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Tolerance of the Trifoliate Citrus Hybrid US-897 (*Citrus reticulata* Blanco × *Poncirus trifoliata* L. Raf.) to Huanglongbing

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Abstract. **Huanglongbing (HLB) is a destructive disease of citrus in most citrus-producing countries worldwide. The disease, presumably caused by phloem-limited bacteria of the genus *Candidatus* Liberibacter, affects all known citrus species and citrus relatives with little known resistance. Typical disease symptoms are the production of abnormal-looking fruit and chlorosis or blotchy mottle of the leaves followed at advanced stages by tree decline and death. Trifoliate orange (*P. trifoliata* L. Raf.) and some of its hybrids reportedly lack distinct disease symptoms despite infection with the pathogen. US-897 is a hybrid of trifoliate orange and 'Cleopatra' mandarin (*C. reticulata* Blanco), the latter being highly susceptible to HLB. This study investigated whether field-grown, naturally infected trees and greenhouse-grown, graft-inoculated seedlings of this genotype display tolerance or resistance to HLB. It was shown that naturally infected US-897 trees exhibited no distinct disease symptoms commonly associated with HLB, except for the occurrence of few mottled leaves in a small percentage of trees. Analysis of fruit and seed from infected trees did not detect any growth reduction or otherwise negative impact on development. Graft-inoculated US-897 seedlings became polymerase chain reaction (PCR)-positive for the pathogen but exhibited a superior performance compared with 'Cleopatra' mandarin seedlings, which displayed severe disease symptoms soon after inoculation. Despite infection, most US-897 seedlings did not develop leaf symptoms typical for HLB. Foliar symptoms observed in a small number of plants at later stages of the disease were faint and difficult to discern. Contrary to 'Cleopatra' seedlings, growth in stem diameter was only moderately reduced or unaffected in infected US-897 seedlings. The superior performance of US-897 plants in greenhouse and field locations suggest tolerance of this genotype to *Ca.* L. asiaticus.**

Citrus HLB, often referred to as "citrus greening," is a destructive disease of citrus that is distributed throughout most citrus-producing countries worldwide, where it generates substantial economic losses (Bové, 2006). The suspected causal agents of HLB, *Candidatus* Liberibacter spp., are fastidious, phloem-inhabiting Gram-negative bacteria (Garnier et al., 1984; Tyler et al., 2009), which are difficult to obtain in pure culture (Davis et al., 2008; Sechler et al., 2009). Three different species of liberibacter are associated with HLB, of which the Asian species, *Ca.* L. asiaticus, is most severe and geographically widespread (Bové, 2006). The African species, *Ca.* L. africanus, and the American species, *Ca.* L. americanus, thus far are restricted to Africa and Brazil, respectively (Garnier et al., 2000; Teixeira et al., 2005). Liberibacters are transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama or the African citrus psyllid *Trioza erytrea* Del Guercio. Transmission can also occur through dodder (*Cuscuta* sp.) or grafting with diseased budwood (Halbert and Manjunath, 2004). Recent studies did not find evidence of transmission of viable bacteria through seed (Albrecht and Bowman, 2009; Hartung et al., 2010). The insect vector *D. citri* was found in Florida in 1998 (Halbert, 1998) followed by the discovery of HLB in South Florida in 2005 (Halbert, 2005). The disease has since become endemic in most parts of the state and is expected to spread to other citrus-producing regions in North America.

Fruit production on HLB-affected trees is reduced, and fruit are often small and misshapen with improper coloration and aborted seeds. Typical leaf symptoms of the disease are an asymmetric blotchy mottling of older leaves (McClean and Schwarz, 1970) and a range of chlorotic patterns, often resembling zinc-deficiency symptoms, which cause the typical appearance of yellow shoots in the tree canopy followed by twig-dieback and tree decline at advanced stages of the disease. Blockage of the translocation stream as a result of infection with liberibacters as demonstrated by Achor et al. (2010) and Schneider (1968) appears to be a major factor of HLB symptom development. The effect of *Ca.* L. asiaticus on transcriptional levels of genes associated with carbohydrate metabolism and other plant metabolic pathways was described by Albrecht and Bowman (2008) and Kim et al. (2009). While no known cure for HLB exists, management strategies include chemical control to reduce psyllid populations, removal of infected trees to eliminate new sources of bacterial inoculum, and the production of pathogen-free nursery plants.

Huanglongbing affects all known citrus species and citrus relatives with little known resistance. Sweet oranges, mandarins, and tangelos were found to be highly susceptible followed by grapefruit, lemon, sour orange, and other commercially important citrus varieties (Folimonova et al., 2009; Halbert and Manjunath, 2004; McClean and Schwarz, 1970; Miyakawa and Zhao, 1990). Citrus trees of the genotypes *C. indica* Tanaka and *C. macroptera* Mont. growing wild in northeastern India were reported to remain symptom-free under psyllid pressure (Bhagabati, 1993). Nariani (1981) identified sweet lime (*C. limetta* Risso) as resistant and several lemon cultivars (*C. limon* L.) as tolerant to HLB with no or only mild symptoms occurring after side-grafting with budwood from infected trees. Several lemon and lime cultivars were also identified as highly tolerant to HLB by Folimonova et al. (2009) after controlled inoculations with *Ca.* L. asiaticus.

No well-defined disease symptoms have been observed in trifoliate orange (*Poncirus trifoliata*) trees and seedlings after graft inoculation with buds from diseased citrus trees originating from South Africa and Taiwan, respectively (McClean and Schwarz, 1970; Miyakawa, 1980). Nariani (1981) observed moderate to severe disease symptoms on this genotype and its trifoliate hybrids 'Carrizo' citrange and 'Troyer' citrange in India (*C. sinensis* L. Osbeck × *P. trifoliata*). McClean and Schwarz (1970) reported that 'Troyer', although displaying some growth reduction, developed only faint or no of leaf symptoms upon infection. Although fruit crop and fruit appearance did not seem to be negatively affected by HLB, 'Troyer' was shown to produce an unusually high number of aborted seeds (McClean and Schwarz, 1970).

The USDA Picos Farm in Fort Pierce, FL, houses a large variety of citrus germplasm. HLB has been endemic at the Picos Farm since 2008 with up to 100% of trees recently confirmed PCR-positive for *Ca.* L. asiaticus in several sectors (personal observations). Whereas most trees at this location display noticeable HLB symptoms, trees of the genotype US-897, a hybrid of *C. reticulata* 'Cleopatra' × *P. trifoliata* released by the USDA (Bowman, 2007), are not visibly affected by

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the disease. The main objectives of this study were to compare infection rate and HLB disease development of field-grown, naturally infected US-897 trees and greenhouse-grown, artificially inoculated US-897 seedlings. Based on the initial field observations, we hypothesized that US-897 displays tolerance to HLB, particularly in comparison with the highly susceptible 'Cleopatra' mandarin, which was included in this study.

Materials and Methods

Field plants. Twenty trees with US-897 (*C. reticulata* 'Cleopatra' × *P. trifoliata*) scion, located at the USDA Picos Farm in Fort Pierce (St. Lucie County, FL), were available for sample collection and symptom evaluation. The trees, planted in Nov. 2004, are on different rootstock varieties and located in an area heavily affected by HLB. Two border trees of the genotype 'Hirado' pummelo (*C. grandis* L. Osbeck), which is susceptible to HLB, are located on the northern side of the US-897 plantings.

Three additional trees with US-897 scion on different rootstock varieties, located at the Whitmore Foundation Farm near Leesburg (Lake County, FL), were selected for fruit and seed comparison. The Whitmore Farm is located 260 km northwest of Fort Pierce and was unaffected by HLB at the time of collection.

Greenhouse plants. For the first experiment, 21 greenhouse-grown 15-month-old US-897 seedlings and 21 greenhouse-grown 15-month-old 'Cleopatra' mandarin (*C. reticulata*) seedlings were used. Fifteen plants of each genotype were inoculated in Dec. 2008 by grafting two bark or bud pieces and two leaf pieces from infected greenhouse-grown 'Valencia' plants, PCR-positive for *Ca.* L. asiaticus and symptomatic for HLB, onto each plant. Six plants of each genotype were inoculated with disease-free tissue pieces obtained from healthy greenhouse-grown 'Valencia' plants to be used as non-infected controls. Inoculations were performed pairwise to ensure that plants from both genotypes received tissue pieces from the same source. For the second experiment, 40 greenhouse-grown 9-month-old seedlings of both genotypes were inoculated in Nov. 2009 as described above. Twelve plants each were inoculated with disease-free tissue pieces and 28 plants were inoculated with infected tissue.

Plants were arranged randomly on the greenhouse bench and kept under natural light conditions at a temperature of 21 to 28 °C. Plants were irrigated as needed and fertilized every 4 weeks using a water-soluble fertilizer mix, 20N–10P–20K (Peters Professional; The Scotts Company, Marysville, OH). Plants were pruned immediately after graft inoculation to promote new leaf growth and enhance HLB disease symptom development. Plants from the first experiment were additionally pruned 24 weeks after inoculation (WAI). Stem diameters were measured at 9 cm (Expt. 1) or 7 cm (Expt. 2) above soil level and below the grafting site.

Evaluation of disease symptoms. Field plants were evaluated in Sept. 2007, Nov. 2008, June 2009, and Nov. 2009 based on the following ranking system: 1 = no apparent leaf symptoms, 2 = leaf symptoms restricted to less than five leaves in one area of the tree canopy, 3 = leaf symptoms extended to five or more leaves in one or more areas but on less than 10% of branches, 4 = extended leaf symptoms on 10% to 30% of branches, 5 = extended leaf symptoms on 30% to 60% of branches, and 6 = extended leaf symptoms on more than 60% of branches. Greenhouse plants were evaluated every 8 weeks (Expt. 1) or 6 weeks (Expt. 2) based on leaf symptoms using the following ranking system: 1 = no apparent leaf symptoms, 2 = less than 25% of all leaves symptomatic, 3 = 25% to 50% of all leaves symptomatic, 4 = 50% to 75% of all leaves symptomatic, and 5 = more than 75% of all leaves symptomatic. Leaf symptoms included yellowing, blotchy mottle, and reduced size combined with severe chlorosis at advanced stages of the disease.

Collection of fruit and seed extraction. Fruit from US-897 trees located at the USDA Picos Farm were collected in Nov. 2009. Four trees, displaying weak leaf symptoms attributed to HLB and consistently PCR-positive for *Ca.* L. asiaticus since Sept. 2007 or Nov. 2008, and four trees without any visible leaf symptoms and consistently PCR-negative before Nov. 2009 were selected for collection. No differences in size, shape, or coloration were observed between fruit from the two sets of trees (Fig. 1). Ten fruit were randomly collected from each tree, resulting in a total of 80 fruit. Fruit peduncles were severed for DNA extraction and PCR analysis. Ten fruit each from three trees located at the Whitmore Foundation Farm, unaffected by HLB, were also collected in Nov. 2009. Seeds were extracted from the fruit, soaked overnight in water containing 0.15 U/mL pectinase (Sigma-Aldrich, St. Louis, MO), rinsed with water, air-dried for 2 d, and weighed. Seeds were assigned to five different seed categories (cat), cat 1 = 0.0 to 9.9 mg, cat 2 = 10.0 to 49.9 mg, cat 3 = 50.0 to 99.9 mg, cat 4 = 100.0 to 149.9 mg, and cat 5 = 150.0 to 200 mg.

Polymerase chain reaction detection of Ca. L. asiaticus in leaves. Six to 10 fully expanded leaves were collected from each field tree in Sept. 2007, Nov. 2008, June 2009, and Nov. 2009. Four to six fully expanded leaves were collected from each greenhouse plant at 8, 16, 24, 32, 40, and 48 WAI (Expt. 1) and at 6, 12, 18, 24, and 30 WAI (Expt. 2). Petioles were severed from leaves and ground in liquid nitrogen with a mortar and pestle. One hundred milligrams 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. Real-time PCR assays were performed using primers HLBas (5′-TCGAGCGCGTATGCAATACG-3′) and HLBr (5′-GCGTTATCCCGTAGAAAAAG GTAG 3′) and probe HLBp (5′-AGACGGGT GAGTAACGCG-3′) developed by Li et al. (2006). Amplifications were performed 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 duplicate in a 20-uL reaction volume using 5 uL DNA. Plants were considered PCR-positive when cycle threshold (Ct) values were below 32. Additional PCR assays were performed on a subset of samples using primers COXf and COXr in combination with probe COXp (Li et al., 2006) to confirm uniformity of DNA preparations and loadings.

Polymerase chain reaction detection of Ca. L. asiaticus in peduncles. DNA was extracted from 2 × 2 × 5-mm peduncle pieces using solutions provided in the REDExtract-N-Amp™ Plant PCR Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. The small size of the peduncles prohibited efficient homogenization and DNA extraction with the method described for leaves. To increase DNA yield, ethanol precipitation was performed according to standard protocols, yielding a final concentration of

Fig. 1. US-897 trees located at the USDA Picos Farm in Fort Pierce polymerase chain reaction (PCR)-negative for *Ca.* L. asiaticus through Nov. 2009 (**A**) and PCR-positive since Nov. 2008 (**B**).

0.3 to 0.6 ng DNA per extraction. Real-time PCR amplifications were performed as described previously.

Statistical analyses. Analysis of variance was performed to determine the effect of *Ca.* L. asiaticus infection on fruit weight, seed number, and seed weight of field trees and on stem diameter increase of greenhouse-grown citrus seedlings followed by multiple comparisons of means using the Tukey's honestly significant difference procedure. All analyses were performed using STATISTICA Version 6.0 (StatSoft, Tulsa, OK).

Results

Field-grown trees

PCR analysis of US-897 trees located at the USDA Picos Farm in Fort Pierce identified one tree (5%) as positive for *Ca.* L. asiaticus in Sept. 2007 (Table 1). In Nov. 2008, seven trees (35%) were PCR-positive, although only one tree displayed a faint mottle on few leaves resembling the blotchy mottle typical for HLB. The average Ct values of positive trees was 25.7. Similar to the previous collection, 35% of trees were identified as positive in June 2009 with three trees (15%) displaying a few lightly mottled leaves. In Nov. 2009, most trees were PCR-positive for *Ca.* L. asiaticus with an average Ct value of 23.9. A faint and difficult to discern mottling of leaves resembling HLB disease symptoms was detected in 30% of the trees but was restricted to a few leaves located on the southeastern edge of the canopy and comprising less than 10% of all branches. In general, trees appeared to be healthy and exhibited a full canopy without noticeable leaf chlorosis or twig dieback. Border trees of the genotype 'Hirado' pummelo displayed conspicuous foliar symptoms (blotchy mottle and leaf chlorosis) throughout the canopy and were PCR-positive at all collection times beginning Sept. 2007.

Fruit and seed evaluation

Fruit production on field-grown US-897 trees was excellent throughout the experiment regardless of infection with *Ca.* L. asiaticus. Fruit from symptomatic trees at the Picos Farm in Fort Pierce and consistently PCR-positive since Sept. 2007/Nov. 2008 did not appear to be different compared with fruit from symptomless trees at the same location (Fig. 1). Peduncles of 63% of the fruit collected from symptomatic trees yielded Ct values below 35 after PCR analysis compared with 28% of fruit collected from symptomless trees. Fruit weight and average seed weight did not differ significantly ($P > 0.05$) between symptomatic and symptomless trees located at the Fort Pierce location (Table 2). Fruit from non-infected trees at the Leesburg location were significantly larger than those from the Fort Pierce Picos Farm, whereas the average seed weight per fruit was significantly lower. The number of seeds per fruit and total seed weight did not differ significantly ($P >$ 0.05) between symptomless and symptomatic trees at the Picos Farm and non-infected trees at the Leesburg Farm. The percentage of seeds was significantly different ($P < 0.05$) between the treatments in four of the five weight categories (Table 3). However, significant differences between symptomless and symptomatic trees at the Fort Pierce location were only found for the largest seeds, which were most numerous in symptomatic fruit (3.7%). Symptomatic fruit also contained the highest number of seeds in the second largest seed category (29.4%). Seeds in the lowest weight category were most abundant in non-infected trees at the Whitmore location (24.3%).

Greenhouse-grown seedlings

Expt. 1. None of the *Ca.* L. asiaticus-inoculated US-897 seedlings displayed any distinct leaf symptoms throughout 32 WAI, although 73% of plants were PCR-positive at that time (Table 4). By the end of the experiment, 93% of the US-897 seedlings were PCR-positive and 18% displayed a faint blotchy mottling in a small percentage of leaves (Fig. 2). The average symptom rank at this time was 1.3, signifying the low degree of symptom expression in this genotype. On the contrary, disease symptom development of 'Cleopatra' seedlings started 16 WAI, at which time 53% were PCR-positive for *Ca.* L. asiaticus (Table 4). At 32 WAI, most 'Cleopatra' plants were noticeably symptomatic and PCR-positive. The average symptom rank of these plants at the end of the experiment was 3.9, meaning that a large proportion of leaves were visually affected by HLB. 'Cleopatra' leaf symptoms generally progressed from an initial yellowing of the leaves to blotchy mottling followed by severe chlorosis and size reduction at advanced stages of the disease. Foliar symptoms were often accompanied by severe stunting of the plants (Fig. 3). No stunting was observed for infected US-897 seedlings. Non-infected control plants of both genotypes did not display any disease symptoms or other growth abnormalities throughout the experiment.

Ct values were generally higher in US-897 seedlings than in 'Cleopatra' seedlings by three or more cycle numbers throughout the study, suggesting lower bacterial levels in this genotype. At 32 WAI, 8 weeks after the second pruning, Ct values were higher in PCR-positive plants from both genotypes compared with Ct values at 24 WAI. Differences were more pronounced in US-897 seedlings. Non-infected control plants were PCR-negative at all time points.

Stem diameters of US-897 seedlings increased by 6% to 8% at 24 WAI and growth was not significantly ($P > 0.05$) different between non-infected and *Ca.* L. asiaticus-infected plants (Fig. 4). Stem growth of 'Cleopatra' seedlings at this time was significantly ($P < 0.05$) lower in infected plants (16% to 20%) compared with non-infected plants (29%). At the end of the experiment, stem diameters of all infected US-897 seedlings had increased by 29%, which was not significantly different from non-infected plants (36%). Only US-897 plants infected since 24 WAI grew significantly less (23%) compared with non-infected plants. On the contrary, stem growth of all 'Cleopatra' seedlings infected at 48 WAI was significantly reduced (30%) compared with non-infected plants (56%). Stem growth of 'Cleopatra' was lowest in plants infected at 24 WAI.

Table 1. Disease symptom development and real-time polymerase chain reaction (PCR) analysis of field-grown US-897 trees naturally inoculated with *Ca.* L. asiaticus.[z]

	Sept. 2007	Nov. 2008	June 2009	Nov. 2009
Percentage of symptomatic trees	—	5	15	30
Symptom type	—	BM	BM	BM
Average symptom rank of symptomatic trees	—	2.0	3.0	2.8
Percentage of PCR-positive trees	5	35	35	85
Average Ct value of PCR-positive trees	N/A	25.7	24.3	23.9
Average symptom rank of PCR-positive trees	—	1.2	1.3	1.6

[z]Symptom ranks were assigned from 1 (no leaf symptoms) to 6 (extended leaf symptoms on more than 60% of branches). Plants were considered PCR-positive when Ct values were below 32.
BM = blotchy mottled leaves; N/A = PCR results are based on conventional PCR according to Albrecht and Bowman (2009) with signal intensities equivalent to Ct less than 32.0.
Ct = cycle threshold.

Table 2. Fruit weight, number of seeds per fruit, and seed weight of fruit from non-infected and *Ca.* L. asiaticus-infected US-897 trees.[z]

Tree type	No.	Fruit wt (g)	Number of seeds per fruit	Total seed wt per fruit (g)	Avg seed wt (mg)
Non-infected[y]	3	62.0 ± 5.7 a	25.2 ± 2.4 a	1.50 ± 0.27 a	58.4 ± 3.6 b
Symptomless and PCR-negative before Nov. 2009[x]	4	44.1 ± 1.2 b	28.8 ± 0.5 a	1.96 ± 0.07 a	68.8 ± 2.1 a
Symptomatic and PCR-positive since Sept. 2007/Nov. 2008[x]	4	46.4 ± 3.6 b	26.8 ± 1.1 a	2.02 ± 0.07 a	76.0 ± 1.2 a
		$P = 0.01944$	$P = 0.23444$	$P = 0.07023$	$P = 0.00231$

[z]Ten fruit were collected from each tree.
[y]Trees located at the Whitmore Foundation Farm in Leesburg (Lake County, FL).
[x]Infected trees; located at the USDA Picos Farm in Fort Pierce (St. Lucie County, FL). Symptoms refer to leaves only. Numbers in each column followed by different letters are significantly different according to Tukey's honestly significant difference test ($P < 0.05$).
No. = number of trees; PCR = polymerase chain reaction.

Table 3. Percentage of seeds in different weight categories (cat) in fruit from non-infected and *Ca.* L. asiaticus-infected US-897 trees.[z]

Tree type	No.	Percentage of seeds: Cat 1 (0.0–9.9 mg)	Cat 2 (10.0–49.9 mg)	Cat 3 (50.0–99.9 mg)	Cat 4 (100.0–149.9 mg)	Cat 5 (150.0–200 mg)
Non-infected[y]	3	24.3 ± 4.0 a	19.7 ± 5.7 a	35.2 ± 2.0 b	19.2 ± 1.6 b	1.6 ± 0.6 b
Symptomless and PCR-negative before Nov. 2009[x]	4	16.7 ± 2.1 ab	15.1 ± 0.6 a	44.5 ± 2.5 a	22.9 ± 3.5 ab	0.9 ± 0.3 b
Symptomatic and PCR-positive since Sept. 2007/ Nov. 2008[x]	4	14.6 ± 0.6 b	11.9 ± 0.6 a	40.5 ± 0.5 ab	29.4 ± 1.0 a	3.7 ± 0.3 a
		$P = 0.05151$	$P = 0.19410$	$P = 0.02757$	$P = 0.05131$	$P = 0.00169$

[z]Ten fruit were collected from each tree.
[y]Trees located at the Whitmore Foundation Farm in Leesburg (Lake County, FL).
[x]Infected trees; located at the USDA Picos Farm in Fort Pierce (St. Lucie County, FL). Symptoms refer to leaves only. Numbers in each column followed by different letters are significantly different according to Tukey's honestly significant difference test ($P < 0.05$). N = number of trees.
PCR = polymerase chain reaction.

Table 4. Disease symptom development and real-time polymerase chain reaction (PCR) analysis of US-897 and 'Cleopatra' mandarin seedlings from Expt. 1 after artificial inoculation with *Ca.* L. asiaticus.[z]

	8 WAI	16 WAI	24 WAI[y]	32 WAI	40 WAI	48 WAI
US-897						
Percentage of symptomatic plants	0	0	0	0	6.7	17.9
Symptom type	—	—	—	—	BM	BM
Average symptom rank of symptomatic trees	—	—	—	—	2.0	2.0
Percentage of PCR-positive plants	6.7	33.3	53.3	73.3	93.3	93.3
Average Ct value of PCR-positive plants	29.7	28.0	24.2	28.2	28.7	24.0
Average symptom rank of PCR-positive plants	1.0	1.0	1.0	1.0	1.1	1.3
'Cleopatra' mandarin						
Percentage of symptomatic plants	0	53.3	53.3	66.7	86.7	86.7
Symptom type	—	YL	YL, BM	YL, BM, CL, ST	YL, BM, CL, ST	YL, BM, CL, ST
Average symptom rank of symptomatic plants	—	2.0	3.6	4.1	3.5	3.9
Percentage of PCR- positive plants	13.3	53.3	60.0	80.0	80.0	86.7
Average Ct value of PCR-positive plants	30.5	22.3	21.7	23.7	24.8	21.4
Average symptom rank of PCR-positive plants	1.0	2.0	3.3	3.0	3.5	3.9

[z]Fifteen plants each of US-897 and 'Cleopatra' mandarin were graft-inoculated with *Ca.* L. asiaticus-infected bark and leaf pieces. Symptom ranks were assigned from 1 (no leaf symptoms) to 5 (more than 75% of all leaves symptomatic). Plants were considered PCR-positive when Ct values were below 32.
[y]Plants were cut back 24 WAI after symptom evaluation and sample collection.
WAI = weeks after inoculation; Ct = cycle threshold; BM = blotchy mottled leaves; CL = small, chlorotic leaves, ST = stunting; YL = yellow leaves.

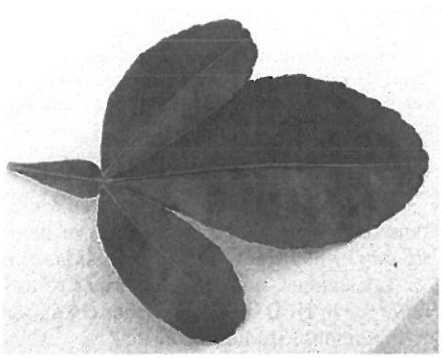

Fig. 2. US-897 leaf with blotchy mottle 48 weeks after inoculation with *Ca.* L. asiaticus.

Expt. 2. None of the US-897 seedlings displayed any distinct leaf symptoms clearly associated with HLB throughout the experiment (Table 5). A small percentage of leaves of several infected plants displayed chlorosis and vein corking. However, the same was observed for non-infected, PCR-negative plants, suggesting that these symptoms were caused by senescence, nutritional deficiencies, or other factors, but not by infection with *Ca.* L. asiaticus. At the end of the experiment (30 WAI), 43% of *Ca.* L. asiaticus-inoculated US-897 seedlings were PCR-positive for the pathogen. Sixty-eight percent of 'Cleopatra' seedlings were PCR-positive at 12 WAI, although no disease symptoms were visible at that time (Table 5). Foliar disease symptom development in these plants started at 18 WAI, at which time leaves turned yellow. At the end of the experiment, 75% of 'Cleopatra' plants were PCR-positive and noticeably symptomatic, displaying severe yellowing, vein corking, and occasional blotchy mottle. The average symptom rank at this time was 4.6, meaning that most leaves were affected by HLB. Non-infected control plants did not display any disease symptoms or other growth abnormalities throughout the experiment.

Similar to the first experiment, Ct values were generally higher in US-897 seedlings by two or more cycle numbers, suggesting lower bacterial levels in this genotype compared with 'Cleopatra'. Non-infected control plants were PCR-negative at all time points.

Stem diameters of US-897 seedlings increased by 19% to 21% from the beginning to the end of the experiment and growth was not significantly ($P > 0.05$) different between non-infected and *Ca.* L. asiaticus-infected plants (Fig. 5). Stem growth of 'Cleopatra' seedlings was significantly ($P < 0.05$) lower in infected plants (65%) compared with non-infected plants (107%) over the same time period.

Discussion

Identification of resistant citrus cultivars is an important step in combating HLB, arguably the most destructive citrus disease found in the United States. Unfortunately, most of the commercially important cultivars as well as many citrus relatives are susceptible to the disease, which is characterized by its negative impact on fruit development and yield and tree decline within a few years after infection. In addition, susceptible plants generally display leaf symptoms such as blotchy mottle and chlorosis upon infection with liberibacters, although varietal differences have been observed (Folimonova et al., 2009; Lopes and Frare, 2008; McClean and Schwarz, 1970; Shokrollah et al., 2009). Trifoliate orange (*P. trifoliata*) and some of its hybrids are among the few genotypes reported, in which distinct disease symptoms are often not apparent despite infection (McClean and Schwarz, 1970; Miyakawa, 1980). We observed that trees of the genotype US-897, a hybrid resulting from a cross between trifoliate orange and the highly susceptible 'Cleopatra' mandarin, located at the USDA Picos Farm in Fort Pierce, looked exceptionally healthy despite severe pressure from HLB. Molecular analyses of these trees using real-time PCR identified 35% as positive for *Ca.* L. asiaticus, the suspected causal agent of HLB in Florida, in Nov. 2008 and June 2009. Most of these trees did not display any disease symptoms, contrary to observations of abundant HLB symptoms on adjacent trees of different genotypes. Although most US-897 trees were confirmed PCR-positive by Nov. 2009, leaf symptoms

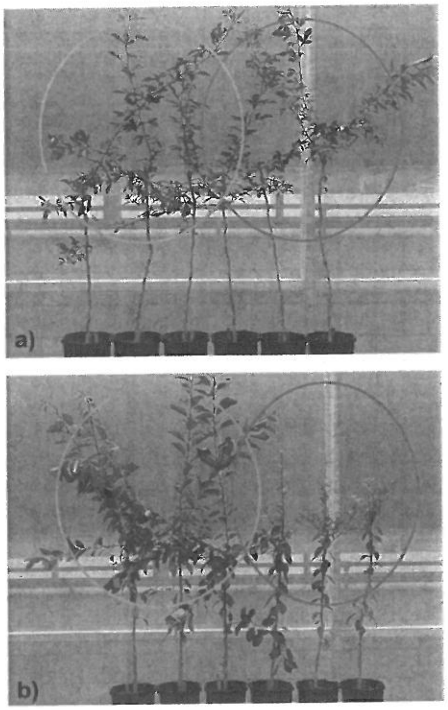

Fig. 3. US-897seedlings (**A**) and 'Cleopatra' mandarin seedlings (**B**) non-infected (green circles) and infected (red circles) with *Ca.* L. asiaticus 32 weeks after inoculation (Expt. 1). Whereas US-897 seedlings did not appear to be affected by huanglongbing, 'Cleopatra' seedlings displayed severe stunting along with yellowing of the leaves.

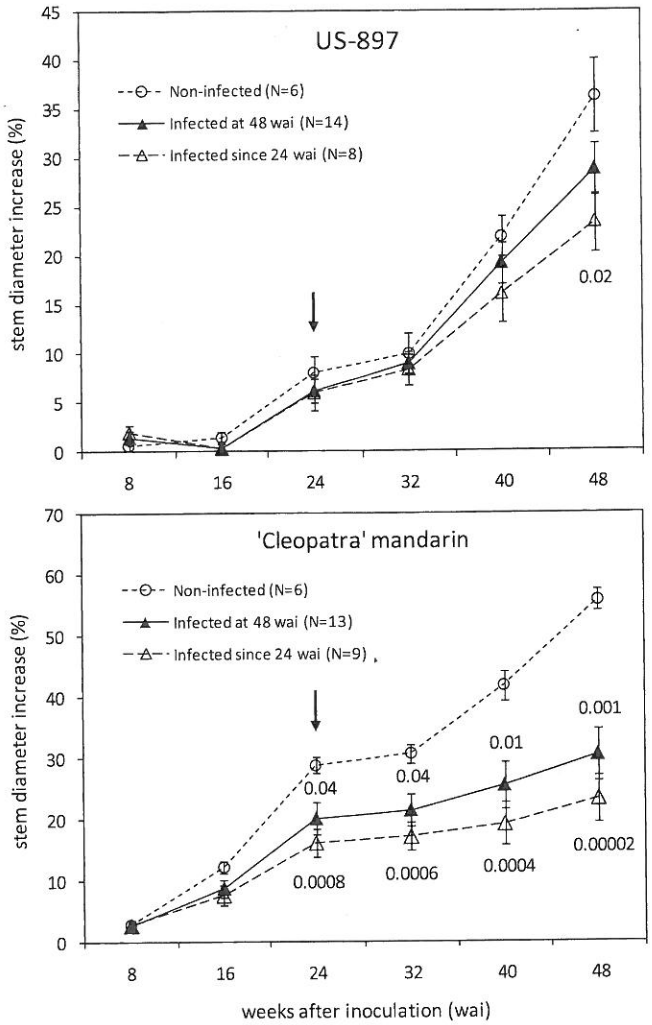

Fig. 4. Stem diameter increase of US-897 and 'Cleopatra' mandarin seedlings non-infected and infected with *Ca.* L. asiaticus from Dec. 2008 to Nov. 2009 (Expt. 1). Vertical bars on data points are ± SE of the mean. *P* values for significant ($P < 0.05$) differences between infected and non-infected plants are indicated. Plants were cut back at 24 weeks after inoculation (WAI) (↓).

were difficult to discern, observed in less than one-third of the trees and restricted to small areas in the tree canopy.

Conflicting observations regarding HLB symptom development in trifoliate genotypes exist. Whereas McClean and Schwarz (1970) and Miyakawa (1980) did not discern any well-defined disease symptoms in trifoliate orange or its hybrids, Nariani (1981) observed moderate to severe disease symptoms in these genotypes. Folimonova et al. (2009) reported inconsistent results for *P. trifoliata* after graft inoculation with *Ca.* L. asiaticus. Trifoliates and their hybrids are deciduous or semideciduous trees with leaf chlorosis preceding an annual leaf drop. As a result, symptoms caused by leaf senescence may be mistaken for symptoms related to infection with liberibacters. Growth and leaf phenotype of trifoliates are also often affected by imbalances of the nutritional state, further complicating symptom detection (personal observation).

In addition to the absence of pronounced foliar disease symptoms, fruit on US-897 did not seem to be affected by HLB. Comparison of fruit and seeds from trees consistently PCR-positive for 1 or more years with those from only recently infected trees did not reveal any significant differences regarding fruit weight, number of seeds per fruit, total seed weight, or average seed weight. On the contrary, seed weight was found to be significantly reduced in various non-trifoliate citrus cultivars upon infection with *Ca.* L. asiaticus (Albrecht and Bowman, 2009). The average weight of fruit from non-infected trees at a field location near Orlando, currently unaffected by HLB, was significantly larger, whereas seeds were smaller, which may have been the result of different environmental conditions. Surprisingly, the percentage of seeds belonging to the smallest weight category was lowest in seeds from trees infected for the longest time. Moreover, the percentage of seeds in the largest weight category was highest in these trees. Slight variations of rootstock types may have contributed to these differences. However, this study did not reveal any trend for high seed abortion in infected trees as was reported by McClean and Schwarz (1970), who found that 73% of seeds were aborted in fruit from infected 'Troyer' citrange trees from South African field locations despite the absence of apparent disease symptoms on fruit and leaves. Fruit from healthy 'Troyer' trees contained 15% of aborted seeds, which is similar to the percentage of seeds from infected US-897 trees belonging to the smallest weight category in the present study.

The lack of prominent HLB-related symptoms or other growth abnormalities in field-grown US-897 trees, despite infection with *Ca.* L. asiaticus, suggests a tolerance of this genotype to HLB that was further examined by greenhouse studies.

Graft inoculation of US-897 seedlings under controlled conditions in the greenhouse confirmed the observations on field-grown, naturally infected US-897 trees, indicating that tolerance of this genotype is not related to different flushing patterns or feeding preference of the insect vectors. Thirty to 32 weeks into the greenhouse experiments, none of the plants developed any disease symptoms typical for HLB, although the majority of plants were PCR-positive for *Ca.* L. asiaticus. In those same experiments, symptom development on leaves of infected 'Cleopatra' mandarin seedlings started 16 to 18 WAI, at which time leaves began to turn yellow. By the end of the experiment, 'Cleopatra' leaf symptoms progressed to severe chlorosis and occasional blotchy mottle and were often accompanied by severe stunting of the plant. Blotchy mottle was detected in a small percentage of infected

Table 5. Disease symptom development and real-time polymerase chain reaction (PCR) analysis of US-897 and 'Cleopatra' mandarin seedlings from Expt. 2 after artificial inoculation with *Ca.* L. asiaticus.[z]

	6 WAI	12 WAI	18 WAI	24 WAI	30 WAI
US-897					
Percentage of symptomatic plants	0	0	0	0	0
Symptom type	—	—	—	—	(VC,[y] CL[y])
Average symptom rank of symptomatic trees	—	—	—	—	—
Percentage of PCR- positive plants	0	3.6	14.3	28.6	42.9
Average Ct value of PCR-positive plants	—	29.0	23.5	24.2	24.6
Average symptom rank of PCR-positive plants	—	1.0	1.0	1.0	1.0
'Cleopatra' mandarin					
Percentage of symptomatic plants	0	0	67.9	71.4	75.0
Symptom type	—	—	YL	YL, BM[x]	YL, VC, BM[x]
Average symptom rank of symptomatic plants	—	—	3.5	3.8	4.6
Percentage of PCR- positive plants	0	67.9	67.9	71.4	75.0
Average Ct value of PCR-positive plants	—	24.7	21.6	21.6	21.1
Average symptom rank of PCR-positive plants	—	1.0	3.5	3.7	4.6

[z]Twenty-eight plants each of US-897 and 'Cleopatra' mandarin were graft-inoculated with *Ca.* L. asiaticus-infected bark and leaf pieces. Symptom ranks were assigned from 1 (no leaf symptoms) to 5 (more than 75% of all leaves symptomatic). Plants were considered PCR-positive when Ct values were below 32.

[y]Identical symptoms were observed in non-infected, PCR-negative plants.

[x]Blotchy mottle was limited to one or two plants.

WAI = weeks after inoculation; Ct = cycle threshold; BM = blotchy mottled leaves; CL = small, chlorotic leaves; VC = vein corking; YL = yellow leaves.

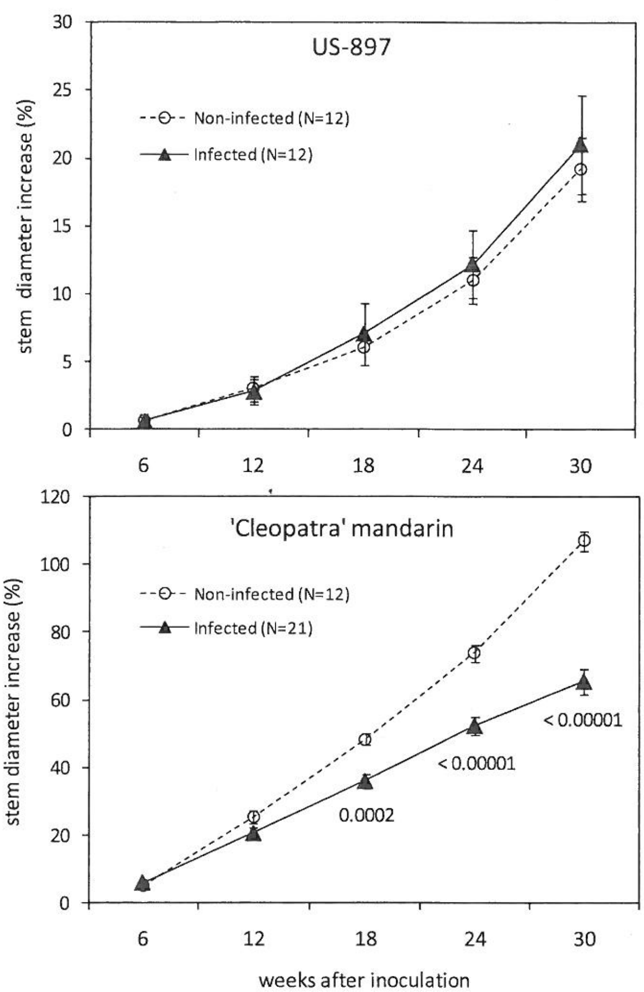

Fig. 5. Stem diameter increase of US-897 and 'Cleopatra' mandarin seedlings non-infected and infected with *Ca.* L. asiaticus from Dec. 2009 to June 2010 (Expt. 2). Vertical bars on data points are ± SE of the mean. *P* values for significant ($P < 0.05$) differences between infected and non-infected plants are indicated.

US-897 seedlings toward the end of the first experiment but was restricted to a few leaves only. Leaf chlorosis and vein corking, sometimes observed in infected and non-infected US-897 seedlings during the second experiment, were likely associated with senescence or other factors and not with HLB.

Non-infected US-897 seedlings typically grow more slowly under greenhouse conditions than non-infected seedlings of 'Cleopatra'. In accordance with the strong foliar symptom expression after inoculation with *Ca.* L. asiaticus, growth of infected 'Cleopatra' seedlings as measured in stem diameter increase was significantly lower compared with non-infected seedlings. Growth reductions of infected US-897 seedlings were not significant or less severe depending on the experiment. In a previous study, 'Troyer' seedlings graft-inoculated with the African strain of liberibacter grew more slowly than untreated controls and only few leaves showed slight mottling (McClean and Schwarz, 1970); however, only two plants were used in that study.

Although the transmission efficiency of *Ca.* L. asiaticus was similarly high (87% to 93%) for US-897 and 'Cleopatra' mandarin seedlings in the first experiment, the rate at which plants became infected was slower in US-897. In Expt. 2, the transmission efficiency was 43% for US-897 seedlings compared with 75% for 'Cleopatra' seedlings and the rate of infection was slower in US-897. Lopes and Frare (2008) observed variation in transmission of *Ca.* L. americanus among different citrus cultivars, whereas Folimonova et al. (2009) observed no variation for *Ca.* L. asiaticus. Time of year and other environmental as well as physiological conditions of the plants may play a role in the different transmission efficiencies observed in individual experiments.

Ct values obtained after real-time PCR analysis of leaves from US-897 and 'Cleopatra' seedlings generally progressed from higher values soon after inoculation to lower values toward the end of the experiments. Complete cutback of plants midway through Expt. 1 only temporarily increased Ct values, indicating that bacterial levels, although initially lower in the newly developing leaves, were re-established to previous levels within several weeks. This observation suggests that pruning as a form of HLB management does not appear to be effective once the bacterium is spread systemically throughout the plant. The ineffectiveness of pruning to control HLB was previously demonstrated for citrus trees infected with *Ca.* L. americanus (Lopes et al., 2007). However, Ct values for US-897 leaves were higher compared with Ct values for 'Cleopatra' leaves throughout both experiments, implying that bacterial populations were lower in the former. This suggests that tolerance of US-897 may be expressed through suppression of bacterial multiplication in the infected tissue. Similarly, Folimonova et al. (2009) did not observe a substantial multiplication of *Ca.* L. asiaticus nor an obvious host response in graft-inoculated 'Carrizo' citrange seedlings.

Poncirus trifoliata and some of its hybrids are known for their resistance to citrus tristeza virus, a phloem-limited pathogen causing phloem necrosis in susceptible rootstocks (Garnsey et al., 1987). Although the mechanism of resistance is still unknown, resistance appears to be associated with the failure of the virus to spread over long distances despite its ability to replicate in the host (Albiach-Marti et al., 2004). In addition, extracts from trifoliate orange have been widely used in folk medicine, and several antibacterial compounds have been identified from fruit and seed (Kim et al., 1999; Rahman et al., 2009). Similar compounds restricting liberibacter development or movement may be present in the phloem, causing resistance or tolerance to HLB in *P. trifoliata* and its hybrids. Whether tolerance to HLB can be imparted to susceptible scions grafted onto these genotypes is currently being investigated in our laboratory.

In summary, field-grown and naturally infected US-897 trees as well as greenhouse-grown graft-inoculated US-897 seedlings exhibited a superior performance in response to infection with *Ca.* L. asiaticus compared with susceptible genotypes. Tolerance is likely not related to lower psyllid pressure or reduced feeding preference of the insects but may possibly be conferred through some antibacterial or other compound(s) associated with the *P. trifoliata* parentage. Identifying these compounds or other mechanisms of defense in trifoliate orange and its hybrids might yield a strategy to combat HLB and will be pursued in future studies.

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