Transmission Efficiency of *Candidatus Liberibacter asiaticus* and Progression of Huanglongbing Disease in Graft- and Psyllid-inoculated Citrus

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Abstract. *Candidatus Liberibacter asiaticus* (Las) is a phloem-limited bacterium associated with huanglongbing (HLB), one of the most destructive diseases of citrus in Florida and other citrus-producing countries. Natural transmission of Las occurs by the psyllid vector *Diaphorina citri*, but transmission can also occur through grafting with diseased budwood. As a result of the difficulty of maintaining Las in culture, screening of citrus germplasm for HLB resistance often relies on graft inoculation as the mode of pathogen transmission. This study evaluates transmission efficiencies and HLB progression in graft-inoculated and psyllid-inoculated citrus under greenhouse and natural conditions in the field. Frequencies of transmission in graft-inoculated greenhouse-grown plants varied between experiments and were as high as 90% in susceptible sweet orange plants 6 to 12 months after inoculation. Transmission frequency in a tolerant *Citrus × Poncirus* genotype (US-802) was 31% to 75%. In contrast, transmission of Las after controlled psyllid inoculation did not exceed 38% in any of four experiments in this study. Whereas the time from inoculation to detection of Las by polymerase chain reaction (PCR) was faster in psyllid-inoculated US-802 plants compared with graft-inoculated US-802 plants, it was similar in graft- and psyllid-inoculated sweet orange plants. HLB symptom expression was indistinguishable in graft- and psyllid-inoculated plants but was not always associated with the number of bacteria in affected leaves. The highest number of Las genomes per gram leaf tissue measured in sweet orange plants was one to four $\times 10^7$ in graft-inoculated plants and one to two $\times 10^7$ in psyllid-inoculated plants. Highest numbers measured in tolerant US-802 plants were one to three $\times 10^6$ and two to six $\times 10^6$, respectively. Compared with artificial inoculation in a greenhouse setting, natural inoculation of field-grown sweet orange trees occurred at a much slower pace, requiring more than 1 year for infection incidence to reach 50% and a minimum of 3 years to reach 100%.

Candidatus Las, a phloem-limited Gram-negative bacterium (Garnier et al., 1984) is the organism associated with citrus HLB in Florida and most other citrus-producing countries around the world (Bové, 2006; Gottwald, 2010). HLB, which is also known as citrus greening or yellow shoot disease, is considered the most destructive disease of citrus at present and threatens citrus production in all affected areas. Fruit on HLB

affected trees remain small, often become misshapen, may develop an undesirable taste, and drop prematurely. Losses to juice orange production in Florida from HLB during production seasons 2006–07 through 2010–11 were estimated to be above 20% (Hodges and Spreen, 2012). Another effect of HLB is the appearance of yellow shoots in the canopy of diseased trees, which are the result of an asymmetric blotchy mottling of leaves or severe chlorosis, often resembling zinc or other nutritional deficiencies (McClean and Schwarz, 1970). At advanced stages of the disease, twig dieback occurs and trees decline. Transmission of Las occurs through the Asian citrus psyllid *Diaphorina citri* (Halbert, 2005; Halbert and Manjunath, 2004), which feeds on plant phloem. Other modes of transmission are through dodder (*Cuscuta* sp.) or grafting with infected budwood (Halbert and Manjunath, 2004). Two other bacterial pathogens, *Ca. L. africanus* (Jagoueix et al., 1994) and *Ca. L. americanus* (Teixeira et al., 2005), are known to cause HLB but are geographically more restricted.

HLB affects all known *Citrus* species and *Citrus* relatives, and most commercial cultivars

are very susceptible (Folimonova et al., 2009; McClean and Schwarz, 1970; Miyakawa, 1980). Because no known cure exists, HLB management strategies consist of insect control, removal of infected trees, enhanced nutritional applications, and the establishment of pathogen-free nursery systems (Hall and Gottwald, 2011). The exact mechanism of disease development is not known, but histological studies identified phloem collapse and blockage of the translocation stream as a probable cause of tree decline (Achor et al., 2010; Schneider, 1968). Other studies described major changes in different metabolic pathways, particularly carbohydrate and phytohormone metabolism, associated with Las infection (Albrecht and Bowman, 2008, 2012a; Fan et al., 2010, 2011; Kim et al., 2009; Rosales and Burns, 2011).

The U.S. Department of Agriculture (USDA) citrus breeding program is one of the largest of its kind and dates back to 1893. In addition to traditional breeding, recent efforts of the program have been directed at the development of transgenic citrus with resistance to diseases, particularly HLB. Evaluation of disease resistance requires the screening of large numbers of plants with efficient and reliable methods. Because attempts to culture *Ca. Liberibacter* spp. have had only limited success thus far (Davis et al., 2008; Sechler et al., 2009), studies designed to screen for HLB resistance often rely on graft inoculation for Las transmission (Albrecht and Bowman, 2011, 2012b; Coletta-Filho et al., 2010; Folimonova et al., 2009; Lopes and Frare, 2008; Shokrollah et al., 2009). In *Ca. Liberibacter* graft inoculation studies, buds, bark pieces, bud sticks, or other tissue types from HLB-affected source plants are used to transmit the pathogen into the phloem of the stem of a healthy plant. Transmission efficiencies are usually high, but this can depend on the type and the amount of tissue used for grafting, the pathogen species, and the time of the year at which inoculations are conducted (Lopes et al., 2009; Lopes and Frare, 2008; McClean, 1970; Schwarz, 1970). In contrast to graft inoculation, transmission of Las through psyllid vector is natural but is experimentally more demanding, and transmission efficiencies largely depend on the numbers of insects used and the life stage at which the psyllid acquired the pathogen (Inoue et al., 2009; Pelz-Stelinski et al., 2010).

The objective of this study was to compare the efficiency of Las transmission and HLB disease progress using graft inoculation methods that are routinely used in our laboratory (Albrecht and Bowman, 2008, 2011, 2012a, 2012b) with transmission efficiencies and disease progress after psyllid inoculation under artificial and natural conditions. Six greenhouse experiments were conducted, which included sweet orange (*Citrus sinensis*) plants and *Citrus × Poncirus trifoliata* plants, of which the latter were generated in vitro through methods used for production of transgenic citrus in our laboratory. In addition, the natural spread of Las was studied in field-grown sweet orange trees in the years

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after the first discovery of HLB in Florida. Knowledge of transmission efficiencies and disease progress rate is essential for the screening of citrus cultivars developed in breeding programs and to study of the effects of HLB using biochemical or molecular biological methods.

Materials and Methods

Plant material

Naturally inoculated field-grown trees.

Rootstock liners were budded with certified disease-free 'Hamlin' sweet orange budwood from the Bureau of Citrus Budwood Registration (Florida Department of Agriculture & Consumer Services, Division of Plant Industry) at 6 to 8 months of age by the inverted T budding method. Rootstocks consisted of 53 different varieties and included 'Cleopatra' mandarin (*C. reticulata*), sour orange (*C. aurantium*), 'Carrizo' citrange (*C. sinensis* × *P. trifoliata*), and 'Swingle' citrumelo (*C. paradisi* × *P. trifoliata*) in addition to new hybrids of mandarin, pummelo (*C. grandis*), and sour orange with *P. trifoliata*. Grafted plants were maintained in the U.S. Horticultural Research Laboratory (USHRL) greenhouses for 8 to 12 months until suitable for field planting. Trees were planted at the USHRL-USDA Farm in St. Lucie County, FL, in June 2005 (Rows 33 and 34) and in Aug. 2007 (Rows 35 and 36). Trees were arranged in rows of 41 to 43 trees at a spacing of 3 m × 8 m in a north to south direction with the north end of the trial adjacent to an area of natural vegetation, primarily native pine. A total of 170 trees were included in the experiment. Trees were irrigated by microsprinklers three times a week in the absence of adequate rainfall. Imidacloprid (Agri Star Macho 2.0 FL; Albaugh Inc., IA) was applied by fertigation in April of every year at a rate of 20 oz/acre (140 g/1000 m²). Additional pesticides such as carbaryl, oil, and copper were applied in spring, summer, and fall and as needed. Fertilizer (9N–2P–9K) was applied monthly by fertigation from March to November of each year at a rate of 70 lb nitrogen (N)/acre (7.8 kg N/1000 m²) annually. On 3-year-old trees liquid fertilizer was supplemented with a dry mix fertilizer (8N–4P–8K) at a rate of 28 lb N/acre/year (3.1 kg N/1000 m²/year).

Graft- and psyllid-inoculated greenhouse-grown plants. Forty 8- to 9-month-old 'Valencia' sweet orange scion T-grafted onto 'Cleopatra' mandarin were used for Expts. 1 and 2. Fifty-six 1- to 2-year-old US-802 ('Siamese' pummelo × *P. trifoliata*) scion, generated from etiolated internode stem segments using standard in vitro plant regeneration procedures (Edriss and Burger, 1984; Zou et al., 2008) and micrografted onto decapitated epicotyls of seed-grown 3- to 5-week-old 'Volkamer' lemon (*C. volkameriana*) or 'Kinkoji' (*C. obovoidea*) rootstocks by wedge grafting, were used for Expts. 3 and 4. Fifty-six 13-month-old 'Valencia' seedlings and fifty-six 15-month-old 'Ridge Pineapple' (*C. sinensis*) seedlings were used for Expts. 5 and 6, respectively. Plants were grown in a mix of peat/perlite/vermiculite

(Pro-Mix BX; Premier Horticulture Inc., Red Hill, PA) in 20 × 32-cm plastic treepots (Stuewe & Sons, Tangent, OR). After inoculations, plants were arranged randomly on the greenhouse benches and kept under natural light conditions at a temperature of 21 to 28 °C. Plants were irrigated as needed and fertilized every 3 weeks using a water-soluble fertilizer mix, 20N–10P–20K (Peters Professional; The Scotts Company, Marysville, OH). Insecticides to control for mites or other greenhouse-born insects were applied as needed.

Inoculation with *Ca. Liberibacter asiaticus*

Natural psyllid inoculation. Field plants were inoculated by psyllid (*D. citri*) populations naturally established at the Picos Farm. The farm has been affected by HLB since the discovery of the disease in Florida in 2005. Studies conducted on citrus trees adjacent to the experimental plants identified large populations of psyllids in late Spring/early Summer of 2005 and 2006 (Hall et al., 2008) and 2007 (Hall and Hentz, 2011).

Artificial graft inoculation. For Expts. 1 and 2, 30 plants were graft-inoculated by grafting two bud and two leaf pieces from infected greenhouse-grown 'Valencia' plants, PCR-positive for Las and symptomatic for HLB, onto each plant. Ten plants were mock-inoculated with disease-free tissue pieces to be used as non-infected controls. Inoculations were conducted in June 2009 (Expt. 1) or in Nov. 2009 (Expt. 2). Plants were pruned at the time of inoculation.

Direct comparison of artificial graft and psyllid inoculation. For Expts. 3 to 6, 16 plants were graft-inoculated by grafting two bud and two leaf pieces (Expts. 3 and 4) or three bark or bud pieces (Expts. 5 and 6) from infected greenhouse-grown 'Valencia' plants, PCR-positive for Las and symptomatic for HLB, onto each plant. Eight plants were mock-inoculated with disease-free tissue pieces to be used as non-infected graft controls. Sixteen plants were inoculated with 15 to 20 psyllids collected from the Picos Farm (Expts. 3 and 4) or, in the absence of adequate psyllid populations at the farm, from a laboratory colony of infected psyllids reared on greenhouse-grown Las-positive citrus plants (Expts. 5 or 6). Eight plants were mock-inoculated with healthy psyllids from a laboratory colony reared on healthy Las-free citrus plants to be used as non-infected psyllid controls. Eight plants received neither grafts nor psyllids to be used as additional controls. Each plant was covered with a clear 60 cm × 15-cm plastic tube. Openings on the side and on the top of each tube were sealed with a mesh screen to allow for air circulation. To allow placing of the plastic tubes, plants were pruned before inoculation leaving green stems and mature leaves to be fed on by psyllids. Previous observations in our laboratory and other studies (Hall et al., 2012) showed that adult psyllids feed on young stems and on leaves of all stages of development.

Psyllids were removed from plants after 1 week and used for DNA extraction and PCR analysis of Las. If the percentage of psyllids with detectable Las levels was less than 30% on average, five to 10 more psyllids were added to each plant for 1 additional week. Cages were removed from plants after psyllid removal and all plants (including plants that had not received any psyllids) were sprayed with Malathion 5EC (Micro Flo Company LLC, Memphis, TN) at a rate of 0.25 mL·L⁻¹ and soil drenched with Admire Pro (Bayer CropScience LP, NC) at a rate of 2.5 mL·L⁻¹. Inoculations were conducted in Oct. 2009 (Expt. 3), Dec. 2009 (Expt. 4), Nov. 2010 (Expt. 5), or Jan. 2011 (Expt. 6). Plants were pruned again at 8 months after inoculation to promote new leaf growth and spread of Las throughout the plant.

Detection of *Ca. L. asiaticus*

Leaves. Six to eight leaves were collected from each field tree every 2 months from Aug. 2007 to June 2010. Trees identified to be infected with Las on three consecutive time points were not analyzed any further. Four to six leaves were collected from each greenhouse-grown plant at 6, 11, and 16 weeks after inoculation (Expt. 1); at 6, 11, 16, 21, and 26 weeks after inoculation (Expt. 2); and at 2, 4, 6, 8, and 12 months after inoculation (Expts. 3 to 6). Only leaves that emerged after the pruning and had not been in contact with psyllids were included in this collection. Plants from Expt. 5 were additionally analyzed at 16 months after inoculation. Petioles and midribs were ground in liquid N 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 to 30 ng DNA per extraction. For the detection of Las in field trees (Aug. 2007 to July 2009), PCRs were performed with the isolated DNA as described in Albrecht and Bowman (2009). For the detection of Las in field trees (Sept. 2009 to June 2010) and in greenhouse-grown plants, real-time PCR assays were performed using primers HLBas and HLBp and probe HLBp developed by Li et al. (2006). 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, Foster City, CA) 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 DNA. Plants were considered PCR-positive when normalized Ct_{Las} values were 32 or less.

Roots. Root tissue was collected from selected 'Valencia' seedlings of Expt. 5 at 16 months after inoculation. Plants were extracted from the potting media and 2 to 3 g of roots, not exceeding 2 mm in diameter, were severed from each plant and rinsed with

tap water. DNA extraction and PCR detection of Las were conducted as described for leaves.

Psyllids. Individual psyllids collected from Expts. 3 to 6 were homogenized in 150 μ L lysis buffer containing 0.1 M Tris-KCl (pH 8.4), 1% Tween 20, and 1% Nonidet P40 with a 2010 Geno/Grinder (SPEX Sample Prep, Metuchen, NJ) for 2 min. DNA was extracted from 100 μ L homogenate at 95 $^{\circ}$ C for 5 min. After extraction, samples were placed on ice for 10 min and centrifuged at 14,000 g for 2 min. For detection of Las, real-time PCR assays were performed using primers HLBas and HLBbr and probe HLBp (Li et al., 2006). Amplifications were performed over 40 cycles using an ABI 7500 Fast real-time PCR system and the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. All reactions were carried out in a 20- μ L reaction volume using 2 μ L DNA.

Las quantification. For quantification of Las in leaves and roots, Ct_{Las} values were converted to copy numbers of Las genomes based on a standard curve created in our laboratory: $y = 12.34 - 0.32x$, where $y = \log$ of Las copy number and $x = \text{normalized } Ct_{Las}$ value. Ct values not determined after 40 cycles were assigned a value of 41. Copy numbers were divided by three to adjust for the three 16s rDNA gene copies present in the Las genome (Duan et al., 2009) and data were expressed as numbers of Las genomes per gram of plant tissue.

Foliar disease symptoms and growth performance

Plants were examined for chlorosis, blotchy mottle, or other foliar abnormalities presumably

associated with HLB at the time of leaf collection. Stem diameters of plants were measured at 8 to 10 cm above the graft union (Expts. 1 and 2), at 2 cm above the graft union (Expts. 3 and 4), or at 4 cm above soil level (Expts. 5 and 6) using a digital caliper. Growth was expressed as percent increase of mean stem diameters from the beginning to the end of the study. For Expts. 5 and 6, biomass removed during pruning was recorded after the pruning at 8 months after inoculation. Foliar disease symptoms and growth of plants from Expt. 5 were additionally evaluated 16 months after inoculation.

Results

Natural psyllid inoculation

At the time of the first sample collection in Aug. 2007, PCR analyses detected Las in 5% and in 28% of trees in Rows 33 and 34 (planted in June 2005), respectively (Fig. 1). The percentage of Las-positive trees increased to 46% and 79% by Apr. 2008, and by Nov. 2008, all trees in Rows 33 and 34 tested PCR-positive for Las. Trees in Rows 35 and 36 (planted in Aug. 2007) did not become Las-positive until Apr. 2008, when 2% and 12% of plants were detected to be infected. By Sept. 2008, 51% and 58% of trees tested positive for the pathogen. During the time from Sept. 2008 to July 2009, the percentage of trees positive for Las did not change considerably, but in Sept. 2009 increased to more than 80%. By June 2010, more than 99% of all trees in Rows 35 and 36 were identified as infected.

Foliar HLB symptoms such as severe chlorosis, often associated with leaf size reduction, and blotchy mottle were detected in all PCR-positive plants, although detection of Las by PCR often preceded manifestation

of HLB symptoms (Table 1). This was particularly evident during the earlier stages of the study from Aug. 2007 to Dec. 2007 (Rows 33 to 34) and from Apr. 2008 to July 2008 (Rows 35 to 36) when the number of trees in which Las was detected by PCR was more than double or triple the number of trees displaying foliar HLB symptoms. Dieback of shoots was observed at the later stages of infection. Trees located in the southern part of each row became infected earlier than trees located in the northern part of each row.

Artificial graft inoculation

In the following, the term "Las-inoculated" is used to define all plants that were inoculated by either graft or psyllid independent of whether the pathogen was detected by PCR.

The percentage of graft-inoculated 'Valencia' plants from Expt. 1 testing positive for Las ranged from 47% at 6 weeks after inoculation (WAI) to 80% at 11 and 16 WAI (Table 2). Although no foliar HLB symptoms were visible at 6 WAI, chlorosis and blotchy mottle developed at 11 WAI in most Las-positive plants and increased in severity by the end of the experiment. The percentage of 'Valencia' plants from Expt. 2 in which Las was detected was 17% at 6 WAI and increased to 60% at 16 WAI. At the end of the experiment (26 WAI), 90% of Las-inoculated plants tested positive. HLB symptoms were not discernible in infected plants until 16 WAI when 17% of plants displayed a light yellowing of leaves. At the later stages of infection, chlorosis (mostly resembling zinc deficiency-like symptoms) and some blotchy mottle was detected in 53% to 63% of Las-positive plants. Growth of stem diameters at the end of Expts. 1 and 2 was 36% and 24% on average but not significantly different

Fig. 1. Percentage of field grown 'Hamlin' trees polymerase chain reaction (PCR)-positive for *Ca. L. asiaticus* from Aug. 2007 to Mar. 2011. Trees in Rows 33 and 34 were planted in June 2005. Trees in Rows 35 and 36 were planted in Aug. 2007. A total of 170 trees (41 to 43 trees per row) were analyzed.

between mock-inoculated and Las-inoculated or PCR-positive plants. From 6 WAI to 16 WAI, the number of Las genomes was considerably higher in plants from Expt. 1 compared with Expt. 2. The number of Las genomes at the end of both experiments was 2.1×10^7 and 1.6×10^7 for PCR-positive plants and 17.4×10^6 and 14.8×10^6 for all Las-inoculated plants.

Comparison of artificial graft and psyllid inoculation

US-802 plants. None of the graft-inoculated US-802 plants from Expt. 3 tested positive for Las during the first 4 months after inoculation (Table 3). The percentage of plants in which Las was detected was 44% at 6 months after inoculation (MAI) and increased to 75% at the end of the experiment (12 MAI). Among the psyllid-inoculated plants, only one (6%) tested positive for Las by 4 MAI. The percentage of Las-positive plants increased to 19% at 6 MAI and remained unchanged until the end of the experiment. Graft- and psyllid-inoculated plants remained HLB symptom-free until 8 MAI when 13% of plants displayed a small number of leathery leaves, which were not observed in mock-inoculated or in control plants. After the pruning at 12 MAI, no foliar abnormalities were observed in any of the plants. Stem diameters of control- and mock-inoculated plants increased 93% to 107% from the beginning to the end of the experiment (Fig. 2). Increases were not significantly different between controls and graft-inoculated PCR-positive plants in contrast to psyllid-inoculated PCR-positive plants for which a significantly ($P < 0.05$) lower increase (57%) was observed. The number of Las genomes in PCR-positive plants was highest at 8 MAI, measuring 9.5×10^5 after graft inoculation and 1.7×10^6 after psyllid inoculation. After pruning at 12 MAI, Las numbers were similar in psyllid- and graft-inoculated

plants (1.8 to 2.2×10^5). Las genome numbers for all inoculated plants varied from 65 to 6.5×10^6 after graft inoculation and from 256 to 3.2×10^6 after psyllid inoculation.

Similar to Expt. 3, none of the graft-inoculated US-802 plants from Expt. 4 tested positive for Las during the first 4 months after inoculation (Table 4). The percentage of Las-positive plants was 13% at 6 MAI and increased to 31% at the end of the experiment. In psyllid-inoculated plants, Las was detected in 19% of plants in the first sampling (2 MAI). The percentage of Las-positive plants increased to 38% at 6 MAI and remained unchanged until the end of the experiment. Graft-inoculated plants remained symptom-free until 6 MAI, but from 8 to 12 MAI, blotchy mottling of leaves was observed in 19% of plants. Light chlorosis was observed in 13% of psyllid-inoculated plants at 4 MAI. In addition to chlorosis, light blotchy mottling and hardening of leaves occurred in 25% to 38% of plants from 6 to 8 MAI. Only one plant (6%) displayed foliar HLB symptoms (blotchy mottle) after pruning at 12 MAI. Stem diameters of control- and mock-inoculated plants increased 100% to 109%

from the beginning to the end of the experiment (Fig. 2). Like Expt. 3, increases were not significantly different between controls and graft-inoculated PCR-positive plants, contrary to psyllid-inoculated PCR-positive plants for which a significantly lower increase (73%) was observed. The number of Las genomes in PCR-positive plants was highest at 8 MAI and measured 3.1×10^6 after graft inoculation and 6.4×10^6 after psyllid inoculation. The number of Las genomes for all inoculated plants ranged from 11 to 7.8×10^5 after graft inoculation and was lower compared with psyllid-inoculated plants in which 9.5×10^4 to 2.4×10^6 Las genomes were detected. After pruning, at 12 MAI, Las numbers were similar in both treatments.

Sweet orange seedlings. The percentage of 'Valencia' seedlings PCR-positive for Las after analysis of leaves ranged from 6% at 2 MAI to 38% at the end of Expt. 5 (Table 5). No HLB disease symptoms were observed during the first 4 months after inoculation. From 6 MAI until the end of the experiment, 88% of graft-inoculated plants displayed stunting, severe chlorosis, reduced leaf size,

Table 2. Percentage of 'Valencia' plants PCR-positive for *Ca. L. asiaticus* (Las), percentage of HLB symptomatic plants, and number of Las genomes 6 to 26 weeks after graft inoculation (Expts. 1 and 2).

	Weeks after inoculation (WAI)				
	6 WAI	11 WAI	16 WAI	21 WAI	26 WAI
Percentage of PCR-positive plants					
Expt. 1	47	80	80	—	—
Expt. 2	17	50	60	77	90
Percentage of plants with HLB symptoms					
Expt. 1	0	53	80	—	—
Expt. 2	0	0	17	53	63
Number of Las genomes ^a (PCR-positive plants)					
Expt. 1	799,047	7,006,422	21,477,084	—	—
Expt. 2	93,107	1,875,719	8,786,702	34,315,932	16,466,212
Number of Las genomes (Las-inoculated plants ^b)					
Expt. 1	373,146	5,605,244	17,371,381	—	—
Expt. 2	17,265	937,941	5,272,256	26,309,135	14,763,102

^aNumber of Las genomes is expressed per gram petiole/midrib. Plants were inoculated in Aug. 2009 (Expt. 1) and Dec. 2009 (Expt. 2).

^bThe term "Las-inoculated" refers to all plants that were graft-inoculated and includes PCR-positive plants and plants with a Ct value of 32 or higher. A total of 30 plants was analyzed per experiment. PCR = polymerase chain reaction; HLB = huanglongbing.

Table 1. Percentage of field grown 'Hamlin' trees PCR-positive for *Ca. L. asiaticus* (PCR+) in comparison with the percentage of trees displaying foliar HLB symptoms (SYM).^a

	Rows 33/34		Rows 35/36	
	PCR+	SYM	PCR+	SYM
15 Aug. 2007	17	7	—	—
10 Oct. 2007	19	7	0	0
5 Dec. 2007	27	12	0	0
5 Feb. 2008	38	37	0	0
3 Apr. 2008	63	46	7	0
5 June 2008	84	59	18	6
31 July 2008	83	54	27	8
25 Sept. 2008	97	87	55	47
21 Nov. 2008	100	98	64	56
16 Jan. 2009	100	100	63	56
13 Mar. 2009	—	—	57	50
13 May 2009	—	—	57	57
15 July 2009	—	—	63	62
24 Sept. 2009	—	—	84	67
23 Nov. 2009	—	—	88	87
27 Jan. 2010	—	—	90	88
25 Mar. 2010	—	—	90	86
2 June 2010	—	—	99	100

^aA total of 86 trees (Rows 33 and 34) and 84 trees (Rows 5 and 36) was analyzed. PCR = polymerase chain reaction; HLB = huanglongbing.

Table 3. Percentage of US-802 plants PCR-positive for *Ca. L. asiaticus* (Las), percentage of HLB symptomatic plants and number of Las genomes 2 to 12 months after graft or psyllid inoculation (Expt. 3).

	Months after inoculation (MAI)				
	2 MAI	4 MAI	6 MAI	8 MAI	12 MAI
Percentage of PCR-positive plants					
Graft-inoculated	0	0	44	69	75
Psyllid-inoculated	0	6	19	19	19
Percentage of plants with HLB symptoms					
Graft-inoculated	0	0	0	13	0
Psyllid-inoculated	0	0	0	13	0
Number of Las genomes ^a (PCR-positive plants)					
Graft-inoculated	—	—	106,530	951,645	219,419
Psyllid-inoculated	—	1,711,304	1,627,620	1,694,088	176,215
Number of Las genomes (Las-inoculated plants ^b)					
Graft-inoculated	96	65	46,613	654,263	164,567
Psyllid-inoculated	256	106,984	305,188	317,799	33,157

^aNumber of Las genomes is expressed per gram petiole/midrib. Plants were cut back after sample collection at 8 MAI.

^bThe term "Las-inoculated" refers to all plants that were graft- or psyllid-inoculated and includes PCR-positive plants and plants with a Ct value of 32 or higher. A total of 16 plants per treatment was analyzed. PCR = polymerase chain reaction; HLB = huanglongbing.

Fig. 2. Stem diameter growth of graft- and psyllid-inoculated US-802 plants from Expt. 3 (top) and Expt. 4 (bottom) 12 months after inoculation (MAI) with *Ca. L. asiaticus* (Las). CTRL = non-inoculated controls (N = 8); Graft-CTRL = mock-inoculated graft controls (N = 8); Graft-PCR+ = graft/Las-inoculated PCR-positive plants (N = 12); Psyllid-CTRL = psyllid-inoculated controls (N = 8); Psyllid-PCR+ = psyllid/Las-inoculated PCR-positive plants (N = 3). Different letters above bars indicate significant differences according to Tukey's honestly significant difference test ($P \leq 0.05$). PCR = polymerase chain reaction.

Table 4. Percentage of US-802 plants PCR-positive for *Ca. L. asiaticus* (Las), percentage of HLB symptomatic plants, and number of Las genomes 2 to 12 months after graft or psyllid inoculation (Expt. 4).

	Months after inoculation (MAI)				
	2 MAI	4 MAI	6 MAI	8 MAI	12 MAI
Percentage of PCR-positive plants					
Graft-inoculated	0	0	13	25	31
Psyllid-inoculated	19	31	38	38	38
Percentage of plants with HLB symptoms					
Graft-inoculated	0	0	0	19	19
Psyllid-inoculated	0	13	25	38	6
Number of Las genomes ^a (PCR-positive plants)					
Graft-inoculated	—	—	442,283	3,134,010	386,787
Psyllid-inoculated	507,016	911,720	3,193,839	6,379,932	431,272
Number of Las genomes (Las-inoculated plants ^b)					
Graft-inoculated	11	11	55,305	783,764	120,940
Psyllid-inoculated	95,183	284,925	1,198,002	2,392,724	161,789

^aNumber of Las genomes is expressed per gram petiole/midrib. Plants were cut back after sample collection at 8 MAI.

^bThe term "Las-inoculated" refers to all plants that were graft- or psyllid-inoculated and includes PCR-positive plants and plants with a Ct value of 32 or higher. A total of 16 plants per treatment was analyzed. PCR = polymerase chain reaction; HLB = huanglongbing.

and leaf drop (Fig. 3), which is a considerably larger percentage of plants than the percentage of plants that tested positive for Las after PCR analysis of leaves. PCR analysis of roots of all HLB symptomatic graft-inoculated plants at 16 MAI detected Las in all plants except for one, which was PCR-positive based on leaf analysis. The percentage of PCR-positive plants after leaf analysis of

psyllid-inoculated plants was 6% at 3 MAI, increased to 19% at 6 MAI, and remained unchanged until the end of the experiment (16 MAI). Stunting, chlorosis, leaf size reductions, and leaf drop were observed only in PCR-positive plants and disease symptoms were indistinguishable from those observed for graft-inoculated plants. Percent increases of stem diameters in all graft-inoculated and

in PCR-positive (based on leaf analysis) graft- and psyllid-inoculated 'Valencia' seedlings at 12 MAI were significantly ($P \leq 0.05$) less (60% to 75%) compared with control- and mock-inoculated (88% to 102%) plants (Fig. 4). Significant differences were also observed for pruned biomass, which measured 30 to 56 g for all graft-inoculated and for PCR-positive graft- and psyllid-inoculated plants compared with 164 to 173 g for control- and mock-inoculated plants at 8 MAI (Fig. 5). Differences in stem diameter increase and pruned biomass at 16 MAI were more extensive but showed a similar pattern (data not shown). The number of Las genomes in leaves of PCR-positive 'Valencia' seedlings ranged from 6.6×10^3 to 3.9×10^7 for graft-inoculated plants and from 4.1×10^5 to 2.3×10^7 for psyllid-inoculated plants (Table 5). Average Las numbers for all graft-inoculated plants were higher than average numbers for all psyllid-inoculated plants from 6 MAI until the end of the experiment. The average number of Las genomes detected in roots was higher (8.5×10^5) in plants that tested negative for Las in leaves than in plants that tested positive for Las in leaves (1.2×10^5). Lowest Las numbers (2.0×10^4) were detected in roots of those plants that tested positive in leaves for the longest time.

The percentage of graft-inoculated 'Ridge Pineapple' seedlings that were PCR-positive for Las ranged from 25% at 2 MAI to 88% at 12 MAI (Table 6). First HLB symptoms were noticeable at 4 MAI when 44% of graft-inoculated plants displayed chlorosis, blotchy mottle, and/or stunting. Disease symptoms increased in severity and by 12 MAI, most plants were symptomatic. The percentage of PCR-positive 'Ridge Pineapple' seedlings after psyllid inoculation was 31% throughout the experiment and plants exhibited chlorosis, leaf drop, blotchy mottle, and stunting. Disease symptoms became more severe with the duration of the experiment and were indistinguishable from those observed in graft-inoculated plants (Fig. 6). Except for the first months after inoculation, the percentage of plants with disease symptoms was slightly higher than the percentage of PCR-positive plants in both graft-inoculated and psyllid-inoculated plants. Stem diameters increased 70% to 72% in control- and mock-inoculated plants from the beginning to the end of the experiment (12 MAI), which was significantly ($P \leq 0.05$) more than observed in graft-inoculated plants independent of PCR detection of Las (Fig. 4). Pruned biomass determined at 8 MAI was significantly higher (190 to 208 g) in control- and mock-inoculated plants than in graft-inoculated plants (81 to 98 g) (Fig. 5). Pruned biomass was lowest in PCR-positive psyllid-inoculated plants (42 g). The number of Las genomes in PCR-positive 'Ridge Pineapple' seedlings ranged from 4.6×10^6 to 1.4×10^7 after graft inoculation and from 3.9×10^5 to 1.1×10^7 after psyllid inoculations and was highest between 4 MAI and 6 MAI. Average Las numbers detected in graft-inoculated plants were higher than numbers

in psyllid-inoculated plants throughout the experiment.

Discussion

As a result of the inability to maintain *Liberibacters* in culture, investigators most often rely on grafting for transmission of the pathogens from diseased to healthy plants. That HLB can be transmitted from diseased to healthy trees through grafting was already observed in 1943 in China and was later confirmed in a series of studies in South Africa and Asia (da Graça, 1991). To investigate whether Las is transmitted with different efficiency in graft- and in psyllid-inoculated citrus and whether the inoculation procedure affects HLB disease severity, we conducted

a series of experiments in field and greenhouse settings.

Incidence of *Ca. Las* infection was analyzed in two sets of field-grown naturally infected ‘Hamlin’ trees, which were either planted in June 2005, 2 months before the first detection of HLB in Florida (Halbert, 2005), or in Aug. 2007 when HLB was endemic at that location. Although disease incidence in both sets of trees was 100% within 3 to 3½ years after planting, trees planted in 2007 reached 50% incidence by 13 months after planting compared with more than 30 months for trees planted in 2005. Similarly, a recent study by Hall et al. (2013) reported little HLB development in sweet orange trees subjected to different psyllid management programs during the first year,

but large percentages of infection within 2 to 3 years of time. Low HLB incidences during the first year of experimentation in commercial citrus groves in Brazil were also observed by Bassanezi et al. (2013) independent of the frequency of symptomatic tree removal. Different rates of disease transmission have been reported in other citrus-growing areas throughout the world, illustrating that disease progress is influenced by the extent of the inoculum reservoir, the local vector populations, the age of the grove at first infection, and other environmental factors (Gottwald, 2010; Gottwald et al., 2007). The faster disease progress rate observed for the ‘Hamlin’ trees planted in 2007 compared with those planted in 2005 in the present study is likely associated with an increased amount of bacterial inoculum around the experimental plots during the years after the first detection of Las at this location. ‘Hamlin’ trees identified as positive for Las displayed blotchy mottle of leaves, severe zinc deficiency-like chlorosis, and shoot dieback typical for HLB (Bové, 2006; Gottwald et al., 2007; McClean and Schwarz, 1970), but PCR detection often preceded symptom development. The pattern of Las detection in all ‘Hamlin’ trees followed a south to north direction, indicating that psyllid populations were introduced from the center of the farm and not from the edge of the planting, which is usually observed in citrus orchards (Gottwald, 2010). According to Hall et al. (2008) and Hall and Hentz (2011), psyllid populations at a site in the center of the farm were large in late spring/early summer of 2005–07, which may have coincided with a prevalence of southerly winds. However, prevailing winds in St. Lucie County, FL, are generally from the north and east, and southerly winds are usually only observed in March (Watts and

Table 5. Percentage of ‘Valencia’ seedlings PCR-positive for *Ca. L. asiaticus* (Las), percentage of HLB symptomatic plants, and number of Las genomes 2 to 16 months after graft or psyllid inoculation (Expt. 5).

	Months after inoculation (MAI)					
	2 MAI	4 MAI	6 MAI	8 MAI	12 MAI	16 MAI
Percentage of PCR-positive plants						
Graft-inoculated	6	13	19	25	38	38 (88) ^z
Psyllid-inoculated	6	13	19	19	19	19
Percentage of plants with disease symptoms						
Graft-inoculated	0	0	88	88	88	88
Psyllid-inoculated	0	6	19	19	19	19
Number of Las genomes ^y (PCR-positive plants)						
Graft-inoculated	6,557	1,291,716	39,227,757	27,408,641	1,233,387	4,556,081
Psyllid-inoculated	409,044	12,774,021	10,902,493	23,364,188	1,876,985	3,728,228
Number of Las genomes (Las-inoculated plants ^y)						
Graft-inoculated	421	161,478	7,355,433	6,852,374	462,561	1,708,603
Psyllid-inoculated	25,576	1,596,823	2,044,542	4,381,036	352,220	699,241

^zPercentage after analysis of roots is given in parentheses.

^yNumber of Las genomes is expressed per gram petiole/midrib. Plants were cut back after sample collection at 8 MAI. The term “Las-inoculated” refers to all plants that were graft- or psyllid-inoculated and includes PCR-positive plants and plants with a Ct value of 32 or higher. A total of 16 plants per treatment was analyzed.

PCR = polymerase chain reaction; HLB = huanglongbing.

Fig. 3. ‘Valencia’ seedlings 12 months after graft inoculation (A–B) or psyllid inoculation (C) with *Ca. L. asiaticus*. Graft-inoculated plants testing positive for Las in leaves are shown in A. Graft-inoculated plants testing negative for Las in leaves but positive for Las in roots are shown in B. A mock-inoculated control plant is shown on the right of each picture for comparison.

Fig. 4. Stem diameter growth of graft- and psyllid-inoculated 'Valencia' (top) and 'Ridge Pineapple' (bottom) seedlings from Expts. 5 and 6 twelve months after inoculation. CTRL = non-inoculated controls (N = 8); Graft-CTRL = mock-inoculated graft controls (N = 8); Graft-LAS = all graft/Las-inoculated plants (N = 16); Graft-PCR+ = graft/Las-inoculated PCR-positive plants (N = 12); Psyllid-CTRL = psyllid-inoculated controls (N = 8); Psyllid-LAS = all psyllid/Las inoculated plants; Psyllid-PCR+ = psyllid/Las-inoculated PCR-positive plants (N = 3). Different letters above bars indicate significant differences according to Tukey's honestly significant difference test ($P \leq 0.05$). Statistical comparisons were made among non-inoculated controls, mock-inoculated graft controls, all graft/Las-inoculated plants, psyllid-inoculated controls, and all psyllid/Las inoculated plants (lowercase letters) and among non-inoculated controls, mock-inoculated graft controls, graft/Las-inoculated PCR-positive plants, psyllid-inoculated controls, and psyllid/Las-inoculated PCR-positive plants (uppercase letters). PCR = polymerase chain reaction.

Stankey, 1980). Analysis of HLB spread in an orchard in French Réunion Island showed that disease movement may also occur through active migration of the psyllid vector and against prevailing winds (Gottwald et al., 1989). The temporary stagnant rate of Las detection between Sept. 2008 and July 2009 in Rows 35 and 36 was likely associated with the low psyllid populations detected near this area during that time (Hall, unpublished data).

Contrary to naturally inoculated field-grown 'Hamlin' trees, for which Las incidences remained below 50% for a minimum of 1 year after planting, graft inoculation of greenhouse-grown 'Valencia' trees resulted in 50% transmission between 6 and 11 weeks after inoculation, and reached 80% to 90% by 16 to 26 weeks after inoculation. Disease symptoms were primarily chlorosis of leaves and appeared several weeks after PCR detection

of Las. High transmission rates of Las in greenhouse-grown sweet orange were also observed in other studies, although results were variable. Coletta-Filho et al. (2010) reported 100% infection at 120 d after graft inoculation of 8-month-old greenhouse-grown 'Valencia' trees. Lopes et al. (2009) reported 55% to 88% of transmission in different graft-inoculated sweet orange cultivars 12 months after inoculation. Percentages of infection in 5-month-old 'Valencia' trees increased from 22% at 3 months after graft inoculation to 82% at 8 months after inoculation (Pereira et al., 2010). In contrast, much lower rates of transmission have been observed for *Ca. L. americanus* (Lopes et al., 2009; Lopes and Frare, 2008) and *Ca. L. africanus* (McClean, 1970; Van Vuuren, 1993). The rate of Las transmission and disease symptom expression in the 'Valencia' trees

of the present study was much accelerated in plants, which were inoculated in June 2009, compared with plants that were inoculated in November of the same year. That transmission rates can vary depending of the season in which inoculations occur was also observed by Coletta-Filho et al. (2010) in a study conducted in Brazil. The authors found that it took less time to reach maximum concentrations of Las when experiments were conducted in an "fall experiment" from April to December than in a "spring experiment" from September to May, which is contrary to our observations of a faster transmission during the warmer months. Lopes and Frare (2008) and Schwarz (1970) also reported higher percentages of *Ca. L. americanus* and *Ca. L. africanus* transmission in graft-inoculated and greenhouse-grown sweet orange trees during the winter. Reasons other than season such as different developmental stage of plants, different levels of viable bacteria in the inoculum source, and different greenhouse conditions may have contributed to the differences in findings between our study and the studies conducted in the southern hemisphere.

In additional experiments, Las transmission and HLB development were examined through direct comparison of graft-inoculated and psyllid-inoculated trifoliate hybrid and sweet orange plants in a greenhouse setting. Contrary to the non-controllable nature of psyllid inoculation in the field setting, psyllid inoculation as conducted in our greenhouses allowed for control of the number of psyllids per plant and the time of inoculation. Depending on the experiment, the percentage of transmission of Las by graft in in vitro-generated US-802 plants increased from 13% to 44% at 6 months after inoculation to 31% to 75% at 12 months after inoculation. In contrast to graft-inoculated plants, the percentage of transmission in psyllid-inoculated plants remained unchanged during that time period and did not exceed 19% or 38%. In addition to the lower rate of Las transmission in US-802 plants compared with sweet orange plants, distinct foliar disease symptoms were rarely observed and were limited to a small number of plants. The lack or inconsistency of HLB symptom expression in *P. trifoliata*-type citrus plants has also been observed in other studies (Albrecht and Bowman, 2011, 2012b; Folimonova et al., 2009; McClean and Schwarz, 1970; Miyakawa, 1980) and is assumed to be associated with tolerance or resistance of these genotypes to HLB. However, stem diameter growth was significantly reduced in infected US-802 plants, but only after psyllid inoculation. This difference in effect on growth may be associated with the faster transmission of Las through psyllid observed in both experiments. However, it cannot be ruled out that the psyllids collected from a natural field site contained an isolate of Las with higher pathogenicity than the isolate used for graft inoculation.

Similar to the previous experiments, transmission of Las in sweet orange seedlings detected after PCR analysis of leaves varied between experiments and was 6% to 38% for

Fig. 5. Pruned biomass of graft- and psyllid-inoculated 'Valencia' (top) and 'Ridge Pineapple' (bottom) seedlings from Expts. 5 and 6 eight months after inoculation. CTRL = non-inoculated controls (N = 8); Graft-CTRL = mock-inoculated graft controls (N = 8); Graft-LAS = all graft/Las-inoculated plants (N = 16); Graft-PCR+ = graft/Las-inoculated PCR-positive plants (N = 12); Psyllid-CTRL = psyllid-inoculated controls (N = 8); Psyllid-LAS = all psyllid/Las inoculated plants; Psyllid-PCR+ = psyllid/Las-inoculated PCR-positive plants (N = 3). Different letters above bars indicate significant differences according to Tukey's honestly significant difference test ($P \leq 0.05$). Statistical comparisons were made among non-inoculated controls, mock-inoculated graft controls, all graft/Las-inoculated plants, psyllid-inoculated controls, and all psyllid/Las inoculated plants (lowercase letters) and among non-inoculated controls, mock-inoculated graft controls, graft/Las-inoculated PCR-positive plants, psyllid-inoculated controls, and psyllid/Las-inoculated PCR-positive plants (uppercase letters). PCR = polymerase chain reaction.

'Valencia' seedlings, or 25% to 88% for 'Ridge Pineapple' seedlings, during the time from 2 to 12 months after graft inoculation. As observed for US-802, the percentage of plants testing positive for Las after inoculation with psyllids did not exceed 19% to 31% and was constant for most of the duration of the experiments. Infected sweet orange plants suffered from severe chlorosis and severe stunting, indistinguishable between graft- and psyllid-inoculated plants. Surprisingly, a large number of graft-inoculated 'Valencia' plants that tested negative for Las in leaves displayed disease symptoms of the same kind and severity. PCR analysis of roots identified Las in all (88%) graft-inoculated symptomatic plants. In citrus, root and shoot growth follows a cyclical pattern in which periods of root growth alternate with flushes of shoot growth (Bevington and Castle, 1985). Active

root growth in the 'Valencia' seedlings at the time of graft inoculation may have forced the bacteria to immediately move downward in the direction of the strongest sink. The resulting phloem and root damage below the inoculation site in some of the plants may have been so extensive that disease symptoms emerged in the canopy despite the absence of the pathogen. Recent studies on greenhouse-grown and field-grown citrus trees showed that Las preferentially colonizes the roots, where it causes extensive damage, before moving to the leaves (Graham et al., 2013; Johnson et al., 2013). Similarly, Samuel (1934) demonstrated that tobacco mosaic virus travels first to the roots of tomato plants after inoculation of leaves before moving up again to the top of the plant. Levy et al. (2011) observed that the direction of movement of *Ca. L. solanacearum* in tomato and

potato follows the movement of carbohydrates in the direction of the strongest sink. It is unclear if Las distribution is restricted to the direction of assimilate flow or if its distribution may also be affected by factors independent of source-sink relationships as observed for phytoplasmas (Christensen et al., 2004).

Compared with bacterial numbers in PCR-positive leaves, Las numbers in roots were generally lower. Lower bacterial numbers in roots than in leaves were also reported by Tatineni et al. (2008) and Trivedi et al. (2009) for field-grown Las-infected sweet orange trees. Li et al. (2009) found similar concentrations of Las in leaves and roots of field-grown citrus trees, but in graft-inoculated greenhouse-grown trees concentrations were 1000-fold lower in roots compared with leaves.

The direct comparison of inoculation methods showed that transmission of Las through psyllid occurred at a lower frequency and was thus less efficient than transmission through grafting. Compared with graft inoculation where large areas of the phloem in the main stem of the host plant are exposed to the pathogen, psyllid inoculation is restricted to the feeding sites of the insects, which, depending on the number of psyllids, may encompass a much smaller area of the phloem and reduce the chance of a successful transmission. In addition, spread of the pathogen throughout the plant may be affected by the distance from the feeding site to the phloem of the main stem. It may be hypothesized that a longer exposure of psyllids or use of larger number of psyllids is necessary for a high percentage of plants to become PCR-positive. This is in accordance with the observations of Pelz-Stelinski et al. (2010) who reported very low transmission efficiencies of Las in citrus after single-psyllid inoculation. The authors observed considerably increased transmission frequencies only after using groups of 100 or more psyllids. Hung et al. (2001) reported later detection of Las in psyllid transmission tests along with later development of HLB symptoms in 'Chinese' box orange and in 'Luchen' sweet orange than in grafting tests, although 100 psyllids were allowed to feed on each plant for a period of 2 weeks. Earlier studies, which did not include molecular detection of the pathogen, showed varying results of psyllid transmission of HLB. Capoor et al. (1974) observed high transmission of HLB using tissue grafts and obtained 100% infection when five or more psyllids were allowed to feed on plants for a period of 24 h. Very low transmission was observed by Huang et al. (1984) in Taiwan, who found only five of 380 plants exposed to adult psyllids exhibiting HLB symptoms. A study by Xu et al. (1985) in China also found low (12.2%) transmission efficiency of HLB by psyllids. Further studies by the same author (Xu et al., 1988) but using modified experimental conditions found that after a 20-d period of acquisition feeding a single adult induced disease symptoms in 80% of 'Ponkan' mandarin seedlings. Modified conditions

Table 6. Percentage of graft- and psyllid-inoculated 'Ridge Pineapple' seedlings PCR-positive for *Ca. L. asiaticus* (Las), percentage of HLB symptomatic plants, and number of Las genomes 2 to 12 months after graft or psyllid inoculation (Expt. 6).

	Months after inoculation (MAI)				
	2 MAI	4 MAI	6 MAI	8 MAI	12 MAI
Percentage of PCR-positive plants					
Graft-inoculated	25	56	56	75	88
Psyllid-inoculated	31	31	31	31	31
Percentage of plants with disease symptoms					
Graft-inoculated	0	44	63	88	94
Psyllid-inoculated	0	44	44	44	44
Number of Las genomes ^z (PCR-positive plants)					
Graft-inoculated	4,565,658	9,008,323	13,975,691	5,003,892	1,070,633
Psyllid-inoculated	2,968,704	11,214,457	10,291,638	4,881,404	385,029
Number of Las genomes (Las-inoculated plants ^y)					
Graft-inoculated	1,141,826	5,068,660	7,861,777	3,752,943	936,805
Psyllid-inoculated	928,532	3,506,844	3,216,725	1,830,603	120,341

^zNumber of Las genomes is expressed per gram petiole/midrib. Plants were cut back after sample collection at 8 MAI. A total of 16 plants per treatment was analyzed.

^yThe term "Las-inoculated" refers to all plants that were graft-or psyllid-inoculated and includes PCR-positive plants and plants with a Ct value of 32 or higher.

PCR = polymerase chain reaction; HLB = huanglongbing.

Fig. 6. 'Ridge Pineapple' seedlings 12 months after graft inoculation (A) or psyllid inoculation (B) with *Ca. L. asiaticus*. A mock-inoculated plant is shown on the right of each picture for comparison.

included the use of psyllids known to have fed on diseased plants and the use of younger seedlings for inoculation. Inoue et al. (2009) and Pelz-Stelinski et al. (2010) found that transmission of Las is higher in psyllids, which acquired the pathogen as nymphs compared with psyllids, which acquired the pathogen as adults. Because psyllids used for inoculation in our study were either reared on infected plants and had thus acquired Las as nymphs or were collected from the field, other factors, in particular the number of psyllids and the age of leaves at the time of inoculation, may have contributed to the low transmission rates in our study.

Coletta-Filho et al. (2010) observed a direct relationship between the concentration of Las in leaves and the expression of HLB disease symptoms. Whereas yellow shoots were observed first and in trees with lower bacterial copy numbers (10^5), blotchy mottle

was observed later and in trees measuring 10^7 Las genomes per gram of tissue. In the experiments conducted here, leaf chlorosis was the first and predominant foliar disease symptom observed in infected sweet orange plants. The number of Las genomes per gram of leaf tissue measured at the time of the first symptom detection varied between experiments but was similar in graft-inoculated (7×10^6 to 4×10^7) and in psyllid-inoculated (1×10^7) sweet orange plants. Las numbers associated with the time of blotchy mottle manifestation in US-802 plants were the same after graft and psyllid inoculation (3×10^6). The minimum concentration of Las required for foliar HLB symptoms to manifest in sweet orange as determined by Trivedi et al. (2009) ranged from 9×10^5 to 7×10^6 Las equivalents per micrograms of plant DNA. The highest Las numbers observed in the sweet orange experiments were one to four $\times$

10^7 in graft-inoculated plants and one to two $\times 10^7$ in psyllid-inoculated plants and were higher than in US-802 plants for which one to three $\times 10^6$ and two to six $\times 10^6$ Las genomes were measured, respectively. The lower number of Las genomes detected in the trifoliate hybrid US-802 is in accordance with the reduced symptom expression observed for this genotype. Compared with the maximum numbers of bacterial genomes measured in leaves of Las-infected citrus plants in this and other studies (Coletta-Filho et al., 2010; Stover and McCollum, 2011; Trivedi et al., 2009), numbers reported by Folimonova et al. (2009) and Li et al. (2009) measured 10^{10} and were thus considerably higher. Pruning reduced bacterial numbers in psyllid-inoculated plants as well as in graft-inoculated plants, which indicates that the movement of Las from the tissue below the pruning site to the emerging flush or the bacterial growth rate is slow. However, previous studies in our laboratory showed that the effect of pruning on bacterial levels is only temporary and that pruning results in much increased numbers of plants with detectable Las levels and disease symptoms (Albrecht and Bowman, 2012b).

In summary, graft inoculation resulted in frequencies of Las transmission as high as 90% in susceptible genotypes within 6 to 12 months after inoculation. In comparison, frequency of Las transmission through controlled psyllid inoculation was lower and did not exceed 38%. The time from inoculation to detection of Las by PCR was faster through psyllids in the tolerant genotype but similar in graft- and psyllid-inoculated susceptible sweet orange plants. The number of infected plants remained constant 6 months after psyllid inoculation, contrary to graft inoculation in which the number of infected plants increased continuously throughout the experiments. Larger numbers of psyllids or continuous exposure for longer periods of time may be necessary for a high percentage of citrus to become PCR-positive. Disease symptom expression was identical in graft- and psyllid-inoculated plants but was not always associated with bacterial numbers in affected leaves. It is concluded that graft inoculation is an efficient method for screening citrus plants for resistance to HLB and under the conditions used in this study was superior to the method of controlled psyllid inoculation. Because of its lesser procedural complexity compared with controlled psyllid inoculations, graft inoculation is particularly suitable for studies requiring large numbers of plants to be screened in preparation for longer-term field trials. Compared with artificial inoculations in the greenhouse, inoculation under natural field conditions does not allow for control of time and extent of inoculation and requires more than 1 year for infection incidence to reach substantial levels. It remains to be investigated if bacterial populations present in naturally occurring psyllids have a higher pathogenicity than the bacterial populations present in psyllids reared on greenhouse-grown infected citrus plants for prolonged periods of time.

Literature Cited

- Achor, D.S., E. Exteberria, N. Wang, S.Y. Folimonova, K.R. Chung, and L.G. Albrigo. 2010. Sequence of anatomical symptom observations in citrus affected with huanglongbing disease. *Plant Pathol.* J. 9:56–64.
- Albrecht, U. and K.D. Bowman. 2009. *Candidatus* Liberibacter asiaticus and Huanglongbing effects on citrus seeds and seedlings. *HortScience* 44:1967–1973.
- Albrecht, U. and K.D. Bowman. 2008. Gene expression in *Citrus sinensis* (L.) Osbeck following infection with the bacterial pathogen *Candidatus* Liberibacter asiaticus causing huanglongbing in Florida. *Plant Sci.* 175:291–306.
- Albrecht, U. and K.D. Bowman. 2011. Tolerance of the trifoliate citrus hybrid US-897 (*Citrus reticulata* Blanco × *Poncirus trifoliata* L. Raf.) to huanglongbing. *HortScience* 46:16–22.
- Albrecht, U. and K.D. Bowman. 2012a. Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus* liberibacter asiaticus. *Plant Sci.* 185-186:118–130.
- Albrecht, U. and K.D. Bowman. 2012b. Tolerance of trifoliate citrus hybrids to *Candidatus* liberibacter asiaticus. *Sci. Hort.* 147:71–80.
- Bassanezi, R.B., J. Belasque, Jr., and L.H. Montesino. 2013. Frequency of symptomatic trees removal in small citrus blocks on citrus huanglongbing epidemics. *Crop Prot.* 52:72–77.
- Bevington, K.B. and W.S. Castle. 1985. Annual root growth pattern of young citrus trees in relation to shoot growth, soil temperature and soil water content. *J. Amer. Soc. Hort. Sci.* 110:840–845.
- Bové, J.M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88:7–37.
- Capoor, S.P., D.G. Rao, and S.M. Viswanath. 1974. Greening disease of citrus in the Deccan Trap Country and its relationship with the vector, *Diaphorina citri* Kuwayama, p. 43–49. In: Weathers, L.G. and M. Cohen (eds.). *Proc. of the 6th Conference of the International Organization of Citrus Virologists (IOCV)*. University of California, Berkeley, CA.
- Christensen, N.M., M. Nicolaisen, M. Hansen, and A. Schulz. 2004. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Mol. Plant Microbe Interact.* 17:1175–1184.
- Coletta-Filho, H.D., E.F. Carlos, K.C.S. Alves, M.A.R. Pereira, R.L. Boscariol-Camargo, A.A. de Souza, and M.A. Machado. 2010. *In planta* multiplication and graft transmission of '*Candidatus* Liberibacter asiaticus' revealed by real-time PCR. *Eur. J. Plant Pathol.* 126:53–60.
- da Graça, J.V. 1991. Citrus greening disease. *Annu. Rev. Phytopathol.* 29:109–136.
- Davis, M.J., S.N. Mondal, H. Chen, M.E. Rogers, and R.H. Brlansky. 2008. Co-cultivation of '*Candidatus* Liberibacter asiaticus' with actinobacteria from citrus with huanglongbing. *Plant Dis.* 92:1547–1550.
- Duan, Y., L. Zhou, D.G. Hall, W. Li, H. Doddapaneni, H. Lin, L. Liu, C.M. Vahling, D.W. Gabriel, K.P. Williams, A. Dickerman, Y. Sun, and T. Gottwald. 2009. Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. *Mol. Plant Microbe Interact.* 22:1011–1020.
- Edriss, M.H. and D.W. Burger. 1984. In vitro propagation of 'Troyer' citrange from epicotyl segments. *Sci. Hort.* 23:159–162.
- Fan, J., C. Chen, R.H. Brlansky, F.G. Gmitter, Jr., and Z.-G. Li. 2010. Changes in carbohydrate metabolism in *Citrus sinensis* infected with '*Candidatus* Liberibacter asiaticus'. *Plant Pathol.* 59:1037–1043.
- Fan, J., C. Chen, Q. Yu, R.H. Brlansky, Z.-G. Li, and F.G. Gmitter, Jr. 2011. Comparative iTRAQ proteome and transcriptome analyses of sweet orange infected by '*Candidatus* Liberibacter asiaticus'. *Physiol. Plant.* 143:235–245.
- Folimonova, S.Y., C.J. Robertson, S.M. Garnsey, S. Gowda, and W.O. Dawson. 2009. Examination of the responses of different genotypes of citrus to huanglongbing (citrus greening) under different conditions. *Phytopathology* 99:1346–1354.
- Garnier, M., N. Danel, and J.M. Bové. 1984. The greening organism is a Gram negative bacterium, p. 115–124. In: Garnsey, S.M., L.W. Timmer, and J.A. Dodds (eds.). *Proc. 9th Conf. Intl. Organ. Citrus Virologists*. IOCV, Riverside, CA.
- Gottwald, T.R. 2010. Current epidemiological understanding of citrus huanglongbing. *Annu. Rev. Phytopathol.* 48:119–139.
- Gottwald, T.R., B. Aubert, and X.-Y. Zhao. 1989. Preliminary analysis of citrus greening (huanglongbing) epidemics in the People's Republic of China and French Reunion Island. *Phytopathology* 70:687–693.
- Gottwald, T.R., J.V. da Graça, and R.B. Bassanezi. 2007. Citrus huanglongbing: The pathogen and its impact. Online. *Plant Health Progress* Online publication.
- Graham, J.H., E.G. Johnson, T.R. Gottwald, and M.S. Irey. 2013. Pre-symptomatic fibrous root decline in citrus trees caused by huanglongbing and potential interaction with *Phytophthora* spp. *Plant Dis.* 97:1195–1199.
- Halbert, S.E. 2005. The discovery of huanglongbing in Florida, p. H-3. In: *Proc. 2nd International Citrus Canker and Huanglongbing Research Workshop*. Florida Citrus Mutual, Orlando, FL.
- Halbert, S.E. and K.L. Manjunath. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. *Fla. Entomol.* 87:330–353.
- Hall, D.G. and T.R. Gottwald. 2011. Pest management practices aimed at curtailing citrus huanglongbing disease. *Outlooks on Pest Management* 22:189–192.
- Hall, D.G., T.R. Gottwald, E. Stover, and G.A.C. Beattie. 2013. Evaluation of management programs for protecting young citrus plantings from huanglongbing. *HortScience* 48:330–337.
- Hall, D.G. and M.G. Hentz. 2011. Seasonal flight activity by the Asian citrus psyllid in east central Florida. *Entomol. Exp. Appl.* 139:75–85.
- Hall, D.G., M.G. Hentz, and R.C. Adair. 2008. Population ecology and phenology of *Diaphorina citri* (Hemiptera: Psyllidae) in two Florida citrus groves. *Environ. Entomol.* 37:914–924.
- Hall, D.G., M.L. Richardson, E.-D. Ammar, and S. Halbert. 2012. Asian citrus psyllid, *Diaphorina citri*, vector of citrus huanglongbing disease. *Entomol. Exp. Appl.* 146:207–223.
- Hodges, A.W. and T.H. Spreen. 2012. Economic impacts of citrus greening (HLB) in Florida, 2006/07–2010/11. Electronic Data Information Source (EDIS) FE903, University of Florida, Gainesville, FL. <<http://edis.ifas.ufl.edu/fe903>>.
- Huang, C.H., M.Y. Tsai, and C.L. Wang. 1984. Transmission of citrus likubin by a psyllid, *Diaphorina citri*. *J. Agr. Res. China* 33:65–72.
- Hung, T.-H., M.-L. Wu, and H.-J. Su. 2001. Identification of the Chinese box orange (*Severinia buxifolia*) as an alternative host of the bacterium causing citrus huanglongbing. *Eur. J. Plant Pathol.* 107:183–189.
- Inoue, H., J. Ohnishi, T. Ito, K. Tomimura, S. Miyata, T. Iwanami, and W. Ashihara. 2009. Enhanced proliferation and efficient transmission of *Candidatus* Liberibacter asiaticus by adult *Diaphorina citri* after acquisition feeding in the nymphal stage. *Ann. Appl. Biol.* 155:29–36.
- Jagoueix, S., J.M. Bové, and M. Garnier. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the *Proteobacteria*. *Intl. J. Syst. Bacteriol.* 44:379–386.
- Johnson, E.G., J. Wu, D.B. Bright, and J.H. Graham. 2013. Association of '*Candidatus* Liberibacter asiaticus' root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. *Plant Pathol.* (in press).
- Kim, J.S., U.S. Sagaram, J.K. Burns, J.-L. Li, and N. Wang. 2009. Response of sweet orange (*Citrus sinensis*) to '*Candidatus* Liberibacter asiaticus' infection: Microscopy and microarray analyses. *Phytopathology* 99:50–57.
- Levy, J., A. Ravindran, D. Gross, C. Tamborindéguy, and E. Pierson. 2011. Translocation of '*Candidatus* Liberibacter solanacearum', the zebra chip pathogen, in potato and tomato. *Phytopathology* 101:1285–1291.
- Li, W., J.S. Hartung, and L. Levy. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *J. Microbiol. Methods* 66:104–115.
- Li, W., L. Levy, and J.S. Hartung. 2009. Quantitative distribution of '*Candidatus* Liberibacter asiaticus' in citrus plants with citrus huanglongbing. *Phytopathology* 99:139–144.
- Lopes, S.A., E. Bertolini, G.F. Frare, E.C. Martins, N.A. Wulff, D.C. Teixeira, N.G. Fernandes, and M. Cambra. 2009. Graft transmission efficiencies and multiplication of '*Candidatus* Liberibacter americanus' and '*Ca. Liberibacter asiaticus*' in citrus plants. *Phytopathology* 99:301–306.
- Lopes, S.A. and G.F. Frare. 2008. Graft transmission and cultivar reaction of citrus to '*Candidatus* Liberibacter americanus'. *Plant Dis.* 92:21–24.
- McClean, A.P.D. 1970. Greening disease of sweet orange: Its transmission in propagative parts and distribution in partially diseased trees. *Phytophylactica* 2:263–267.
- McClean, A.P.D. and R.E. Schwarz. 1970. Greening or blotchy-mottle disease of citrus. *Phytophylactica* 2:177–194.
- Miyakawa, T. 1980. Experimentally-induced symptoms and host range of citrus likubin (greening disease). *Ann. Phytopath. Soc. Japan* 46:224–230.
- Pelz-Stelinski, K.S., R.H. Brlansky, T.A. Ebert, and M.E. Rogers. 2010. Transmission parameters for *Candidatus* Liberibacter asiaticus by Asian citrus psyllid (Hemiptera: Psyllidae). *J. Econ. Entomol.* 103:1531–1541.
- Pereira, F.M.V., D.M.B.P. Milori, A.L. Venâncio, M.D.S.T. Russo, P.K. Martins, and J. Freitas-Astúa. 2010. Evaluation of the effects of *Candidatus* Liberibacter asiaticus on inoculated citrus plants using laser-induced breakdown spectroscopy (LIBS) and chemometrics tools. *Talanta* 83:351–356.
- Rosales, R. and J.K. Burns. 2011. Phytohormone changes and carbohydrate status in sweet orange fruit from huanglongbing-infected trees. *J. Plant Growth Regul.* 30:312–321.
- Samuel, G. 1934. The movement of tobacco mosaic virus within the plant. *Ann. Appl. Biol.* 21:90–111.

- Schneider, H. 1968. Anatomy of greening-diseased sweet orange shoots. *Phytopathology* 58:262–266.
- Schwarz, R.E. 1970. Seasonal graft-transmissibility and quantification of gentisoyl glucoside marker of citrus greening in the bark of infected trees. *Phytophylactica* 2:115–120.
- Sechler, A., E.L. Schuenzel, P. Cooke, S. Donnua, N. Thaveechai, E. Postnikova, A.L. Stone, W.L. Schneider, V.D. Damsteegt, and N.W. Schaad. 2009. Cultivation of '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' associated with huanglongbing. *Phytopathology* 99:480–486.
- Shokrollah, H., T.L. Abdullah, K. Sijam, S.N.A. Abdullah, and N.A.P. Abdullah. 2009. Differential reaction of citrus species in Malaysia to huanglongbing (HLB) disease using grafting method. *Amer. J. Agr. Biol. Sci.* 4: 32–38.
- Stover, E. and G. McCollum. 2011. Incidence and severity of huanglongbing and *Candidatus Liberibacter asiaticus* titer among field-infected citrus cultivars. *HortScience* 46:1344–1348.
- Tatineni, S., U.S. Sagaram, S. Gowda, C.J. Robertson, W.O. Dawson, T. Iwanami, and N. Wang. 2008. In planta distribution of '*Candidatus Liberibacter asiaticus*' as revealed by polymerase chain reaction (PCR) and real-time PCR. *Phytopathology* 98:592–599.
- Teixeira, D.C., A.J. Ayres, E.W. Kitajima, F.A.O. Tanaka, J.L. Danet, S. Jagoueix-Eveillard, C. Saillard, and J.M. Bové. 2005. First report of a huanglongbing-like disease of citrus in Sao Paulo State, Brazil, and association of a new liberibacter species, '*Candidatus Liberibacter americanus*', with the disease. *Plant Dis.* 89:107.
- Trivedi, P., U.S. Sagaram, J.-S. Kim, R.H. Bransky, M.E. Rogers, L.L. Stelinski, C. Oswalt, and N. Wang. 2009. Quantification of viable *Candidatus Liberibacter asiaticus* in hosts using quantitative PCR with the aid of ethidium monoazide (EMA). *Eur. J. Plant Pathol.* 124:553–563.
- Van Vuuren, S.P. 1993. Variable transmission of African greening to sweet orange, p. 264–268. In: Moreno, P., J.V. da Graça, and L.W. Timmer (eds.). *Proc. 12th Conference of the International Organization of Citrus Virologists (IOCV)*. University of California, Riverside, CA.
- Watts, F.C. and D.L. Stankey. 1980. Soil survey of St. Lucie County area, Florida. U.S. Dept. of Agriculture, Soil Conservation Service, Washington, DC. <<http://ufdc.ufl.edu/UF00025728/00001>>.
- Xu, C.F., K.B. Li, C. Ke, and J.Z. Liao. 1985. On the transmission of citrus yellow shoot by psylla and observation with electron microscopy. *Acta Phytopathologica Sin.* 15:241–245.
- Xu, C.F., Y.H. Xia, K.B. Li, and C. Ke. 1988. Further study of the transmission of citrus huanglongbin by a psyllid, *Diaphorina citri* Kuwayama, p. 243–248. In: Timmer, L.W., S.M. Garnsey, and L. Navarro (eds.). *Proc. 10th Conference of the International Organization of Citrus Virologists (IOCV)*. University of California, Riverside, CA.
- Zou, X., D. Li, X. Luo, K. Luo, and Y. Pei. 2008. An improved procedure for *Agrobacterium*-mediated transformation of trifoliolate orange (*Poncirus trifoliata* L. Raf.) via indirect organogenesis. *In Vitro Cell. Dev. Biol. Plant* 44:169–177.