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TRAILERS TRANSPORTING ORANGES TO PROCESSING PLANTS MOVE ASIAN CITRUS PSYLLIDS

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ABSTRACT

Huanglongbing (citrus greening) is one of the most serious diseases of citrus. Movement of the disease occurs as a result of natural transmission by the insect vector and by movement of infected plant material. We demonstrate here that *Diaphorina citri* Kuwayama, the vector of citrus greening pathogens, can be transported in trailers of unprocessed fruit. Several samples of *D. citri* collected from trailers of oranges were positive for citrus greening pathogens, indicating that the disease can spread widely with the movement of the mature fruit as a result of moving the vectors. While movement of disease and the vector through plant materials into new areas has been recognized, our findings emphasize the need to evaluate the importance of long distance movement of psyllids on unprocessed fruit, even in the absence of vegetative tissue.

Key Words: *Diaphorina citri* Kuwayama, Asian citrus psyllid, citrus greening disease, huanglongbing, *Citrus*, fruit movement

RESUMEN

La "Huanglongbing" (enverdecimiento de los citricos) es una de las enfermedades mas sérias de los cítricos. El movimiento de la enfermedad sucede como resultado de la transmissión natural por el insect vector y por el movimiento de material de plantas infectadas. Demonstramos que *Diaphorina citri* Kuwayama, un vector de los patógenos del enverdecimiento de los cítricos, puede ser transportado en remolques con frutas no procesadas. Varias muestras de *D. citri* recolectadas de remolques de naranjas resultaron positivas para los patógenos del enverdecimiento de los cítricos, lo cual indica que la enfermedad puede esparcirce ampliamente con el movimiento de frutas maduras como resultado del traslado de los vectores. Mientras que el movimiento de la enfermedad y el vector por medio del movimiento de material de plantas en nuevas areas ha sido reconocido, nuestros hallazgos enfatizan la necesidad de evaluar la importancia del movimiento del sílido por largas distancias sobre frutas no procesadas, aun en la ausencia de tejido vegetativo.

Huanglongbing (citrus greening disease), associated with phloem-limited bacteria in the genus *Candidatus* Liberibacter, is one of the most important citrus diseases worldwide, causing significant economic losses to global citriculture (Bové 2006; Gottwald et al. 2007). Natural spread of the disease is primarily by the psyllid vectors *Diaphorina citri* Kuwayama (Asian citrus psyllid) and *Trioza erytreae* (del Guercio) (African citrus psyllid). The disease also can be transmitted by grafting, dodder (a parasitic plant), and possibly by seed (Halbert & Manjunath 2004). *Diaphorina citri* was reported for the first time in the United States in Florida in 1998 (Halbert et al. 2002), and the disease was found to be established in Florida in Aug 2005 (Halbert 2005; Bové 2006).

In the United States, there are 4 major commercial citrus growing states: Florida, Texas, Arizona, and California. *Diaphorina citri* was reported in Texas in 2001 (French et al. 2001). *Diaphorina citri* was detected for the first time in California in San Diego County near the border with Mexico on 27-VIII-2008 by California Department of Food and Agriculture (CDFA) Agricultural Technician Rosalio Peña and CDFA Agricultural Pest Control Specialist Dan Arena. It was identified by CDFA Systematic Entomologist Dr. Alessandra Rung on 29-VIII-2008 (Brian Taylor,

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personal communication; CDFA 2008). *Diaphorina citri* nymphs positive for citrus greening pathogens were intercepted in baggage from India at a FedEx facility in Fresno in Jul 2009 (CDFA 2009). The insects were feeding on curry leaves (*Bergera koenigii* L.) that were detected by a sniff dog. Other than this interception, no citrus greening has been detected in California. To date, no citrus greening or citrus psyllids have been detected in Arizona. Both *D. citri* and citrus greening disease were reported from Louisiana in Jun 2008 (Hummel & Ferrin 2008). In Apr 2009, citrus greening was recorded in South Carolina (Harden & Kent 2009), and in Jun 2009, the disease was reported in Georgia (Gomes 2009). To date, in the United States, only Florida, Louisiana, South Carolina, and Georgia are known to have the citrus greening pathogens established, but isolated infected plants smuggled from other places may remain undetected until vectors spread the pathogens. So far, *Ca*. Liberibacter asiaticus (LAS) is the only species of citrus greening pathogen known to occur in the USA.

Movement of the psyllid vectors infected with LAS has been shown to occur on plants marketed by garden stores and nurseries in Florida (Manjunath et al. 2008). Spread of the insect vector and the associated disease also occurs through transportation of potted citrus plants by the general public (unpublished observations, Susan Halbert).

Florida Department of Agriculture and Consumer Services Division of Plant Industry (DPI) plant inspection supervisor Kenneth L. Hibbard intercepted numerous *D. citri* adults in boxes of unprocessed fruit shipped from the Bahamas to Ft. Pierce, Florida, for processing in 2001 (Halbert & Núñez 2004). At that time, DPI assisted in inspections of citrus fruit arriving from Bahamian groves. The objective of the present study was to determine whether Asian citrus psyllids were moving on shipments of unprocessed fruit within the state of Florida, and if so, whether the psyllids were infected with citrus greening pathogens.

Materials and Methods

Seven loaded trailers of oranges were inspected at 2 processing plants in Hendry County, Florida on 27-III-2006 (Fig. 1). Typically, oranges are picked and loaded into trailers for transport to processing facilities that extract orange juice. Processing plants may obtain fruit from a wide area depending on contracts and market factors. We chose trailers with sides made from metal webbing (as opposed to solid sides) so that both the sides and the tops of the loads could be inspected. Any *D. citri* found on the oranges were hand collected with aspirators. They were counted and preserved in Gainesville and sent to USDA, Riverside, CA, where they were tested by quantitative realtime PCR assays to determine if they were carrying LAS.

Most psyllids were tested in aliquots of 2-6 insects per extraction; however, there were 2 extractions with 50 psyllids per extraction. DNA was extracted from psyllids according to the procedure described in Manjunath et al. (2008), with some modifications, and tested for the presence of LAS. Briefly, the psyllids were air-dried for 10 min, trans-



Fig. 1. Trailers of field-picked oranges at an orange juice processing plant in Florida.

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ferred to a 1.5-mL microfuge tube containing 300 µL of extraction buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% SDS) and 20 units of Proteinase K (New England Biolabs), and ground finely in a mini bead beater (Biospec Inc.) in the presence of 1 zinc-plated ultra smooth bead (Daisy products, #631). The samples were homogenized with the bead beater at maximum speed for five min. After brief centrifugation, the samples were incubated either at 50°C for 3 h or overnight at 37°C. The nucleic acids were extracted with 300 µL of phenol chloroform-isoamyl alcohol (25:24:1), followed by a second extraction with 300 µL of chloroform-isoamyl alcohol (24:1). The aqueous phase was ethanol precipitated, and the resulting DNA pellet was dissolved in 20-50 µL of sterile water and stored at -20°C.

A TaqMan-based real time PCR assay was used for the detection of *Ca*. L. asiaticus in psyllids as described by Manjunath at al. (2008). A hexachlorofluorescein (HEX) labeled probe was used in combination with forward and reverse primers to amplify a fragment of the *wingless* gene (GenBank accession number AF231365) from the genome of *D. citri*. This served as an internal control to ensure extraction of good quality insect DNA. Primers and probes used to amplify the HLB associated bacterium, LAS, have been described by Li et al. (2006). Samples with a cycle threshold (ct) value of 30 and below for the bacterial probe were considered as positive for LAS. A ct value of 31-34 was counted as "suspect" sample and a ct value of 35 and above indicated absence of LAS.

Results

Live adult *D. citri* were found in all 7 trailers. In total, 509 psyllids were collected from the 7 trailers. Collections per trailer ranged from 23 to 268 individuals. Live psyllids could be found both on the sides and on the tops of all loads, and up to 30 cm below the surface of the oranges, probably indicating that the insects were distributed throughout the loads. The psyllids were observed to be resting on the fruit itself rather than on the minimal amount of foliage that was present in the loads.

A total of 116 extractions were done with varying numbers of psyllids (2 to 50) per extraction. Four extractions, obtained from 3 different loads, were clearly positive for citrus greening pathogens (ct values of up to 30). Two aliquots of 6 insects, 1 aliquot of 3 insects, and 1 aliquot of 50 insects tested positive for LAS. Another set of 9 extractions were considered suspects, with ct values for the bacterial probe ranging from 31 to 34. LAS-positive psyllids were found in loads from both processing plants (Table 1). One hundred and three extractions were negative (ct values >34).

Discussion

Our results indicate that Asian citrus psyllids can be transported with harvested citrus fruit. Furthermore, some *D. citri* found in loads of oranges tested positive for citrus greening pathogens. One of the LAS-positive loads was traced to a block where citrus greening disease was confirmed later. This block was inspected in Jan 2007, but the trees were asymptomatic at that time. By Aug 2007, however, the grower had found citrus greening in that block. This particular block was among the first in the large grove to manifest symptoms of citrus greening. Symptoms in the block of mature orange trees were discov-

Table 1. Numbers of *Diaphorina citri* Kuwayama collected from trailers of oranges at 2 citrus juice processing plants (A and B) in Hendry County and quantitative realtime PCR test results for citrus greening pathogens; Mar 2006.

Processing Plant—trailer No.[1]	Number of psyllids collected	Number of extractions tested	Number of insects per extraction	Number of extractions positive[2]	Number of extractions suspect[2]	Number of extractions negative[2]
A—1	23	5	4-5	0	0	5
A—2	55	1	50	0	0	1
A—2 continued	(same)	1	5	0	0	1
A—3	31	7	4-5	0	1	6
A—4	41	7	5-6	1	0	6
B—1	47	8	5-6	1	0	7
B—2	268	1	50	1	0	0
B—2 continued	(same)	71	3-5	1	5	65
B—3	44	15	2-3	0	3	12

[1]Seven collections were made from the 2 processing plants, A and B. A—1 through A—4 represent 4 trailers, and B—1 through B—3 represent 3 trailers.

[2]Samples with cycle threshold (CT) values ≤30 were considered positive, and samples with CT values of 31-34 were considered suspect. Samples with CT values >34 were considered negative.

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ered more than 1 year after positive psyllids were found on oranges picked from that block (Manjunath et al. 2008).

The known distributions of Asian citrus psyllid and citrus greening are growing. The psyllid has been found recently in southern parts of California where both quarantine and vector suppression programs are underway (CDFA 2008, 2009). To date, no samples collected from the outdoor environment in California have tested positive for LAS. Texas has 32 counties with sporadic psyllid populations but none with recorded citrus greening. Florida has 32 counties with reported citrus greening in the landscape and several more with finds in retail venues. *Diaphorina citri* is established throughout the Florida peninsula, and there are records from retail venues of *D. citri* and citrus greening in several counties in the Florida panhandle. *Diaphorina citri* also is widespread in the Caribbean. Citrus greening has been reported in Cuba (Albrigo 2008), the Dominican Republic (Garrido Jansen 2008), Belize (Manjunath et al., in press 2010), and Mexico (Anonymous 2009). The status of many other localities in the Caribbean Basin with respect to citrus greening disease is not known.

In order to minimize the accidental introduction of greening into the greening-free states of Arizona, Texas, and California, it is important to consider all possible pathways of entry for the pathogen and the vector. Recently, USDA APHIS (Animal and Plant Health Inspection Service) issued federal orders to restrict movement of citrus and other related plants from Florida (O'Dell & Harless 2008). However, there are other possible sources of introduction for both *D. citri* and citrus greening pathogens. An effective regulatory program designed to protect the US citrus industry against *D. citri* and citrus greening must ensure that all possible means of entry for the pathogens are restricted. There is a considerable amount of citrus fruit imported from other citrus growing areas of the world. The United States imports citrus fruit from Mexico and the Bahamas, both of which have reported the presence of *D. citri* (Halbert & Núñez 2004), and Mexico also has reported citrus greening (Anonymous 2009). Interceptions made by the USDA at the border indicate that *D. citri* has been present in Mexico since 1996 (Halbert & Núñez 2004). Our results and Ken Hibbard's discovery of psyllids on Bahamian fruit arriving in Fort Pierce indicate that there is a risk of importing live *D. citri* on shipments of bulk fruit from outside the United States. Additionally, if citrus greening becomes established widely in Mexico, there is a risk that the pathogen could be transported inadvertently with imported unprocessed fruit.

USDA records indicate that in Fiscal Year (FY) 2007 (the Federal Fiscal Year runs from Oct 1-Sep 30), there were 38 loads of citrus (13,812,970 kg) shipped from the Bahamas to the USA (all destinations). In FY 2008, there were 28 loads (23,852,814 kg). In the past, Bahamian fruit was imported into Fort Pierce, FL unprocessed and infested with psyllids, presumably from the Bahamas (Halbert & Manjunath 2004; Halbert & Núñez 2004). More recently, it has not been possible for DPI to obtain permission to sample this fruit for psyllids because the Department of Homeland Security, rather than the USDA, performs the inspections.

The situation regarding import of fruit from Mexico is much more complex (Table 2). There is no interception record for *D. citri* on fruit from Mexico, but the thoroughness of fruit inspections is not known. For Fiscal Years 2007 and 2008, 140 citrus fruit shipments were rejected for various reasons such as transporting prohibited product, contamination, presence of pests, etc. Eighty-six of the rejected or destroyed shipments were refused because of the use of non-compliant wood packing material. Another 534 shipments were transit shipments received and exported to another country.

TABLE 2. DISPOSITION OF CITRUS FRUIT RECEIVED FROM MEXICO IN FISCAL YEARS 2007 AND 2008.

	FY 2007[1]		FY 2008	
Disposition	Kilograms	Number of shipments	Kilograms	Number of shipments
Rejected or returned	1,192,974	83	929,497	57
Action taken (problem fixed), released	32,218	2	0	0
Regular inspection, release	41,767,409	5,830	28,071,138	4,126
NARP[2], inspected	13,397,325	936	15,348,053	892
NARP, released (no inspection)	310,286,481	16,985	362,945,935	20,015
Shipped to another country (transist only in the USA)	2,345,766	315	1,237,570	219
Total	369,022,173	24,151	408,532,193	25,309

[1]Federal Fiscal Year (FY) runs from Oct 1 through Sep 30.
[2]National Agricultural Release Program.

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A total of 48,786 Mexican citrus fruit shipments remained in the USA. Of these, 9,956 shipments received regular inspections, 2 more were allowed after a paperwork discrepancy was resolved, and 38,828 shipments were admitted under the NARP (National Agricultural Release Program). Of the NARP shipments, 37,000 of them apparently were not inspected. This amounts to about 95% of total shipments and just over 96% of the total by weight.

It is not clear from the data if the Mexican citrus fruit shipments consisted of unprocessed field picked fruit (as was the case with the fruit arriving from the Bahamas in 2001), or whether the fruit was washed, waxed, and ready for sale. Some citrus fruit requires treatment for fruit flies, and other shipments come in refrigerated containers. Both treatments should eliminate most *D. citri*. Probably even minimal processing, such as washing, would eliminate the risk of psyllids on the fruit, but this requires further study.

Regulations are subject to change. The records cited above represent a snapshot in time. A flier created in Mar 2007 by the USDA/APHIS indicates that the rate of inspection for bulk citrus at that time was 10%. In 2008, a memorandum from the USDA requested that all citrus fruit being exported from Mexico to the USA be sent through packinghouse cleaning procedures prior to shipping in order to mitigate risk of disseminating *D. citri*. The new procedure took effect on Feb 1, 2008. Our results underscore the importance of re-evaluating regulations based on new information.

Dead *D. citri* were found on the top surface of some loads that we inspected in the present study. In Florida, fruit trailers must pass through citrus canker decontamination spray loops as they leave the grove where the fruit was picked. It is possible that these decontamination sprays killed some of the insects on the tops of the loads, but live *D. citri* could be found below the surface layer of oranges. Further investigations are needed to determine whether treatments, covering trailers during transport, or other measures, would best prevent movement of *D. citri* with loads of unprocessed citrus fruit.

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