Graft Transmission Efficiencies and Multiplication of '*Candidatus Liberibacter americanus*' and '*Ca. Liberibacter asiaticus*' in Citrus Plants

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ABSTRACT

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In Brazil '*Candidatus Liberibacter asiaticus*' and '*Ca. L. americanus*' cause huanglongbing (also known as greening), the most destructive citrus disease. A shift in pathogen prevalence was observed over time, with a disproportional increase in '*Ca. L. asiaticus*' occurrence. Graft transmission experiments were used for a comparative study of both species using budsticks from symptomatic branches of field-affected trees as inoculum. The plants were inoculated with '*Ca. L. asiaticus*' or '*Ca. L. americanus*' alone, or simultaneously with both species. Symptom manifestation and conventional and quantitative real-time polymerase chain

reaction were used for plant evaluations. '*Ca. L. americanus*' was detected mainly in symptomatic plants and '*Ca. L. asiaticus*' was detected in symptomatic plants as well as in infected plants prior to symptom manifestation. Transmission percentages varied from 54.7 to 88.0% for '*Ca. L. asiaticus*' and 10.0 to 45.2% for '*Ca. L. americanus*' in two experiments. In co-inoculated plants, 12.9% contained '*Ca. L. americanus*' only, 40.3% contained '*Ca. L. asiaticus*' only, and 19.3% contained both species. Average bacterial titers for '*Ca. L. asiaticus*' and '*Ca. L. americanus*', in log cells per gram of leaf midrib, were 6.42 and 4.87 for the experimental plants and 6.67 and 5.74 for the field trees used as the source of inoculum. The higher bacterial populations of the '*Ca. L. asiaticus*'-infected plants provided an explanation for the disproportional increase in field prevalence of this species over time, based on the greater likelihood for pathogen transmission by the insect vector.

'*Candidatus Liberibacter asiaticus*' and '*Ca. L. americanus*' cause citrus huanglongbing disease (HLB) in Brazil (2,20). The pathogens colonize the phloem (9), are disseminated in the field by the psyllid *Diaphorina citri* (1,24), and can affect all commercial cultivars of mandarin and sweet orange (12), regardless of rootstock and age of the trees. The pathogens also have been detected in *Murraya paniculata* (orange jasmine) trees in rural and urban areas (14,15) but their importance as reservoirs of inoculum to citrus trees has not yet been elucidated.

Citrus HLB is a destructive disease. Symptoms include mottled leaves, deformed fruit, aborted seed, leaf and fruit drop, and shoot dieback (3). In the presence of sources of inoculum and the insect vector, the increase of incidence of newly symptomatic trees in affected areas can be dramatic. In a 2-year-old orchard surrounded by heavily affected adult trees, the disease incidence increased from 0.6 to 27.4% in 9 to 10 months (5). The disease was first reported in March 2004 in two municipalities and, as of August 2008, has been reported in 208 municipalities of São Paulo, Minas Gerais, and Paraná States. HLB management has been conducted through the continuous removal of symptomatic trees to reduce sources of inoculum and constant applications of insecticides to reduce vector populations (21). Low disease incidence and absence of other affected areas in the vicinities are factors that, in some farms, have contributed decisively to the success of the control.

Since the report on the presence of HLB in the State of São Paulo in 2004, Fundecitrus has engaged in an HLB suppression program by carrying out field surveys, assisting the growers through training to recognize HLB symptoms and learn about control measures, and performing free diagnostic polymerase chain reaction (PCR) tests. Analysis of the laboratory data has shown a change in the prevalence of '*Liberibacter* spp.', with a decrease in '*Ca. L. americanus*' occurrence from 98 to 20% in less than 4 years (13). This change in pathogen prevalence also has been observed in specific farms.

Since 2004, several graft inoculation experiments have been carried out in our greenhouses. Recently, the conditions favorable for '*Ca. L. americanus*' transmission, the reaction of multiple citrus species to the disease, and the efficiency of pathogen propagation from individual buds were determined (12). Here, we present the results of a comparative study that demonstrates that '*Ca. L. americanus*' and '*Ca. L. asiaticus*' differ in graft transmission efficiency and in the concentration of bacterial cells that can be attained in graft- and vector-inoculated citrus plants.

MATERIALS AND METHODS

Plants, sources of inoculum, and inoculations. Experiments were conducted in greenhouses essentially as described previously (12). Inoculated plants were 6-month-old sweet orange (*Citrus sinensis*) plants of 'Hamlin', 'Natal', 'Pera', and 'Valencia' (experiment 1) or 'Natal' (experiments 2 and 3), all grafted on Rangpur lime rootstock (*C. limonia*). All plants were produced in 4-liter plastic bags in screened nurseries free of psyllid vectors and fertilized as necessary. The sources of inoculum for experiment 1 were symptomatic branches of 10 5-year-old Valencia

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sweet orange trees from a farm located in Descalvado, São Paulo, and the sources of inoculum for experiments 2 and 3 were symptomatic branches of 10 4-year-old Natal sweet orange trees from a farm located in Matão, São Paulo. The plants were affected by '*Ca. L. americanus*' or '*Ca. L. asiaticus*' as previously determined by conventional PCR at Fundecitrus and, later, by quantitative real-time PCR (qPCR) carried out at the Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain. Branches of approximately 1.5 cm in diameter were removed from the trees, cut into 2- or 4-cm-long budsticks, and grafted on opposite sides of the scion of the experimental plants, 10 to 15 cm above the bud union. In order to use similar amounts of affected tissues as sources of each '*Liberibacter* spp.', the plants inoculated with only one species received two 2-cm long budsticks, and those inoculated with both species received two 4-cm long budsticks. The controls received budsticks from healthy trees maintained in screened greenhouses. Experiment 1 started in March 2006 with the objective of assessing '*Liberibacter* spp.' aggressiveness in the four most important Brazilian sweet orange cultivars. It contained 30 inoculated plants plus 5 controls of each citrus cultivar. Those plants, for which the budstick died, were regrafted in June 2006 with inoculum from trees of the same original blocks. Experiment 2 started in May 2006 with the objective of assessing '*Liberibacter* spp.' multiplication in single and co-infected Natal plants. It contained 80 plants for each treatment plus 10 controls that were grafted once. Experiment 3 started in June 2007 with the objective of assessing symptom and infection progress in single and co-infected Natal plants. It included groups of 10 plants (5 symptomatic and 5 asymptomatic) selected 5 months after inoculation from a set of 30 plants inoculated with one or both '*Liberibacter* spp.'

Plant evaluations. The experimental plants were observed regularly for symptom development where the presence of blotchy mottled leaves and leaves with symptoms similar to those caused by mineral deficiencies were recorded and photographed. Field trees and experimental plants were also evaluated by conventional and qPCR of total DNA extracted from 0.5 g of leaf midribs using a modified version of the cetyltrimethylammonium bromide method of Murray and Thompson (17) as described by Teixeira et al (18). The leaves of the field trees that served as sources of inoculum were collected just prior to collection of tissue for graft inoculations, and the leaves of the experimental plants were collected at 12 months (experiments 1 and 2) or 5, 6, and 7 months (experiment 3) after inoculation. Yield and purity of the DNA samples were estimated with a Lambda EZ 201 spectrophotometer (Perkin Elmer). The concentration was adjusted to 10 ng/μl for qPCR assays. All DNA samples were kept at -20°C. Conventional PCR assays included the two primer pairs GB1/GB3 and A2/J5, routinely used at Fundecitrus for detection of '*Ca. L. americanus*' (18) and '*Ca. L. asiaticus*' (7), respectively. Although GB1/GB3 targets the 16S rDNA region, A2/J5 targets the β operon of ribosomal proteins. PCR reagents and amplification conditions were as described (18). qPCR assays included the newly developed *rpl*-primer pairs *f-rp/LAs/r-rp/LAs* and *f-rp/IJAm/r-rp/IJAm* for detection and quantification of '*Ca. L. asiaticus*' and '*Ca. L. americanus*', respectively (19). In '*Liberibacter* spp.', as in other eubacteria (16), the *rplKAJL* gene cluster is linked to the *rpoBC* operon, and occurs as single-copy genes (22) (Wulff et al., unpublished data). qPCR assays were performed in a StepOne Plus thermocycler (Applied Biosystems). The reaction cocktail consisted of 25 μl of reaction mixture containing 1× Power SYBR green master mix (Applied Biosystems), 0.15 μM each primer, and 50 ng of total DNA. In order to obtain high endpoint fluorescence and low cycle thresholds, the annealing temperature had to be optimized. Final amplification conditions consisted of one step at 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 63°C for 1 min for both '*Liberibacter* spp.' To obtain the melting curve data, after the PCR

reaction, the temperature increased by 0.5°C every 10 s from 69 to 95°C with continuous measurement of fluorescence. Data acquisition and analysis were performed with the StepOne Plus 2.0 software (Applied Biosystems). Melting curves peaks were at 75.4°C for '*Ca. L. americanus*' and 77.4°C for '*Ca. L. asiaticus*'. qPCR analysis was conducted on one to four symptomatic plants for each cultivar included in experiment 1, and on all symptomatic and asymptomatic plants plus the controls included in experiments 2 and 3. Conventional PCR was performed once and qPCR twice for each sample. The SAS system (SAS Institute, Cary, NC) was used for statistical analysis of the data. The χ^2 test was used for comparing observed proportions of infected and noninfected plants within '*Liberibacter* spp.' and citrus cultivars, and Tukey's studentized range test was used for comparing means of qPCR cycle threshold (Ct) data, with significance level of $P = 0.05$.

'*Liberibacter* spp.' DNA quantification. Amplified products of both '*Liberibacter* spp.' were obtained through conventional PCR using the primers *f-rp/LAs/r-rp/LAs*, which give an amplicon of 161 bp for '*Ca. L. asiaticus*', and *f-rp/IJAm/r-rp/IJAm*, which give an amplicon of 127 bp for '*Ca. L. americanus*' (20). For both primer pairs, PCR was run for 40 cycles of 95°C for 30 s, 67°C for 30 s, and 72°C for 30 s, preceded by a denaturation step of 94°C for 3 min and followed by an elongation step of 72°C for 5 min. Reaction mixtures of 25 μl contained 500 ng of total DNA, 150 μM each *rpl*-primer, 200 μM dNTP, 300 μM MgCl₂, and 1.25 U of *Taq* DNA polymerase (Invitrogen) in the reaction buffer supplied by the manufacturer. Amplified products were purified using Wizard SV Gel kit and PCR Clean-up System (Promega Corp.), inserted into the vector pGem-T easy (Promega Corp.), and cloned in *Escherichia coli* DH5α. Transformants were selected for ampicillin resistance, X-gal, or IPTG. The white colonies were grown in Luria-Bertani medium supplemented with ampicillin at 100 μg/ml. Plasmid DNA was prepared with Wizard Miniprep kit (Promega Corp.), linearized with the restriction enzyme *EcoRI*, confirmed by electrophoretic analysis in a 1.2% agarose gel, and sequenced using T7/Sp6 primers. The identity of the sequences was confirmed by BLAST search and alignment. The amount of plasmid DNA was estimated by UV densitometry. Conversion of micrograms of double-stranded (ds)DNA to pmoles was performed by considering the average molecular weight of a DNA base pair (660 Da) and the number of bases of the dsDNA fragment (Nb). The following mathematical formula was applied: pmol of dsDNA = μg (of dsDNA) × (10⁶ pg/1 μg) × (1 pmol/660 pg) × (1/Nb). Avogadro's constant (6.023 × 10²³) was used to estimate the number of plasmid DNA copies. Tenfold serial dilutions of the plasmid DNA copies were prepared from 5.0 × 10¹⁰ to 5.0 × 10¹ for '*Ca. L. americanus*' and from 3.7 × 10¹⁰ to 3.7 × 10¹ for '*Ca. L. asiaticus*', divided into aliquots, and stored at 4°C until use. Dilutions from 5.0 × 10⁸ to 5.0 × 10² for '*Ca. L. americanus*' and 3.7 × 10⁸ to 3.7 × 10² for '*Ca. L. asiaticus*' were employed to generate the standard curve. To determine the theoretical sensitivity and the reliability of the qPCR, three repetitions of the assay were undertaken using the 10-fold serial dilutions of the previously prepared plasmid DNA copies.

RESULTS

The aggressiveness of both '*Liberibacter* spp.' was assessed through analysis of symptom development on inoculated plants, graft-transmission efficiencies, and the estimated amounts of bacterial cells that accumulated on symptomatic and asymptomatic plants after different time intervals. In general, approximately 4 months after inoculation, a few inoculated plants of all cultivars tested exhibited a slight chlorosis on young and mature leaves on the upper portions of the branches. The number of symptomatic plants and the intensity of the chlorosis increased over time. The chlorosis progressed to the characteristic HLB blotchy mottling or

to symptoms similar to those induced by iron, manganese, and zinc deficiencies, as described (12). On plants infected by ‘*Ca. L. asiaticus*’, however, the mottling was less noticeable and the zinc deficiency less intense. Advanced symptoms also included leaves with curved margins and protuberant and corky veins, regardless the pathogen used as inoculum. Symptom progress on Natal sweet orange inoculated simultaneously with both ‘*Liberibacter* spp.’ followed the same trend, with no apparent additional or synergistic effects of double infection.

In experiment 1, significantly higher transmission percentages ($\chi^2 = 108.04$, $P < 0.0001$) and lower Ct values were observed for plants inoculated with ‘*Ca. L. asiaticus*’ for all four cultivars 12 months after inoculation compared with those inoculated with ‘*Ca. L. americanus*’ (Table 1).

In experiment 2, the percentage of transmission varied depending on whether the plants were inoculated with one or simultaneously with both ‘*Liberibacter* spp.’, and the percentage of pathogen detection varied depending on the PCR method used (Table 2). Conventional PCR detected the pathogen in 54.7 and 40.3% of the plants inoculated with ‘*Ca. L. asiaticus*’ or ‘*Ca. L. americanus*’ alone, respectively, and in 69.4% of the plants inoculated with both pathogens, of which 40.3% contained only ‘*Ca. L. asiaticus*’, 12.9% only ‘*Ca. L. americanus*’, and 16.1% both species. qPCR detected the pathogen in 88.0 and 45.2% of the plants inoculated with ‘*Ca. L. asiaticus*’ or ‘*Ca. L. americanus*’ alone, respectively, and in 91.9% of the plants inoculated with both pathogens, of which 51.6% contained only ‘*Ca. L. asiaticus*’, 9.7% only ‘*Ca. L. americanus*’, and 30.6% both species. Overall, conventional PCR detected ‘*Ca. L. asiaticus*’ in 55.5% (76/137) and ‘*Ca. L. americanus*’ in 34.7% (43/124), and qPCR detected ‘*Ca. L. asiaticus*’ in 85.4% (117/137) and ‘*Ca. L. americanus*’ in 42.7% (53/124) of all inoculated and co-inoculated plants in experiment 2. qPCR detected ‘*Ca. L. asiaticus*’ in addition to ‘*Ca. L. americanus*’ in two plants in which only ‘*Ca. L. americanus*’ was detected by conventional PCR. The percentage of plants infected with ‘*Ca. L. asiaticus*’ was significantly higher than those infected with ‘*Ca. L. americanus*’ ($\chi^2 = 72.99$, $P < 0.0001$). Average Ct values also differed significantly in plants inoculated

individually with one or the other pathogen but not in plants inoculated simultaneously with both pathogens.

Experiment 3 was conducted for a more detailed assessment of symptom and infection progress in graft-inoculated Natal sweet orange plants. Groups of 10 plants, including 5 symptomatic and 5 asymptomatic (Table 3), were selected from a set of 30 graft-inoculated plants. In the group inoculated with ‘*Ca. L. americanus*’ alone, at 5 months after inoculation, three of the five symptomatic plants exhibited only mottling, one mottling plus deficiency, and one just deficiency. The pathogen was detected in all plants exhibiting mottling and in the plant exhibiting just mineral deficiency. At 6 months, the appearance of the plants had not changed. The pathogen was detected in all symptomatic plants and in the plant showing just mineral deficiency, in which the pathogen titer increased from 1.49, as determined 1 month earlier, to 6.16 log cell/g of tissue. At 7 months, the mottling had not progressed to any new leaves in plant no. 1. Plant no. 5 exhibited mottling in addition to mineral deficiency, and plant nos. 3 and 4 exhibited mineral deficiency in addition to mottling. The pathogen was detected in all symptomatic and in two asymptomatic plants but at higher Ct values. Comparison of means for Ct and titer values among evaluation dates did not reveal any significant differences.

In the group inoculated with ‘*Ca. L. asiaticus*’ alone, at 5 months after inoculation, three of the five symptomatic plants exhibited only leaf mottling, one leaf mottling plus mineral deficiency, and one just mineral deficiency. However, the pathogen was detected in all plants in this group, with lower Ct values and consequently higher bacterial titers when compared with the group inoculated with ‘*Ca. L. americanus*’ alone. At 6 months, the symptoms associated with plant nos. 1 to 4 did not change and only plant no. 5 exhibited mineral deficiency. An additional plant (no. 8) also exhibited only mineral deficiency. Again, the pathogen was detected in all plants. Seven months after inoculation, all plants were showing symptoms, with six exhibiting leaf mottling and mineral deficiency and four only mineral deficiencies. There were no significant differences between means for the Ct and titer values among evaluation dates.

TABLE 1. Transmission efficiencies and cycle threshold (Ct) values of ‘*Candidatus Liberibacter americanus*’ and ‘*Ca. L. asiaticus*’ in graft-inoculated young sweet orange plants, 12 months after inoculation

Cultivar	Transmission ^y		Ct values in symptomatic plants ^z	
	‘ <i>Ca. L. americanus</i> ’	‘ <i>Ca. L. asiaticus</i> ’	‘ <i>Ca. L. americanus</i> ’	‘ <i>Ca. L. asiaticus</i> ’
Hamlin	3 (10.0)	22 (73.3)	24.14 (22.22–26.07) A	19.42 (18.70–19.83) B
Natal	5 (16.7)	22 (73.3)	24.95 (22.67–27.39) A	20.12 (19.81–20.32) B
Pera	5 (16.7)	21 (70.0)	24.97 A	20.92 (19.59–21.73) B
Valencia	7 (23.3)	20 (66.7)	24.32 (22.70–25.29) A	20.00 (19.60–20.33) B
Mean	5 (16.7)	21.3 (70.8)	24.54 (22.22–27.39) A	20.11 (18.70–21.73) B

^y Number and percentage (in parentheses) of plants that tested positive by conventional polymerase chain reaction (PCR); χ^2 value for transmission percentages was 72.99 ($P < 0.0001$).

^z Average and range of quantitative real-time PCR Ct values obtained in DNA samples for one to four plants for each citrus cultivar. Average Ct values with different letters are significantly different ($P < 0.05$) for the comparison between ‘*Liberibacter* spp.’ within each cultivar.

TABLE 2. Transmission efficiencies and cycle threshold values of ‘*Candidatus Liberibacter americanus*’ and ‘*Ca. L. asiaticus*’ in young sweet orange plants graft inoculated individually or simultaneously with both ‘*Liberibacter* spp.’ (Both) 12 months after inoculation

Inoculum	Total	Pathogen detected	Transmission ^y		Average Ct values ^z
			PCR	qPCR	
‘ <i>Ca. L. americanus</i> ’ alone	62	‘ <i>Ca. L. americanus</i> ’	25 (40.3)	28 (45.2)	25.15 (22.20–33.51) a
‘ <i>Ca. L. asiaticus</i> ’ alone	75	‘ <i>Ca. L. asiaticus</i> ’	41 (54.7)	66 (88.0)	22.92 (18.87–32.24) b
Both	62	‘ <i>Ca. L. americanus</i> ’	8 (12.9)	6 (9.7)	25.54 (23.50–30.22) a
		‘ <i>Ca. L. asiaticus</i> ’	25 (40.3)	32 (51.6)	24.84 (19.50–33.99) a
		Both	10 (16.1)	19 (30.6)	25.29 (21.99–34.99) for ‘ <i>Ca. L. americanus</i> ’ a 24.34 (19.68–29.43) for ‘ <i>Ca. L. asiaticus</i> ’ a

^y Number and percentage (in parentheses) of plants that tested positive by conventional polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR); χ^2 value for transmission percentages was 108.04 ($P < 0.0001$).

^z Averages and ranges of qPCR cycle threshold (Ct) values obtained in DNA samples of infected plants. Means with different letters are significantly different ($P < 0.05$).

In the group inoculated simultaneously with '*Ca. L. americanus*' and '*Ca. L. asiaticus*', at 5 months after inoculation, two of the symptomatic plants exhibited mottling, one mottling and mineral deficiency, and two showed only mineral deficiency. Both pathogens were detected in DNA samples of these plants but with higher Ct values and corresponding lower cell titers for '*Ca. L. americanus*'. '*Ca. L. asiaticus*' alone was detected in three additional asymptomatic plants. At 6 months, no new mottled leaves developed on plant nos. 1 or 2 and the remaining plants were as they appeared 1 month earlier. Both '*Liberibacter* spp.' were detected in all symptomatic and in three asymptomatic plants. '*Ca. L. asiaticus*' alone was detected in the remaining three asymptomatic plants. At 7 months, plant nos. 2 and 3 exhibited mottling and deficiency and, with exception of plant nos. 1, 7, and 10, which remained asymptomatic, all others exhibited only mineral deficiency symptoms. Plant nos. 1, 9, and 10 were infected only with '*Ca. L. asiaticus*' and the remaining plants were infected with both pathogens. Comparisons of means among evaluation dates revealed a significant increase in Ct values for '*Ca. L. americanus*' in doubly infected plants, from 5 to 7 months post-inoculation. Comparison of means between pathogens within evaluation date revealed, as observed in experiment 1, significantly higher Ct values for '*Ca. L. americanus*' on all dates.

A combined analysis of all Ct values of '*Ca. L. americanus*' and '*Ca. L. asiaticus*' is shown in Table 4. In the experimental

plants, '*Ca. L. asiaticus*' reached an average Ct of 22.35 and estimated titer of 6.42 log cells/g of leaf midrib, and '*Ca. L. americanus*' reached an average Ct of 25.82 and titer of 4.87 log cells/g of leaf midrib. In the naturally affected, field-grown trees used as the source of inoculum for graft inoculations, '*Ca. L. asiaticus*' reached average Ct of 22.36 and titer of 6.67 log cells/g of leaf midrib, and '*Ca. L. americanus*' reached an average Ct of 25.22 and titers of 5.74 log cells/g of leaf midrib. Mean comparison of pathogen Ct values within time after inoculation showed significant lower Ct values for '*Ca. L. asiaticus*'.

DISCUSSION

Since the development of primers for '*Ca. L. americanus*' detection (18), free diagnostic services to the growers have been provided by Fundecitrus in Brazil. From August 2004 to January 2008, over 12,000 samples were processed and evaluated by PCR. Analysis of these data, grouped in periods of 6 months, showed a consistent decrease in the proportion of samples infected with '*Ca. L. americanus*' with a consequent increase in the proportion of samples infected with '*Ca. L. asiaticus*' (13). For example, from August 2004 to January 2005, 331 samples from farms of 64 municipalities were processed. Of the 137 samples in which the HLB pathogen was detected, 131 (95.6%) were positive for '*Ca. L. americanus*', 4 (2.9%) for '*Ca. L. asiaticus*', and 2 (1.5%) for

TABLE 3. Symptoms, quantitative real-time polymerase chain reaction (qPCR) cycle threshold (Ct) values, and the related bacterial concentrations observed in Natal sweet orange plants, graft inoculated individually with '*Candidatus Liberibacter americanus*' or '*Ca. L. asiaticus*' or simultaneously with both '*Liberibacter* spp.' (Both) at 5, 6, and 7 months after inoculation

Plant number	5 Months			6 Months			7 Months		
	Symptom ^w	Ct ^x	Titer ^y	Symptom	Ct	Titer	Symptom	Ct	Titer
<i>'Ca. L. americanus'</i>									
1	mt, –	22.09	5.79	mt, –	24.12	6.24	–, df	23.88	6.24
2	mt, df	27.70	4.34	mt, df	24.43	5.92	mt, df	25.04	5.63
3	mt, –	27.56	3.95	mt, –	23.06	6.28	mt, df	26.68	5.17
4	mt, –	21.75	6.16	mt, –	26.98	5.13	mt, df	27.32	5.12
5	–, df	37.06	1.49	–, df	23.98	6.16	mt, df	25.47	5.51
6	–, –	–	–	–, –	–	–	–, –	32.49	3.51
7	–, –	–	–	–, –	–	–	–, –	34.17	2.91
8-10	–, –	–	–	–, –	–	–	–, –	–	–
Average	...	27.23	4.35	...	24.51	5.95	...	27.86	4.87
<i>'Ca. L. asiaticus'</i>									
1	mt, –	17.77	7.47	mt, –	18.75	7.31	mt, df	20.51	7.20
2	mt, –	17.61	7.42	mt, –	17.93	7.44	mt, df	20.11	7.22
3	mt, df	17.52	7.36	mt, df	17.92	7.44	mt, df	19.78	7.47
4	–, df	19.36	6.78	–, df	18.74	7.27	mt, df	19.97	7.34
5	mt, –	19.76	6.97	–, df	18.77	7.13	–, df	20.57	7.15
6	–, –	20.17	6.39	–, –	19.26	6.77	–, df	20.85	7.05
7	–, –	17.67	7.29	–, –	18.70	6.94	–, df	20.51	7.09
8	–, –	22.41	6.17	–, df	19.12	7.45	–, df	19.51	7.13
9	–, –	21.55	6.50	–, –	22.22	6.37	mt, df	21.06	6.95
10	–, –	17.66	7.42	–, –	21.62	6.64	mt, df	20.82	6.84
Average	...	19.15	6.98	...	19.30	7.08	...	20.33	7.15
Both ^z									
1	mt, –	22.42, 19.73	5.77, 6.50	–, –	28.03, 20.51	4.75, 6.87	–, –	–, 20.64	–, 7.14
2	mt, df	23.49, 22.16	5.91, 6.27	–, df	25.89, 20.08	5.71, 7.33	mt, df	26.10, 19.48	5.51, 7.37
3	mt, –	24.95, 18.68	5.51, 7.26	mt, –	25.38, 21.70	5.87, 6.90	mt, df	25.84, 21.96	5.66, 6.75
4	–, df	23.48, 17.04	5.73, 7.53	–, df	26.56, 21.45	5.52, 6.96	–, df	25.77, 21.79	5.54, 6.66
5	–, df	22.05, 19.45	6.29, 6.99	–, df	26.67, 22.32	5.43, 6.65	–, df	25.48, 22.31	5.63, 6.51
6	–, –	–, 18.95	–, 6.14	–, –	29.08, 19.66	4.73, 7.40	–, df	25.33, 21.57	5.81, 6.85
7	–, –	–, 16.75	–, 7.12	–, –	–, 20.84	–, 7.04	–, –	36.80, 20.24	2.40, 7.12
8	–, –	–, –	–, –	–, –	–, 20.23	–, 7.04	–, df	32.70, 20.59	3.67, 7.12
9	–, –	–, –	–, –	–, –	33.31, 24.47	3.23, 5.77	–, df	–, 23.99	–, 6.11
10	–, –	–, 24.38	–, 5.66	–, –	–, 21.61	–, 6.81	–, –	–, 20.11	–, 7.02
Average	...	23.28, 19.64	5.84, 6.68	...	27.84, 21.28	5.03, 6.88	...	28.29, 21.34	4.89, 6.86
Control									
1 to 5	–, –	–, –	–, –	–, –	–, –	–, –	–, –	–, –	–, –

^w Pattern of leaf symptoms: mt = blotchy leaf mottling, df = chlorosis similar to that induced by mineral deficiency, and – = no symptoms.

^x qPCR Ct values; – = undetectable fluorescence.

^y Pathogen titer: estimated log₁₀ of the number of '*Liberibacter* spp.' cells per gram of leaf midrib based on a Ct/DNA standard quantification curve; – = undetectable values.

^z First and second numbers refer to data obtained for '*Ca. L. americanus*' and '*Ca. L. asiaticus*', respectively.

both 'Liberibacter spp.' In this period, 'Ca. L. americanus' was present in 39 and 'Ca. L. asiaticus' in 3 municipalities, all located in the central and southern regions of São Paulo State, where probably both 'Liberibacter spp.' were first introduced. From August 2007 to January 2008, 2,103 samples from farms of 115 municipalities were processed. Of the 1,288 samples in which the HLB pathogen was detected, 253 (19.6%) were positive for 'Ca. L. americanus', 1,024 (79.5%) for 'Ca. L. asiaticus', and 11 (0.9%) for both 'Liberibacter spp.' 'Ca. L. americanus' was present in 50 and 'Ca. L. asiaticus' in 70 municipalities, located in most regions of the state except for the extreme of the northern and northwestern regions. The current prevalence of 'Ca. L. asiaticus' over 'Ca. L. americanus' has been observed also in field experiments in farms where 'Ca. L. americanus' was originally the main 'Liberibacter sp.' present (R. B. Bassanezi, *personal communication*). These observations, in addition to data already reported (13,23), suggest that 'Ca. L. americanus' and 'Ca. L. asiaticus' have distinct biological characteristics that result in different rates of natural transmission. This faster temporal and spatial progress of 'Ca. L. asiaticus', which started at low incidence levels and supplanted 'Ca. L. americanus' in less than 4 years, indicates that 'Ca. L. asiaticus' has a competitive advantage over 'Ca. L. americanus', contrary to what was originally hypothesized (5).

In this work we demonstrate that 'Ca. L. americanus' and 'Ca. L. asiaticus' differ in at least three probably interrelated aspects: efficiencies of graft transmission from infected to healthy tissues, patterns of infection and symptom development, and concentration of bacterial cells that may accumulate in midribs of infected citrus leaves. 'Ca. L. asiaticus' was transmitted more efficiently and attained 35 times higher titers in the infected experimental plants than 'Ca. L. americanus'. On branches of affected field-grown trees used as the source of inoculum, the differences were lower (8.6-fold) but followed the same trend, as observed in another study that used a distinct qPCR protocol (10). These differences in cell titers could be the main reason for the different transmission rates observed in this study and be an important factor influencing the shift in pathogen prevalence in the State of São Paulo over time. Higher titers would increase the chances for pathogen acquisition and transmission by the insect vector, as has been described for the *Cucumber mosaic virus* in tomato by *Aphis gossypii* (4) and for the xylem-limited bacterium *Xylella fastidiosa* in grapevines by sharpshooters (6). Although strongly suggestive, this assumption needs to be further investigated in experiments involving the insect vector *D. citri* for 'Liberibacter spp.' transmission.

An interference of one bacterium in the multiplication ability of the other bacterium in the phloem of infected leaves has not been clarified in this study. The result obtained in experiment 2 (Table 2) showing differences among Ct values for 'Ca. L. americanus' and 'Ca. L. asiaticus' in singly but not in doubly infected plants

would suggest a positive interference. However, no difference was observed when singly and doubly infected plants were compared within the group of co-inoculated plants in experiment 2 (Table 2), or when similar comparisons were made using the data from experiment 3 (Table 3).

Differences in pathogen ability to multiply in the citrus phloem could also be a factor responsible for the differences in infection development observed in plants in experiment 3, although this aspect would certainly be better assessed if the plant evaluations had been initiated earlier. Although, in the group of plants inoculated with 'Ca. L. americanus', the incidence of infected plants increased from 50 to 70% from 5 to 7 months postinoculation, in the group inoculated with 'Ca. L. asiaticus', all plants were already infected by the pathogen at 5 months postinoculation. In plants inoculated with 'Ca. L. americanus', the pathogen was present at detectable levels mainly in those showing mottled leaves. In asymptomatic plants, the pathogen was not present or was present at very low populations, contrary to that observed in 'Ca. L. asiaticus'-infected plants. Failure to detect 'Ca. L. americanus' in asymptomatic tissues had already been reported with the use of conventional PCR in graft-inoculated, greenhouse-grown young plants (12), and also with the use of qPCR in a naturally infected, field-grown adult tree (19). In plants inoculated with 'Ca. L. asiaticus', the pathogen was also detected in asymptomatic tissues prior to symptom development. Asymptomatic and symptomatic tissues contained similar pathogen cell titers (6.72 and 7.27 cell/g of tissue, respectively) and the population did not increase with time; therefore, these might be the 'Ca. L. asiaticus' population levels that trigger symptom expression in citrus. In a similar study conducted with pathogen isolates from Florida, using a PCR protocol different than the one used in the present study, the pathogen also was detected in asymptomatic plants 1 and 2 months postinoculation but apparently at lower cell titers (11).

'Ca. L. asiaticus' and 'Ca. L. americanus' also differed in the overall symptomatology induced on leaves of the graft-inoculated citrus plants, as has been observed in growth chamber experiments (S. A. Lopes, *unpublished data*). Despite the higher cell titer, the typical leaf mottling and strong symptoms of mineral deficiency, particularly of zinc, observed in plants infected with 'Ca. L. americanus' were absent or less noticeable in plants infected with 'Ca. L. asiaticus'. This lack of a clear blotchy mottle pattern is analogous to that observed in field-grown trees, especially in early infections and during the summer season. Because of similarities to symptoms induced by other pathogens or by mineral deficiencies, the absence of a clear blotchy mottling on leaves has created difficulties for the growers in correct identification of HLB-affected trees. From a comparative and evolutionary perspective, the higher cell titer associated with less intense symptom expression may indicate that 'Ca. L. asiaticus' is more adapted than 'Ca. L. americanus' to live in the citrus

TABLE 4. Overall analysis of cycle threshold (Ct) values for 'Candidatus Liberibacter americanus' and 'Ca. L. asiaticus' obtained by quantitative real-time polymerase chain reaction (qPCR) from leaf samples of singly and doubly infected potted plants and singly infected field-grown trees

Source, time after inoculation	'Ca. L. americanus'		'Ca. L. asiaticus'	
	No. of plants ^y	Average Ct ^z	No. of plants	Average Ct
Graft-infected potted plants				
5 months	10	25.25 (21.75–37.06) A	18	19.37 (16.75–24.38) B
6 months	12	26.46 (23.06–33.31) A	20	20.29 (17.92–24.47) B
7 months	14	28.08 (23.88–36.80) A	20	20.81 (19.48–23.99) B
12 months	63	25.13 (21.99–34.99) A	134	23.27 (18.70–33.99) B
Naturally infected field trees				
Unknown	10	25.22 (22.01–28.13) A	10	22.36 (19.48–27.35) B
Mean	...	25.37 (21.01–36.80) A	...	22.35 (16.75–33.99) B

^y Total number of plants that tested positive by qPCR.

^z Averages and range of qPCR Ct values. Means with different letters are significantly different ($P < 0.05$). Comparison between 'Ca. Liberibacter spp.' within each inoculation time.

phloem, which could be the result of a longer host–pathogen co-evolution process than that involving ‘*Ca. L. americanus*’, in which lower concentrations of bacteria have caused stronger plant reaction.

In this work, we presented data on the infection progress of the HLB bacteria present in Brazil in artificially graft-inoculated young plants. Unfortunately, no data are available on infection progress of these pathogens in insect-inoculated, field-grown trees. Also, nothing is known about the role of asymptomatic tissues as source of inoculum or the spatial distribution of trees with latent infections in relation to the symptomatic trees, as was determined in a citrus orchard in Florida (8). Research on these aspects of the HLB problem are essential for generating information to improve the practices currently adopted for disease control, which is strongly dependent on the elimination of sources of inoculum and on the reduction of vector populations.

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