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INCIDENCE OF HUANGLONGBING-ASSOCIATED 'CANDIDATUS LIBERIBACTER ASIATICUS' IN *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE) COLLECTED FROM PLANTS FOR SALE IN FLORIDA

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, was reported for the first time in Florida in June 1998, and huanglongbing (HLB, citrus greening), vectored by *D. citri*, was detected in Florida for the first time in Aug 2005. In Florida, the only known HLB pathogen is 'Candidatus Liberibacter asiaticus' (Las). After HLB was known to be established in Florida, the psyllid vectors found in regulatory samples from plants for sale were tested for the pathogen by real-time qPCR. Approximately 1,200 regulatory samples were tested between Aug 2005 and Aug 2009. Samples came from venues in 44 of Florida's 67 counties. Most of the samples came from citrus, but about 11% came from *Murraya exotica*, a popular ornamental plant and close relative of citrus. Approximately 9.7% of the psyllid samples tested were positive for Las. Numbers of samples and proportion of positive samples varied by year and by county.

Key Words: huanglongbing, HLB, citrus greening, *Diaphorina citri*, citrus, regulations

RESUMEN

Diaphorina citri Kuwayama fue registrado por primera vez en la Florida en junio de 1998, y la enfermedad Huanglongbing (HLB, enverdecimiento de los cítricos) se detectó en el estado por primera vez en agosto del 2005. En Florida, el único patógeno conocido de HLB es 'Candidatus Liberibacter asiaticus' (=Las). Después de que HLB se estableció en la Florida, los vectores psílidos encontrados en las muestras de las plantas reguladas para la venta fueron probados para el patógeno en tiempo real de qPCR. Aproximadamente 1,200 muestras regulatorias fueron examinadas entre agosto del 2005 y agosto del 2009. Las muestras procedían de 44 de los 67 condados de la Florida. La mayoría de las muestras procedían de cítricos, pero aproximadamente el 11% fueron de *Murraya exotica*, una planta ornamental popular que se relaciona con los cítricos. Aproximadamente el 9.7% de las muestras fueron positivas para el patógeno "Las". El número de muestras y la proporción de muestras positivas variaron según el año y por condado.

Palabras clave: Huanglongbing, HLB, enverdecimiento de los cítricos, *Diaphorina citri*, cítricos, regulaciones

Huanglongbing (HLB, also known as citrus greening) is the most destructive disease of citrus in the world (Gottwald et al. 2007). It was discovered in Florida in 2005 (Gottwald et al. 2007) and has spread throughout Florida's citrus growing counties. In Florida, a phloem-limited bacterium, 'Candidatus Liberibacter asiaticus' (Las) is associated with HLB. The Florida vector is *Diaphorina citri* Kuwayama, one of at least 8 species of psyllids that feed on citrus (Halbert & Manjunath 2004). *Diaphorina citri* was discovered in Florida

in 1998 (Halbert et al. 2002). Although the insect was too widespread to eradicate, the infested area was quite small (coastal Palm Beach and coastal northern Broward counties). The limited distribution indicated that the discovery of the vector occurred very shortly after it became established (Halbert et al. 2008a, 2008b).

The HLB syndrome has been known for about a century. The first convincing description of HLB can be found in Husain and Nath (1927) in India. A more formal description of the disease was pub-

lished by Lin (1956a, 1956b) in China. In both cases, the vector was *D. citri*. *Diaphorina citri* clearly is native to the Indian subcontinent, but there is considerable debate about the origin of the disease (Halbert & Manjunath 2004; Beattie et al. 2008). What is clear is that movement of citrus plants across international borders over a period of several decades led to the dissemination of the pathogen and its vector. In Brazil, *D. citri* was introduced without the pathogens that cause HLB. *Diaphorina citri* was reported in Brazil in 1942 (Costa Lima 1942) and persisted in the absence of HLB for several decades. HLB was reported in Brazil for the first time in 2004 (Teixeira, et al. 2005) and has caused considerable damage to citrus there since its discovery. The discovery of HLB in Brazil was the first report in the Western Hemisphere. In Florida, HLB was discovered 7 yr after the discovery of the psyllid, and the disease had spread into several counties by the time it was found (Halbert et al. 2008a, 2008b). In a very short period of time, HLB spread throughout the citrus-growing regions of the state.

In an effort to understand the possible causes for the rapid spread of HLB in Florida, we conducted large-scale psyllid surveys, testing the psyllid vectors for the presence or absence of Las (Manjunath et al. 2008). We demonstrated that much can be learned about the distribution of HLB by testing *D. citri* for the presence of Las. In particular, we learned that psyllids positive for Las can be found mo to yr prior to development of symptoms on the plants. In this paper, we summarize the results of 4 yr of testing regulatory samples of *D. citri* collected from plants for sale in Florida. The purpose of this survey was to discover whether Las-positive psyllids could be found on plants for sale in Florida, and to determine the effect of the regulations implemented for control of *D. citri* and HLB in nursery and retail environments.

MATERIALS AND METHODS

Collection of Psyllids

The Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) is a regulatory agency. DPI plant inspectors regularly visit venues where plants are sold and collect samples of potential pest insects and plant pathogens. Sampled plant pests are sent to the DPI laboratory in Gainesville for further scrutiny, diagnosis and verification. Florida regulations require that citrus plants for sale (retail and commercial production) must be inspected at intervals varying from 30 to 60 d. If citrus psyllids are found, they are submitted to the Gainesville DPI laboratory for verification. Plants that have psyllid colonies that include nymphs older

than first instars are subject to official quarantine until the insects are eradicated. Prior to 1 Jan 2008, *Murraya exotica* L.; Sapindales: Rutaceae, plants also were inspected. After that date, *M. exotica* has not been propagated in Florida as a result of more stringent regulations that restrict the sale and movement of citrus relatives that are potential hosts of *D. citri* and HLB (FDACS/DPI 2008).

On 1 Sep 2006 Florida regulations began requiring that citrus nursery stock be treated with an imidacloprid-based soil drench (ISD) within 30 d prior to shipment to a venue for retail sale. A tag indicating the date of treatment is required. Florida regulations specified that the tags expired in 6 mo. This expiration date was determined based on an estimate from the University of Florida/IFAS about how long the pesticide should be effective, presuming that it was applied correctly. Florida retail citrus nursery inspectors are required to include the registration number of the propagating nursery and the ISD date in their paperwork submitted with citrus psyllid samples. Sometimes, plants with expired tags are re-treated at the retail venue, either by the retailer, or by personnel from the propagating nursery.

The routine inspections described above produced about 1200 samples of *D. citri* over a 4-yr period from Aug 2005, when HLB was discovered in Florida, through Aug 2009. These samples were verified according to quarantine regulations and subsequently packaged and sent to the USDA National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) in Riverside, CA for DNA analysis for Las.

Samples of *D. citri* were preserved in 95% ethanol. Nymphs and adults were separated into different vials. Each sample received a log number and was entered into the DPI Entomology regulatory database. Upon arrival at the NCGRCD, the samples were logged into an extraction database, and stored at -20 °C until processed.

DNA Testing

DNA extraction from psyllids was done as previously described by Manjunath et al. (2008). Psyllid nymphs were extracted individually, whereas adults were extracted usually in groups of 5. The DNA, after completing the extraction, was stored at -20 °C in sterile water.

Extracted DNA was analyzed by qPCR using the Taqman probe and primers in a multiplex real-time qPCR assay (Manjunath et al. 2008). The Taqman probe and primers for the psyllid wingless gene were included in each qPCR assay when testing psyllid DNA to monitor the quality of DNA extraction (Manjunath et al. 2008).

RESULTS

Between Aug 2005 and Aug 2009, we analyzed 1,186 psyllid samples. Forty four of Florida's 67 counties were represented in the samples (Fig. 1). Although counties were not sampled evenly, there was a fair representation from many Florida counties.

Samples of *D. citri* were obtained from a variety of venues, including general retail nurseries, large discount garden centers, commercial citrus nurseries (nurseries that propagate citrus), and Foundation Budwood Blocks (Fig. 2). There was a marked decrease in numbers of psyllid samples collected from commercial citrus-propagating nurseries and Foundation Budwood Blocks in 2008, when the new Florida regulations became mandatory. These new regulations required citrus to be propagated in secure facilities, protected from psyllids. Frequency and intensity of inspections for commercial citrus nurseries also increased. *Murraya exotica* production was included in the regulations (FDACS/DPI 2008).

About 88% (1,031) of the *D. citri* samples were collected from citrus plants (Fig. 3). Another 11%

(125) were from *M. exotica*, and 1.5% (18) were obtained from other citrus relatives. Twelve samples had no host data. Most of the *M. exotica* samples were collected in 2005-2006. Early in 2008, *M. exotica* became unavailable commercially in Florida. The new regulations made this plant prohibitively expensive to propagate.

Las Status of Samples

Overall, about 9.7% of the 1,186 tested *D. citri* samples collected from plants for sale were positive for Las. Numbers of *D. citri* samples submitted and percentages of positive samples varied by year (Fig. 4).

Las was found in psyllids collected from citrus, *M. exotica* and *Berberis koenigii* L.; Sapindales: Rutaceae, (Fig. 5). Las is difficult to detect in *M. exotica* plants, but nevertheless, significant numbers of positive psyllid samples were collected from these plants (Fig. 5). Most of the positive samples from *M. exotica* were collected in the Miami area (Miami-Dade County), the location of the major *M. exotica* propagation industry; however, 2 of the positive samples were collected

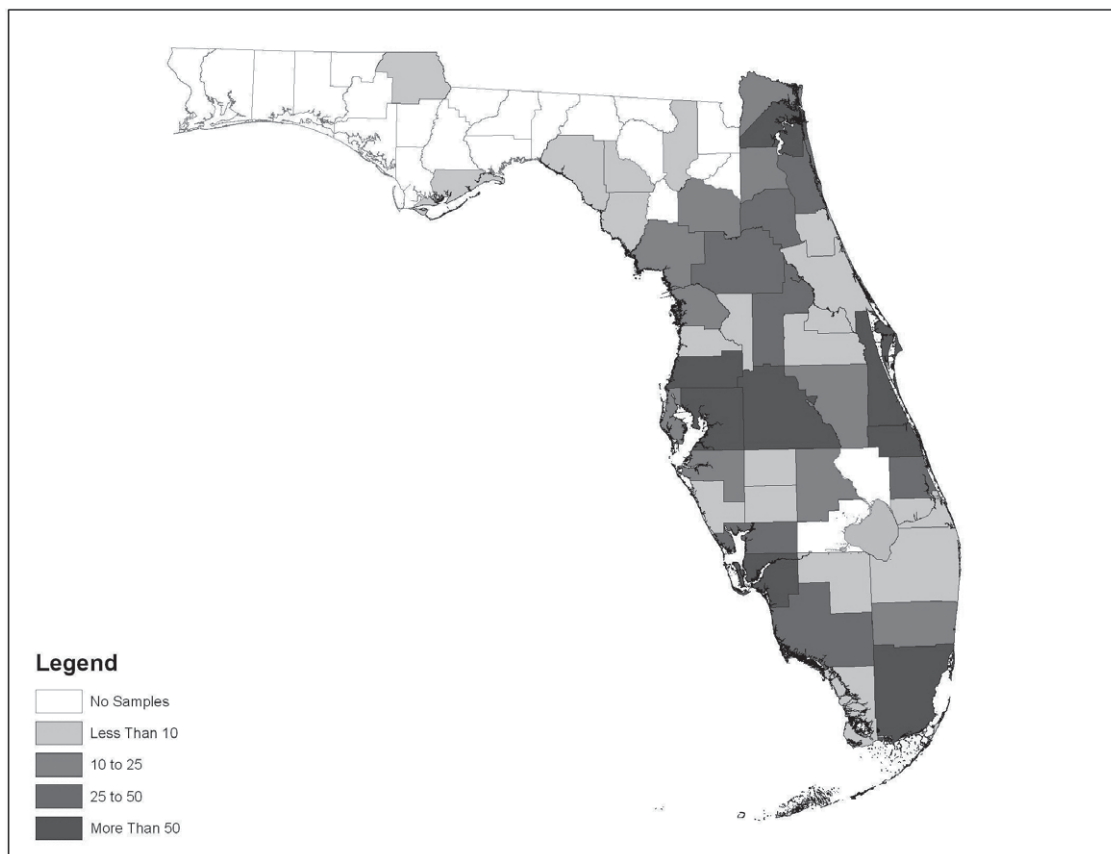


Fig. 1. Numbers of samples of *Diaphorina citri* Kuwayama collected from plants for sale in Florida by county (2005-2009). Map: Matthew Albritton, DPI.

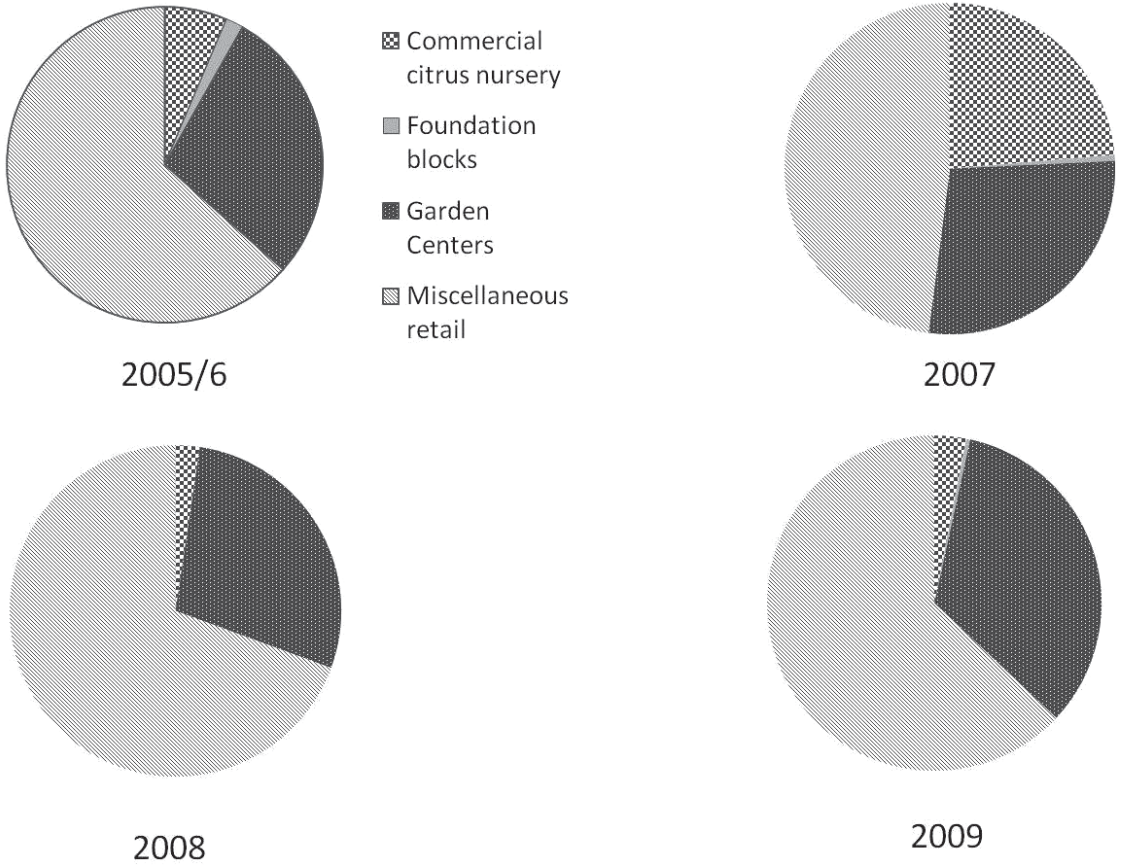


Fig. 2. Relative numbers of samples of *Diaphorina citri* Kuwayama collected on plants for sale at various types of nursery businesses in Florida (2005-2009). Legend begins at the 12:00 position and proceeds clockwise. Total numbers of samples by year: 2005/6 – 501, 2007 – 338, 2008 – 85, 2009 – 262.

from retail stores in locations where *M. exotica* was not being propagated. In one of those 2 cases (Highlands County), nymphs in the sample were positive.

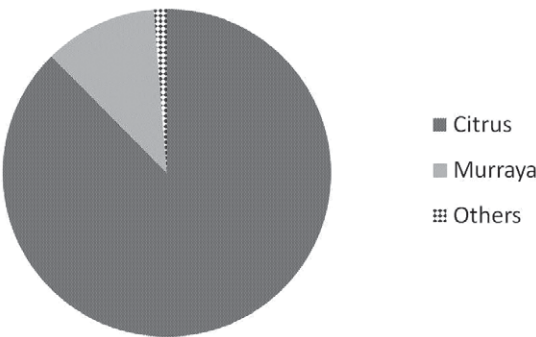


Fig. 3. Host sources of *Diaphorina citri* Kuwayama collected from plants for sale in Florida (2005-2009). Legend begins at the 12:00 position and proceeds clockwise. There were 1,186 total samples, but 12 samples had no listed host.

The proportion of positive *D. citri* samples varied depending on the county in which the

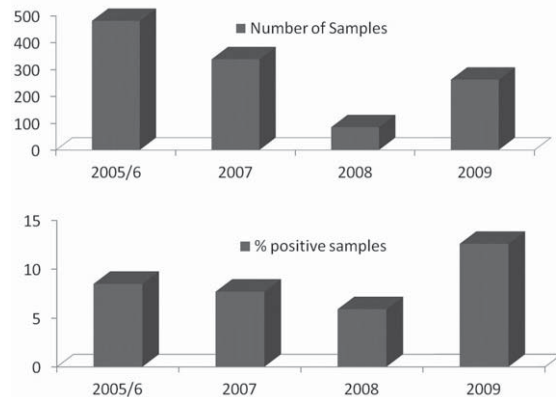


Fig. 4. Numbers of samples (top) and percent samples positive for ‘*Candidatus Liberibacter asiaticus*’ (bottom) of *Diaphorina citri* Kuwayama collected from plants for sale in Florida (2005-2009).

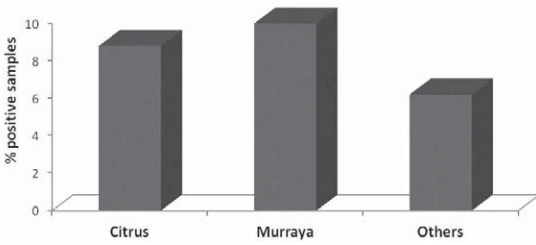


Fig. 5. Relative numbers of samples of *Diaphorina citri* Kuwayama that were positive for 'Candidatus Liberibacter asiaticus' collected from various host plants (2005-2009). Total samples from each group: citrus – 1031, *Murraya* – 125, others – 18. Twelve samples had no listed host.

sample was collected (Fig. 6). For the first 5 yr after its discovery in Florida, HLB incidence in Florida roughly followed a gradient from highest incidence in the southeast (except in the Everglades, where there is very little citrus) to lower levels in the northwest (FDACS/DPI maps 2005-2011—only latest one on line; Halbert et al. 2008b). The proportion of Las-positive *D. citri* on

plants for sale does not correspond to the state-wide relative incidence of HLB in the landscape. As expected, Monroe and Miami-Dade Counties, on the southern tip of the Florida peninsula, had a high proportion of Las-positive samples, but some counties farther north also have high relative numbers of Las-positive samples but a relatively low incidence of HLB in the landscape.

In some counties, there were more positive *D. citri* samples from plants for sale than would be expected based on numbers of infected plants in the landscape. In Marion County, in north-central Florida, 17% (7 of 41) of *D. citri* samples from plants for sale were positive, whereas DPI records show that only 4 Las-positive plants were found in the landscape in Marion County through 2009. Las-positive psyllids were found on citrus plants for sale in Levy and Nassau Counties in Northern Florida, but no positive plants have been found in the landscape in those counties to date. Positive plants were found for the first time in Duval County on 1 May 2009 at a retail store. The discovery of Las-positive psyllids usually did not coincide with the discovery of symptomatic, positive plants. In fact, for the

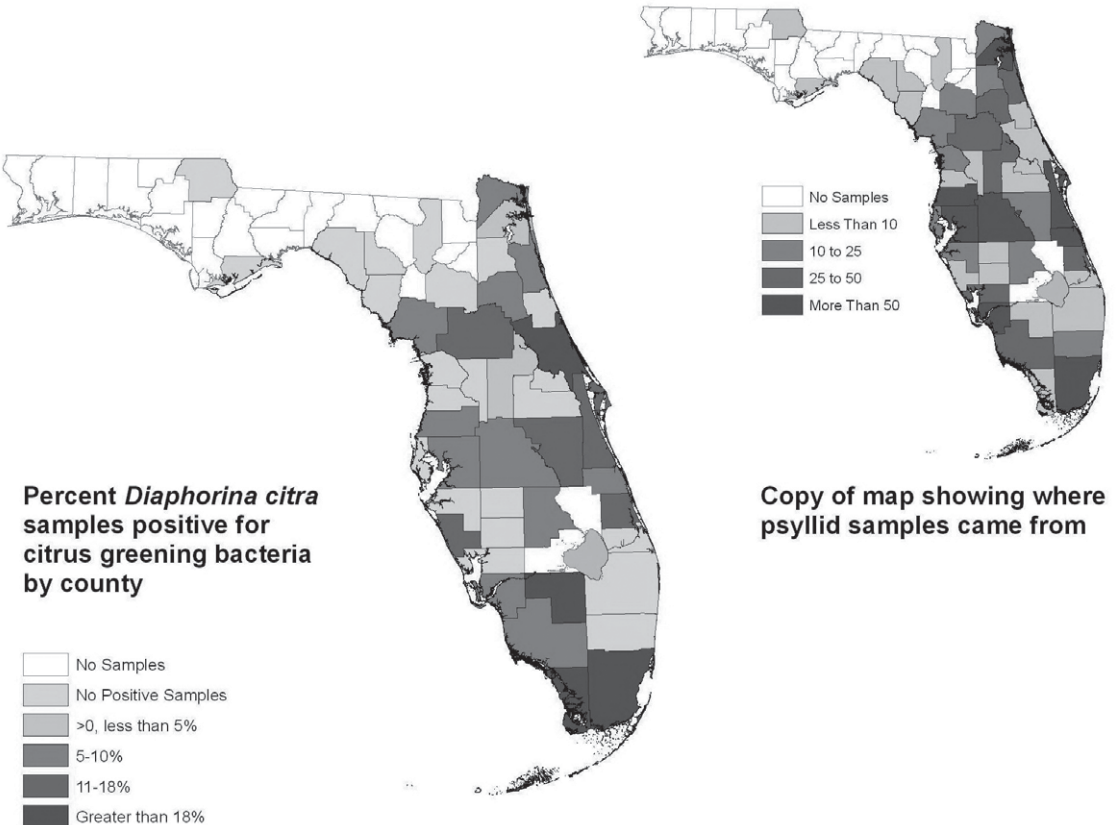


Fig. 6. Proportion of samples of *Diaphorina citri* Kuwayama collected from plants for sale in Florida that were positive for 'Candidatus Liberibacter asiaticus' by county. A map showing the numbers of samples per county (copy of Fig. 1) is included for comparison. Map: Matthew Albritton, DPI.

16 venues where we found positive psyllids, and DPI records document the presence of Las-positive citrus plants, symptomatic positive plants were found an average of 267 d after the collection of the positive psyllids.

DISCUSSION

Effect of Imidacloprid Soil Drench (ISD).

The ISD became a mandatory requirement in Florida after 2006. Part of the requirement is to put a tag on the plant that indicates the date of the ISD treatment. We analyzed the 2009 data set, which is relatively complete with respect to inclusion of ISD dates on the accompanying sample paperwork, to determine whether the policy had an effect on numbers of infected *D. citri* samples. The fact that we found any psyllids at all indicates some difficulty with the pesticide treatment, because the intent of the ISD is to keep the plants free of *D. citri*. Of 259 samples collected in 2009 with complete ISD information on the accompanying documentation, 150 samples had paperwork that indicated plants had current (treated within the recommended 6 mo) ISD tags, 45 psyllid samples were from plants with expired tags, and 64 samples had paperwork that indicated the ISD tags were missing.

A slightly higher proportion of the positive and inconclusive samples were collected from plants with expired or non-existent ISD tags. Fifty-five percent of positive and inconclusive samples came from plants with expired or missing tags, as opposed to 39% for negative samples. A slightly lower proportion of samples from plants with current ISD tags tested positive for Las. Twelve percent of samples from plants with current tags were positive for Las, as opposed to 15% of samples from plants with expired or missing tags. These results could be due to chance, or they could be explained by the relatively longer time that plants with expired or non-existent tags spent at the venue, exposed to infective vectors.

Based on our findings, the ISD requirement is not working as well as it should, because if it were working, there would not be psyllids on plants for sale. It is not possible to determine from our data whether the ISD is having an effect on acquisition or transmission of Las. The answer to that question will require controlled experiments in a laboratory environment. Laboratory experiments also are needed to sort out whether the problems are due to application issues or to inherent limitations of the product.

Sources of Retail Infections.

It is very difficult to trace or account for HLB infections in retail stores. Potential sources of in-

oculum include liners (rootstock plants) and budwood, the propagating nurseries, the retail nursery itself, and the local environment. Inspectors are required to record the registration number for the propagating nursery on their specimen reports. Las-positive psyllid samples were obtained from plants originating at many different propagating nurseries. Thus, the source of HLB pathogens cannot be traced to any single propagator. Few positive samples were found at commercial (propagating) nurseries. The numbers of psyllid-infested plants at commercial nurseries declined markedly after the new regulations were implemented in 2008 (Fig. 2).

Long-Distance Dissemination of HLB Pathogens in Florida.

HLB spread much faster in Florida than in Brazil. Most citrus industries cannot pinpoint accurately when HLB began to spread. In Florida, however, there is a fairly firm date for the beginning of our experience with this pathosystem (Halbert et al. 2008a, 2008b). *Diaphorina citri* had very limited distribution when it was discovered in 1998. Most likely, spread of the HLB pathogens began in Miami-Dade County in late 1999 or early 2000 when the psyllids arrived there for the first time. In Brazil, it has been estimated that the geographic range of HLB expanded by approximately 20 km per year (Gottwald et al. 2007). In Florida, its range expanded at approximately 50-60 km per year (Halbert et al. 2008a, 2008b), a rate about 3 times that in Brazil.

In earlier papers (Halbert et al. 2008a, 2008b), we show circumstantial evidence indicating that psyllids can fly much farther than the 1.5 km that is reported in the literature. It also was demonstrated (Halbert et al. 2010) that infective insects can move potentially long distances on unprocessed fruit in trailers. However, data we present here suggests that distribution of both psyllids and HLB also could have occurred through retail sales of compromised plant material or plants that harbored Las-positive psyllids.

Murraya exotica, a popular landscape ornamental plant and an excellent host of *D. citri*, was produced in large numbers in south Miami-Dade County prior to 2008 (Fig. 7). *Murraya exotica* also is a host of the HLB pathogens (Zhou et al. 2007). Thus, for about 8 yr (2000-2008) after *D. citri* populations established in the area and probably encountered the HLB pathogens, production of *M. exotica* continued without much regulation. Unfortunately, separate records were not maintained to document the numbers of *M. exotica* plants that were propagated and exported out of the area, but undoubtedly, numbers were in the tens of thousands of potted plants per year.

Murraya exotica played an important role in the distribution of *D. citri* throughout Florida



Fig. 7. Small, weedy *Murraya exotica* L. nursery in Miami-Dade County, FL, USA with HLB-positive citrus in the fencerow (circled). This nursery had an inventory of 4500-5000 pots between 2004 and 2006. Photograph: Susan Halbert.

(Halbert et al. 2002). After the insects were distributed widely in Florida, little attention was paid to psyllid infestations on *M. exotica* plants for sale prior to the discovery of HLB in late summer, 2005. Over 15% (12 of 78) of *D. citri* samples from *M. exotica* plants for sale tested in 2005 and 2006 were positive for Las. An unknown number of pots of *M. exotica* that might have carried Las-positive psyllids left south Miami-Dade County prior to the implementation of new regulations in 2008, when the plants were not produced anymore. It is possible that some Las-positive psyllids on potted *M. exotica* were distributed widely through efficient networks maintained by large discount retailers.

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