Incidence of *Candidatus* Liberibacter asiaticus Infection in Abandoned Citrus Occurring in Proximity to Commercially Managed Groves

SIDDHARTH TIWARI, HANNAH LEWIS-ROSENBLUM, KIRSTEN PELZ-STELINSKI, AND LUKASZ L. STELINSKI[1]

Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850

J. Econ. Entomol. 103(6): 1972–1978 (2010); DOI: 10.1603/EC10149

ABSTRACT Huanglongbing is one of the most devastating diseases of citrus (*Citrus* spp.). One management tactic against huanglongbing is aggressive management of the vector, the Asian citrus psyllid (*Diaphorina citri* Kuwayama), with insecticide applications. However, *D. citri* in abandoned groves are not controlled and therefore pose a risk of reinfestation for nearby commercial citrus. These abandoned groves could serve as a reservoir for the vector, as well as a source of the presumed causal agent for huanglongbing in Florida, *Candidatus* Liberibacter asiaticus (Las). The current study was conducted to determine the degree to which Las is present in abandoned Florida citrus groves and to compare relative inoculum levels in nearby managed and abandoned groves during times of the year when *D. citri* are abundant (June, July, and August). In addition, the movement of Las by dispersing *D. citri* adults from inner and edge rows of abandoned grove plots to the corresponding rows of managed plots was quantified during the same 3 mo. The results of the current study confirmed the presence of Las in both *D. citri* and plant tissue in abandoned groves at statistically equivalent levels to those in nearby managed groves. The mean number of *D. citri* adults dispersing from abandoned to managed grove plots ranged from 7.25 ± 1.70 to 70.25 ± 21.25 per 4-d intervals. Of those, the mean number of dispersing *D. citri* adults that were carrying the Las pathogen ranged from 1.00 ± 0.58 to 1.50 ± 0.50. Our results indicate that abandoned citrus groves are a significant source of *Ca.* Las and that dispersing *D. citri* move this pathogen into nearby managed groves.

KEY WORDS abandoned citrus, Asian citrus psyllid, *Candidatus* Liberibacter asiaticus, citrus greening, Huanglongbing

Huanglongbing (HLB) is one of the most destructive and economically important diseases of citrus throughout the world (Halbert and Manjunath 2004, Manjunath et al. 2008). HLB disease is presumably caused by any of three species of fastidious phloem-inhabiting gram-negative bacteria: *Candidatus* Liberibacter asiaticus (Las), *Candidatus* L. americanus (Lam), or *Candidatus* L. africanus (Laf) (Garnier et al. 1984, Jagoueix et al. 1996). The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), vectors Las in Asia and the Americas and Lam in Brazil, and the South African citrus psyllid *Trioza erytreae* (Del Guercio) (Hemiptera: Psyllidae), vectors Laf in Africa.

HLB causes yield reduction, fruit drop, reduced fruit quality and, ultimately, tree death. Currently, there is no cure for HLB. Management programs for HLB include using disease-free planting materials, removal of infected trees and aggressive management of *D. citri* (Halbert and Manjunath 2004). The HLB infection rate in Florida is estimated to be 1.6%, with higher infection rates in the southern and eastern parts of the state (Morris et al. 2009). It has been projected that at the current rate of disease spread, all commercial Florida citrus plantings will be infected within 6–12 yr (Stover et al. 2008).

HLB spread within a citrus grove has been attributed to the movement of *D. citri* from infected trees to healthy trees (Gottwald et al. 1991a,b). Within a grove, the rate and range of HLB spread is directly dependent on the dispersal range of *D. citri.* Likewise, the spread of HLB from one grove to another is probably dependent on the dispersal range of *D. citri* (Halbert and Manjunath 2004). Movement of *D. citri* between abandoned and managed groves has been found to be bidirectional; however, the movement is biased, with more *D. citri* moving from abandoned groves into managed groves (Boina et al. 2009). *D. citri* adults are able to disperse between abandoned and managed groves separated by a distance of 60–100 m within 48 h (Boina et al. 2009). However, no information exists on the dispersal range of Las-infected *D. citri* between groves.

[1] Corresponding author, e-mail: stelinski@ufl.edu.

Table 1. Location of abandoned and managed citrus grove pairs sampled in central Florida for *Candidatus* Liberibacter asiaticus infection in *D. citri* and citrus trees

Grove	Latitude	Longitude
1	28° 06.613′ N	81° 43.788′ W
2	28° 28.451′ N	81° 38.498′ W
3	28° 05.656′ N	81° 3.350′ W
4	28° 06.978′ N	81° 43.868′ W
5	28° 07.402′ N	81° 42.952′ W
6	28° 06.883′ N	81° 42.823′ W
7	28° 06.784′ N	81° 42.584′ W

Abandoned groves are not treated with insecticides or managed to limit the presence and spread of Las, which allows these groves to potentially serve as a continual source of *D. citri* infestation and HLB infection. Several agencies and scientific panels have assessed the risk created by abandoned citrus located in the vicinity of commercial groves. These agencies have suggested that abandoned groves may provide a continuous source of *D. citri* and Las and have recommended possible removal and destruction of abandoned citrus (FHSPR 2006, CHRP 2007, USDA 2009). One of the goals of these agencies has been to identify abandoned citrus groves across the state. The area of abandoned citrus in Florida increased 6.5% from 2008 to 2009, totaling ≈57,650 ha (USDA 2008, Morris et al. 2009, USDA 2009). One of the major contributing factors for the increasing acreage of abandoned citrus is the escalating spread of this infection (Morris et al. 2009). Despite the increasing prevalence of abandoned citrus, the incidence of Las infection in *D. citri* populations and citrus trees in these potential reservoirs has not been previously investigated. We predicted that Las infection in abandoned citrus should be low due to tree decline. New leaf growth (flush), which is required for *D. citri* oviposition (Halbert and Manjunath 2004), is significantly reduced at these sites, which should result in little or no acquisition or spread of Las by developing *D. citri*. The objectives of this study were to determine whether abandoned citrus groves in Florida represent a significant source of Las inoculum and to quantify the movement of Las-infected *D. citri* from abandoned into managed grove plots.

Materials and Methods

To determine the potential impact of abandoned groves on commercial citrus, we investigated seven pairs of abandoned and managed groves in central Florida (Table 1). Each pair of abandoned and managed groves was separated by ≈100 m. Managed groves typically received six to eight insecticide sprays per year, with routine mowing and other disease management programs. Abandoned groves were devoid of irrigation, fertilization and pest management programs for at least 3 yr.

DNA Extraction. Leaves were collected from each pair of abandoned and managed groves during August 2009. Each grove was divided into four transects and 25 trees from each transect were selected based on visual HLB symptoms. Five leaves were selected from each tree (based on visible HLB symptoms), placed into airtight bags, and transported in a cooler to the laboratory. Petiole and midrib tissues from the leaves of five trees were combined, and subsequently 100-mg subsamples were ground under liquid nitrogen for DNA extraction. Petiole and midrib tissue was chosen because it contains substantial amounts of phloem, and Las is phloem limited. Total DNA was extracted from plant tissue using the DNeasy plant kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Samples were eluted in 50 μl of buffer AE (QIAGEN) and stored in sterile 1.5-ml microcentrifuge vials at −20°C for use in quantitative real-time polymerase chain reaction (qPCR) assays.

Approximately 100 *D. citri* adults were collected monthly from each grove described above during June, July, and August 2009. Each grove was divided into four transects, and ≈25 *D. citri* adults were collected from each transect using an aspirator. Adults were transported in a cooler to the laboratory, where they were stored individually within sterile 1.5-ml microcentrifuge tubes containing 80% ethanol at −20°C until DNA was extracted. Adults were pooled in groups of 10 for each grove and collection date, resulting in 10 samples for each site per collection date. Pooled adults were homogenized in a buffer solution (QIAGEN) by using a sterile pestle and lysed overnight at 56°C in a hybridization oven (model 136400, Boekel Scientific, Feasterville, PA) before extraction of total DNA. DNA was extracted from each batch using the DNeasy blood and tissue kit (QIAGEN) following the manufacturer's protocol, with modifications for the extraction of bacterial DNA from arthropods. Samples were eluted in 35 μl of buffer AE and stored in sterile 1.5-ml microcentrifuge tubes at −20°C for use in qPCR assays.

qPCR Assays. qPCR assays of *D. citri* and plant samples were performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) by using a multiplex TaqMan qPCR assay developed for detection of *Ca.* Las (Li et al. 2006). qPCR was chosen because of the inconsistent detection of Las in *D. citri* and plant tissues by conventional PCR and other methods (Halbert and Manjunath 2004, Li et al. 2006). Amplification of *D. citri* samples, conducted in duplicate, contained the following: 1 μl of template DNA, 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 235 nM each of target primers (LasF, 5′-TCGAGCGCGTATGCAATACG-3′; LasR, 5′-GCGTTATCCCGTAGAAAAAGGTAG-3′; GenBank accession L22532; Li et al. 2006), internal control primers specific to the *wingless* (*wg*) gene (GenBank accession AF231365; WgF, 5′-GCTCTCAAAGATCGGTTTGACGC-3′; WgR, 5′-GCTGCCACGAACGTTACCTTC-3′; Thao et al. 2000), and 118 nM each probe (WGp, JOE-5′-TTACTGACCATCACTCTGGACGC3′-BHQ2; Duan et al. 2009; HLBp, FAM-5′-AGACGGGTGAGTAACGCG-BHQ1; Li et al. 2006; Integrated DNA Technologies, Inc., Coralville, IA). Similarly, qPCR amplification of plant samples, conducted in duplicate, contained the following: 1 μl of tem-

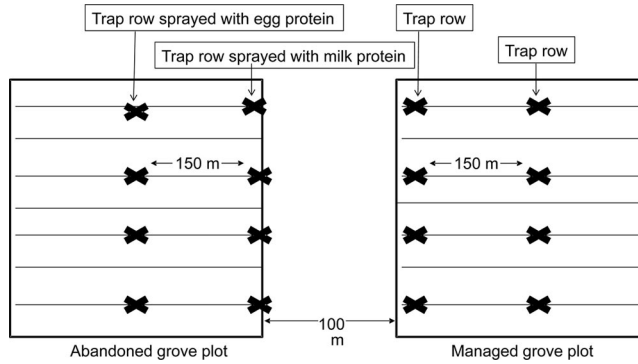

Fig. 1. Schematic layout of a plot used to quantify the movement of Las-infected *D. citri* from abandoned into managed grove plots. In total, there were four sets of such plots, each plot set serving as a replicate.

plate DNA, 12.5 μl of TaqMan Universal PCR Master, 218 nM each of target primers (LasF and LasR), internal control primers specific to the plant cytochrome oxidase (COX) gene (GenBank accession CX297817) (CoxF, 5′-GTATGCCACGTCGCATTCCAGA-3′ and CoxR, 5′-GCCAAAACTGCTAAGGGCATT-3′) (Li et al. 2006), and 136 nM each probe HLBp and COXp (JOE5′-ATCCAGATGCTTACGCTGG-3′BHQ2) (Li et al. 2006; Integrated DNA Technologies, Inc.). DNA amplifications were conducted in 96-well MicroAmp reaction plates (Applied Biosystems). qPCR reactions consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each 96-well plate containing *D. citri* samples included a no template control, a positive control (Las DNA in DNA extractions from *D. citri*) and a negative control (no Las DNA in DNA extractions from *D. citri*). Likewise, plates containing plant samples included a no template control, a positive control (Las DNA in DNA extractions from plant) and a negative control (no Las DNA in DNA extractions from plant). Samples were considered positive for *wg* gene or Las or COX gene if the cycle quantification (C_q) value determined by the ABI 7500 real-time software (version 1.4, Applied Biosystems) was 35 or less.

Dispersal of Las-Infected *D. citri* Adults From Abandoned to Managed Grove Plots. A field study was conducted to quantify the movement of Las-infected *D. citri* from abandoned into managed grove plots. Abandoned and managed grove plots were selected from a site that also was used for plant tissue and *D. citri* sampling. Abandoned and managed grove plots were separated by ≈100 m, and each consisted of ≈200 'Valencia' orange, *Citrus* × *sinensis* (L.) Osbeck, trees on a 10- by 11-m spacing. Each plot was replicated four times with 40 m spacing between replicates (Fig. 1). The experiment was conducted three times, corresponding with the June, July, and August collections of *D. citri*.

D. citri adults in abandoned plots were marked with protein in situ following the protein marking technique described by Boina et al. (2009). The two proteins used in this study were bovine casein (All Natural Whole Milk, Publix Super Markets, Lakeland, FL) and chicken egg albumin (All Whites, Papetti Foods, Elizabeth, NJ). Each protein was sprayed onto a specified row of trees in each abandoned plot. The two rows defined for protein marking were edge and inner rows. The edge row was the first row of trees facing the managed grove plots, and the inner row was the row of trees that was approximately in the center of the grove (150 m from both borders). The edge row of each abandoned plot was sprayed with a 20% dilution of whole milk in water, with Silwet L-77 (Helena Chemicals, Collierville, TN) added at the rate of 2,000 ppm. The inner row of each abandoned plot was sprayed with a 10% dilution of egg white in water, with Silwet L-77 added at the rate of 2,000 ppm. These assignments were chosen at random. The protein solutions were applied using a hand gun sprayer (model 5275016; Fimco Industries, North Sioux City, SD) at ≈250 psi (Boina et al. 2009). The spray was applied until there was visible leaf runoff. After the protein markers were applied, four Pherocon AM yellow sticky traps (Trécé, Adair, OK) were placed in both the edge and inner rows of each abandoned and managed plot (Fig. 1). All traps were evenly spaced throughout their respective row and positioned in the tree canopy at a height of ≈2 m.

Traps were removed 4 d after application of protein sprays. *D. citri* adults were removed individually from the traps using clean, disposable wooden toothpicks (Hearthmark, LLC, Munice, IN) and placed into 1 ml of protein extraction buffer (Tris-buffered saline, pH 8.0, and 0.5 g/liter EDTA [Sigma-Aldrich, St. Louis, MO]) for 3–5 min in separate 1.5-ml centrifuge tubes. Adults were then removed from the buffer using clean wooden toothpicks and placed into new 1.5-ml centrifuge tubes containing 1 ml of 80% ethyl alcohol. Samples were stored at −20°C for later analysis by qPCR. Trap location was recorded for each *D. citri* captured. Extracts from field-collected *D. citri* were analyzed for both milk and egg proteins by using an indirect enzyme linked immunosorbent assay as described in Boina et al. (2009). Individual *D. citri* collected from traps in the managed plots, which were positive for either protein, were subjected to qPCR as described above for detection of Las. Uninfected *D.*

Table 2. Mean ± SEM cycle quantification (C_q) of *Candidatus* Liberibacter asiaticus-infected plant samples and *D. citri* adults collected from seven pairs of abandoned and managed groves

	Abandoned		Managed	
	Las	COX	Las	COX
Plant sample	27.72 ± 1.69	22.38 ± 0.11	31.53 ± 1.03	21.84 ± 0.06
Positive control	23.51 ± 0.17	25.18 ± 2.30	24.13 ± 0.29	22.45 ± 0.28
	Las	*wg*	Las	*wg*
D. citri	32.52 ± 2.17	25.59 ± 0.08	25.95 ± 3.45	23.60 ± 0.11
Positive control	29.10 ± 1.78	25.26 ± 1.78	30.44 ± 4.03	28.64 ± 2.61

Candidatus Liberibacter asiaticus-infected plant samples represent the mean value of 280 plant tissue samples collected from 1,400 trees belonging to seven pairs of abandoned and managed groves. *D. citri* adults represent the mean value of 420 *D. citri* samples comprising 4,200 total adults collected from seven pairs of abandoned and managed groves.

citri adults, with no exposure to a protein marker and serving as controls for both assays, were collected from a greenhouse culture maintained in Lake Alfred, FL, and were described in Wenninger et al. (2008).

Statistical Analyses. The incidence of Las-infected plant samples in abandoned and managed groves was compared using a paired *t*-test ($P < 0.05$; PROC TTEST, SAS Institute 2005). The mean number of Las-infected *D. citri* samples occurring in abandoned and managed groves was compared using two-way analysis of variance (ANOVA) followed by Fisher protected least significant difference (LSD) mean separation test (PROC GLM, SAS Institute 2005). Grove type (managed and abandoned) and month served as main effects.

Two-way ANOVA followed by Fisher protected LSD mean separation test was used to compare the total number of protein-marked *D. citri* adults and the number of Las-infected *D. citri* adults moving from either row of abandoned into managed plots. For each analysis, dispersal type (four levels) and month (three levels) served as main effects. Four possible outcomes of *D. citri* dispersal were included in the analysis: 1) inner row of the abandoned plots to the inner row of managed plots, 2) inner row of the abandoned plots to edge row of the managed plots, 3) edge row of the abandoned plots to the inner row of the managed plots, and 4) edge row of the abandoned plots to the edge row of the managed plots.

Results

C_q values from qPCR assays of Las-positive plant tissue samples from abandoned and managed groves with their respective positive controls are presented in Table 2. Two hundred and eighty plant tissue samples collected from 1,400 trees belonging to seven pairs of abandoned and managed groves were analyzed by qPCR. The mean Las infection rate in plant samples was numerically greater in abandoned (3.6%) than managed groves (1.4%) (Fig. 2a); however, the difference was not statistically significant ($t = -0.93$, df = 12, $P = 0.3691$).

Cq values from qPCR assays of Las-positive *D. citri* from abandoned and managed groves with their respective positive controls are presented in Table 2. Four hundred and twenty *D. citri* samples comprising 4,200 total adults collected from seven pairs of abandoned and managed groves were analyzed by qPCR. The mean Las infection rate in *D. citri* samples was numerically greater in abandoned (1.9%) than managed (1.2%) groves (Fig. 2b); however, the difference was not statistically significant ($F = 0.38$; df = 1, 36; $P = 0.5441$). The mean ± SEM number of Las-infected samples found in abandoned and managed groves was higher during the month of August (1.00 ± 0.53 and 0.29 ± 0.28, respectively) than in June (0.14 ± 0.14 and 0.14 ± 0.13) or July (0.00 ± 0.00 and 0.29 ± 0.28); although month was not a significant main effect ($F = 2.04$; df = 2, 36; $P = 0.1446$). Las-infected *D. citri* adults were never collected from the same grove on more than one occasion, with the exception of one managed grove, where Las-infected *D. citri* adults were found during two consecutive sampling periods.

In total, 204, 281, and 29 *D. citri* adults were marked in the abandoned plots and recaptured in the managed plots over 4-d intervals during June, July, and August, respectively. Overall, the mean number of *D. citri* adults dispersing during June or July was significantly greater than during August ($F = 5.02$; df = 2, 9; $P = 0.0344$; Fig. 3). The majority of recaptured *D. citri*

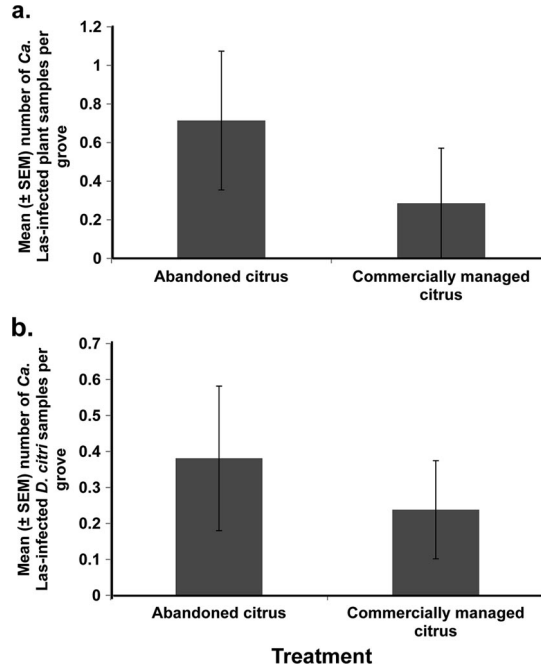

Fig. 2. Mean ± SEM number of *Ca.* Liberibacter asiaticus-infected plant tissue (a) and *D. citri* (b) samples from seven pairs of abandoned and managed citrus groves in central Florida. For plant tissue samples, 20 DNA samples were analyzed from each grove, and each DNA sample consisted of pooled plant tissues from five trees. For *D. citri* samples, 10 DNA samples were analyzed from each grove and each DNA sample consisted of 10 *D. citri* adults.

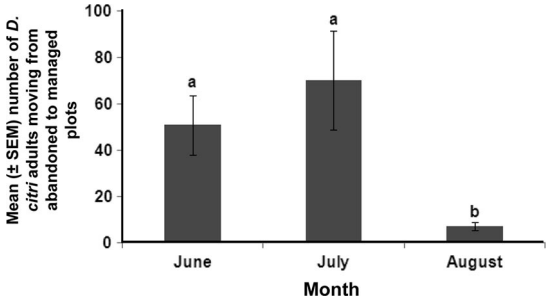

Fig. 3. Mean ± SEM number of *D. citri* adults dispersing from abandoned into managed grove plots during June, July, and August.

moved from the inner rows of abandoned plots to the edge rows of managed plots (Fig. 4). This occurred during each month. Las-infected *D. citri* adults comprised 2.9% of the total *D. citri* dispersing from abandoned into managed plots. Most Las-infected *D. citri* moved from the inner row of the abandoned plots to the inner row of the managed plots, followed by those moving from the inner row of abandoned plots to the edge row of managed plots (Fig. 4).

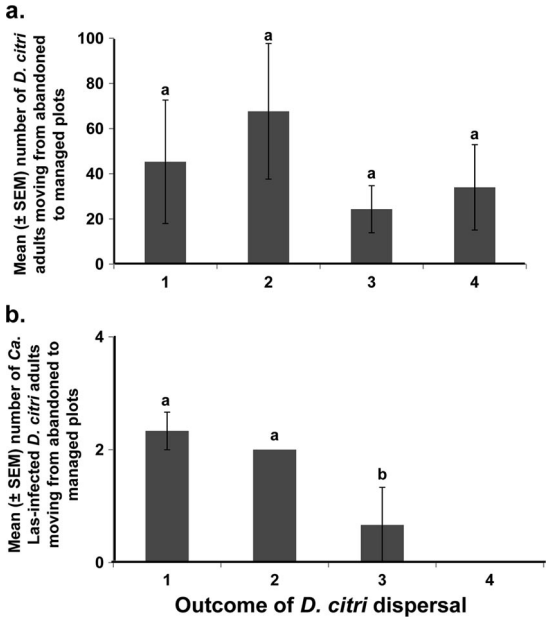

Fig. 4. Mean ± SEM total number of marked *D. citri* (a) and mean number of *Ca.* Liberibacter asiaticus-infected *D. citri* (b) found moving from abandoned into managed citrus grove plots: 1 refers to the movement of adults from the inner row of the abandoned to the inner row of the managed plots, 2 refers to the movement of adults from the inner row of the abandoned to the edge row of the managed plots, 3 refers to the movement of adults from edge row of the abandoned to the inner row of the managed plots, and 4 refers to the movement of adults from the edge row of the abandoned to the edge row of the managed plots.

Discussion

Ca. Liberibacter asiaticus is present in both citrus trees and *D. citri* adults in abandoned Florida citrus groves at rates that are comparable with those found in managed groves. Although trees in the abandoned groves used in our study were in a state of severe decline compared with those in the managed groves, they harbored comparable levels of Las infection. Although fewer psyllids were found in abandoned groves, our results indicate that the percentage of collected samples with *Ca.* Las inoculum tended to be slightly greater in abandoned than managed groves. Las-infected *D. citri* adults dispersed at least 400 m over 4-d intervals from inner rows of abandoned grove plots to inner rows of the managed groves. These results confirm that abandoned citrus groves act as reservoirs of the bacterium that causes huanglongbing, its vector, and also serve as sources of *D. citri* infestation and potential Las infection for nearby commercial groves.

Results from the current study should assist regulatory agencies in deciding the fate of abandoned citrus. Abandoned citrus has not been considered a significant reservoir of HLB, given the limited growth of leaf flush on unmanaged trees. Despite limited habitat for egg laying and feeding, *D. citri* in these sites harbored Las inoculums levels comparable with those found in managed sites. Abandoned groves harboring *D. citri* populations will probably require removal or control for successful area wide management of HLB in Florida. Management programs conducted on an area wide basis also may reduce short-range movement of Las-infected *D. citri* and thus reduce the spread of the HLB pathogen.

Commercially managed citrus groves receive six to eight insecticide applications per year (Srinivasan et al. 2008) and other routine maintenance, such as mowing and disease management sprays. Therefore, the biased movement of *D. citri* from abandoned into managed groves (Boina et al. 2009) is probably due to the presence of more citrus flush in managed groves, which serve as oviposition sites and food for developing nymphs. The short-range movement of *T. erytreae* also is influenced by the availability of new flush (Catling 1969, and Samways and Manicom 1983). Immigrating Las-infected *D. citri* adults that settle and feed on new flush in managed groves could result in rapid multiplication of Las within healthy flushing trees. Multiplication of Las is greater on the distal end of citrus branches, especially where new growth occurs regularly (Teixeira et al. 2008).

The dispersal capabilities of *D. citri* have not been experimentally quantified; however, it has been speculated that *D. citri* can disperse from 90 to 145 km (Gottwald et al. 2007) up to 470 km (Sakamaki 2005). *D. citri* was found to disperse up to 100 m within 3 d between abandoned and managed groves in our previous investigation (Boina et al. 2009). The abovementioned studies on the dispersal capabilities of *D. citri* have not distinguished between the movement of Las-infected and uninfected *D. citri.* At this point, it is unknown whether the presence of Las may alter the

dispersal behavior of *D. citri* or whether presence of Las in host plants may alter the dispersal of *D. citri*. However, the psyllid *Cacopsylla picta* (Förster), which vectors *Ca.* Phytoplasma mali in apple (*Malus* spp.), is known to be attracted by the odor of phytoplasma-infected apple trees (Mayer et al. 2008). Determining the dispersal range capabilities of *D. citri*, specifically of Las-infected *D. citri*, is critical to thoroughly understand the potential impacts of abandoned citrus on commercial citrus production. In addition, determining the effect of HLB infection on the dispersal behavior of uninfected and Las-infected *D. citri* should improve the understanding of this arthropod-pathogen interaction. Also, this information should help establish effective area wide management programs, determine useful quarantine boundaries and develop guidelines for management or removal of abandoned citrus groves.

Acknowledgments

We thank J. Burns for access to real-time PCR equipment. We appreciate the technical assistance of A. Hoyte, I. Jackson, R. Blanco, and M. Flores. We thank Drs. Nian Wang, Pankaj Trivedi, Susan Halbert, and Wendy Meyer for critical review of this manuscript, and we also appreciate the comments from two anonymous reviewers. We thank the Florida Citrus Production Research Advisory Council for financial support.

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Received 23 April 2010; accepted 3 September 2010.