Effect of Thermotherapy on the Acquisition of Candidatus Liberibacter Asiaticus by the Asian Citrus Psyllid (Hemiptera: Liviidae)

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Effect of thermotherapy on the acquisition of *Candidatus* Liberibacter asiaticus by the Asian citrus psyllid (Hemiptera: Liviidae)

Alicia J. Kelley[1], and Kirsten S. Pelz-Stelinski[1,]*

Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the most detrimental insect pest of citrus crops due to its role as a vector of *Candidatus* Liberibacter asiaticus (Las), the bacterial causal agent of huanglongbing, also known as citrus greening disease. Trees infected with Las decline rapidly and fruit production decreases until eventual tree death. Few treatment options for infected trees are available for disease management. A technique called "thermotherapy" is under development to reduce bacterial titers in infected trees; however, the effect of these treatments on the transmission cycle of Las is not known. Field and laboratory assays were conducted to determine whether thermotherapy treatment reduced Las acquisition by *D. citri*. Trees in the field were treated with a mobile heat treatment system. Potted trees in the laboratory were treated in a steam chamber. We monitored acquisition rates in *D. citri* following thermal treatment of Las-positive *Citrus sinensis* (L.) (Rutaceae). Psyllid acquisition and Las titer in thermotherapy-treated trees were compared with untreated Las-positive and untreated Las-negative trees. Our results confirmed the efficacy of whole-tree thermotherapy on Las in potted citrus trees. In contrast, thermotherapy did not significantly reduce plant Las titers or acquisition of Las by *D. citri* under field conditions. These results suggest that further development of field application methods is needed to determine the utility of thermotherapy as a tool for huanglongbing management.

Key Words: citrus greening; huanglongbing; transmission; *Diaphorina citri*

Resumen

El cítrico psílido asiático, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), es la plaga de insecto más perjudicial de los cultivos de cítricos debido a su papel como vector de *Candidatus* Liberibacter asiaticus, el agente bacteriano que causa el huanglongbing, también conocido como la enfermedad del enverdecimiento del cítrico. Los árboles infectados con *Candidatus* Liberibacter asiaticus se disminuyen rápidamente y todavía producen frutos. Hay pocas opciones disponibles para el tratamiento de los árboles infectados para el manejo de la enfermedad. Se está desarrollando una técnica llamada "termoterapia" para reducir el nivel de bacterias en los árboles infectados; sin embargo, no se conoce el efecto de estos tratamientos sobre el ciclo de transmisión de *Candidatus* Liberibacter asiaticus. Se realizaron ensayos de campo y de laboratorio para determinar si el tratamiento de termoterapia reduce la adquisición de *Candidatus* Liberibacter asiaticus por *D. citri*. Los árboles en el campo fueron tratados con un sistema de tratamiento térmico móvil. Los árboles en maceta en el laboratorio fueron tratados en una cámara de vapor. Monitoreamos la taza de adquisición en *D. citri* después del tratamiento térmico de *Citrus sinensis* (L.) (Sapindales: Rutaceae) positivo de *Candidatus* Liberibacter asiaticus. Se compararon la adquisición por los psílidos y el nivel de *Candidatus* Liberibacter asiaticus en árboles tratados con termoterapia con los positivos de *Candidatus* Liberibacter asiaticus sin tratamiento y de los negativos de *Candidatus* Liberibacter asiaticus sin tratamiento. Nuestros resultados confirmaron la eficacia de la termoterapia de árboles enteros sobre *Candidatus* Liberibacter asiaticus en cítricos sembrados en macetas. En contraste, la termoterapia no redujo significativamente el título o la adquisición de *Candidatus* Liberibacter asiaticus por *D. citri* bajo condiciones de campo. Estos resultados sugieren que se requiere un mayor desarrollo de los métodos de aplicación en el campo para determinar la utilidad de la termoterapia como una herramienta para el manejo de huanglongbing.

Palabras Clave: enverdecimiento del cítrico; Huanglongbing; transmisión; *Diaphorina citri*

Candidatus Liberibacter asiaticus (Las) (Rhizobiales: Rhizobiaceae) is a gram-negative, phloem-limited alphaproteobacterium, and the putative causal agent of citrus greening disease, or huanglongbing (Bové 2006). This disease is responsible for severe damage to citrus groves worldwide. Symptoms of this disease include blotchy, mottled leaves, premature fruit drop, reduced fruit size, and bitter, misshapen fruits. The tree produces less marketable fruit, ultimately becoming unproductive within 7 to 10 yr (Gottwald 2010).

Current management strategies for huanglongbing focus on the propagation of young nursery stock, maintaining tree health, removing inoculum sources (infected trees), and area-wide insecticide sprays to control its insect vector, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Brlansky & Rogers 2007; Rogers & Shawer 2007; Dewdney et al. 2018). Insecticides are an attractive option due to the immediate mortality rate they provide, and the use of these chemicals has the benefit of simultaneously controlling other insect citrus pests, such as leaf miners and root weevils. However, the use of insecticides increases grower costs, and has many negative impacts on the environment. Developing alternative control methods is necessary in order to reduce the use of these chemicals and the development of resistance among insect populations (Chen & Stelinski 2017).

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Although these treatments minimize the dispersal of Las, even a single psyllid can transmit the pathogen. Despite rigorous efforts to reduce psyllid abundance, Las has spread throughout the citrus-growing regions of the USA since its initial introduction to Florida in 2005 (French et al. 2001; Kumagai et al. 2013). *Diaphorina citri* are highly mobile, especially during spring and summer months, and can transmit the bacteria rapidly throughout a grove even when Las incidence is low (Hall & Hentz 2011; Lewis-Rosenblum et al. 2015). Citrus trees can remain asymptomatic for 1 to 2 yr before displaying symptoms of huanglongbing. During this asymptomatic latent period, infected citrus serves as an inoculum source for *D. citri*. Psyllids also inoculate the flush, thus providing an immediate source of Las for the nymphs that develop there (Lee et al. 2015). In addition, D. citri infected with Las disperse more frequently and for longer distances than uninfected psyllids, which further increases the rate of Las transmission to new plant hosts (Martini et al. 2015).

Once a tree is infected, there are few options for growers to treat the bacteria directly to improve the production and lifespan of the tree. Long-term hunaglongbing management will rely on the development of resistant citrus varieties. Some resistance has been observed in the genus *Citrus* (Ramadugu et al. 2016), but the use of these lines in resistant crosses is still under investigation. RNA interference (RNAi) is also an appealing option for huanglongbing management. Non-virulent strains of *Citrus tristeza virus* (CTV), another phloem-limited citrus pathogen, can be engineered as a vector to induce the expression of RNA interference silencing genes targeting psyllid development (Hajeri et al. 2014). While promising, the research into transgenic and resistant varieties is many years away from commercial application.

Heat treatment of Las-infected trees, or "thermotherapy," uses controlled applications of heat for a specific time and temperature, thereby minimizing damage to the host, and at the same time reducing pathogen titers. Thermotherapy has been used successfully for management of bacterial diseases in several crops, and may be efficacious against Las due to the heat sensitivity of this pathogen (Lopes et al. 2009). Under laboratory conditions, Las-infected trees exposed to temperatures of 40 to 42 °C for 48 h experienced a reduction in pathogen titers to low or undetectable levels (Hoffman et al. 2012). Multiple studies have demonstrated the efficacy of thermotherapy, including some combination treatments using heat and antibiotics (Ehsani et al. 2013; Al-jumaili & Ehsani 2015; Yang et al. 2016a, b). The efficacy of thermotherapy under field conditions is still undetermined. Likewise, the effects of thermotherapy on the vector efficiency of *D. citri* are unknown. Citrus trees can serve as an inoculum reservoir, even if Las is not detectable (Lee et al. 2015). *Diaphorina citri* acquire Las within as few as 5 h of feeding on infected citrus (Xu et al. 1988). *Diaphorina citri* that feed on infected hosts as nymphs are far more likely to acquire Las, and subsequently inoculate new trees than are adults (Inoue et al. 2009; Pelz-Stelinski et al. 2010; Ammar et al. 2016). Thermotherapy may reduce the availability of Las to *D. citri*, thereby inhibiting acquisition. Alternatively, heat-stressed trees may be unable to defend against re-inoculation from psyllids, allowing Las to multiply rapidly and serve as new inoculum to psyllids.

The goal of this study was to investigate the effect of thermotherapy on acquisition of Las by *D. citri*. We hypothesized that reductions in the titer of Las in trees receiving thermal treatment should be associated with a reduction of Las acquisition by *D. citri*. *Candidatus* Liberibacter asiaticus titers in the leaf tissue of thermotherapy-treated trees were monitored over the course of 1 yr. We also measured the rate of pathogen acquisition in both adult and nymphal *D. citri* over time.

Materials and Methods

FIELD STUDY

Thermotherapy trials were conducted in a research grove located at the University of Florida Citrus Research and Education Center (Lake Alfred, Florida, USA) (28.216700°N, 81.183300°W). Three-yr-old *Citrus sinensis* (L.) (Rutaceae) 'Hamlin' sweet orange trees ('Swingle' rootstock) were screened for the presence of Las status using quantitative real-time polymerase chain reaction (qPCR) analysis. Trees were considered positive if the cycle threshold value was less than 36. Twenty-four Las-positive trees and 10 Las-negative trees were selected for the experiments. The site was regularly mowed, but no other management inputs (i.e., pesticides, biological control agents) were applied during the course of this study. Fruit was not harvested from trees for the duration of the study.

Twelve Las-positive trees were randomly selected to receive thermotherapy treatment. The remaining 12 Las-positive trees and 10 Las-negative trees remained untreated. The thermal treatments were applied per methods described by Al-jumaili & Ehsani (2015) on 14 Jul 2015. Briefly, the tree canopy was enclosed in a canvas tent, and the interior of the tent was heated with pressurized steam until the temperature reached 55 °C. This temperature was maintained for 30 s. A second treatment of thermotherapy was applied to the same thermotherapy-treated trees (minus 1 tree that died from Las infection) on 14 Dec 2015, 153 d after the first treatment.

Acquisition Assays

Uninfected adult *D. citri* used in acquisition assays were collected from a Las-free colony maintained on *Citrus sinensis*, 'Swingle' citrumelo (*Citrus paradisi* Macfayden × *Poncirus trifoliata* (L.)), and *Murraya paniculata* (L.) (all Rutaceae). Environmental conditions were maintained at 26 to 29 °C and 60 to 80% RH on a 16:8 h (L:D) photoperiod. Psyllids are tested for Las every 2 to 3 mo to confirm Las-free status.

Adult psyllid acquisition trials were conducted as described by Pelz-Stelinski et al. (2010). Briefly, 25 to 30 adult *D. citri* from a Las-free colony were enclosed on tree shoots with fine nylon mesh sleeve cages for a 9 d acquisition access period. Following the 9 d period, a maximum of 10 psyllids were collected and stored in 80% ethanol at −80 °C for subsequent Las detection. This assay was completed once prior to thermal treatments, and every 2 to 3 mo post initial treatment for 1 yr, resulting in a total of 4 sampling periods. Acquisition assays were conducted on the same tree branches for each trial, when possible; alternatively, a branch close to the original branch site was used.

The effect of thermal treatment on acquisition of Las by developing *D. citri* was assessed by identifying flush infested with *D. citri* nymphs. Infested branches were bagged, and a maximum of 10 psyllids were collected 2 to 3 wk later, or when adults emerged. Insects were placed in 80% ethanol and stored at −20 °C for subsequent processing and Las detection. Collections were made every 2 to 3 mo post-treatment for 1 yr, resulting in 4 sampling periods. Tree shoots were chosen according to nymph presence and were not consistent between sampling periods.

DNA Isolation and Quantification of *Candidatus* Liberibacter asiaticus in *D. citri*

Genomic DNA was isolated using the "Blood & Tissue" DNeasy kit (Qiagen, Valencia, California, USA) for extraction of nucleic acids, with modifications as described in previous studies (Pelz-Stelinski et al. 2010; Pelz-Stelinski & Killiny 2016). Quantitative real-time polymerase

chain reactions were conducted with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, California) as described previously by Pelz-Stelinski et al. (2010). Briefly, 25 µL reactions, containing 0.2 µM each of Las primers (LasF: 5'-TCGAGCGCGTATGCGAATAC-3'; LasR: 5'-GCGTTATCCCGTAGAAAAAGGTAG-3') and probe (5'-/56-FAM/AGACGGGTGAGTAACGCG/3BHQ_1/-3') in PerfeCTa quantitative real-time polymerase chain reaction ToughMix, Low ROX (Quanta Biosciences, Beverly, Massachusetts, USA). All quantitative real-time polymerase chain reactions were performed in duplicate under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All plates included a non-template control in triplicate (Ambion Nuclease-Free Water, ThermoFisher Scientific, Waltham, Massachusetts, USA).

Diaphorina citri collected from adult acquisition assays were evaluated by nested-quantitative real-time polymerase chain reactions (Coy et al. 2014). Prior to quantitative real-time polymerase chain reaction, samples were amplified by conventional polymerase chain reactions in 20 µL reactions containing 0.5 µM each of external Las primers (Ex-LasF: 5'-TGACGTTGGAAGATGTTTGTAGC-3'; Ex-LasR: 5'-ACGCAGGCTCATCTCTCTCC-3'), 0.2 µM dNTPs, 0.03U per µL TaKaRa Taq™ DNA Polymerase (TaKaRa Bio USA, Inc., Mountain View, California) in 2 µL Mg+ Plus 10× Buffer, and 1 µL of template DNA. Amplification reactions consisted of 2 cycles of 94 °C for 90 s, 62 °C for 45 s, and 72 °C for 20 s, followed by 34 cycles of 94 °C for 30 s, 62 °C for 45 s, and 72 °C for 20 s, and a final extension of 72 °C for 3 min. The polymerase chain reaction products were diluted to 1:100 in 0.5 mL microcentrifuge tubes with Nanopure water for use in quantitative real-time polymerase chain reactions.

DNA Isolation and Quantification of *Candidatus* Liberibacter asiaticus in Leaf Tissue

Three leaves were sampled from the tree at the time of each psyllid collection, resulting in 8 total leaf tissue collections (4 during adult acquisition sampling, 4 during nymph acquisition sampling). Leaves were selected from the branches with caged psyllids. Leaf samples were stored at −80 °C until subsequent DNA processing. Midribs were cut from leaf samples, and 100 mg of plant tissue was subsampled. DNA isolation was conducted using the DNeasy Plant Mini Kit (Qiagen, Valencia, California) as described previously (Li et al. 2006; Pelz-Stelinski et al. 2010; Pelz-Stelinski & Killiny 2016). Isolated plant DNA was quantified to nanograms per microliter with a NanoDrop 2000 (Thermo Scientific, Wilmington, Delaware, USA), and diluted to a concentration of 10 ng per µL for quantitative real-time polymerase chain reactions.

Candidatus Liberibacter asiaticus detection in plant tissue was conducted as described previously (Chu et al. 2016). *Candidatus* Liberibacter asiaticus titers were quantified using a plasmid-based standard curve. Briefly, Las polymerase chain reaction products were ligated into plasmids, and transformed into competent cells using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA). Extracted plasmid DNA was quantified with a NanoDrop 2000, and diluted to a starting concentration of 0.05 ng per µL. A standard curve consisting of 5 serial 10-fold dilutions was included on each quantitative real-time polymerase chain reaction plate.

GREENHOUSE STUDY

Potted 'Valencia' on 'Swingle' (15 trees, approx. 1 m tall) were placed in screen cages with Las-exposed *D. citri* obtained until a systemic Las infection developed in the trees (approx. 4 mo). Infected *D. citri* were collected from (1) a laboratory colony in which psyllids are cultured on Las-positive *Citrus sinensis* (L.) Osbeck, *Swingle citrumelo*, and *Citrus macrophylla*, maintained at 26 to 29 °C and 60 to 80% RH on a 16:8 h (L:D) photoperiod; (2) Las-infected *C. sinensis* trees in a research grove located in Lake Alfred, Florida (28.131000°N, 81.717100°W). Leaves were sampled from each tree and screened with quantitative real-time polymerase chain reaction prior to treatment to establish initial Las infection status. Adult *D. citri* used in whole-tree thermotherapy acquisition assays were collected from our Las-free laboratory culture described previously.

Five Las-positive trees (cycle threshold < 36) were randomly selected for treatment with whole-tree thermotherapy. Trees were heated in a mobile steam chamber (1.8 × 1.8 × 3.0 m.) at 40 °C for 48 h. After treatment, trees were sprayed with Magna-Bon CS 2005 (Magna-Bon Agricultural Control Solutions, Okeechobee, Florida, USA) (250 ppm) to prevent secondary fungal infection. Five Las-positive trees remained untreated and served as positive controls. An additional 5 trees, which were maintained in an insect-free greenhouse and were never exposed to Las, were used as negative controls. Cages were placed in an environmentally controlled greenhouse at 26 to 29 °C and 60 to 80% RH on a 16:8 h (L:D) photoperiod. Three leaves were collected from each tree prior to thermotherapy treatment, and at 42 and 190 d post-treatment, for a total of 3 sampling periods.

DNA Isolation and Quantification of *Candidatus* Liberibacter asiaticus in Leaf Tissue

Candidatus Liberibacer asiaticus titers were quantified using a plasmid-based standard curve as described previously. In addition, a plasmid-based standard curve was created for the plant cytochrome oxidase gene (Cox), a common housekeeping gene. The standard curves for Las and cytochrome oxidase gene were included on each plate in triplicate. Quantitative real-time polymerase chain reactions remained the same, except for the addition of 0.2 µM each of cytochrome oxidase gene primers (CoxF: 5'-GTATGCCACGTCGCATTCCAGA-3'; CoxR: 5'-GCCAAAACTGCTAAGGGCATTC-3') and probe (CoxP: 5'-/56-JOEN/ATCCAGATGCTTACGCTGG/3BHQ-2/-3').

STATISTICAL ANALYSIS

Field Study

Psyllids tested for Las with quantitative real-time polymerase chain reaction were considered positive if cycle threshold < 36; psyllids tested with nested-quantitative real-time polymerase chain reaction were considered positive if cycle threshold < 19 (Coy et al. 2014). The percentage of Las-positive *D. citri* was calculated per replicate, and the average percentage was calculated per treatment group for each collection period. An analysis of variance (ANOVA) with post-hoc multiple comparisons (Tukey's HSD test at $P < 0.05$) was used to compare the mean percentage of Las-positive *D. citri* between the 3 treatment groups for each collection period.

Candidatus Liberibacter asiaticus titer in plant tissues was determined using the ABI 7500 Real-Time PCR System software. *Candidatus* Liberibacter asiaticus copy numbers per mL were calculated using the DNA quantification equation described in previous publications (Whelan et al. 2003; Dossi et al. 2014). Mean Las copy numbers were determined from duplicate quantitative real-time polymerase chain reactions for each sample and subjected to $\log_{10}(x+1)$ transformation to meet the condition of normality. The effect of thermal treatment on Las titer in leaf tissue over time was determined using analysis of covariance (ANCOVA). Linear regression with Pearson's correlation co-efficient was conducted to determine if the mean psyllid acquisition correlated with mean Las titer in leaf tissue for each treatment group at each sampling period. All statistical tests were conducted in R Studio (R Core Team 2013).

Greenhouse Study

Titers of Las and cytochrome oxidase gene in plant tissue were quantified using the ABI 7500 Real-Time PCR System software. Las/cytochrome oxidase gene copy numbers per microliter were calculated using the DNA quantification equation described previously (Whelan et al. 2003; Dossi et al. 2014). *Candidatus* Liberibacter asiaticus copy number divided by cytochrome oxidase gene copy number were used to calculate the quantity of Las DNA relative to plant DNA. The mean Las/cytochrome oxidase gene copy number was $\log_{10}(x+1)$ transformed, where *x* was the average copy number. *Candidatus* Liberibacter asiaticus titers in leaf tissue were analyzed using Kruskal-Wallis rank sum tests ($\alpha = 0.05$) due to non-normality of data. Comparisons of Las/cytochrome oxidase gene among treatment groups were conducted separately for each sampling period (pre-treatment, 42 d, and 190 d post-treatment). All statistical tests were conducted in R Studio (R Core Team 2013).

Results

FIELD STUDY

Candidatus Liberibacter asiaticus acquisition was significantly greater among *D. citri* adults confined on positive control trees than negative control trees during the second ($F_{2,27} = 8.323$; $P = 0.003$), third ($F_{2,29} = 3.194$; $P = 0.047$), and fourth ($F_{2,28} = 4.617$; $P = 0.014$) sampling periods. *Candidatus* Liberibacter asiaticus acquisition also was greater among *D. citri* adults on thermotherapy-treated trees than on negative control trees during the second sampling period ($F_{2,27} = 8.323$; $P = 0.007$). There were no other significant differences in adult acquisition of Las (Fig. 1). Acquisition of Las during nymphal development did not differ significantly during the first, second, and fourth sampling periods. During the third sampling period, acquisition of Las by nymphs from positive control and thermotherapy-treated trees was significantly higher than negative control trees ($F_{2,15} = 6.32$; $P = 0.008$; $P = 0.045$, respectively) (Fig. 2).

Candidatus Liberibacter asiaticus acquisition by *D. citri* nymphs and adults was positively correlated with leaf Las titer (nymphs: $R^2 = 0.35$; Pearson's correlation coefficient = 0.59; $F = 5.315$; $P = 0.044$ [Fig. 4]; and adults: $R^2 = 0.45$; Pearson's correlation coefficient = 0.67; $F = 8.279$; $P = 0.017$ [Fig. 5]). Negative control trees had significantly lower Las titers over time as compared with positive control ($F_{2,225} = 2.96$; $P < 0.001$) and heat-treated trees ($P < 0.001$). *Candidatus* Liberibacter asiaticus titers were not significantly different between positive control and heat-treated trees ($P = 0.39$) (Fig. 3).

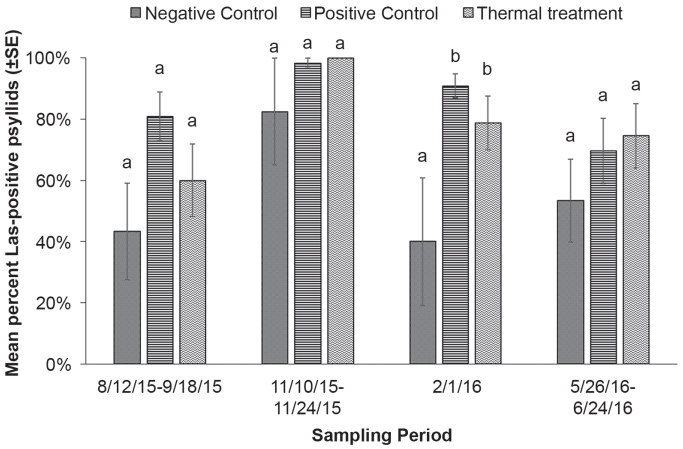

Fig. 2. Mean percent (± SE) of *Candidatus* Liberibacter asiaticus-positive *Diaphorina citri* following nymphal acquisition. ANOVA was conducted separately for each sampling period to compare means between treatment groups. Treatments with different letters within a sampling period indicate significant differences within a sampling period (Tukey's HSD, $\alpha = 0.05$). Thermal therapy treatments were applied on 14 Jul 2015 and 14 Dec 2015.

GREENHOUSE STUDY

The titer of Las in leaves increased in the untreated Las-positive trees (positive controls) over time (Fig. 6). *Candidatus* Liberibacter asiaticus-positive trees that received thermotherapy exhibited reduced Las titers from the pre-treatment period to the 42 d assessment, and the low Las titer was maintained for 190 d. Untreated Las-negative trees (negative controls) remained negative throughout the study. Pathogen titers among treatment groups were not significantly different during the pre-treatment sampling period ($\chi^2 = 0.095$; $P = 0.95$), 42 d post-treatment ($\chi^2 = 5.33$; $P = 0.070$), or 190 d post-treatment ($\chi^2 = 4.75$; $P = 0.093$).

Discussion

This is the first study to examine the effect of thermotherapy on psyllid acquisition of Las. Although Las titers in heat-treated trees

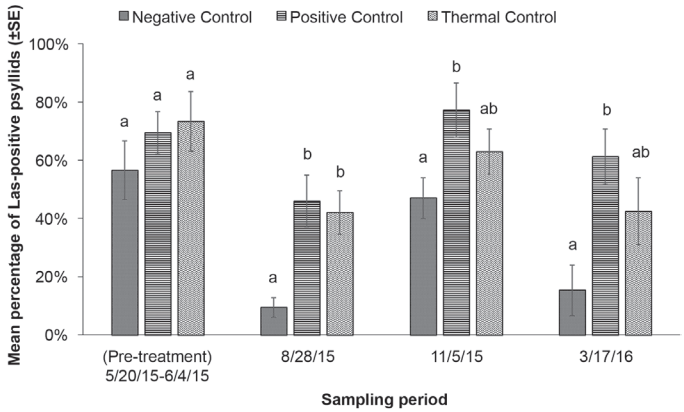

Fig. 1. Mean percent (± SE) of *Candidatus* Liberibacter asiaticus-positive adult *Diaphorina citri* following a 9-d acquisition access period on trees. ANOVA was conducted separately for each sampling period to compare means between treatment groups. Treatments with different letters within a sampling period indicate significant differences within a sampling period (Tukey's HSD, $\alpha = 0.05$). Thermal therapy treatments were applied on 14 Jul 2015 and 14 Dec 2015.

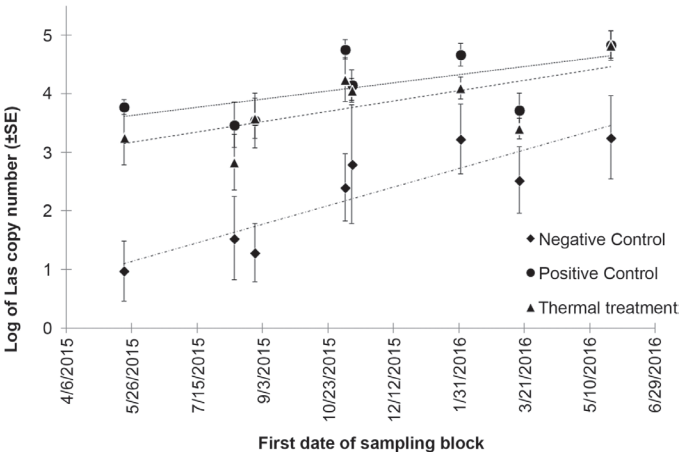

Fig. 3. Mean *Candidatus* Liberibacter asiaticus titer (± SE) in trees for each treatment group on the first date leaf samples were collected for each sampling period.

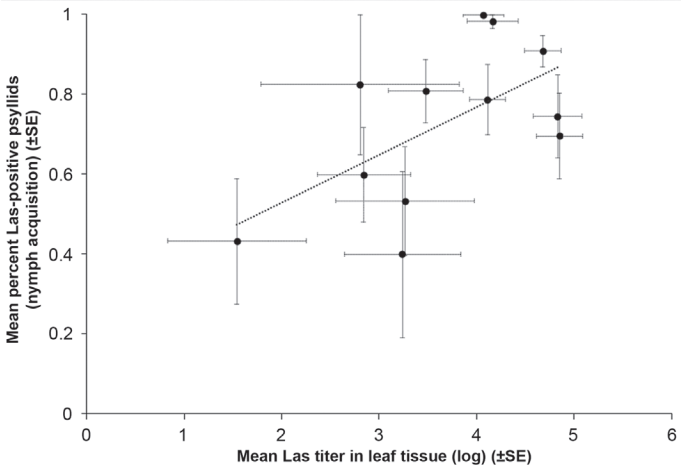

Fig. 4. Mean nymph acquisition (± SE) compared to mean titer (± SE) of *Candidatus* Liberibacter asiaticus in leaf tissue. Means calculated per treatment group per sampling period. y = 0.1197x + 0.2898.

remained lower than untreated Las-infected citrus, the trees did not exhibit significantly reduced Las titers in leaf tissue over time in field experiments. *Candidatus* Liberibacter asiaticus acquisition rates were correlated with titers of the pathogen in plants. This suggests that Las acquisition efficiency reflects the availability of inoculum in host plants. Thus, methods targeting Las reduction for huanglongbing management will require concomitant insecticide applications for success, particularly if bactericidal treatments are not completely effective.

Whole-tree thermotherapy of potted citrus in greenhouse experiments did reduce Las titers, which is consistent with previous studies with *Citrus paradisi* 'Macfayden' trees cultivated in similar pots (Hoffman et al. 2012). This suggests that treating only the aboveground portion of the tree at 55 °C for 30 s is not adequate to replicate the efficacy of thermotherapy observed in potted trials.

The percentage of *D. citri* that acquired Las from treated trees did not decrease relative to untreated trees in the field study. Psyllid acquisition rates followed similar patterns as leaf titer, in which the mean adult and nymph acquisition rates correlated to the mean Las titer in leaves (Figs. 4, 5). Thus, psyllid acquisition is directly related to, and affected by, the Las titer in the trees. Importantly, this suggests that

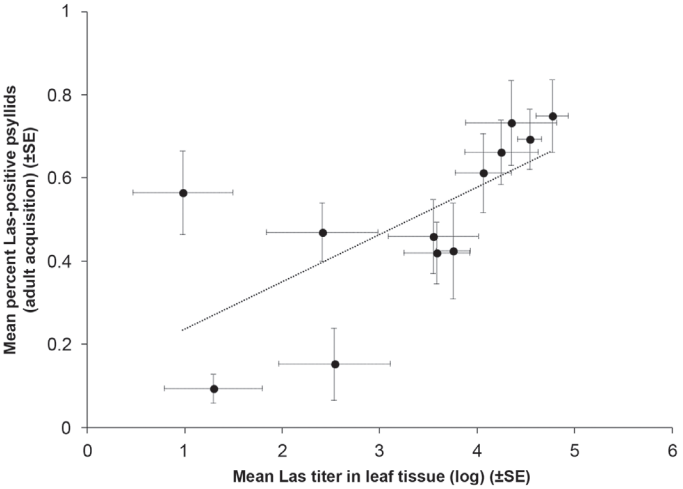

Fig. 5. Mean adult acquisition (± SE) compared to mean titer (± SE) of *Candidatus* Liberibacter asiaticus in leaf tissue. Means calculated per treatment group, per sampling period. y = 0.1138x + 0.1237.

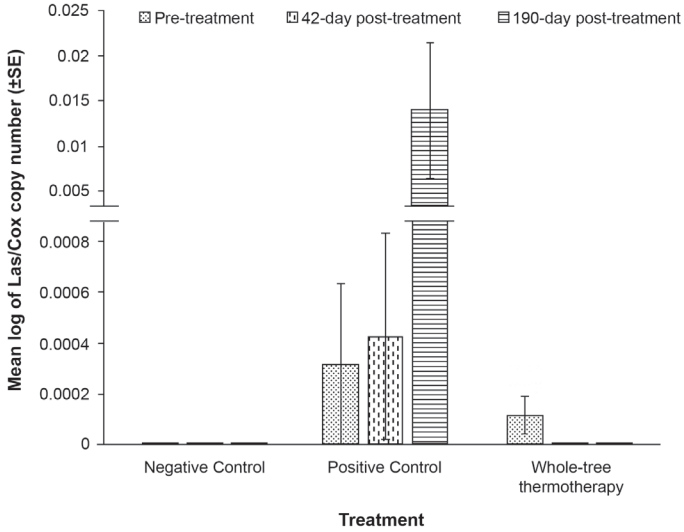

Fig. 6. Mean log of *Candidatus* Liberibacter asiaticus/cytochrome oxidase gene titer (± SE) in trees among each treatment group in whole-tree thermotherapy study for pre-treatment, 42 d, and 190 d sampling periods. Dotted bars = pre-treatment; vertical dash bars = 42 d post treatment; horizontal line bars = 190 d post treatment.

under field conditions, heat-treated trees still serve as a source of inoculum for *D. citri* to acquire and spread Las.

The field study required psyllid populations to survive to collect acquisition data; therefore, the grove used in this research was not managed for insect pests. It is possible that psyllids were able to re-inoculate the tree faster than the effects of thermotherapy could be observed. However, a healthy tree remains asymptomatic for 6 mo to 2 yr after initial inoculation (Gottwald 2010), and assessments in this study were every 2 to 3 mo after treatment. Thus, it is unlikely that the efficacy of thermotherapy would be obscured by the amount of Las that was inoculated by wild psyllid populations. In addition, residual dead bacteria in the tree may result in higher calculations of Las titer (Trivedi et al. 2009). Although the effect of dead bacterial fragments also was noted by Hoffman et al. (2012), significant titer reductions were observed 30 d post-treatment in their study. This research did not find a significant decrease in Las 119 d after the first treatment, nor 164 d after the second treatment.

There are several challenges to overcome before thermotherapy can succeed on a commercial scale. Currently it is only possible to treat the aboveground portion of the tree in the field, and so the root system remains infected with Las. Because trees in the field are much larger than the potted varieties used in laboratory tests, it is currently not known what optimal temperature and time of exposure is necessary to achieve the same results (Ehsani et al. 2013). Furthermore, it is difficult to control the application of thermotherapy under field conditions. Enclosing single trees under a plastic tarp without the use of supplemental heat is an alternative thermotherapy technique that relies on a greenhouse effect to raise internal temperatures of the enclosure. However, this method is weather dependent, and can take 6 to 7 h to complete the treatment (Ehsani et al. 2013). Thus, mobile heat treatments such as the one used in this study are under development to allow better control of heat and time (Al-jumaili & Ehsani 2015). Although the ability to customize the conditions of thermotherapy is desirable, this presents further challenges. A manned vehicle has time limitations for efficient operation and cannot be left unsupervised in the field. Additionally, longer treatment times would increase the cost per tree. This complicates the improvement of the heat to time ratio since the treatment time necessary to produce efficacy may be cost prohibitive.

Application of thermotherapy in combination with other control techniques, such as antimicrobial treatment, may improve efficacy in huanglongbing management programs. Studies have demonstrated that chemo-thermotherapy decreases Las more than thermotherapy alone treatments in potted trees (Yang et al. 2016b), although this has not been confirmed under field conditions. Further research is required to optimize thermotherapy application under field conditions. Although this study showed no effect of thermotherapy in an unmanaged grove, its efficacy in a managed grove with low psyllid populations has yet to be determined. In addition, thermotherapy may improve the efficacy of chemical treatments in the field, which has the potential to prolong the productive life of a tree, and could provide temporary relief to growers until a resistant citrus variety is available.

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