection is negligible. In addition, current injection technology and equipment is labor intensive and costly and may not be able to scale up to cope with hundreds of thousands of '*Ca. L. asiaticus*'-infected trees. Development of an automated trunk injection system with high efficiency is needed for large-scale application in commercial agricultural settings.

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