Colonization of Citrus Seed Coats by '*Candidatus Liberibacter asiaticus*': Implications for Seed Transmission of the Bacterium

Mark E. Hilf

U.S. Department of Agriculture, Agricultural Research Service, United States Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945.

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ABSTRACT

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Huanglongbing is an economically damaging disease of citrus associated with infection by '*Candidatus Liberibacter asiaticus*'. Transmission of the organism via infection of seeds has not been demonstrated but is a concern since some citrus varieties, particularly those used as rootstocks in commercial plantings are propagated from seed. We compared the incidence of detection of '*Ca. Liberibacter asiaticus*' DNA in individual fruit peduncles, seed coats, seeds, and in germinated seedlings from 'Sanguenelli' sweet orange and 'Conners' grapefruit fruits sampled from infected trees. Using real-time quantitative PCR (qPCR) we detected pathogen DNA in nucleic acid extracts of 36 and 100% of peduncles from 'Sanguenelli' and from 'Conners' fruits, respectively. We also detected pathogen DNA in extracts of 37 and 98% of seed coats and in 1.6 and 4%

of extracts from the corresponding seeds of 'Sanguenelli' and 'Conners', respectively. Small amounts of pathogen DNA were detected in 10% of 'Sanguenelli' seedlings grown in the greenhouse, but in none of 204 extracts from 'Conners' seedlings. Pathogen DNA was detected in 4.9% and in 89% of seed coats peeled from seeds of 'Sanguenelli' and 'Conners' which were germinated on agar, and in 5% of 'Sanguenelli' but in none of 164 'Conners' seedlings which grew from these seeds on agar. No pathogen DNA was detected in 'Ridge Pineapple' tissue at 3 months post-grafting onto 'Sanguenelli' seedlings, even when pathogen DNA had been detected initially in the 'Sanguenelli' seedling. Though the apparent colonization of 'Conners' seeds was more extensive and nearly uniform compared with 'Sanguenelli' seeds, no pathogen DNA was detected in 'Conners' seedlings grown from these seeds. For either variety, no association was established between the presence of pathogen DNA in fruit peduncles and seed coats and in seedlings.

Huanglongbing (HLB) (also known as citrus greening) is a disease of citrus associated with infection by three species of α -proteobacteria, '*Candidatus Liberibacter asiaticus*', '*Ca. Liberibacter americanus*', and '*Ca. Liberibacter africanus*' (2,9,28,29). In addition, two '*Ca. Phytoplasma* spp.' have been associated with HLB-like symptoms in China (4) and Brazil (30), though it is not clear the extent to which these organisms contribute to the epidemiology of the disease. Disease symptoms on leaves include a blotchy-mottle or more extensive chlorosis which can resemble certain nutrient deficiencies, highly chlorotic young shoots, stunting, and a thinning of the canopy as the disease progresses. In extreme cases, the tree dies from the infection. Yield on affected trees can be reduced and trees may produce smaller fruit of unacceptable commercial quality. Most if not all commercial citrus varieties are susceptible and commercial losses due to reduced fruit quality, reduced yield, and deleterious effects on tree growth can be substantial. For additional information on these topics, the historical and horticultural aspects and epidemiology of the disease, several recent and extensive reviews are available (2,9,34).

The genome of '*Ca. Liberibacter asiaticus*' has been sequenced by two research groups (6,33), greatly increasing the available information on the genetic and the derived biochemical and

physiological characters of this organism. Attempts have been made to develop axenic cultures of the three aforementioned species of liberibacter but the results have been mixed (5,25).

Long-distance (global) transmission of these pathogens is by movement of infected citrus propagation material and local short distance or tree-to-tree movement can occur by two species of psyllid (Hemiptera: Psyllidae). '*Ca. Liberibacter asiaticus*' and '*Ca. Liberibacter americanus*' are vectored by *Diaphorina citri* (Kuwayama) (3,35) and '*Ca. Liberibacter africanus*' is vectored by *Trioza erytreae* (Del Guercio) (20). *D. citri* is present now in eight states in the continental United States, including Florida, California, Arizona, and Texas (8,22).

In the United States, '*Ca. Liberibacter asiaticus*' was identified in Florida in 2005 (11) and is now widespread in Florida but has not been detected in California, Texas, or Arizona, the three other commercially important citrus-producing areas in the United States. In addition to the movement of infected live seedlings or mature citrus material for propagation or the movement of psyllids carrying the bacterium, vertical transmission through citrus seed with the subsequent distribution of the seed to noninfested areas is another possible mode of increasing the distribution of '*Ca. Liberibacter asiaticus*'.

In the United States and other major citrus-producing areas, citrus scion varieties for commercial or other uses are propagated vegetatively almost universally to maintain the horticultural characteristics of the mature plant. However, rootstock varieties to which the scion material is grafted are propagated mainly from seed. In the United States, rootstock seedlings usually are grown locally since citrus-producing states enforce regulations which do not allow the importation and distribution of citrus plant material without rigorous testing for specific pathogens under quarantine, a process which may take years. The seedborne status of '*Ca.*

Corresponding author: M. E. Hilf; E-mail address: mark.hilf@ars.usda.gov

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Liberibacter asiaticus' has not been clarified so the movement of citrus seeds from infested areas of '*Ca. Liberibacter asiaticus*' to noninfested areas is a concern.

Several studies have been published in recent years that addressed this aspect of liberibacter biology. Shokrollah et al. (27) found no molecular or pathological evidence for seed transmission of '*Ca. Liberibacter asiaticus*' in the mandarin (*Citrus reticulata*) cultivar Limau Madu although their study population of 20 seedlings was small. Albrecht and Bowman (1) used polymerase chain reaction (PCR) to assay for '*Ca. Liberibacter asiaticus*' DNA in extracts from over 1,000 citrus seedlings germinated from seeds from fruit from infected trees in the field in Florida. Extracts from five seedlings yielded the appropriate amplicon upon the initial analysis several months after germination, but the amplicon was not produced in subsequent samplings as the seedlings grew and none of these seedlings showed foliar symptoms of HLB. In their study the peduncles of each fruit were tested so it was known at the beginning of the study if the vascular tissue going into a particular fruit contained '*Ca. Liberibacter asiaticus*'.

Hartung et al. (12) extracted and planted seed from symptomatic fruit from infected trees but their quantitative PCR analyses found no '*Ca. Liberibacter asiaticus*' DNA in extracts of several hundred seedlings monitored over several years of growth. Graham et al. (10) removed seed coats from seeds prior to germination so a match could be made between the detection of pathogen DNA in the seed coat (23) and the seedlings and Shatters (26) germinated seeds on agar under sterile conditions in addition to germinating seeds in soil. Similar to what was found in the Albrecht and Bowman (1) study, Graham et al. (10) and Shatters (26) each detected pathogen DNA in extracts from a small percentage of seedlings. However, no additional information was presented and none has come forth indicating if these few seedlings had active seedborne infections of '*Ca. Liberibacter asiaticus*' or whether these also may have been spurious positive results as found in the Albrecht and Bowman (1) study.

Several of these studies had a unique component: (i) testing peduncles of fruit from which seeds were collected (1), (ii) removing seed coats to correlate detection of the pathogen in seed coats and seedlings (10), and (iii) germinating seeds under sterile conditions as well as in soil under standard greenhouse conditions (26).

We developed a study to address the question of seedborne transmission of '*Ca. Liberibacter asiaticus*' that incorporated the unique aspects of the aforementioned studies. We selected infected trees of 'Sanguenelli' sweet orange (*Citrus sinensis* L.) and 'Conners' grapefruit (*C. paradisi* Macf.) and collected fruit from these trees. Seeds of each variety were either (i) destructively sampled with removal of the seed coat with separate testing of seed coat and seed for the pathogen, (ii) planted in soil and germinated under greenhouse conditions with testing of the seedlings, or (iii) sterilized with subsequent removal and testing of the seed coat and germination of the seeds on agar under sterile conditions and testing for the pathogen with comparison of what is detected in the seed coat and in the seedlings.

This report describes an interesting and significant difference in the results for the 'Sanguenelli' and 'Conners' varieties and discusses the possible relevance of this difference to the question of seed transmission of '*Ca. Liberibacter asiaticus*'.

MATERIALS AND METHODS

'Sanguenelli' sweet orange and 'Conners' grapefruit source trees. Single infected trees of each variety with leaves showing characteristic HLB-like blotchy-mottle symptoms were in a collection of citrus germplasm on United States Horticultural Research Laboratory property in Fort Pierce, FL. Originally these trees were propagated in the greenhouse at the A.H. Whitmore Foundation Farm, Lake County, Florida and were determined to

be free from *Citrus tristeza virus*, *Citrus psorosis virus*, and *Citrus exocortis viroid* before they were planted in Fort Pierce during August 1999. They are presumed not to have been infected with '*Ca. Liberibacter asiaticus*' at the time of planting as the bacterium had not been detected in Florida at that time. These trees likely were infected by psyllid inoculation but at an unknown time after planting. Foliar and fruit samples of 'Sanguenelli' were collected at the end of March through the beginning of April 2009. The fruit were derived from flowers from the spring 2008 bloom. The foliar and fruit samples of 'Conners' were collected in November 2009. The fruits were derived from blooms in the spring of 2009. Fruits were collected with attached peduncles when possible. The canopy of each tree was visually divided into quarters and foliar samples were collected from each quarter for extraction of nucleic acids from mid-ribs and assaying for '*Ca. Liberibacter asiaticus*' DNA by quantitative real-time PCR (qPCR). None of the 'Conners' fruits showed the classic bicolor fruit symptoms of HLB while several, but definitely a minority, of 'Sanguenelli' fruits showed this classic symptom.

Division of 'Sanguenelli' and 'Conners' seeds for analysis.

Many citrus varieties are seedless to near-seedless because of consumer or industry preference. 'Conners' is not a commercially grown grapefruit variety in part because its fruits are seedy. 'Sanguenelli' also is not a commercial variety, but like many sweet orange varieties, the number of seeds per fruit is small. The range of the number of viable seeds in an individual 'Sanguenelli' fruit used in this study was 2 to 14 seeds, whereas the range for an individual 'Conners' fruit was 33 to 64 seeds. Seeds from the two varieties were divided into three groups for (i) "destructive analysis" of seeds, (ii) germination of seeds in soil in the greenhouse, and (iii) germination on agar under sterile conditions. Because the seed count per individual fruit of 'Sanguenelli' was generally low, groups of 'Sanguenelli' fruit were selected and seeds from these fruit were dedicated to one phase of the analysis as this provided an appropriate number of seeds. In contrast, because of the much higher seed count per fruit, seeds from an individual 'Conners' fruit were used in all three phases of the analysis, with 10 seeds removed from each fruit for analysis of the separated seed coat and seed and for germination on agar, with the remaining seeds germinated in the greenhouse.

Greenhouse germination of 'Conners' and 'Sanguenelli' seedlings.

Before planting, an outer mucilaginous layer was removed from the seeds by washing in 2% (vol/vol) household dishwashing detergent for 30 min followed by thorough rinsing with distilled water. Seeds were then treated overnight at 25°C with a dilute solution (110 µl in 250 ml of water) of ADEX-G Pectic Enzyme Solution (Presque Isle Wine Cellars, North East, PA) and thoroughly rinsed with water. Seeds were treated next with a solution of 8-hydroxyquinoline (0.01 g/ml) for 30 min at 25°C, rinsed twice with distilled water, once with sterile water, and then air-dried.

Seeds were evaluated for overall appearance and size and seeds which were expected to germinate were planted in Pro Mix Mycorise Pro soil mix (Premier Tech Horticulture, Rivière-du-Loup, Quebec, Canada) in Cone-tainer cells (Stuewe & Sons, Inc., Tangent, OR) and remained in the greenhouse for the duration of the experiment. Seedlings were fertilized weekly with a general 20-10-20 liquid fertilizer. Greenhouses were cooled by evaporative cooling and the areas around both intake and exhaust portals were enclosed by insect proof screening in the house containing the 'Sanguenelli' sweet orange seedlings. Because of space limitations, the 'Conners' grapefruit seedlings were germinated in a house without screening of intake and exhaust portals, but in both houses scouting for insect pests was performed on a regular basis and pesticides were applied as needed. Though common now in the field in Florida, neither adults nor juveniles of *Diaphorina citri*, the psyllid vector of '*Ca. Liberibacter asiaticus*', were detected in either greenhouse during this study.

Grafting ‘Ridge Pineapple’ sweet orange to ‘Sanguenelli’ seedlings germinated in the greenhouse. ‘Sanguenelli’ seedlings that were germinated in the greenhouse in spring of 2009 were grafted with buds from 1-year-old ‘Ridge Pineapple’ sweet orange seedlings in spring of 2010 to look at potential transmission of bacteria from the ‘Sanguenelli’ stock to the ‘Ridge Pineapple’ scion. The surviving ‘Ridge Pineapple’ buds were grown out and sampled at 75 to 105 days post-grafting for the presence of ‘*Ca. Liberibacter asiaticus*’ DNA.

Germination of ‘Sanguenelli’ and ‘Conners’ seedlings on agar. As was done for germination of seeds in soil in the greenhouse, seeds for germination on agar were visually assessed and healthy appearing seeds were chosen. Seeds were treated as described in the section on greenhouse-grown seedlings. In addition, in a laminar flow hood, the seed coat was removed from each seed for extraction of nucleic acid. Individual seeds were placed in a 2-ml microcentrifuge tube and treated with 95% ethanol for 5 min and then rinsed once with sterile distilled water followed by treatment with a solution of 10% (vol/vol) household bleach for 30 min followed by three rinses in sterile distilled water. Treated seeds were placed in Magenta boxes on 0.8% agar (Sigma-Aldrich, St. Louis, MO) supplemented with Murashige and Skoog basal salt mixture with vitamins (*PhytoTechnology Laboratories*, Shawnee Mission, KS). Seeds were germinated in an incubator maintained at 25°C with a 16/8 h light/dark regime for the duration of the experiment. Cultures were assessed at least weekly and contaminated cultures were removed from the experiment.

DNA extractions, conventional and quantitative real-time qPCR assays. Sample pulverization and extraction of nucleic acids were performed with a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK) with 0.2 g of tissue in a 2.0-ml microcentrifuge in the presence of the appropriate extraction buffer. Leaf mid-vein tissue was pulverized for 60 s and tougher tissues such as seed coats and fruit peduncles were pulverized for 3 min. Extraction of nucleic acids from pulverized tissues was done primarily with a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. In some cases, tissue was pulverized in buffer composed of 0.1 M Tris, pH 8.0, 50 mM EDTA, 0.5 M NaCl, and 10 mM 2-mercaptoethanol with the extracted nucleic acid purified using an sodium dodecyl sulfate-sodium acetate protocol according to Irey et al. (15). DNA concentrations were standardized and 25 ng was used in each amplification reaction.

The detection of ‘*Ca. Liberibacter asiaticus*’ sequences in nucleic acid extracts was performed by qPCR with primer and probe oligonucleotides specific for a portion of the ‘*Ca. Liberibacter asiaticus*’ 16S rRNA gene as described in Li et al. (18). Duplicate 20- μ l reactions containing 25 ng of DNA, 0.25 μ M each of the forward and reverse primers and 0.15 μ M probe and 2 $\times$ TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) were subject to one cycle of denaturation at 95°C for 20 s followed by 40 cycles of denaturation for 3 s at 95°C and annealing/extension at 60°C for 30 s using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The presence of target template in a sample was confirmed if the cycle threshold (Ct) value did not exceed 36.0 (32). Samples with amplification results indicated as “undetermined” were assigned a Ct of 40.0 for the sake of data comparison and discussion. For the sake of discussion, a nucleic acid extract in which ‘*Ca. Liberibacter asiaticus*’ DNA was detected is considered positive, whereas lack of detection indicates a negative sample. In all qPCR analyses, duplicated samples of nucleic acid extracts from foliar tissues of ‘*Ca. Liberibacter asiaticus*’ infected and noninfected trees maintained in insect-proof greenhouses were included as positive and negative controls.

In some instances, in addition to qPCR, conventional PCR was used to assess the presence of ‘*Ca. Liberibacter asiaticus*’ DNA in nucleic acid extracts. Several primer pairs were used to amplify

target sequences by conventional PCR: (i) 5'-CACCGAAGATATGGACAACA-3' (+)/5'-GAGGTTCTTGTGGTTTTTCTG-3' (–), which amplifies a 226-bp fragment of the ‘*Ca. Liberibacter asiaticus*’ *secE* gene (14), GenBank Accession EF164805); (ii) 5'-CTTACCAGCCCTTGACATGTATAGGA-3' (+)/5'-TCCCTATAAGTACCCAACATCTAGGTAAA-3' (–) which are called USHRL-CL1f and USHRL-CL1r primers which amplify a 195-bp fragment of the ‘*Ca. Liberibacter asiaticus*’ 16S rRNA gene (21); (iii) 5'-TCGAGCGCGTATGCAATACG-3' (+)/5'-CTACCTTTTCTACGGGATAACGC-3' (–) which are the forward and reverse primers, respectively, used in the qPCR protocol devised by Li et al. (18) and which amplify a 75-bp fragment of the 16S rRNA gene; and (iv) 5'-GGTCCATTGGCAAATTGCGTTATGG-3' (+)/5'-CCTTGCAATACCCACGGGTCC-3' (–) which amplify a 503-bp fragment of the ‘*Ca. Liberibacter asiaticus*’ metalloprotease gene (GenBank Accession no. EF164804). The cycling parameters for the first three primer pairs were one cycle at 94°C for 60s, followed by 30 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s and one cycle at 72°C for 10 min. The cycling parameters for amplification of the zinc metalloprotease gene were the same except the initial cycle was 94°C for 2 min.

DNA amplified by conventional PCR was evaluated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and visualized with a Kodak Gel Logic 200 Imaging System (Eastman Kodak, Rochester, NY).

RESULTS

Detection of ‘*Ca. Liberibacter asiaticus*’ DNA in foliar samples from the ‘Sanguenelli’ sweet orange and ‘Conners’ grapefruit trees. Because of an expected inconsistent distribution of ‘*Ca. Liberibacter asiaticus*’ within the canopy of the tree, the canopy was visually divided into four vertical quadrants. Ten and twenty foliar samples with apparent HLB-like symptoms were collected from the four vertical quarters of the canopies of the ‘Sanguenelli’ and ‘Conners’ trees, respectively. The DNA of ‘*Ca. Liberibacter asiaticus*’ was detected with qPCR in extracts from 4/10 (40%) of the ‘Sanguenelli’ samples, with Ct values of 23.1 to 25.1 and in extracts from 14/20 (70%) of ‘Conners’ samples, with Ct values of 24.4 to 29.4. In addition, to corroborate the qPCR results, the correctly sized amplicons were amplified from these positive samples by conventional PCR using the zinc metalloprotease, CL1, *secE*, and qPCR forward and reverse primers (no probe) as described in the Materials and Methods (data not shown). Although all foliar samples from ‘Sanguenelli’ and ‘Conners’, respectively, did not contain detectable ‘*Ca. Liberibacter asiaticus*’ DNA, these molecular data corroborate the foliar symptoms observed and indicate that each tree is infected with the bacterium.

Detection of ‘*Ca. Liberibacter asiaticus*’ DNA in peduncles, seed coats and seeds of ‘Sanguenelli’ sweet orange and ‘Conners’ grapefruit. Sixty fruits were collected from the ‘Sanguenelli’ tree and 13 from the ‘Conners’. Fifty-five ‘Sanguenelli’ fruits had attached peduncles which were tested for ‘*Ca. Liberibacter asiaticus*’ DNA. Five ‘Sanguenelli’ fruits were missing the peduncle, so tissue from the central axis of the fruit was tested, as extracts from this tissue were shown in previous studies to have detectable levels of ‘*Ca. Liberibacter asiaticus*’ DNA (19,23).

In this part of the study, peduncles were removed from each fruit for analysis, seeds were removed from the fruits and seed coats removed from the seeds with the seed coat and seed (embryo and cotyledon tissue) tested separately for ‘*Ca. Liberibacter asiaticus*’ DNA. For this part of the study, 20 ‘Sanguenelli’ fruits were selected, which yielded 128 seeds and 10 seeds from each of 12 ‘Conners’ fruits were collected for destructive analysis. Based on size and appearance, seeds were visually assessed as viable or nonviable relative to their germinating if planted. A

normal range of appearance of seeds from both ‘Sanguenelli’ and ‘Conners’ is represented in Figure 1A by eight ‘Conners’ seeds. Seeds similar to numbers 1 to 5 were considered to be viable for germination, whereas seeds similar to numbers 6, 7, and 8 were considered nonviable for germination (Fig. 1A). Even though seeds similar in appearance to 3 and 4 had a brownish color to a portion of the seed coat, the seed coat was intact and the seed felt firm and relatively plump. Seeds similar to number 5 were smaller than seeds 1 to 4, but the seed coat was a healthy appearing tan to white and the seed coat appeared intact and the seed firm. The seed coats of seeds 6, 7, and 8 were a brownish color and seeds were not firm or were shriveled. For analysis, nonviable seeds were processed whole for extraction of DNA, whereas the seed coat was removed from viable seeds and the seed coat and seed were processed and analyzed separately (Fig. 1B).

Since the large number of samples prohibited listing each individual sample mean, the column head “Range of Ct values” in Tables 1 and 2 presents the range of these individual sample means for samples which were judged positive for the pathogen DNA based on the Ct value. The value listed under the column heading “Mean Ct” is the mean derived from the indicated range of individual positive sample means with indicated standard deviations. In some instances it is pertinent to present and discuss individual sample means and these will be indicated as such in the text.

Pathogen DNA was detected in extracts from 8/20 (40%) ‘Sanguenelli’ peduncles (Table 1). The range of mean Ct values was 22.7 to 33.1 with an overall mean of 26.2. ‘*Ca. Liberibacter asiaticus*’ DNA was detected in extracts of seed coats from 47/128

(37%) ‘Sanguenelli’ seeds recovered from these 20 fruit (Table 1). Thirty-nine of these positive seed coats were from fruit with a positive peduncle and eight seed coats were from fruit with no ‘*Ca. Liberibacter asiaticus*’ DNA detected in peduncle extracts. Pathogen DNA was not consistently detected in seed coats from the same fruit. Pathogen DNA was detected in all seed coats from only three fruits, in at least one seed coat from nine fruits, and in none of the seed coats from eight fruits, even though pathogen DNA was detected in the peduncles of two of these eight fruits. The level of pathogen DNA detected in seed coat extracts was also variable, with a range of Ct values of 22.2 to 35.9 with an overall mean of 27.7.

‘*Ca. Liberibacter asiaticus*’ DNA was detected in only 1.6% (2/128) of the seed extracts from these 20 ‘Sanguenelli’ fruits. The two Ct values were 29.1 and 35.5. Since the incidence of pathogen DNA was higher in seed coats, the lower incidence and higher Ct values for the seeds suggest that in either or both instances, amplification may have been from DNA from remnant seed coat which was not completely removed.

The presence of pathogen DNA was assessed in nonviable seeds collected from the ‘Sanguenelli’ fruits; typically, nonviable seeds were smaller than the designated viable seeds collected from fruits of both varieties (Fig. 1A). Twenty-three nonviable seeds were collected from these 20 ‘Sanguenelli’ fruits and ‘*Ca. Liberibacter asiaticus*’ DNA was detected in extracts from four seeds, two seeds each from two fruits (Table 1). The range of Ct values was 26.2 to 30.1 with an overall mean of 27.6. All seed coats from the viable seeds from these two fruits also were positive for ‘*Ca. Liberibacter asiaticus*’ DNA.

Fig. 1. A, Range of appearance of seeds recovered from fruit from an infected ‘Conners’ grapefruit tree. From left to right, seeds 1 to 5 were considered viable for germination in soil in the greenhouse and on agar. Seeds 6, 7, and 8 were considered nonviable and were not planted. **B,** Seeds and seed coats as they appear after separation for separate analysis.

TABLE 1. Quantitative polymerase chain reaction (qPCR) results for detection of ‘*Candidatus Liberibacter asiaticus*’ DNA in extracts from viable and nonviable seeds from 20 fruits of ‘Sanguenelli’ sweet orange and 12 fruits of ‘Conners’ grapefruit

Cultivar	Tissue	Results		
		Positives	Range of Ct values ^y	Mean Ct ^z
‘Sanguenelli’	Peduncle	8/20	22.7–33.1	26.2 ± 3.5
	Seed coat	47/128	22.2–35.9	27.7 ± 5.3
	Seed	2/128	29.1, 35.5	32.3 ± 4.5
	Seed	4/23	26.2–30.1	27.6 ± 1.7
	Nonviable			
‘Conners’	Peduncle	12/12	23.8–28.9	26.7 ± 1.4
	Seed coat	117/120	20.5–35.9	26.6 ± 3.5
	Seed	5/120	30.9–35.1	33.4 ± 1.6
	Seed	34/85	26.3–34.7	30.3 ± 1.8
	Nonviable			

^y Only cycle threshold (Ct) values for samples evaluated as positive for ‘*Ca. Liberibacter asiaticus*’ DNA are included in this range.

^z Mean Ct ± indicated standard deviations.

In contrast to 'Sanguenelli' fruits, '*Ca. Liberibacter asiaticus*' DNA was detected in extracts from 12/12 peduncles from 'Conners' fruits, with Ct values of 23.8 to 28.9 (Table 1). The qPCR analysis of 120 seed coats from these 12 fruits showed 97.5% (117/120) of seed coats were positive for DNA of '*Ca. Liberibacter asiaticus*' (Table 1). As was seen for 'Sanguenelli' fruits, the number of 'Conners' seeds which were positive for '*Ca. Liberibacter asiaticus*' DNA was small at 5/120 (4%). Four of these positive seeds were from one fruit and Ct values were 35.1, 34.4, 33.2, and 30.9 with Ct values from the corresponding seed coats of 22.9, 23.3, 23.4, and 21.2, respectively. There was one additional positive seed from another fruit with a Ct value of 33.2 and a Ct of 24.4 for the corresponding seed coat. As was found for the 'Sanguenelli' positive seeds, the Ct values for the five positive 'Conners' seeds were much higher than for the corresponding seed coats.

'*Ca. Liberibacter asiaticus*' DNA was detected in 40% (34/85) of nonviable seeds collected from 8 of the 12 'Conners' fruits (Table 1). The Ct values for nonviable 'Conners' seeds were generally higher than those from 'Sanguenelli' fruits with a range of 26.3 to 34.7 and an overall mean of 30.3. Not all nonviable seeds from a designated 'Conners' fruit had detectable pathogen DNA, even if pathogen DNA was detected in all seed coats from viable seeds from that fruit. All nonviable seeds from four of the eight fruits had detectable '*Ca. Liberibacter asiaticus*' DNA, whereas pathogen DNA was detected in only a portion of the nonviable seeds from the other four fruits.

Germination of 'Sanguenelli' and 'Conners' seeds in the greenhouse and detection of '*Ca. Liberibacter asiaticus*' DNA in germinated seedlings. One hundred thirteen 'Sanguenelli' seeds considered viable for germination were recovered from a group of 16 fruits and planted in the greenhouse on 2 April 2009. Nineteen seeds (17%) either failed to germinate or the seedlings did not survive to be sampled. Harvesting of tissue for analysis occurred during a period from 110 to 137 days post-planting (dpp) (Table 2). Although 94 seeds germinated and grew successfully, due to the polyembryonic character of citrus in which clonal nucellar embryos and zygotic embryos can develop from the same seed (17), some seeds produced more than one seedling, so foliar samples from a total of 136 seedlings were collected and tested.

Of the 136 'Sanguenelli' seedlings germinated and grown in the greenhouse, 122 (90%) had no detectable '*Ca. Liberibacter asiaticus*' DNA when sampled at 110 to 137 dpp (Table 2), whereas assays of extracts from 14 seedlings yielded Ct values indicating the presence of pathogen DNA. These Ct values ranged from 30.8 to 35.9 with an overall mean of 33.5. These seedlings germinated from seeds from only three fruits, with eight, five, and one seedling derived from seeds from these three fruits, with Ct

values of 30.8 to 34.4, 33.9 to 35.9, and 34.9, respectively, for the extracts from the seedlings.

Seedlings were observed for any aberrations in growth or for foliar symptoms reminiscent of those used to diagnose the disease in mature trees. Figure 2A to C shows seedlings which represent the variability seen in the appearance of greenhouse germinated 'Sanguenelli' seedlings near the time seedlings were assessed for the presence of '*Ca. Liberibacter asiaticus*' DNA. Figure 2A and B shows two seedlings from the same fruit; one is stunted with some foliar blotchy-mottle similar to symptoms observed in mature trees, while the other is more normal in appearance and size. Figure 2C shows a seedling which is extremely stunted with extensive chlorosis similar to what is seen in some shoots on mature field trees. No pathogen DNA was detected in extracts from any of these seedlings. The 'Sanguenelli' seedlings which tested positive for pathogen DNA showed a similar range in phenotypes, with no apparent association of the phenotype of the seedlings and the detection of pathogen DNA in nucleic acid extracts.

Two hundred seventy-three 'Conners' seeds were collected from the 12 fruits and planted in soil in the greenhouse on 18 November 2009. In this experiment, multiple seedlings arising from the same seed were harvested together and analyzed as a pooled sample.

Generally seeds were slow to germinate compared with seeds from fruits from an uninfected 'Duncan' grapefruit source (a variety indistinguishable from 'Conners') planted at the same time (data not shown) and germination was poor for all seeds from some fruits. Sixty-nine seeds failed to germinate or seeds germinated but seedlings did not survive the study period. Seedlings of two fruits were harvested at 187 dpp as many of these seedlings were stunted and showed little or no growth with distorted and chlorotic leaves and it was not certain if they would survive a longer incubation period. All other seedlings were sampled at 218 dpp.

The germinated 'Conners' seedlings showed a range of phenotypes similar to those observed in 'Sanguenelli' seedlings, but in general, the chlorosis and stunting of 'Conners' seedlings were more frequent and more severe than that seen with the 'Sanguenelli' seedlings. Figure 2D shows two seedlings from the same fruit, one of which is stunted and highly chlorotic (right) whereas the other appears healthy and dark green (left). Figure 2E and F shows the extreme chlorosis and stunting seen in two seedlings derived from seeds from two different fruits.

Irrespective of the appearance of the seedlings, no '*Ca. Liberibacter asiaticus*' DNA was detected in extracts from tissues collected from the 204 'Conners' grapefruit seedlings which were grown in the greenhouse (Table 2). Since these seeds were planted with the seed coat intact, it is not known if '*Ca. Liberibacter*

TABLE 2. Quantitative polymerase chain reaction (qPCR) results for detection of '*Candidatus Liberibacter asiaticus*' DNA in extracts from foliar samples of 'Sanguenelli' sweet orange and 'Conners' grapefruit seedlings germinated in soil in the greenhouse and in vitro on agar from seed harvested from fruit from infected trees

Source	Cultivar	Tissue	PCR results		
			Positives	Range of Ct values ^w	Mean Ct ^x
Greenhouse	'Sanguenelli'	Peduncle	8/16	23.5–31.3	26.2 ± 2.7
		Seedlings	14/136	30.8–35.9	33.5 ± 1.8
	'Conners'	Peduncle ^y	12/12	23.8–28.9	26.7 ± 1.4
		Seedlings	0/204	N/A ^z	N/A
Agar	'Sanguenelli'	Peduncle	5/24	24.9–33.7	29.1 ± 3.8
		Seed coat	5/102	22.1–35.3	30.4 ± 5.9
		Seedlings	7/139	31.4–35.6	34.3 ± 1.6
	'Conners'	Peduncle	12/12	23.8–28.9	26.7 ± 1.4
		Seed coat	106/120	21.4–35.8	27.3 ± 3.3
		Seedlings	0/168	N/A	N/A

^w Only cycle threshold (Ct) values for samples evaluated as positive for '*Ca. Liberibacter*' DNA are included in this range.

^x Mean Ct ± indicated standard deviations.

^y Values for peduncles are the same for both data sets because all 'Conners' seeds are derived from the same fruits.

^z N/A: not applicable. No positive DNA samples were identified so no values are entered.

asiaticus' colonized these seed coats to the extent found for the seeds characterized in Table 1, even though both sets of seeds were from the same fruits. Even though '*Ca. Liberibacter asiaticus*' DNA was detected in the peduncles of all 'Conners' fruits used in this study (Table 2), there was no apparent association between the detection of pathogen DNA in the peduncle and in seedlings germinated from seeds from these fruits.

Results of grafting 'Ridge Pineapple' sweet orange buds to 'Sanguenelli' seedlings germinated in the greenhouse. Buds were selected from 16 1-year-old 'Ridge Pineapple' seedlings for grafting to 'Sanguenelli' seedlings (Table 2). Extracts of DNA from all 'Ridge Pineapple' source trees were tested for '*Ca. Liberibacter asiaticus*' before samples for budding were taken and pathogen DNA was not detected in any source tree (data not shown). Grafts were made on 30 March 2010 and regrafting of any buds that did not survive was done during the first week of May 2010. Foliar samples of the 'Ridge Pineapple' scion were harvested on 13 July 2010 at 105 days post-budding for the initial set of plants and at approximately 74 days for the rebudded plants.

Of 80 'Sanguenelli' seedlings budded, 68 seedlings (57 from March and 11 from May) were harvested and tested. Twelve

'Ridge Pineapple' buds either did not survive or the bud survived but was not grown out when foliar tissue was collected.

At the time of the initial budding in March tissue was collected from each 'Sanguenelli' seedling and extracts were tested for pathogen DNA. Interestingly, the extract from tissue from one seedling yielded a Ct of 32.4 (data not shown), indicating the presence of pathogen DNA, yet no pathogen DNA was detected in extracts of this seedling when initially tested at 110 dpp in 2009 (Table 2). Seven 'Sanguenelli' seedlings which were grafted with 'Ridge Pineapple' buds originally tested positive for pathogen DNA (Table 2), but samples taken from these plants immediately prior to budding with 'Ridge Pineapple' indicated no pathogen DNA was present (data not shown). When analyzed at the indicated times, no extracts from any budded 'Ridge Pineapples' tissues were positive for pathogen DNA, indicating that if any bacteria were present in the 'Sanguenelli' stock plant they did not move to colonize the budded tissue (data not shown).

Detection of '*Ca. Liberibacter*' DNA in extracts from 'Sanguenelli' and 'Conners' seedlings germinated on agar. As a comparison to seedlings grown in soil in the greenhouse, seeds from 'Sanguenelli' and 'Conners' fruits were germinated on agar

Fig. 2. Variable appearance of 'Sanguenelli' and 'Conners' seedlings germinated in soil in the greenhouse. Two 'Sanguenelli' sweet orange seedlings germinated from seeds from the same fruit, **A**, one stunted with chlorosis and **B**, the other a healthy appearing seedling. **C**, Seedling with extreme stunting and chlorosis. **D**, Two 'Conners' grapefruit seedlings germinated from seeds from the same fruit, one healthy appearing (left) and the other stunted with chlorosis (right). **E and F**, Two seedlings germinated from seeds from different 'Conners' fruits showing similar extreme stunting and chlorosis.

under sterile conditions. One hundred and two viable ‘Sanguenelli’ seeds collected from 24 fruits and 120 viable ‘Conners’ seeds (10 seeds from each of the 12 fruits) were selected for germination on agar as outlined in Materials and Methods. Seed coats were removed from all seeds, and DNA was extracted and tested for ‘*Ca. Liberibacter asiaticus*’ DNA.

In general, the appearance of seedlings was similar for seedlings of both varieties, and in contrast to the seedlings grown in the greenhouse, seedlings did not display the chlorosis or severe stunting and deformation seen in greenhouse grown seedlings (Fig. 2). Overall, seedlings germinated on agar did not grow as tall during the study period as some greenhouse grown seedlings did, possibly due to the differences in lighting and temperature between the greenhouse and growth chamber.

The 102 ‘Sanguenelli’ seeds germinated on agar yielded 139 seedlings and seven (5%) were rated positive for ‘*Ca. Liberibacter asiaticus*’ DNA; the Ct values were relatively high with a narrow range of values, from 31.4 to 35.6 with a mean of 34.3 (Table 2). The seven positive seedlings germinated from seeds from three fruits, with two fruits each yielding three positive seedlings. Extracts from 5/24 peduncles (21%) from the source fruits had detectable pathogen DNA but none of the positive seedlings germinated from seeds from these fruits. Extracts from 5/102 seed coats (5%) were positive for pathogen DNA, but no positive seedlings germinated from seeds with positive seed coats. The range of Ct values, 24.9 to 33.7 and 22.1 to 35.3, was broader and the overall mean Ct values, 29.1 and 30.4, for the peduncles and seed coats, respectively, were lower than those for the positive seedlings (Table 2). Overall, pathogen DNA was detected in a small but significant percentage of ‘Sanguenelli’ seedlings germinated on agar, but the Ct values indicated a lesser amount of pathogen DNA in samples from these seedlings than in equivalent seed coat and peduncle samples, and more importantly, the presence of pathogen DNA in these seedlings was not associated with the detection of pathogen DNA in the corresponding seed coats or in the peduncle of the source fruit.

The ‘Conners’ seedlings grown on agar were destructively harvested with half of the seedlings from each fruit harvested from 45 to 52 dpp and the remaining harvested from 96 to 136 dpp (Table 2). The 120 ‘Conners’ seeds germinated on agar yielded 168 seedlings; pathogen DNA was not detected in extracts from any seedling (Table 2). In contrast to the low percentage (5%) of extracts of ‘Sanguenelli’ seed coats with detectable pathogen DNA, extracts from 88% (106/120) of seed coats from ‘Conners’ seeds which germinated on agar were rated positive for ‘*Ca. Liberibacter asiaticus*’ DNA (Table 2). The range of Ct values (21.4 to 35.8) for the ‘Conners’ samples was similar to that for the ‘Sanguenelli’ samples but the overall mean (27.3) was lower than for the ‘Sanguenelli’ samples (30.4). Additionally, all peduncles (12/12) from the ‘Conners’ source fruits had detectable pathogen DNA (Table 2). Similar to what was found for the ‘Sanguenelli’ seedlings germinated on agar, the detection of pathogen DNA in the source fruit peduncles and in seed coats of ‘Conners’ was not associated with detection of pathogen DNA in germinated seedlings.

DISCUSSION

We examined vertical transmission of ‘*Ca. Liberibacter asiaticus*’ in seedlings of ‘Conners’ grapefruit and ‘Sanguenelli’ sweet orange germinated from seeds from fruits obtained from infected trees. This manuscript addresses the question of the spread of ‘*Ca. Liberibacter asiaticus*’ via seed transmission in citrus and expands our previous understanding of the association of the presence of the organism in seeds and the occurrence of seed transmission. Pathogen DNA was not detected in 372 ‘Conners’ grapefruit seedlings germinated in either the greenhouse or on agar under controlled conditions but it was detected in 21 out of

275 ‘Sanguenelli’ seedlings germinated in either the greenhouse or on agar under the same controlled conditions. The chosen assay method detects only pathogen DNA so it cannot be stated with certainty that the pathogen DNA detected in these ‘Sanguenelli’ seedlings was derived solely from intact bacterial cells, though this is the usual interpretation of a positive assessment using the qPCR assay. Interestingly, a second assay of seven of the positive ‘Sanguenelli’ seedlings which were germinated in the greenhouse failed to confirm the initial detection of pathogen DNA, suggesting that if vertical transmission occurred in these seedlings, the infection failed to sustain itself as the seedlings grew. Also, the Ct values for foliar samples from these ‘Sanguenelli’ seedlings were within a narrower range and were consistently higher than those for other tissues assayed suggesting a lesser amount of pathogen DNA.

Even though pathogen DNA was detected in all ‘Conners’ fruit peduncles and in 93% of seed coats, no vertical transmission was detected. This is in contrast to the detection of pathogen DNA in only 33% of ‘Sanguenelli’ fruit peduncles and in only 23% of seed coats tested; in addition, none of the seed coats associated with the seven positive ‘Sanguenelli’ seedlings from the agar cultures had detectable pathogen DNA. In this study, there clearly was no association between the detection of pathogen DNA in fruit peduncles or seed coats, so these parameters likely would not be effective for predicting vertical transmission in citrus.

In an earlier study, Tatineni et al. (23) detected ‘*Ca. Liberibacter asiaticus*’ DNA in extracts of seven of seven samples each of peduncles and seed coats, but not in the seven seeds from which the seed coats were derived. So it was not surprising that pathogen DNA was detected in these tissues in this study, although the near uniform incidence of ‘*Ca. Liberibacter asiaticus*’ DNA in peduncles and seed coats of ‘Conners’ grapefruit was an unexpected contrast to the lesser incidence in these tissues of the ‘Sanguenelli’ sweet orange variety. This difference could reflect a basic difference in the extent of colonization of tissues of ‘Sanguenelli’ and ‘Conners’ which may be characteristic of sweet oranges and grapefruit in general but this needs further research. It is interesting to note, though, both varieties shared the characteristic of uneven distribution of the pathogen in samples from the tree canopy, a noted characteristic of infection of citrus with ‘*Ca. Liberibacter asiaticus*’.

Even though a number of ‘Sanguenelli’ and ‘Conners’ seedlings in this study grew poorly and were stunted and chlorotic, no evidence indicated this was due to an infection of the seedling by ‘*Ca. Liberibacter asiaticus*’. No symptoms typical of HLB in mature trees were seen in seedlings in two prior studies which evaluated several thousand seedlings (1,12). But since it is not clear that an infection of young seedlings via seed has been verified, it is uncertain what symptoms would be expressed in these very young seedlings. In some cases, the altered appearance and growth of the ‘Sanguenelli’ and ‘Conners’ seedlings noted in this study could be an indirect effect due to the colonization of the seed coat, but there were no seeds from fruit from noninfected ‘Sanguenelli’ and ‘Conners’ planted concurrently for comparison.

Several recent studies on seed transmission used PCR to test for ‘*Ca. Liberibacter asiaticus*’ in seedlings grown from seeds from fruit from infected trees. Graham et al. (18) used the same qPCR assay (10) to study 415 ‘Pineapple’ sweet orange seedlings that they germinated from seeds from fruit from infected trees and found seven that gave what the authors viewed as questionable or positive Ct values, but this number was reduced to three upon a second assay. From 2006 to 2008, the authors assayed over 1,500 seedlings and identified 16 seedlings which yielded Ct values which the authors felt were low enough to warrant follow up work, such as psyllid transmission studies from these plants. The results of these additional studies are not available, so it is not known if the seedlings the authors identified maintained stable infections of ‘*Ca. Liberibacter asiaticus*’.

Shatters reported a summary of a study in which putative symptoms were observed and '*Ca. Liberibacter asiaticus*' DNA was detected in extracts from a small percentage of 'Duncan' grapefruit seedlings ('Duncan' is a variety very similar to if not indistinguishable from 'Conners') grown in soil in the greenhouse and on agar under sterile conditions (26). He noted that the highest levels of bacterial DNA were in roots of the sterile grown seedlings, a tissue in which Tatineni et al. (23) also found significant amounts of pathogen DNA. It is not clear from this 2008 summary if these seedlings were maintained for a longer period and sampled again to confirm the continued presence of pathogen DNA.

Hartung et al. (12) used the same qPCR assay as in this study and found no evidence of seed transmission to 723 seedlings of seven species of *Citrus* (including a variety of sweet orange and one of grapefruit) and from *Murraya paniculata*, a citrus relative, even though the seeds which were germinated were collected from symptomatic fruit which were located on branches which were tested and found infected with the pathogen. This study did not analyze for the presence of the pathogen in either the peduncles of these fruit or in seed coats.

Using conventional PCR, Albrecht and Bowman (1) analyzed extracts of 1,117 seedlings germinated from seeds from fruit from infected trees and detected '*Ca. Liberibacter asiaticus*' DNA in extracts from 2 of 69 young seedlings of 'Sun Chu Sha' mandarin (*Citrus reticulata*) and in three of 431 'Valencia' sweet orange seedlings tested at 5 and 2 months after planting, respectively. The authors noted that in all cases where pathogen DNA was detected in extracts from seedlings the amplicon levels were low and no amplicons were obtained from extracts from tissue samples taken from these seedlings at later dates. This result is similar to that observed in this study for the 'Sanguenelli' seedlings germinated in the greenhouse which yielded high and questionable Ct values when initially tested, but which tested negative for the pathogen at a later date. In both cases, the detection of low levels of pathogen DNA in initial samples and then its lack of detection in subsequent samples may reflect an incidence of laboratory contamination which was not repeated at the later dates. Or, the data may reflect unsuccessful colonization of embryos by the bacterium and the assays detected the remnant DNA, as suggested by Albrecht and Bowman (1).

In the field, citrus trees are likely infected by direct inoculation of the bacterium into the phloem by psyllids. In an extensive review, Bové (2) discusses the electron microscopic evidence that indicates '*Ca. Liberibacter asiaticus*' colonizes sieve tubes in mature phloem exclusively. In the developing seed, the seed vascular bundle in the outer integument is the likely source for the bacterial DNA detected in seed coats in this study and in the study by Tatineni et al. (23). The developing seed would behave as a sink for photosynthate movement through the phloem so bacterial colonization of the phloem in the seed coat vascular bundle is reasonable if the bacteria are introduced into the vascular system elsewhere (since direct inoculation of phloem tissue in the seed coat is not likely) and subsequently move through the phloem to colonize the phloem in the vascular bundle in the developing seed.

Based on the Ct values of the qPCR tests, moderate to large amounts of pathogen DNA were detected in 93% (224/240) of nucleic acid extracts of seed coats of 'Conners' grapefruit seeds assayed in this study, even though no pathogen DNA was detected in extracts of 372 seedlings germinated from these seeds. The qPCR results imply a large population of cells in these seed coats, most likely in the vascular bundle as indicated above, but the presence of the bacteria in the seed coat was on its own not sufficient to lead to infection of the embryo.

As shown in earlier microscopic studies (24) and as discussed by Albrecht and Bowman (1) there is no vascular continuity between maternal seed tissue and the developing embryo so a path by which '*Ca. Liberibacter asiaticus*' would colonize the devel-

oping embryo remains undefined. If '*Ca. Liberibacter asiaticus*' colonizes mature phloem to utilize the contents as a nutrient source then it seems unlikely that colonization of the embryo by this path would occur since mature phloem tissue is not present in the embryo.

A recent ultrastructural study of citrus seedlings infected with '*Ca. Liberibacter asiaticus*' by graft inoculation showed that sieve tubes in younger asymptomatic tissue had higher numbers of bacteria than sieve tubes in older, symptomatic tissue, even though both tissues had equivalent Ct values indicating equivalent levels of bacterial DNA and hence supposedly equivalent levels of bacterial cells (7). The authors speculated that Ct values from DNA extracts of older tissue may be derived primarily from DNA from fragmented dead cells, whereas the Ct values from younger tissues may be derived primarily from DNA from intact live cells. This speculation is supported by results from Trivedi et al. (31), who used ethidium monoazide with qPCR to estimate that only 17 to 31% of '*Ca. Liberibacter asiaticus*' cells in samples of symptomatic citrus tissue are viable.

Recent other electron microscope studies of '*Ca. Liberibacter asiaticus*' in infected tissues of citrus (16) and dodder (*Cuscuta indecora*) (13) serve to highlight that microscopic analyses either have not been done or the results have not been included in published studies on seed transmission of '*Ca. Liberibacter asiaticus*'. Instead, studies including this one relied upon qPCR to establish indirectly the presence of bacteria by detection of bacterial DNA. In this study we established that infected 'Conners' grapefruit and perhaps similar seedy white grapefruits produce abundant seeds which qPCR data suggests are consistently colonized by large numbers of '*Ca. Liberibacter asiaticus*'. Microscopic studies are needed to locate and identify '*Ca. Liberibacter asiaticus*' cells in seed tissues and to verify that intact bacterial cells are the source of the pathogen DNA detected in seeds and seedlings in this and previous studies. To that end we started fluorescence in situ hybridization analysis using probes complementary to the '*Ca. Liberibacter asiaticus*' 16S rRNA gene and found large numbers of bacteria in the phloem of the vascular bundle in thin sections of immature 'Conners' seeds collected in July and August 2010 (data not shown). We are continuing these studies as seeds mature with the progression of the growing season. The current study indicates 'Conners' and possibly other seedy grapefruits or related varieties will be ideal subjects for these studies because of the apparent consistent presence of the pathogen in seeds.

It seems that the concern regarding seed transmission is that infected seeds unknowingly would be transported to areas that currently do not have '*Ca. Liberibacter asiaticus*' and plants grown from these seeds will act as sources of inoculum in the new location. The current practice of harvesting seeds removes them from the fruit and then they are stored, usually with refrigeration, for periods which can be several months before they are planted. Would '*Ca. Liberibacter asiaticus*' cells remain viable in seeds during long periods of storage? Since the organism is not easily cultured (5,25) determining the viability of bacterial cells which are detected in seeds is difficult.

It seems clear that studies conducted to date that have relied solely on qPCR for pathogen detection to determine whether '*Ca. Liberibacter asiaticus*' is transmitted by seed in citrus cannot derive a conclusive answer to this question. Results from these studies with qPCR indicate that further research is needed but that new approaches and methodologies are needed to address more thoroughly the question of seed transmission of this pathogen in citrus.

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