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Research article

Transmission Rates of '*Ca.* Liberibacter asiaticus' by Asian Citrus Psyllid Are Enhanced by the Presence and Developmental Stage of Citrus Flush

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Abstract

Asian citrus psyllid (*Diaphorina citri* Kuwayama) transmits a bacterium '*Candidatus* Liberibacter asiaticus' (CLas) putatively responsible for a devastating citrus disease known as Asiatic huanglongbing (HLB) (citrus greening disease). The psyllid and disease have invaded many citrus-growing regions including the United States, where the disease is seriously jeopardizing the Florida citrus industry. We recently concluded research that showed CLas transmission rates are increased when citrus flush is present. Flush is any new leaf growth ranging in development from first emergence up until the leaves are fully expanded yet still tender. In an experiment with seedlings of a rootstock cultivar 'US-942', a 1-wk infestation of 20 Asian citrus psyllids from an infected colony resulted in 53–60% of seedlings becoming infected when flush was present compared with only 7% when no flush was present. In a second experiment with 'US-942', 77–97% of seedlings became infected when flush was present compared with 40% when no flush was present. A similar experiment with 'Valencia' sweet orange resulted in 23, 80, and 3% seedlings becoming infected when young, older, or no flush was present, respectively. Young plants are therefore more likely to contract HLB if flush is present, with older flush promoting higher infection rates under the conditions of this study. Based on this finding, healthy citrus should be protected from Asian citrus psyllid infestations throughout a flush. To evaluate germplasm for CLas resistance, inoculations using infected Asian citrus psyllid would best be achieved if flush is present.

Key words: *Diaphorina citri*, huanglongbing, citrus greening

The Asian citrus psyllid (*Diaphorina citri* Kuwayama) is an important pest of citrus in North America and other geographic locations because it transmits '*Candidatus* Liberibacter asiaticus' (CLas), a bacterium putatively responsible for a devastating citrus disease known as Asiatic huanglongbing (HLB), also called citrus greening (Bové 2006). The disease causes most commercial citrus genotypes to severely decline in health and productivity (Gottwald 2010). Sweet orange and grapefruit genotypes are particularly susceptible to the disease. There is no cure for HLB. The psyllid and disease have invaded many citrus-growing regions around the world including the United States, where the disease is seriously jeopardizing the Florida citrus industry (Hall et al. 2013a). Aggressive control of Asian citrus psyllid has been advocated as a critical component of a management program for HLB (Bové 2006), and some observations support this contention (Qureshi et al. 2014, Boina and Bloomquist 2015). However, empirical observations in Florida indicate HLB may be slowed but not prevented in groves subjected to intensive insecticide programs, and in a recent study, intensive use of insecticides failed to prevent introduction and spread of HLB in newly planted citrus (Hall et al. 2013b). Solutions to the HLB problem continue to be desperately needed.

Considerable research efforts are currently being made by USDA-ARS citrus breeders and entomologists to develop and identify citrus germplasm with resistance or tolerance to CLas. Although citrus can be manually inoculated by grafting trees with CLas-infected budwood, the USDA-ARS inoculation program uses CLas-infected Asian citrus psyllid because this is what occurs normally and because traits conferring resistance to the vector might contribute to HLB resistance (e.g., see Ammar et al. 2013, 2014). Colonies of Asian citrus psyllid developing on infected citrus were established in greenhouses or controlled environmental chambers, and individuals from these colonies are used to inoculate germplasm. This is accomplished using a two-step inoculation program. First, individual plants are caged for 1–2 wk with 20 Asian citrus psyllids from a

CLas-infected colony. Second, the plants are held for 6 mo in a greenhouse with an open infestation of Asian citrus psyllid developing on CLas-infected source plants.

With respect to the caged inoculation step, the choice of an infestation density of 20 Asian citrus psyllids per seedling was somewhat arbitrary but was hypothesized to be better than using only several Asian citrus psyllid per seedling for two reasons. First, not all individual Asian citrus psyllids within a colony developing on CLas-infected plants acquire the pathogen, and the percentage testing polymerase chain reaction (PCR) positive for CLas has varied over time, averaging around 50% but ranging from as low as 15% to as high as 90% using our procedures (D.G.H., unpublished data). Other researchers have noted that not all adult Asian citrus psyllids from a colony on diseased plants test positive (Lee et al. 2015). It is for this reason that we sometimes use the phrase 'CLas-exposed' Asian citrus psyllid. Second, normally only small percentages (≤12%) of individual Asian citrus psyllids transmit CLas to healthy citrus seedlings, although much higher percentages of these psyllids (up to 90%) may prove CLas positive in PCR tests (Pelz-Stelinski et al. 2010, Ammar et al 2011, Ukuda-Hosokawa et al. 2015).

The principal goal of the USDA-ARS program is to successfully inoculate as many plants as possible. Success rates could be negatively or positively influenced by a number of different factors, including environmental conditions. Another variable of interest is whether the presence of flush on a tree influences the success rate. Citrus trees naturally produce numerous shoot flushes of new leaf growth each year, and young trees may continually produce at least some new growth. In addition to naturally occurring flushes, flush can be promoted by trimming plants or by applying fertilizer. A flush shoot may be defined as any shoot with immature leaves but can range from as small as newly breaking buds of tiny feather leaves to fully elongated shoots with expanded, tender leaves (Hall and Albrigo 2007). The reproductive biology of Asian citrus psyllid is closely tied to the emergence and development of flush shoots, as feather flush leaves are required for oviposition, and relatively young leaves are required for the development of nymphs (Husain and Nath 1927). Plants subjected to our inoculation procedure sometimes have had flush of various stages of development and sometimes no flush at all. The importance of flush in the asymptomatic spread of HLB has been noted (Lee at al. 2015). Because of the close association between Asian citrus psyllid and flush, we thought it possible that transmission rates might be influenced by the presence or absence of flush.

The objective of research presented here was to determine if the presence of flush growth on citrus seedlings of two cultivars influences transmission rates of CLas by Asian citrus psyllid.

Materials and Methods

To assess the influence of flush on transmission rates of CLas by Asian citrus psyllid, individual seedlings with no flush, with young flush, or with older flush were caged with 'CLas-exposed' Asian citrus psyllid for 1 wk as described below, and percentages of seedlings contracting the disease were assessed 6 mo later. Two citrus cultivars were studied, 'US-942' (*Citrus reticulata* L. Blanco × *Poncirus trifoliata* L. Raf.) and 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck]. 'US-942' is CLas-susceptible but HLB-tolerant (Albrecht and Bowman 2012), while 'Valencia' is CLas-susceptible and shows pronounced growth suppression and HLB symptoms following infection. Seeds of the two genotypes were planted and resulting seedlings maintained in a greenhouse dedicated to producing plants in the absence of Asian citrus psyllid and other citrus pests.

Source of Infected Asian Citrus Psyllid

A colony of 'CLas-exposed' Asian citrus psyllid was established during 2007, using adults obtained from a colony of healthy Asian citrus psyllid reared on CLas-free *Citrus macrophylla* Wester. The healthy colony has previously been described (Hall et al. 2015). The adults were transferred into cages (BugDorm-44590F, 47.5L by 47.5W by 93H cm, MegaView Science Education Services Co., Ltd., Taichung, Taiwan) containing potted citrus plants (rough lemon *Citrus jambhiri* Lushington, or citron *Citrus medica* L.) infected by CLas and showing HLB symptoms. These host plants were graft-inoculated using budwood from a potted lemon plant infected by CLas (previously inoculated using budwood from an infected 'Lisbon' lemon tree in a commercial grove in Saint Lucie County, FL, see Albrecht and Bowman 2008). A number of cages containing Asian citrus psyllid on CLas-infected citrus have subsequently been maintained in walk-in chambers (4L by 3W by 2.4H m, cage bottoms on benches 79 cm above the chamber floor); the chambers are set at 25°C, 75% relative humidity (RH), and 14 h daily illumination (400 watt mercury vapor lamps, one lamp 23 cm above each rearing cage). Plants in the cages are trimmed to stimulate new flush growth; ~50 adults are introduced and allowed to feed and oviposit for several days, after which they are removed from the cages. Eggs hatch and nymphs develop to the adult stage, at which point they can be used in experiments. A colony cage is cleaned after each generation of Asian citrus psyllid by washing the plant, trimming it, and reintroducing 'CLas-exposed' Asian citrus psyllid from other cages of infected plants. Alternatively, after a generation, most adults are removed, the plant is trimmed again, and adults remaining in the cage continue to feed and oviposit, producing another generation. The colonies are routinely confirmed by qPCR to be infected by CLas and, because percentages of Asian citrus psyllid testing positive vary over time, Asian citrus psyllid for inoculations are taken from colonies with the highest percentages.

Flush Experiment 1

An experiment was conducted to determine if the presence or absence of flush on a young 'US-942' seedling influences transmission rates of CLas by Asian citrus psyllid. Three treatments were studied: seedlings without any flush; seedlings with young flush shoots (<1 cm in length) with new tiny leaves; and seedlings with older flush (large, soft, nearly or fully expanded leaves). Seedlings were generated by planting seeds on 22 May 2014 in individual plastic cells (3.8 cm dia. by 21 cm; SC-10 super cell Cone-tainers, Stuewe and Sons, Tangent, OR) containing steamed potting mix (Pro-Mix BX, Premier Horticulture, Inc., Quakertown, PA). After planting, the Cone-tainers were watered on an as-needed basis (when the soil started to appear dry) and fertilized weekly with a general purpose 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, OH) water-soluble fertilizer mix.

On 16 September, a large group of the young seedlings was trimmed to stimulate flush. This was accomplished by clipping ~2.5 cm off the top of each seedling. A second group was trimmed on 25 September, and a third group was trimmed on 8 October. The experiment was started on 8 October using seedlings trimmed on 8 October for the no-flush treatment, seedlings trimmed on 25 September for the young-flush treatment, and seedlings trimmed on 16 September for older-flush treatment. In addition to older flush, some of the seedlings trimmed on 16 September had new young

flush shoots, which we removed. Three groups (replications) of 10 seedlings for each treatment were selected and transferred from the greenhouse to a laboratory bench under ambient conditions (23°C, 53% RH) and 14 h daily illumination provided by light-emitting diode lamps (EnduralLED #17E26PAR38-E1, PAR38 22°/17W/white/3000K, Philips Lighting Co., Somerset, NJ). Twenty 'CLas-exposed' adults (from a colony of Asian citrus psyllid on infected citron, 63% Asian citrus psyllid positive for CLas based on a CT threshold of 36, which is discussed below) were aspirated into a small glass vial; this vial was placed onto the soil beside a seedling; the vial's lid was removed; and a plastic, ventilated cylinder (37 mm dia. by 305 mm; Richardson and Hall 2013) was quickly placed over the seedling to confine the psyllids as they escaped the vial. The open bottom end of each cylindrical cage was pressed down into the Cone-tainer to secure the cage. Screen was glued to the open top end of each cage to prevent Asian citrus psyllid from escaping. The seedlings were arranged in a randomized complete block design in Cone-tainer racks. The psyllids on each seedling were removed 7 d later, stored at −80°C, and later processed for DNA extraction and quantitative real-time polymerase chain reaction (qPCR). Immediately after Asian citrus psyllids were removed, the seedlings were treated with carbaryl (2.6 ml per liter of water, Sevin XLR Plus, Bayer CropScience LP, Research Triangle Park, NC) in a fume hood and left in the hood until the next day, when they were moved to an environmental chamber (25°C, 75% RH, and 14 h daily illumination). The seedlings were treated a second time with carbaryl in the fume hood 5 d later, and the next day they were moved to a greenhouse where they were maintained for 6 mo. Because some oviposition occurred on seedlings with young flush, the two carbaryl treatments were applied to eliminate young nymphs. The seedlings were periodically treated in the greenhouse with foliar applications of pesticides for general pest control (mites, whiteflies, and thrips) using abamectin (1.3 ml/liter, Epimek 0.15EC, Syngenta Crop Protection, LLC, Greensboro, NC) and oil (4 ml/liter, Omni Supreme Spray, Helena Chemical Company, Collierville, TN) on 9 November 2014, cyflumetofen (1.1 ml/liter, Nealta, BASF, Research Triangle Park, NC) on 12 January and 7 May 2015, and carbaryl (2.6 ml/liter) on 6 April 2015. Leaf samples (six to eight mature leaves) were taken from each seedling at 1 and 6 mo after the Asian citrus psyllid infestation ended; petioles and parts of the midrib were severed and then stored at −20°C until extraction of DNA.

Flush Experiment 2

This experiment evaluated the same three flush treatments as the first experiment and followed the same basic procedures. Two genotypes were studied, 'US-942' (HLB-tolerant) and 'Valencia' sweet orange (HLB-sensitive). Seeds of the two genotypes were planted on 11 September 2014. Seedlings of each genotype were trimmed on 12 January (for the older-flush treatment), 23 January (for the young-flush treatment), and 3 February (for the no-flush treatment). Asian citrus psyllid from infected colonies (five different colonies on a mix of lemon and citron plants, an average of 69% Asian citrus psyllid positive for CLas based on a CT threshold of 36, discussed below) were introduced onto the seedlings on 3 February. The infestations were terminated on 10 February, all live psyllids from each seedling were stored for qPCR, the seedlings were treated with carbaryl (2.6 ml/liter) twice as in the first experiment, and then they were moved to the greenhouse for 6 mo. The seedlings were periodically treated in the greenhouse with foliar applications of pesticides for general pest control using carbaryl (2.6 ml/liter) on 6 April 2015, cyflumetofen (1.1 ml/liter) on 7 May 2015, and carbaryl (2.6 ml/liter) and oil (26 ml/liter) on 9 June, 1 July, and 5 August 2015. After 6 mo in the greenhouse, the seedlings were rated for HLB symptoms (1 = symptomless, 2 = moderate symptoms, 3 = severe symptoms). Mature leaves (six to eight per seedling) were collected, and petioles and parts of the midrib were severed and saved for qPCR assays. The above-ground portion of each seedling was weighed using a digital scale. Fibrous roots not exceeding 2 mm in diameter were extracted from the potting mix, washed with tap water, and blotted dry. Leaf and root tissue were stored at −20°C until extraction of DNA.

Statistical Analyses

Analyses of variance on parametric data were conducted using PROC ANOVA, PROC GLM, or PROC TTEST (SAS Institute 2010). Mean comparisons among treatments were investigated using Tukey's HSD test. Percentage data were arcsine-transformed (Gomez and Gomez 1984). Mean disease ratings were computed and subjected to a generalized linear mixed model using PROC GLIMMIX (SAS Institute 2010) and compared by least squares means (LSMEANS option in SAS). Correlation analyses (Pearson's coefficient) were conducted between plant weight and CLas titer (the latter based on CT values from qPCR) using PROC CORR (SAS Institute 2010). All statistical tests were conducted at the 0.05 level of significance.

Quantitative Real-Time Polymerase Chain Reaction

The HLBaspr primer/probe set (Li et al. 2006) was used to determine the presence and relative titer of CLas in psyllid and plant samples. For psyllid samples from each of the two experiments, each Asian citrus psyllid was processed individually, and each DNA sample was tested for CLas as described below. For leaf and root samples, DNA was extracted from each sample and tested for CLas as described below. Psyllid and plant samples were considered CLas positive when the CT value was <36. CT values reflect titers of the pathogen, with low CT values associated with high titers and high CT values associated with low titers.

For qPCR of psyllid samples, all sample processing and DNA extraction took place in a laminar flow hood. DNA was extracted from psyllids using a crude extraction method derived from De Barro and Driver (1997). Each psyllid was placed in a 1.2-ml tube (eight-tube strips, Macherey Nagel, Düren, Germany) containing 150 µl of lysis buffer (5% 1M KCl, 5% 1M Tris at pH 8.4, 0.45% Tween20, 0.45% NP40, and 89.1% autoclaved DI water) and three 2.3-mm chrome steel beads (#11079123, BioSpec Products, Inc., Bartlesville, OK). Tubes were placed in a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ) and homogenized at 1,600 rpm for 4 min. Samples were centrifuged at 6,000 *g* for 1 min and 100 µl of supernatant was transferred to a clean 200 µl PCR tube (eight-tube strips). Samples were heated at 95°C for 5 min, cooled to 4°C, briefly centrifuged, and stored at −20°C until subjected to PCR analysis. In each qPCR reaction, 2 µl of crude psyllid extract was used. Extracted DNA was assayed for the presence of CLas using the HLBaspr probe/primer set as described above. PCR reactions were conducted in a 20-µl reaction volume using Taqman Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) containing 0.4 mM each of forward and reverse primer and 500 nM of probe. The temperature program for the HLBaspr primers was 95°C for 5 min, followed by 50 cycles of 95°C for 3 s, followed by 60°C for 30 s. All qPCR reactions were run on an ABI 7500 real-time PCR system and analyzed with 7500 Software v2.0.1 (Applied Biosystems).

Table 1. Transmission of CLas by Asian citrus psyllid to citrus seedlings ('US-942') with young flush, older flush, or no flush, plants infested for 1 wk by 20 adults from a colony of 'CLas-exposed' Asian citrus psyllid

Flush treatment	Percentage Asian citrus psyllid infected per plant	Mean CT value among infected Asian citrus psyllid	1 mo after infestations		6 mo after infestations	
			Percentage plants infected	Mean CT value of infected plants	Percentage plants infected	Mean CT value of infected plants
Young	48.5a	30.6a	0.0a	–	53.3a	23.9a
Older	55.9a	30.5a	16.7a	32.0a	60.0a	23.7a
None	48.1a	31.2a	3.3a	35.1a	6.7b	24.1a
F	1.9	1.2	1.6	1.3[a]	41.0	3.2
df	2, 4	2, 4	2, 4	14	2, 4	2, 3
P	0.26	0.38	0.30	0.22	0.002	0.18

Plants were infested during October 2014. A CT threshold of <36 was used as the indicator of whether a psyllid or plant was infected.

Means in the same column followed by the same letter are not significantly different ($P = 0.05$), Tukey's HSD test for all variables except CT value of plants after 1 mo, which was based on a *t*-test.

[a] *t* value.

For qPCR of plant samples, leaf and root tissues were ground in liquid nitrogen with a mortar and pestle and 100 mg of ground tissue was used for DNA extraction. DNA was extracted using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, yielding 20–30 ng/µl DNA per extraction. For detection of CLas, real-time PCR assays were performed using the HLBaspr probe/primer set as described above. For normalization, all samples were assayed using primers COXf and COXr and probe COXp (Li et al. 2006). Amplifications were performed over 40 cycles using an ABI 7500 real-time PCR system (Applied Biosystems) and the QuantiTect Probe PCR Kit (Qiagen) according to the manufacturer's instructions. All reactions were carried out in a 20-µl reaction volume using 5 µl of extracted DNA.

Results and Discussion

In the first flush experiment, after the 1-wk inoculation period, an average of 51% of the Asian citrus psyllid from each seedling tested positive for CLas. There were no significant differences among flush treatments with respect to the percentage of positive Asian citrus psyllid per seedling, and there were no significant differences among the flush treatments with respect to CT values associated with infected psyllids (Table 1). Few seedlings tested CLas positive 1 mo after infestation, and there were no significant differences among flush treatments with respect to the percentage of seedlings testing positive or CT value associated with infected seedlings (Table 1). After 6 mo, substantial percentages of seedlings tested positive but only for seedlings which had flush during the Asian citrus psyllid infestation. Among seedlings that had no flush during the infestations, only 6.7% plants tested positive after 6 mo. Regardless of the percentage of seedlings that contracted the pathogen, plants testing positive after 6 mo had high titers of CLas, and there were no significant differences in CT values among the three flush treatments. The results of this experiment indicated that a 1-wk infestation of 20 'CLas-exposed' Asian citrus psyllid per 'US-942' seedling resulted in a plant becoming infected 53–60% of the time, provided at least some flush was present during the infestation. This is based on infestations where around 10 Asian citrus psyllid tested CLas positive, with CT values in the range of 30–31.

In the second flush experiment over both citrus cultivars, an average of 69% of the psyllids from each seedling tested positive for CLas, and CT values indicated titers of the pathogen were relatively high in these psyllids (Table 2). There were no significant differences among flush treatments with respect to percentages of psyllids testing positive for CLas. For 'US-942', there were no significant differences among flush treatments in CLas CT values associated with infected Asian citrus psyllid, but for 'Valencia' there were significant differences with Asian citrus psyllid from seedlings with older flush having significantly higher CT values (=lower CLas titers) than Asian citrus psyllid from seedlings without flush. This difference was biologically insignificant with respect to the results of the experiment. Six months after Asian citrus psyllid were removed from seedlings, qPCR assays on leaf samples indicated that greater percentages of seedlings became infected when flush was present and especially when older flush was present (Table 2). These results were reflected by the results of qPCR assays on roots. However, in both cultivars, CT values were higher for roots compared with leaves, especially in 'US-942' where CT values differed by seven- to eightfold, which may be associated with the tolerance to HLB observed for this rootstock cultivar (Albrecht and Bowman 2012). Higher percentages of 'US-942' seedlings generally contracted the pathogen than 'Valencia' seedlings, particularly under the no-flush and young-flush treatments, but CT values indicated that CLas titers were generally higher in the 'Valencia' seedlings, especially in root tissue. Based on a standard curve developed in our laboratory (Albrecht and Bowman 2012), mean CLas titers calculated from CT values were 8 and 1,086 CLas genomes per mg root tissue for 'US-942' and 'Valencia', respectively, from older flush inoculations. Plant weights and disease ratings indicated that 'US-942' was more tolerant of CLas than 'Valencia'. There were no significant differences among flush treatments in either plant weights or disease ratings for 'US-942', but there were for 'Valencia' where disease severity was most pronounced under the older-flush treatment. Significant correlations were found between plant weight and CT values associated with leaves for 'US-942' ($r = 0.47$, $P \leq 0.0001$, $N = 90$) and especially 'Valencia' ($r = 0.78$, $P \leq 0.0001$, $N = 90$). These results showed that HLB severity increases as CLas titer increases.

The highest level of inoculation success among 'US-942' seedlings occurred during the second flush experiment under the older flush treatment, where 97% of seedlings contracted the pathogen based on leaf samples—the Asian citrus psyllid infestation responsible consisted of a mean per seedling of about 12 CLas-infected adults, with an average CT value of 28.6. The Asian citrus psyllid infestation during the first experiment consisted of fewer infected Asian citrus psyllid with lower CLas titers than the infestation during the second experiment, which may help explain why greater

Table 2. Transmission of CLas by Asian citrus psyllid to citrus seedlings ('US-942' or 'Valencia') with young flush, older flush, or no flush, plants infested for 1 wk by 20 adults from a colony of 'CLas-exposed' Asian citrus psyllid

Flush	Asian citrus psyllid infestation		Leaf samples		Root samples		Mean plant weight (g)	Disease rating
	Percent Asian citrus psyllid infected per plant	Mean CT value among infected Asian citrus psyllid	Percent plants infected	Mean CT value of infected plants	Percent plants infected	Mean CT value of infected plants		
'US-942'								
Young	70.9a	27.9a	76.7b	24.6a	80.0a	32.8a	13.4a	1.1a
Older	63.5a	28.6a	96.7a	24.7a	86.7a	32.0a	13.0a	1.0a
None	73.7a	28.1a	40.0c	24.9a	36.7b	32.5a	18.5a	1.0a
F	2.0	0.8	55.6	0.3	17.4	1.0	6.9	0.1
df	2, 4	2, 4	2, 4	2, 4	2, 4	2, 4	2, 4	2, 4
P	0.25	0.52	0.001	0.75	0.01	0.42	0.05	0.89
'Valencia'								
Young	73.0a	27.1ab	23.3b	22.0a	16.7b	25.6a	18.6b	1.5b
Older	66.3a	27.9a	80.0a	22.3a	76.7a	25.4a	10.2c	2.5a
None	68.7a	26.2b	3.3c	22.7a	6.7b	28.6a	24.8a	1.1b
F	0.8	10.7	68.2	0.7	28.6	12.0	25.1	19.6
df	2, 4	2, 4	2, 4	2, 2	2, 4	2, 1	2, 4	2, 4
P	0.52	0.02	0.001	0.60	0.004	0.2	0.006	0.009

Plants were infested during February 2015; 6 mo later plants were rated for damage and analyzed for CLas. A CT threshold of <36 was used as the indicator of whether a psyllid or plant was infected according to leaf and root samples.

For each cultivar, means in the same column followed by the same letter are not significantly different ($P = 0.05$), Tukey's HSD test for all variables except 'disease rating,' which was based on least squares means.

percentages of 'US-942' seedlings contracted CLas during the second experiment. Similar to 'US-942', transmission rates to 'Valencia' were also highest under the older flush treatment, where 80% of seedlings developed the disease following Asian citrus psyllid infestations averaging about 12 infected adults, with an average CT value of 27.9.

One important conclusion from this research is that young plants are more likely to contract HLB if flush is present, with older flush promoting higher infection rates than young flush within the first 6 mo after inoculation. The presence of flush would therefore be expected to accelerate disease development in a grove. Based on this finding, healthy citrus should be protected from Asian citrus psyllid infestations throughout the emergence and development of a flush. For the USDA-ARS inoculation program under which high transmission rates are desirable, the results of this research showed that seedlings should be trimmed to stimulate flush before caging them with infected psyllids, and some growth of this flush should be allowed to occur before infesting the seedlings.

Citrus with flush may be more susceptible to inoculation of CLas by Asian citrus psyllid than seedlings without flush as a result of morphological or chemical differences between mature and immature leaves. It has been speculated that the thick-walled 'fibrous ring' (sclerenchyma) around the phloem may act as a barrier to Asian citrus psyllid stylet penetration into the phloem, and this ring is more prominent in mature leaves (Ammar et al. 2013). Using electrical penetration graph (EPG) assays, Luo et al. (2015) showed that some aspects of Asian citrus psyllid feeding activity were significantly influenced by leaf age. Serikawa et al. (2012) reported that, based on EPG, Asian citrus psyllid feeding on young leaves probed more often and for longer durations than Asian citrus psyllid feeding on mature leaves. Differences have been noted in feeding sites chosen by Asian citrus psyllid on mature and immature leaves, with adults more likely to feed on adaxial small veins and abaxial mid veins in mature leaves and on abaxial small veins in immature leaves (Ammar et al. 2014). In addition to morphological differences, there may be chemical composition differences between mature and immature leaves that affect CLas transmission rates. For example, some leaf flavonoids at certain concentrations have been shown to act as insect phagostimulants while at different concentrations can inhibit feeding (Simmonds 2001)—perhaps there are flavonoid differences between mature and immature citrus leaves. There may also be leaf volatiles that affect Asian citrus psyllid feeding activities. Citrus flush has been reported to have an array of volatiles that attract adult Asian citrus psyllid (Patt and Sétamou 2010), and some of these might stimulate Asian citrus psyllid feeding and thus increase the likelihood of CLas transmission.

The research also supported that some genotypes such as 'US-942' may be susceptible to CLas infection but at the same time more tolerant to HLB disease than other CLas-susceptible genotypes such as 'Valencia'. The observation of much lower bacterial titers in the roots of infected seedlings of HLB-tolerant 'US-942' suggests that there may be some association between reduced titer and tolerance that is deserving of further investigation.

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