Quantitative Distribution of '*Candidatus Liberibacter asiaticus*' in Citrus Plants with Citrus Huanglongbing

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Accepted for publication 29 September 2008.

ABSTRACT

Li, W., Levy, L., and Hartung, J. S. 2009. Quantitative distribution of '*Candidatus Liberibacter asiaticus*' in citrus plants with citrus huanglongbing. *Phytopathology* 99:139-144.

Citrus huanglongbing (HLB), or greening disease, is strongly associated with any of three nonculturable gram-negative bacteria belonging to '*Candidatus Liberibacter* spp.' '*Ca. Liberibacter* spp.' are transmitted by citrus psyllids to all commercial cultivars of citrus. The diseases can be lethal to citrus and have recently become widespread in both São Paulo, Brazil, and Florida, United States, the locations of the largest citrus industries in the world. Asiatic HLB, the form of the disease found in Florida, is associated with '*Ca. Liberibacter asiaticus*' and is the subject of this report. The nonculturable nature of the pathogen has hampered research and little is known about the distribution of '*Ca. L. asiaticus*' in infected trees. In this study, we have used a quantitative polymerase chain reaction assay to systematically quantify the distribution of '*Ca. L. asiaticus*' genomes in tissues of six species of citrus either identified in the field during survey efforts in Florida or propagated in a greenhouse in Beltsville, MD. The populations of '*Ca. L. asiaticus*' inferred from the distribution of 16S rDNA sequences specific for '*Ca. L. asiaticus*' in leaf

midribs, leaf blades, and bark samples varied by a factor of 1,000 among samples prepared from the six citrus species tested and by a factor of 100 between two sweet orange trees tested. In naturally infected trees, above-ground portions of the tree averaged 10^{10} '*Ca. L. asiaticus*' genomes per gram of tissue. Similar levels of '*Ca. L. asiaticus*' genomes were observed in some but not all root samples from the same plants. In samples taken from greenhouse-inoculated trees, levels of '*Ca. L. asiaticus*' genomes varied systematically from 10^4 genomes/g at the graft inoculation site to 10^{10} genomes/g in some leaf petioles. Root samples from these trees also contained '*Ca. L. asiaticus*' at 10^7 genomes/g. In symptomatic fruit tissues, '*Ca. L. asiaticus*' genomes were also readily detected and quantified. The highest levels of '*Ca. L. asiaticus*' in fruit tissues were found in the locular membranes and septa (10^8 genomes/g), with 100-fold lower levels of '*Ca. L. asiaticus*' in the meso and pericarp of such fruit. Our results demonstrate both the ubiquitous presence of '*Ca. L. asiaticus*' in symptomatic citrus trees as well as great variation between individual trees and among samples of different tissues from the same trees. Our methods will be useful in both the management and scientific study of citrus HLB, also known as citrus greening disease.

Citrus huanglongbing (HLB), also known as citrus greening disease (6), poses the greatest pathogenic threat to citrus production worldwide (10). The disease has long been endemic only in Asia, Africa, the Indian subcontinent, the Mascarene islands, and the Arabian peninsula (5), but recently was found to be widespread in both Brazil (26) and Florida (17). A gram-negative bacterium belonging to the α subdivision of the proteobacteria (14) is consistently associated with the disease but, because the bacterium has never been isolated, the name of the organism has provisional ('*Candidatus*') status in nomenclature and Koch's postulates have never been completed for this pathogen and disease. With this caveat noted, we will hereafter refer to '*Candidatus Liberibacter* spp.' as pathogens that cause citrus HLB. The disease is caused by three forms of the bacterium: '*Ca. Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*'. These bacteria cause disease in Asia, Africa, and Brazil, respectively, and can be distinguished by differences in the sequence of the rRNA operon (14–16,26). The disease can be spread efficiently by vector psyllids *Trioza erytreae* and *Diaphorina citri* (22,24) and through the propagation of infected plant materials (3,21).

Infected citrus orchards are usually destroyed or become unproductive in 5 to 8 years (2). The HLB pathogens are currently on the list of select agents (28). *D. citri* has become established in Florida since its introduction in 1998 (9) and in Texas in 2001 (7). Because of the wide distribution of the vector and the distribution and amount of disease present in Florida, HLB is now of great concern to the entire U.S. citrus industry.

HLB disease is difficult to distinguish initially from nutrient deficiencies or other plant diseases. Serologically based detection methods are not available. Accurate identification of '*Ca. Liberibacter* spp.' is greatly needed for an effective regulatory response, to facilitate management of infected trees, and to contribute toward the development of a '*Ca. Liberibacter* spp.'-free nursery system. Conventional polymerase chain reaction (PCR) assays have been developed for the HLB pathogens (12,13,15,25,27). Consistent detection of the pathogens in infected plants or vector insects remains problematic for these standard-format PCR assays. The presumed low concentration and uneven distribution of the pathogens in host plants and the complications of testing vector insects, as well as PCR inhibitors present in citrus extracts (11), may make the pathogens difficult to detect. Real-time PCR (17,20,29), and loop-mediated isothermal amplification (LAMP) (23) have recently been developed for detection or differentiation of '*Ca. Liberibacter* spp.' Although conventional and real-time or quantitative PCR (qPCR) assays are accepted as confirmatory tests for symptom-based HLB surveys in various locations, such as the states of São Paulo, Brazil and Florida, United States, the

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doi:10.1094/PHYTO-99-2-0139

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potential of these assays to contribute to the knowledge of 'Ca. Liberibacter spp.' biology in host plants or vector insects has not been realized.

We have previously developed a real-time qPCR assay for 'Ca. Liberibacter spp.' that is multiplexed with a plant mitochondrial cytochrome oxidase (COX) gene assay as an internal control for extract quality and quantity (17). We have shown that this assay is more sensitive and robust than conventional PCR assays (18) and the assay has also been validated for DNA extracts from different citrus species and different citrus tissues, and for samples obtained from different geographical regions. In the course of that validation work, a "grand universal regression equation" was developed that accounts for all of these sources of variability and relates the crossing threshold of the assay to the concentration of genome targets in the extracts. This equation makes it possible to quantify 'Ca. L. asiaticus' genome targets in any citrus extract using the qPCR assay (19). The distribution of 'Ca. L. asiaticus' in infected plants has never been thoroughly characterized though it is thought, based primarily on electron microscopy and graft transmission data, that the pathogen is unevenly distributed within infected plants (8). Apart from the obvious biological importance of the distribution of the pathogen in planta, knowledge of the distribution of the pathogen in infected plants would inform the choice of plant tissues for sampling and assay. We have used quantitative PCR to study the distribution of 'Ca. L. asiaticus' in infected plants and as a proxy to estimate population levels of the pathogen.

MATERIALS AND METHODS

Sampling of different host plant tissues. Plant samples were collected from symptomatic trees discovered in the course of 2005–06 surveys for HLB disease in Florida and sent under permit to the CPHST laboratory in Beltsville, MD. For above-ground tissues, three trees of each species listed (Table 1) were sampled. Root samples were taken from one symptomatic field-grown tree each of Persian lime (*Citrus latifolia* Tan.), Palestine sweet lime (*C. limettioides* Tan.), and lemon (*C. limon* (L.) Burm.), or three trees each of sweet orange (*C. sinensis* (L.) Osbeck), Mexican lime (*C. aurantifolia* Swing.), and Combava (*C. hystris* DC.) grown in a containment greenhouse. Field-grown trees were naturally infected and greenhouse-grown trees had been previously inoculated with 'Ca. L. asiaticus' by grafting. Trees were uprooted and root samples were collected. Samples of approximately 200 mg were taken from midribs, petioles, leaf blades, and green stem bark (field-grown trees) or mature bark (greenhouse-grown trees) and roots in triplicate. Fruit samples were collected from symptomatic branches that had previously been confirmed positive for 'Ca. L. asiaticus' by reverse-transcription PCR. The lemon and Palestine sweet lime fruit were

themselves symptomatic at the time they were collected; the sweet orange and Persian lime fruit were not symptomatic when tested. Five fruit were dissected for each species. Three 200-mg subsamples of each tissue type from each of the five fruit within a species were collected. All samples were cut in sections approximately 1 mm wide and the total DNA present (plant + pathogen) was extracted using a Fast Prep (MP Biomedical, Solon, OH) bead mill as described previously (17) using the Plant DNeasy extraction system (Qiagen). Sample DNA was eluted with 200 µl of T₁₀ E₁ buffer, pH 8.0. The concentration of DNA in extracts was estimated with a SPECTRA max PLUS384 spectrophotometer (Molecular Devices, Sunnyvale, CA), and adjusted to 50 ng/µl with Tris-EDTA buffer.

Distribution of 'Ca. L. asiaticus' in greenhouse-grown trees. Graft-inoculated sweet orange trees (*C. sinensis* L.) were maintained in the greenhouse with a fertigation solution composed of Peters fertilizer (nitrogen/phosphorus/potassium, 20:5:20) calibrated to deliver nitrogen at 100 ppm. The fertigation solution also contained chelated iron at 6 ppm and CuSO₄ at 1 ppm. Temperatures were maintained by heat in cold weather and by a combination of evapotranspiration with cooling pads and 30% shade cloth in hot weather. Pesticides were applied to control insect pests. Three trees, which had been graft inoculated 5 years earlier and maintained in a containment greenhouse, were destructively sampled for the presence of 'Ca. L. asiaticus'. The trees were symptomatic and had previously tested positive for the presence of 'Ca. L. asiaticus' by conventional and qPCR. The graft unions between the 'Ca. L. asiaticus' source bud and the trees were marked and bark samples were taken as 1-cm "rings" at 10-cm increments above and below the graft union. Leaf blade, midrib, and bark samples were taken in triplicate, as available, at 10-cm intervals above and below the graft union, which was approximately 10 cm above the soil line. Leaf blades were sampled with a cork borer to remove leaf disks and midribs were removed from leaves with a razor blade prior to extraction. Extracts were prepared as above.

Quantification of 'Ca. L. asiaticus' genomes in plant extracts. qPCR assays were performed as reported previously in a SmartCycler II (Cepheid, Sunnyvale, CA) using the target primer-probe sets HLBaspr plus a plant cytochrome oxidase (COX)-based primer-probe set (COXfpr). All reactions were performed in triplicate and each run contained one negative and one positive control. The data were analyzed using the SmartCycler software, version 2.0D, to obtain the crossing threshold (Ct) values. The qPCR data (Ct values) were subjected to analysis of variance, and means were separated using the Tukey test. The mean Ct values were then entered into the grand universal regression equation for calculating 'Ca. L. asiaticus' genome equivalents in citrus extracts (19): $Y = 13.82 - 0.2866X$. This equation, which yields the number of 16S rDNA targets specific for 'Ca. L. asiaticus' present

TABLE 1. Detection and quantification of 'Candidatus Liberibacter asiaticus' genomes by quantitative polymerase chain reaction (qPCR) in DNA extracts of leaves and stems of huanglongbing (HLB)-infected citrus plants

Citrus cultivar	Mean Ct values of qPCR ^y				Genome/g ^z
	Midrib, petiole	Leaf blade	Bark	Mean	
Sweet orange 2	18.44 a	18.68 a	18.02 a	18.38 a	1.78×10^{11}
Lemon	20.26 b	21.08 b	17.53 a	19.52 a	8.41×10^{10}
Sour orange	19.34 a	20.10 a	22.16 b	19.52 a	4.23×10^{10}
Sweet orange 1	22.86 a	22.21 a	28.15 b	24.41 b	3.33×10^9
Sweet lime	28.78 b	27.93 b	18.24 a	24.98 b	2.29×10^9
Pumelo	25.45 a	26.02 a	28.92 b	26.80 b	6.89×10^8
Mean	22.52 a	22.68 a	22.17 a	...	...
Genome/gm	1.12×10^{10}	1.05×10^{10}	1.46×10^{10}	...	...

^y Mean crossing threshold (Ct) values followed by the same letter for different tissues of a citrus species or different plant tissue or different citrus cultivars are not significantly different ($P = 0.05$).

^z For multiplex qPCR with primer-probe sets HLBaspr and COXfpr (17), 1 µl of a 200-µl DNA elution obtained from 200 mg of HLB-infected fresh tissues was used. The bacterial population was obtained based on the grand universal regression equation for citrus plants: $Y = 13.82 - 0.2866X$ (19) and two rRNA operons (containing the PCR target 16S rRNA) per cell of the bacterium (16).

per microliter of plant extract, was used to calculate the number of '*Ca. L. asiaticus*' genome equivalents present per gram of tissue sampled based on the estimate that the genome of '*Ca. L. asiaticus*' has two copies of this target per genome (16).

RESULTS

'*Ca. L. asiaticus*' in above-ground tissues of field-infected citrus trees. Three symptomatic trees each of five citrus species were sampled and subjected to quantitative PCR: sweet orange, pumelo, sweet lime, lemon, and sour orange. The observed levels of '*Ca. L. asiaticus*' genomes varied over three orders of magnitude, from approximately 7×10^8 genome equivalents/g of tissue (pumelo) to approximately 2×10^{11} genome equivalents/g of tissue (sweet orange from location 2) (Table 1). Samples were taken within each tree from leaf blades, leaf midribs, and bark. On average, there was no significant difference in '*Ca. L. asiaticus*' genome equivalents detected based on the tissue type sampled, although variation was observed among tissue types taken from individual trees (Table 1). On average, each tissue type sampled contained approximately 10^{10} '*Ca. L. asiaticus*' genomes per gram of tissue sampled.

'*Ca. L. asiaticus*' in roots of field- and greenhouse-infected citrus trees. '*Ca. L. asiaticus*' genomes were readily detected in roots of greenhouse-inoculated and -grown citrus trees of three citrus species. The average populations of '*Ca. L. asiaticus*' genomes in the three species ranged from approximately 1×10^{10} to 6×10^{10} genome equivalents per gram of root tissue (Table 2). A similar population of '*Ca. L. asiaticus*' genomes was observed in one of the field-grown trees sampled but the pathogen was not detected in the roots of the two other field-grown trees sampled.

'*Ca. L. asiaticus*' in fruit from symptomatic field-grown trees. Five fruit were collected from each citrus cultivar. The branches from which the fruit were harvested were symptomatic and confirmed positive for '*Ca. L. asiaticus*' by qPCR; however, only the lemon and Palestine sweet lime fruit were themselves symptomatic (data not shown). '*Ca. L. asiaticus*' genomes were consistently detected in symptomatic fruit (Table 3; Fig. 1) but were not detected in the asymptomatic Persian lime fruit. Populations of '*Ca. L. asiaticus*' genomes were not uniform with respect to tissue sampled in the fruit, ranging between approximately 10^5 and 10^9 genome equivalents/g (Table 3). Populations of '*Ca. L. asiaticus*' genomes were not different (approximately 2 to 4×10^7 genome equivalents/g) on average in the lemon and Palestine sweet lime fruit (Table 3). Populations of '*Ca. L. asiaticus*' genomes were higher in the locular membrane and septum than in the peduncle, pericarp, or central axis (Table 3; Fig. 1).

TABLE 2. Detection and quantification of '*Candidatus Liberibacter asiaticus*' genomes by quantitative polymerase chain reaction (qPCR) in DNA extracts of roots of Huanglongbing (HLB)-infected citrus plants

Location, species ^x	Mean Ct value of qPCR ^y	Genomes/g of fresh tissues ^z
Field		
Persian lime	19.96	6.30×10^{10}
Palestine sweet lime	0	0
Lemon	0	0
Greenhouse		
Sweet orange	22.38	1.26×10^{10}
Mexican lime	20.14	5.60×10^{10}
Combava	18.24	1.95×10^{10}

^x Plant locations and citrus species. Field = in the field in Florida and greenhouse = in greenhouses in Beltsville, MD.

^y Ct = crossing threshold.

^z For multiplex qPCR with primer-probe sets HLBspr and COXfpr (17), 1 μ l of a 200- μ l DNA elution obtained from 200 mg of HLB-infected fresh tissues was used. The bacterial population was obtained based on the grand universal regression equation for citrus plants: $Y = 13.82 - 0.2866X$ (19) and two rRNA operons (containing the PCR target 16S rRNA) per cell of the bacterium (16).

'*Ca. L. asiaticus*' in systematically sampled greenhouse-grown trees. The leaf midribs of greenhouse-grown trees had consistently higher populations of '*Ca. L. asiaticus*' genomes than the leaf blades at all sampling points. The midribs also contained higher populations of '*Ca. L. asiaticus*' genomes than bark in the lower portions of the trees but, in the higher portions of the trees, the population levels were comparable between the midribs and bark samples (Fig. 2; Table 4). Branches with leaves were not available in the region near or below the graft union; therefore, only bark samples were available for these regions. The pathogen's genome was detected in all sampled points of leaf blades, midribs, and stem bark above and in all root samples below the soil (Table 4; Fig. 2). The population of '*Ca. L. asiaticus*' genomes was lowest at the point of the graft union (4×10^4 genome equivalents/g) and increased both below and above the graft union approximately 1,000- to 10,000-fold (Figs. 2 and 3).

DISCUSSION

DNA of the pathogen '*Ca. L. asiaticus*' was readily detected in all six of the field-grown trees sampled (Table 1). The apparent population levels of the pathogen varied among hosts over a range of three log units from approximately 7×10^8 (pumelo) to approximately 2×10^{11} (sweet orange location 2) genomes per gram of tissue sampled (Table 1). The apparent population levels between the two sweet orange trees samples also varied 100-fold. Samples were composed of midribs, petioles, leaf blades, and bark. Within a particular species, significant population differences were observed among the three tissue types. However, these differences were not consistent across all species or trees tested. Note that the concentration of '*Ca. L. asiaticus*' genomes was generally quite consistent between leaf blade and midrib or petiole samples from a given tree, varying less than one log unit. When the tissue source data were analyzed across all trees tested, there was no statistically significant difference among tissue types as a source of material for testing.

'*Ca. L. asiaticus*' DNA was also readily detected in the roots of infected trees (Table 2). The field-grown Persian lime tree roots contained 6×10^{10} '*Ca. L. asiaticus*' genomes per gram of tissue sampled which is comparable with the levels observed in the above-ground portions of trees (Table 1). This tree was not sampled as part of the data shown in Table 1; however, two other field-grown trees, Palestine sweet lime and lemon, were included in Table 1. Although '*Ca. L. asiaticus*' was not present at detectable levels in the roots of these trees, '*Ca. L. asiaticus*' was

TABLE 3. Detection and quantification of '*Candidatus Liberibacter asiaticus*' genomes by quantitative polymerase chain reaction (qPCR) in DNA extracts of fruit tissues of Huanglongbing (HLB)-infected citrus plants

Fruit tissue	Mean Ct values of qPCR ^y			Genomes/g ^z
	Lemon	Lime	Mean	
Locular membrane	24.50 a	31.18 ab	27.84 a	3.45×10^8
Septa	29.40 b	28.38 a	28.89 a	1.75×10^8
Endocarp	30.98 b	30.15 a	30.56 ab	6.00×10^7
Peduncle	30.28 b	33.37 b	31.82 b	2.50×10^7
Central axis	39.48 c	29.29 a	34.38 c	4.65×10^6
Pericarp/mesocarp	37.92 c	34.61 b	36.26 d	1.35×10^6
Mean	32.09 a	31.16 a	...	...
Genomes/g	2.1×10^7	3.89×10^7	...	...

^y Mean crossing threshold (Ct) values followed by the same letter for different tissues of a citrus species or different tissues or different citrus species are not significantly different ($P = 0.05$).

^z For multiplex qPCR with primer-probe sets HLBspr and COXfpr (17), 1 μ l of 200- μ l DNA elution obtained from 200 mg of HLB-infected fresh tissues was used. The bacterial population was obtained based on the grand universal regression equation for citrus plants: $Y = 13.82 - 0.2866X$ (19) and two rRNA operons (containing the PCR target 16S rRNA) per cell of the bacterium (16).

present in the bark of the above-ground portion of the trees at very high levels. It is likely that these trees may represent a relatively early stage of disease progression, in which the pathogen is moving toward but has not yet reached and colonized the roots. The greenhouse-grown sweet orange, Mexican lime, and particularly the combava trees all supported large populations of '*Ca. L. asiaticus*' in their roots (Table 2). These trees were, of course, small and had been inoculated by grafting several years

earlier; therefore, ample time had passed to allow complete colonization of the roots. The long-term presence of '*Ca. L. asiaticus*' in citrus trees can lead to significant populations of the pathogen in the roots. This may explain the fact that pruning of infected branches does not control the disease.

Fruit were collected from symptomatic branches of field-grown trees. The branches from which the fruit were harvested also tested positive for '*Ca. L. asiaticus*'. When symptomatic lemon and Palestine sweet lime fruit were tested, '*Ca. L. asiaticus*' genomes were readily detected in all tissue types tested, with population levels varying from 10^5 to 10^9 genomes per gram of tissue, and averaging 10^7 genomes/g (Table 3). The population levels observed in the fruit were, on average, approximately 1,000-fold less than the populations observed in the midribs, leaf blades, bark, and roots (Tables 1 and 3). The details of the distribution of the pathogen within the lemon and Palestine sweet lime differed (Table 3). Relatively high populations of '*Ca. L. asiaticus*' genomes were observed in the locular membranes and the septa of both fruit. Although the pathogen was detected in both the central axis and the peel of the fruit, the levels in these tissues were 100-fold lower than in the septa and locular membrane. These differences were significant (Table 3; Fig. 1A). When the estimated populations of '*Ca. L. asiaticus*' were averaged across the several tissue types tested, the populations in symptomatic lemon and Palestine sweet lime fruit were not different (Table 3; Fig. 1B). The asymptomatic sweet orange fruit were determined to be in the early stages of colonization, with '*Ca. L. asiaticus*' detectable in the peduncle (1.4×10^6 genomes/g) and central axis (1.6×10^7 genomes/g). However even at this early stage of colonization, the pathogen was multiplying preferentially in the fruit septum (1.8×10^9 genomes/g). '*Ca. L. asiaticus*' was not detected in the pericarp or mesocarp, endocarp, or locular membranes of these presymptomatic sweet orange fruit.

Potted greenhouse-grown trees were also destructively and systematically sampled as part of this study (Table 4). These trees had been inoculated with '*Ca. L. asiaticus*' 5 years earlier and had consistently tested positive for '*Ca. L. asiaticus*' by conventional and qPCR. Despite the destructive nature of HLB, these plants had survived, presumably because of their careful maintenance in a containment greenhouse. In these trees, the populations of '*Ca. L. asiaticus*' genomes were much higher in the midribs than in the leaf blades (Table 4), and varied systematically within these trees over more than four log units (Figs. 2 and 3). The highest levels of the pathogen were detected in the midribs of mature leaves (20

Fig. 1. Distribution of '*Candidatus Liberibacter asiaticus*' genomes in tissues of greenhouse-grown sweet orange trees inoculated by bud grafting with '*Ca. L. asiaticus*' strain B 239. The colors indicate the mean concentration of '*Ca. L. asiaticus*' genome equivalents per gram of tissue sampled at the sampling sites indicated.

Fig. 2. Distribution of '*Candidatus Liberibacter asiaticus*' genomes in tissues of five symptomatic lime (left) and lemon (right) fruit sampled. The colors indicate the mean concentration of '*Ca. L. asiaticus*' genome equivalents per gram of tissue sampled. The peduncles are drawn in the upper corners of the figure. Distribution of '*Ca. L. asiaticus*' genomes in fruit of lime and lemon averaged over 10 fruit sampled.

to 70 cm above the graft union). Interestingly, the population of ‘*Ca. L. asiaticus*’ was scarcely detectable at the point of graft inoculation and increased rapidly to approximately 10^6 genomes/g throughout the other regions of the trunk, both above and below this site and into the root tissues (Figs. 2 and 3).

We have shown that ‘*Ca. L. asiaticus*’ is ubiquitously distributed throughout symptomatic citrus trees. This result does not conflict with the widely held belief that the distribution of ‘*Ca. L. asiaticus*’ in planta is irregular, based in part on previous PCR assay results. Because the qPCR assay used in this study is approximately 100 times more sensitive than the conventional PCR assay for the same targets (18,29), the pathogen may not have been detected using the conventional PCR assay in many of our samples that were determined to have relatively low levels of ‘*Ca. L. asiaticus*’.

We have estimated populations of ‘*Ca. L. asiaticus*’ genome equivalents to be as high as 10^{10} and, in one case 10^{11} per gram of tissue sampled (Table 1). These apparent populations of ‘*Ca. L. asiaticus*’ are higher than might be expected based on previous studies of HLB by electron microscopy. Electron microscopy is not a quantitative technique and necessarily samples only small portions of tissue whereas our method sampled much larger sample volumes, favoring detection of bacteria with a nonuniform distribution.

There are also aspects of any qPCR assay which should be kept in mind, because the number of DNA targets present in a cell will tend to be greater than the number of cells present in any sample. First, qPCR does not measure cell numbers directly but, rather, genome equivalents based on amplification of the 16S rRNA encoding genes of the ‘*Ca. L. asiaticus*’ genome. ‘*Ca. L. asiaticus*’ is a member of the α subdivision of the proteobacteria (14). We have used the value of two rRNA operons per genome equivalent (16). Therefore, if ‘*Ca. L. asiaticus*’ has more than two rRNA operons per genome, we will overestimate the number of genomes present in a sample. Second, when growing exponentially, bacteria may have more than one genome per cell. Nothing is known about the growth kinetics of ‘*Ca. L. asiaticus*’ in our sampled plants but if more than one genome is present per cell, it would also cause an overestimation of the number of cells present.

The qPCR-based method used cannot distinguish between living and dead cells, and nonliving cells are not included in population estimates enumerated by dilution-plating methods used for culturable bacteria. In addition to the viable cells, our

estimate would include DNA remaining from ‘*Ca. L. asiaticus*’ cells that have died. ‘*Ca. Phytoplasma spp.*’ are similar to ‘*Ca. L. asiaticus*’ in that both organisms are phloem limited in plants and are not culturable. Christensen et al. (4) have applied qPCR methods based on 16SrDNA sequence to the quantification of ‘*Ca. Phytoplasma spp.*’ in plants. They were able to detect ‘*Ca. Phytoplasma spp.*’ 16SrDNA at a level of greater than 10^9 genome equivalents per gram of *C. roseus* (100 mg of plant tissue sampled; 100- μ l extract volume; 10^{-6} dilution; 1 μ l per assay). Confocal microscopy of infected plant tissue with vital stains revealed that a large portion of the ‘*Ca. Phytoplasma spp.*’ cells detected were not viable when examined (4).

Spiroplasma citri, like ‘*Ca. L. asiaticus*’, is an insect-transmitted and phloem-limited bacterium that causes citrus stubborn disease. Fruit produced on trees infected by both ‘*Ca. L. asiaticus*’ and *S. citri* are notably symptomatic, and both pathogens induce seed abortion (1,5). We have described the thorough colonization of lemon and lime fruit tissues by ‘*Ca. L. asiaticus*’. Overall, the distribution of the pathogen was similar in the two fruit cultivars, though the population of ‘*Ca. L. asiaticus*’ was much higher in the central axis of lime versus lemon fruit (Table 3; Fig. 1A). Sweet orange fruit that were not yet symptomatic also had significant

Distribution of a Ca Las in Artificially Inoculated Trees

Fig. 3. Distribution of ‘*Candidatus Liberibacter asiaticus*’ genomes in bark tissues of greenhouse-grown sweet orange trees inoculated by bud grafting with ‘*Ca. L. asiaticus*’ strain B 239. The soil line was between positions –10 and –20 cm below the graft inoculation site, represented as ‘0’ in this figure.

TABLE 4. Distribution of ‘*Candidatus Liberibacter asiaticus*’ genomes in greenhouse-grown sweet orange inoculated with ‘*Ca. L. asiaticus*’-infected budwood

Sampling site	Mean Ct value of qPCR ^y			Bacterial population (‘ <i>Ca. L. asiaticus</i> ’/g of fresh tissues) ^z		
	Blade	Midribs	Bark	Blade	Midribs	Bark
H100cm	42.24	33.13	28.60	2.59×10^4	1.06×10^7	2.10×10^8
H90cm	35.36	28.63	27.09	2.43×10^6	2.06×10^8	5.69×10^8
H80cm	33.31	27.54	26.90	9.38×10^6	4.23×10^8	6.45×10^8
H70cm	30.58	24.35	27.78	5.69×10^7	3.47×10^9	3.61×10^8
H60cm	29.89	22.42	30.02	8.96×10^7	1.24×10^{10}	8.23×10^7
H50cm	29.06	24.93	35.45	1.55×10^8	2.37×10^9	2.51×10^6
H40cm	28.88	23.62	37.36	1.79×10^8	5.62×10^9	6.48×10^5
H30cm	28.26	23.57	37.42	2.52×10^8	5.80×10^9	6.23×10^5
H20cm	29.86	23.79	36.57	9.14×10^7	5.02×10^9	1.09×10^6
H10cm	...	...	38.39	...	...	3.51×10^5
Budding	...	...	41.43	...	...	4.42×10^4
H-10cm	...	...	35.71	...	...	2.06×10^6
H-20cm	...	...	35.55	...	...	2.14×10^6
H-30cm	...	...	36.31	...	...	1.30×10^6
H-40cm	...	...	34.38	...	...	4.63×10^6
H-50cm	...	...	30.93	...	...	4.51×10^7
H-60cm	...	...	31.45	...	...	3.20×10^7

^y Ct = crossing threshold and qPCR = quantitative polymerase chain reaction.

^z For multiplex qPCR with primer-probe sets HLBaspr and COXfpr (17), 1 μ l of 200- μ l DNA elution obtained from 200 mg of huanglongbing-infected fresh tissues was used. The bacterial population was obtained based on the grand universal regression equation for citrus plants: $Y = 13.82 - 0.2866X$ (19) and two rRNA operons (containing the PCR target 16S rRNA) per cell of the bacterium (16).

populations of ‘*Ca. L. asiaticus*’ in the central axis. Populations of ‘*Ca. L. asiaticus*’ in both cultivars of symptomatic fruit were highest in the fruit septa and locular membranes, with lower populations of the pathogen in the peel (Fig. 1B). Seed from infected fruit are among the best sources of tissue for the isolation of *S. citri* (1). We are currently investigating the interaction of ‘*Ca. L. asiaticus*’ and citrus seed.

ACKNOWLEDGMENTS

The research was supported as part of the project 71681-V7R01 financed by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service PPQ-CPHST and by project 1275-22000-251-00D of USDA–Agricultural Research Service. We thank personnel in the Department of Plant Industry, Gainesville, FL and the APHIS-PPQ-CHRP in Florida for providing plant materials, and C. Paul for technical and graphical assistance.

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