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Diverse DNA extraction methods and PCR primers for detection of Huanglongbing-associated bacteria from roots of 'Valencia' sweet orange on sour orange rootstock

Madhurababu Kunta, Zenaida Viloria, Hilda S. del Rio, Eliezer S. Louzada*

Texas A&M University-Kingsville Citrus Center, 312N. International Blvd, Weslaco, TX 78599, USA

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ABSTRACT

Fibrous root samples from Huanglongbing symptomatic (S) and asymptomatic (AS) 'Valencia' sweet orange trees (*Citrus sinensis*) grafted onto sour orange (*C. aurantium*) rootstock were analyzed using different DNA extraction procedures and primers to detect *Candidatus* Liberibacter asiaticus. Root sampling procedure was modified from the conventional use of soil probe to reduce fatigue, and to facilitate sample collection. DNA extraction kits including Qiagen DNeasy, Mo Bio PowerSoil and PowerPlant generated root DNA suitable for polymerase chain reaction (PCR) diagnostic assays. Quantitative PCR (qPCR) using HLBaspr primers and probe on root DNA extracts from a total of 206 samples from 60 AS trees showed unreliable results for the presence of *Ca.* L. asiaticus. Except for one sample, all the remaining samples gave negative qPCR result using LJ900fpr primers and probe. Further analysis of roots samples from S and AS trees indicate that HLBaspr is not suitable for *Ca.* L. asiaticus diagnosis in root samples. Leaf samples from all 60 AS trees showed negative qPCR result with HLBaspr. Conventional PCR (cPCR) using OI1/OI2c primers on 10 randomly selected root samples, from the 206 samples, produced non-specific amplification products which were easily distinguished from products specific to *Ca.* L. asiaticus seen in the positive control. The same root samples did not produce any amplification product in cPCR using A2/J5 primers. Any of the above mentioned cPCR or qPCR assays could accurately detect *Ca.* L. asiaticus in S leaf samples of *Ca.* L. asiaticus-infected trees. Root samples from 10 (5 S and 5 AS) additional trees analyzed by cPCR using A2/J5 and Las606/LSS primers and qPCR using LJ900fpr and CQULA (CQULA04F/CQULA04R, and TaqMan probe CQULAP10) clearly showed that all these primers are efficient in detecting *Ca.* L. asiaticus in root samples. There was no difference in frequency and consistency of *Ca.* L. asiaticus detection for root samples collected near the tree trunk compared to the roots collected about 1.8 m from the trunk. *Ca.* L. americanus was not detected in either root or leaf samples.

1. Introduction

HLB is the most devastating citrus disease worldwide, known to cause significant economic damage to citrus industries in Asian countries for more than hundred years (Aubert, 1992; Bové, 2006). In Florida, the disease has already caused approximately $3.6 billion dollar in economic losses (Hodges and Spreen, 2012) and loss of thousands of acres of citrus production. Texas is the third largest US state in domestic citrus production after Florida and California, where commercial citrus contributes to more than $250 million to the state's economy with an estimated 1900 jobs. It was estimated that HLB may reduce the value of Texas citrus production by 20 percent after two years of infection and up to 60 percent after five years (Rosson et al., 2007). HLB is present in Florida, Georgia, Louisiana, South Carolina, Texas and California (Gottwald, 2010; Halbert, 2005; Kumagai et al., 2013; Kunta et al., 2012). The disease was also reported from Mexico which has close proximity to several US citrus producing states (North American Plant Protection Organization, 2009).

HLB is associated with three Gram-negative phloem inhabiting Liberibacter species, '*Ca.* Liberibacter asiaticus', '*Ca.* L. africanus' and '*Ca.* L. americanus' (Bové, 2006; Teixeira et al., 2008). The bacterium is vectored by insect vectors *Diaphorina citri* Kuwayama, the Asian citrus psyllid (ACP) (Capoor et al., 1967; Martinez and Wallace, 1967), in the Asian countries, USA and Brazil, and *Trioza erytreae*

Abbreviations: S, symptomatic; AS, asymptomatic; HLB, Huanglongbing; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; cPCR, conventional polymerase chain reaction.

* Corresponding author. Tel.: +1 956 447 3367; fax: +1 956 969 0649.
E-mail address: eliezer.louzada@tamuk.edu (E.S. Louzada).

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Fig. 1. The tools (Fiskars garden knife) and protective wear (eye wear, rubberized gloves, and knee pads) recommended in the collection of fibrous root samples from citrus trees. The fibrous root samples are mostly found within 6 inches from soil surface.

Del Guercio, the African citrus psyllid (McClean and Oberholzer, 1965), in Africa. ACP was first found in Florida in 1998 and had spread to more than 31 citrus producing counties by 2000 (Halbert and Manjunath, 2004), and it has been present in Texas for over a decade (French et al., 2001).

Several HLB disease management and control strategies were reported such as antibiotics, rootstocks/interstocks, heat, and a combination of chemical compounds (Belasque et al., 2010; Hoffman et al., 2012; Shokrollah et al., 2011; Zhang et al., 2010). However, early detection and initiation of rapid disease management by controlling psyllid populations and removal of infected trees are essential in preventing further spread of the disease (Gottwald, 2010).

Ca. L. asiaticus is unevenly distributed in the vascular system of infected citrus plants (Aubert, 1992; Bové, 2006; Garnier and Bové, 1983) and different plant tissue in different plant parts contain varied levels of bacterial populations (Tatineni et al., 2008; Li et al., 2009). Moreover, there is a long latency periods before symptom appearance in aerial parts of the infected trees.

Greenhouse experiments showed that *Ca.* L. asiaticus systemically moves through the plant phloem from site of infection to different plant parts (Tatineni et al., 2008) and presence of *Ca.* L. asiaticus in citrus tree roots considerably changes the relative proportions of the bacterial community associated with the roots (Trivedi et al., 2010). Ann et al. (2004) showed that *P. parasitica*-induced root rot was enhanced by Likuben (HLB is also known as Likubin disease) bacterium (LB) infection and LB-induced symptoms. This study also revealed that there was an enhancement of LB-induced symptoms by *P. parasitica* in citrus trees. Moreover, it was recently reported that HLB causes pre-symptomatic fibrous root decline, which was observed independently of *Phytophthora* (Graham et al., 2013). Detection of *Ca.* L. asiaticus in root samples of asymptomatic infected sweet orange is more reliable than leaf samples, however; root sampling is very labor intensive which makes this method impractical for area wide field surveys (Johnson et al., 2014).

Current HLB diagnostic procedure is based on qPCR on leaf samples, and the methods for DNA extraction and qPCR are well established (Jagoueix et al., 1996), however, Fujikawa and Iwanami (2012) reported that the HLBaspr primers used has a great potential to produce false positives due to similarity of the region where the primers where designed to several bacteria, and they designed an improved primers based on 16S rDNA for conventional PCR of leaf samples. Despite of few reports on HLB detection using root samples (Johnson et al., 2014), the conventional method for root sample collection using soil probes is very cumbersome making it improper for surveys (Johnson et al., 2014). Furthermore, the presence of PCR inhibitor from soil in DNA extracts can interfere with PCR reaction (Tatineni et al., 2008).

The objectives of this research is to improve root sampling protocol, evaluate different DNA extraction methods using three commercially available kits and to determine the efficacy of different primers and PCR methods for HLB detection in root and leaf samples from both asymptomatic and symptomatic 'Valencia' sweet orange trees on sour orange rootstock from three different locations in South Texas. To our knowledge, this is the first in depth study examining root samples for *Ca.* L. asiaticus and *Ca.* L. americanus diagnosis in high pH calcareous soils on trees grafted on sour orange rootstocks.

2. Material and methods

2.1. Sampling of asymptomatic trees and soil (collection 1)

Thirty-nine apparently healthy, completely asymptomatic (AS) 'Valencia' sweet orange trees (6 years old) grafted onto sour orange rootstock, from a commercial orchard in San Juan (SJ), Texas, were chosen as source for roots and leaf samples. Each tree was divided into four quadrants, and leaf and root samples were collected in each quadrant totaling 156 leaf samples and 156 root samples. Fibrous superficial roots (Fig. 1) were collected using a garden knife (Item: 70796935J, Fiskars, Madison, WA). These trees were separated by seven rows of trees from the first *Ca.* L. asiaticus-infected sweet orange reported in Texas (Kunta et al., 2012). Moreover, eight root and leaf samples (two per tree) were collected from four additional trees separated by 34 rows from the initial HLB-positive tree. From another group of 21 AS Valencia sweet orange trees, of similar age, grafted on sour orange rootstock, [9 trees from Southern Research Farm (SRF), Weslaco, TX, and 12 from Monte Alto (MA), TX], two root sample and two leaf samples per tree were collected. Additionally, one root sample from five *in-vitro* grown sterile sour orange seedlings and a total of 11 soil samples including three each of soil samples from commercial potting soil mixtures Sunshine® and Metro-Mix (Sun Gro Horticulture, Agawam, MA), from pots of 1-year old greenhouse grown sour orange seedlings, and two soil samples adhered to the fibrous roots from asymptomatic trees in SJ were also collected.

2.2. Sampling of both symptomatic and asymptomatic trees (collection 2)

Five AS and five S trees were divided into four quadrants and one leaf sample per quadrant per tree was collected. For S trees, leaves were collected from symptomatic branches.

2.3. Quadrant sampling of roots at different distances from trunk and leaves at different tiers (collection 3)

In this round of sample collection, a total of eight leaf samples per tree, four from upper tier (2.13 m) and four from lower tier canopy (0.9 m) were randomly collected from the same group of five AS and five S trees described in collection 2. Furthermore, four root samples per tree (one per quadrant), at the dripline (at least 1.8 m from the trunk), and four close to the tree trunk (less than 30 cm away) were collected. Each of the four root samples per tree were analyzed as individual samples. The PCR test results for the root samples collected close to the tree trunk were compared with the results obtained with the samples collected at dripline.

2.4. DNA isolation

Total DNA was isolated from leaves using Qiagen DNeasy Plant Mini Kit from 200 mg chopped tissue of midribs with petioles. The tissue was placed in a 2 mL lysing matrix A tube (MP Biomedicals, Santa Ana, CA) with extraction buffer and pulverized for 3 min using a Mini-Beadbeater-96 (Biospec Products Inc, Bartlesville, OK). The extract of total DNA was eluted in 100 μL nuclease-free water. Moreover, total DNA was isolated from chopped root samples after thoroughly washing under running tap water for 10 min followed by rinsing in running reverse osmosis water, and from soil using either Qiagen, PowerSoil or Power Plant (MoBio) kit following manufacturer's recommendations.

2.5. Quantitative PCR (qPCR) detection of Ca. L. asiaticus and Ca. L. americanus

For the detection and quantification of *Ca.* L. asiaticus and *Ca.* L. americanus, multiplex qPCR assays (Li et al., 2006) were performed using HLBaspr primer-probe set on 2 μL total DNA extract in a 25 μL reaction using a SmartCycler II (Cepheid, Sunnyvale, CA) or ABI 7500 Fast thermocycler (Life Technologies). A citrus mitochondrial cytochrome oxidase (COX)-based primer-probe set, COXfpr (Li et al., 2006), was used as a positive internal control. Reactions containing known positive control DNA, healthy plant DNA and non-template water control were also performed. The presence of the target sequences in the DNA extracts were confirmed based on the threshold cycle (C_t) values obtained. Some of the root DNA extracts that gave a positive test result ($C_t < 37$) were subjected to qPCR targeting *Ca.* L. asiaticus *hyv*I and *hyv*II multiple tandem repeats using LJ900 primers (Morgan et al., 2012). Since it was recently reported (Tomimura et al., 2009) that the bacteriophage-type DNA polymerase gene could not be amplified from some Japanese *Ca.* L. asiaticus isolates, qPCR using CQULA primers and probe set (CQULA04F/CQULA04R, and TaqMan probe CQULAP10) was conducted to amplify *Ca.* L. asiaticus specific sequence of rplJ/rplL ribosomal protein gene (Wang et al., 2006) to further validate the results obtained.

2.6. Conventional PCR (cPCR) detection of Ca. L. asiaticus and Ca. L. americanus

The qPCR results were further confirmed by cPCR using OI1/O12c primers (Jagoueix et al., 1996) and Las606/LSS primers (Fujikawa and Iwanami, 2012; Fujikawa et al., 2013) that amplify 16S ribosomal DNA (rDNA) sequences of *Ca.* L. asiaticus. Additionally, PCR amplification of *Ca.* L. asiaticus ribosomal protein genes of β operon was conducted using the A2/J5 primers (Hocquellet et al., 1999). Root samples were tested using GB1/GB3 primers that amplify 16S ribosomal DNA (rDNA) sequences to detect *Ca.* L. americanus (Teixeira et al., 2005). The PCR amplification products were separated by electrophoresis on 1% agarose gels, ethidium-bromide stained, visualized under UV light, and photographed using Biospectrum imaging system (UVP, Upland, CA). Thin slices of agarose gel containing the amplicon DNA were cut; DNA was purified using Qiaquick Gel Extraction Kit (Qiagen),cloned into pcr4 TOPO vector (Invitrogen), transformed into *Escherichia coli* cells, and sequenced at MCLAB (MCLAB, San Francisco, CA). The nucleotide sequences were analyzed for similarities at NCBI database using Blastn program and deposited at the NCBI database. All primers and probes used in this study are listed in Table 1.

Table 1
Description of different primers and probes used in this study.

Primer name	Target area	Reference
OI1/OI2c	16S rDNA of *Ca.* L. asiaticus	Jagoueix et al. (1996)
Las606/LSS	16S rDNA of *Ca.* L. asiaticus	Fujikawa and Iwanami (2012)
A2/J5	Ribosomal protein genes of β operon of *Ca.* L. asiaticus	Hocquellet et al. (1999)
GB1/GB3	16S rDNA of *Ca.* L. americanus	Teixeira et al. (2005)
HLBaspr	16S rDNA of *Ca.* L. asiaticus	Li et al. (2006)
COX fpr	Plant cytochrome oxidase	Li et al. (2006)
LJ900fpr	*hyv*I and *hyv*II multiple tandem repeats of *Ca.* L. asiaticus	Morgan et al. (2012)
CQULA	rplJ/rplL ribosomal protein gene of *Ca.* L. asiaticus	Wang et al. (2006)
HLBampr	16S rDNA of *Ca.* L. americanus	Li et al. (2006)

3. Results

3.1. Root sample collection procedure

It was very convenient and less time taking to use Fiskars garden knife compared to soil probe in collecting the fibrous root samples which are within less than six inch depth from the ground surface. This knife with an extra-long handle and a soft-molded grip provides excellent control and comfort and is useful in quickly collecting the root samples with reduced hand fatigue, especially from heavy clay soils.

3.2. Q-PCR and cPCR assays to detect Ca. L. asiaticus in roots and leaves of field-grown asymptomatic sweet orange trees (trees from collection 1).

All root samples that were collected from asymptomatic sweet orange trees in different South Texas citrus orchards of San Juan (SJ) mature trees (164 samples), South Research Farm (SRF) in Weslaco (18 samples), and Monte Alto (MA) orchard (24 samples) consistently produced HLBaspr qPCR positive results with C_t values ranging from 22.90 to 31.78. However, all the leaf samples from the same trees produced negative test results. When these root DNA extracts were tested with LJ900fpr primers, all of them produced negative result, except one sample from SJ with a C_t value of 26.67. This indicates that HLBaspr seems to be amplifying a different product than the 16 S ribosomal gene from *Ca.* L. asiaticus. To verify if the template DNA being amplified comes from soil organisms we extracted DNA, using PowerSoil kit, from 11 soil samples including six commercial (3 each of Sunshine® and Metro-Mix), three from pots of greenhouse grown sour orange seedlings, and two soil samples from SJ field. All soil samples produced positive qPCR results with C_t values between 32 and 36 using HLBaspr primers and probe.

Table 2
Comparison of quantitative PCR threshold cycle (C_t) values obtained using HLBaspr and LJ900fpr primers and probe on DNA samples extracted with three different commercially available kits for the fibrous feeder root samples collected from 'Valencia' sweet orange trees on sour orange rootstock in San Juan, (SJ), South Research Farm (SRF), and Monte Alto (MA).

Sample ID	Qiagen		PowerSoil		PowerPlant	
	HLBaspr[a]	LJ900fpr[a]	HLBaspr[a]	LJ900fpr[a]	HLBaspr[a]	LJ900fpr[a]
SJ 1	30.4	–	25.5	–	27.4	–
SJ 2	24.9	–	24.0	–	23.9	–
SJ 3	–	–	23.8	–	24.2	–
SJ 4	24.3	–	23.6	–	22.7	–
SRF 1	27.7	–	31.7	–	28.4	–
SRF 2	27.1	–	28.5	–	27	–
SRF 3	30.1	–	27.8	–	28.2	–
SRF 4	32.6	–	27.5	–	27	–
MA 1	26.7	–	27.1	–	26.5	–
MA 2	27.7	–	26.3	–	29	–
MA 3	27.3	–	25.9	–	–	–

[a] The threshold cycle (C_t) values are shown and (–) indicates that presence of *Ca.* L. asiaticus was not determined, C_t value >37.

Root DNA extracted from five *in-vitro* grown sterile sour orange seedlings showed negative result.

To exclude the possibility that DNA extraction method was the cause of the problem we extracted DNA from 11 random root samples collected from three locations including SJ (4 samples), SRF (4 samples), and MA (3 samples) using Qiagen, PowerSoil, and Power Plant kits. All these samples produced positive qPCR results using HLBaspr primer-probe set for the presence of *Ca.* L. asiaticus regardless of extraction method used, but they produced *Ca.* L. asiaticus negative results using LJ900fpr (Table 2). In an attempt to find the origin of the product being amplified, we performed conventional PCR using the *Ca.* L. asiaticus 16S primers OI1/OI2c, and the *Ca.* L. asiaticus β-operon primer A2/J5 on 10 of the 11 samples above. No amplicon of the right size for the presence of *Ca.* L. asiaticus (positive control, lane +) was observed with OI1/OI2c primers, but non-specific amplification of products of 728 and 519 base pairs were produced (Fig. 2); however, the same samples did not produce any amplification product using A2/J5 primers. A homology search for the nucleotide sequence obtained from the 728 bp non-specific amplicon (Genbank accession: KM201256) at the National Center for Biotechnology Information (NCBI) using nucleotide BLAST (blastn) did not show identities to any nucleotide sequence in the database, however, protein database (blastx) showed identities to beta-glucosidase from several proteobacteria. The 519 bp amplicon sequence (Genbank accession: KM201257) did not show any significant similarity both at blastn and blastx searches. We do not know the specific organism from which these false positive results are being generated. All the samples showed negative qPCR results for the presence of *Ca.* L. americanus as no amplification was observed for any of the samples using GB1/GB3 primers.

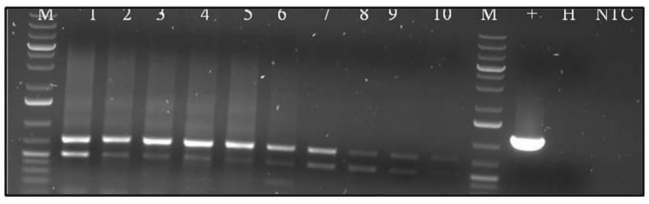

Fig. 2. Non-specific amplification products of sizes 728 bp and 519 bp obtained in the conventional PCR using OI1/OI2c primers on the DNA extracts from the fibrous root samples of apparently healthy sweet orange trees without any visual HLB leaf symptoms. *M* = 1 kb plus molecular marker (Fermentas), Lanes 1–10: root samples collected from sweet orange trees in different citrus orchards of Lower Rio Grande Valley, + = *Ca.* L. asiaticus PCR positive control DNA, H = healthy leaves, and NTC = non template water control.

3.3. PCR assays on roots and leaves from symptomatic and asymptomatic trees (trees from collections 2 and 3)

Since the results obtained with root qPCR and cPCR assays described above is for the first time reported herein, we selected five S and five AS trees to confirm the results and at the same time to find the best primer combination for HLB diagnosis using root samples. From S trees, leaves were collected from symptomatic branches (collection 2). Quantitative PCR on all leaf DNA extracts using HLBaspr and LJ900fpr (Table 3) or cPCR using A2/J5 and OI1/O12c (Fig. 3) assays gave very clear results differentiating infected trees from non-infected trees, except one AS tree that the leaf sample showed qPCR Ct values of 34.1 and 35.02 with HLBaspr and LJ900fpr, respectively. This shows that these primers are very reliable for HLB diagnoses using leaf samples from symptomatic branches. From the same S and AS trees above, DNA was extracted from roots samples using Qiagen and PowerSoil DNA isolation kits. cPCR results using OI1/OI2c and A2/J5 primers compared with qPCR results using LJ900fpr and HLBaspr showed variability in sensitivities of detection (Table 4). Moreover, *Ca.* L. asiaticus was detected from 2 AS trees using qPCR while the same samples produced negative results in cPCR. Since HLB distribution in leaves is uneven,

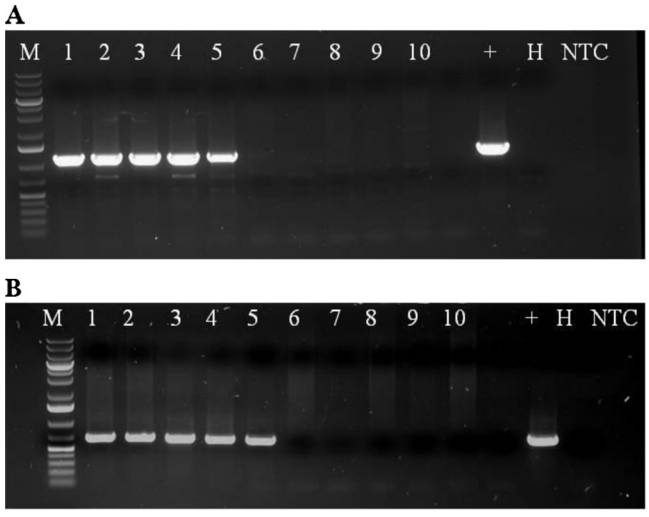

Fig. 3. The specific conventional PCR amplification products of sizes 1160 bp and 703 bp obtained by using OI1/OI2c primers (A) and A2/J5 primers (B), respectively, on DNA extracts of HLB symptomatic and asymptomatic sweet orange leaves. Panels A&B: *M* = 1 kb plus DNA ladder (Fermentas), lanes 1–5 = symptomatic leaves, lanes 6–10—asymptomatic leaves, + = *Ca.* L. asiaticus PCR positive control DNA, H = healthy leaves, and NTC = non template water control.

Table 3
Conventional PCR using A2/J5 and OI1/OI2c primers and quantitative PCR using HLBaspr and LJ900fpr performed on DNA samples extracted from sweet orange leaves with classic HLB mottle symptom and leaves without any visual symptoms. For qPCR, the threshold cycle (Ct) values were incorporated in the table.

Sample ID[a]	cPCR[b]		qPCR[c]	
	A2/J5	OI1/OI2c	HLBaspr	LJ900fpr
Symptomatic				
R12-T1	+	+	22.40	21.68
R12-T7	+	+	15.10	17.89
R12-T11	+	+	16.10	20.05
R12-T15	+	+	16.30	19.28
R13-T02	+	+	18.80	20.08
Asymptomatic				
R12-T8	–	–	34.10	35.02
R12-T10	–	–	–	–
R12-T12	–	–	–	–
R12-T13	–	–	–	–
R12-T19	–	–	–	–

[a] Sample identification where R–Row number, T–Tree number.
[b] cPCR result where (+) expected amplification product obtained and (–) no amplification product was obtained.
[c] The threshold cycle (C_t) values are shown and (–) indicates that presence of *Ca*. L. asiaticus was not determined, C_t value >37.

we divided each of the 10 trees, same as above, in four quadrants and collected from each Tree 8 random leaf samples (two from each quadrant, from upper and lower canopy) to better estimate the HLB levels in the aerial part of the 5 S and 5 AS trees. Furthermore, in each quadrant root samples were collected close and far from the trunk.

None of the leaf samples produced PCR products using A2/J5 or qPCR using HLBaspr, including AS leaves collected randomly from S trees. On the contrary, of 20 root samples collected close to the trunk (Table 5) from the 5 S trees, all of them produced the expected 500 bp cPCR amplicon for the 16 S ribosomal *Ca*. L. asiaticus gene, using Las606/LSS primers and 703 bp cPCR amplicon using A2/J5 primers characteristic of the β-operon gene from *Ca*. L. asiaticus. All samples were HLB positive by qPCR using CQULA (CQULA04F/CQULA04R, and TaqMan probe CQULAP10) and LJ900fpr. Out of the 20 samples collected far from the trunk (Table 6) for the 5 S trees, all produced cPCR products using A2/J5 primers and Las606/LSS primers and all were positives by qPCR using LJ900fpr and CQULA. For the 5 AS trees out of 20 samples collected close to the trunk, 8 produced the characteristic cPCR amplicon of the β-operon gene from *Ca*. L. asiaticus using A2/J5 primers while 11 showed positive results using Las606/LSS, while by qPCR 15 were positive using LJ900fpr and 11 positive using CQULA (Table 5). From the 20 AS samples collected far from the trunk (Table 6), 8 produced amplicons by cPCR using A2/J5 primers while 11 were positive using Las606/LSS and only 12 were HLB positive using either LJ900fpr or CQULA, respectively, in qPCR. The 500 bp amplicons produced in cPCR using Las606/LSS (GenBank accession number KF777103) showed 100% identities to *Ca*. L. asiaticus nucleotide sequences at NCBI's GenBank database. All root samples above, regardless of being from S or AS trees gave qPCR positive results using HLBaspr primers and probe as expected considering that as discussed previously; this primer and probe set is not reliable for HLB diagnostics using root samples since it amplifies products non-related to *Ca*. L. asiaticus.

All the DNA extracts used in this study, irrespective of DNA extraction method, produced Ct values in the range of 19–22 for COX gene. In all cPCR reactions using OI1/OI2c performed in roots, regardless of S or AS trees, close or far from the trunk, independent of tree location, amplicons of lower size than the expected 1160 bp were produced. All the samples including roots and leaves showed negative qPCR results for the presence of *Ca*. L. americanus with no amplification observed.

4. Discussion

Currently, symptomatic leaf samples are widely used for *Ca*. L. asiaticus detection in citrus; however, it is important to note that the disease symptom expression by leaves will depend on several environmental and host factors and significantly varies during different seasons of the year. In Texas, we have noticed that classic leaf

Table 4
Comparison of quantitative PCR using LJ900fpr and HLBaspr and conventional PCR using OI1/OI2c and A2/J5 to detect '*Candidatus* Liberibacter asiaticus' from fibrous root samples collected from sweet orange trees with visual HLB symptomatic leaves (symptomatic) and trees without visual symptomatic leaves (asymptomatic). Samples were collected at the drip line (at least 1.8 m from the trunk).

Sample ID[a]	cPCR[b]		qPCR[c]		Sample ID[a]	cPCR[b]		qPCR[c]	
	OI1/OI2c	A2/J5	HLBasrp	LJ900fpr		OI1/OI2c	A2/J5	HLBaspr	LJ900fpr
Symptomatic					**Asymptomatic**				
R12-T1-Q	+	+	26.19	25.40	R12-T8-Q	–	–	30.67	–
R12-T7-Q	+	+	28.33	27.25	R12-T10-Q	–	–	–	–
R12-T11-Q	+	–	29.44	27.76	R12-T12-Q	–	–	36.32	–
R12-T15-Q	+	–	28.63	28.40	R12-T13-Q	–	–	28.41	–
R13-T2-Q	+	+	26.45	24.50	R12-T19-Q	–	–	–	–
R12-T1-PS	–	+	26.02	25.40	R12-T8-PS	–	–	22.71	–
R12-T7-PS	–	+	26.05	26.20	R12-T10-PS	–	–	22.4	31.70
R12-T11-PS	–	+	25.38	24.50	R12-T12-PS	–	–	23.00	30.90
R12-T15-PS	+	+	25.58	24.80	R12-T13-PS	–	–	23.15	–
R13-T2-PS	+	+	25.18	22.90	R12-T19-PS	–	–	22.71	–

[a] Sample identification where R—row number, T—tree number.
[b] cPCR result where (+) expected amplification product obtained and (–) no amplification product was obtained.
[c] The threshold cycle (C_t) values are shown and (–) indicates that presence of *Ca*. L. asiaticus was not determined, C_t value >37.

Table 5
Conventional PCR on fibrous feeder root samples collected close to the tree trunk (≤30 cm) of trees with symptomatic leaves and asymptomatic leaves using A2/J5 and Las606/LSS primers amplifying β-operon gene and 16S-rDNA and quantitative PCR using LJ900fpr and CQULA (CQULA04F/CQULA04R primers, and CQULAP10 probe) targeting *Candidatus* L. asiaticus prophage *hyv*I and *hyv*II multiple tandem repeats and *rplJ*/*rplL* ribosomal protein gene, respectively.

Symptomatic trees					Asymptomatic trees				
Sample ID[a]	cPCR[b]		qPCR[c]		Sample ID[a]	cPCR[b]		qPCR[c]	
	A2/J5	Las606/LSS	LJ900 fpr	CQULA		A2/J5	Las606/LSS	LJ900fpr	*CQULA
R12-T1-1	+	+	26.30	31.06	R12-T8-1	–	+	24.89	33.91
R12-T1-2	+	+	25.85	30.47	R12-T8-2	–	+	27.33	34.60
R12-T1-3	–	+	27.19	34.96	R12-T8-3	–	+	25.27	35.75
R12-T1-4	+	+	25.33	29.21	R12-T8-4	+	+	23.74	29.87
R12-T7-1	+	+	24.88	28.20	R12-T10-1	+	+	26.25	30.84
R12-T7-2	+	+	25.12	32.12	R12-T10-2	+	+	25.02	29.48
R12-T7-3	+	+	23.48	27.38	R12-T10-3	+	+	23.27	26.72
R12-T7-4	+	+	23.15	30.32	R12-T10-4	+	+	23.18	27.38
R12-T11-1	+	+	25.82	34.17	R12-T12-1	–	–	35.14	–
R12-T11-2	+	+	23.31	31.97	R12-T12-2	–	–	33.73	–
R12-T11-3	+	+	23.51	33.14	R12-T12-3	–	–	34.90	–
R12-T11-4	+	+	24.62	29.44	R12-T12-4	–	–	30.55	–
R12-T15-1	+	+	25.75	27.08	R12-T13-1	+	+	29.90	33.69
R12-T15-2	+	+	25.09	27.44	R12-T13-2	+	+	25.37	31.85
R12-T15-3	+	+	25.60	29.29	R12-T13-3	–	–	–	–
R12-T15-4	+	+	25.73	31.48	R12-T13-4	+	+	24.60	26.73
R13-T2-1	+	+	29.20	33.01	R12-T19-1	–	–	–	–
R13-T2-2	+	+	31.90	34.06	R12-T19-2	–	–	38.41	–
R13-T2-3	+	+	25.84	28.08	R12-T19-3	–	–	–	–
R13-T2-4	+	+	27.63	30.32	R12-T19-4	–	–	–	–

[a] Sample identification where R—row number, T—tree number.
[b] cPCR result where (+) expected amplification product obtained and (–) no amplification product was obtained.
[c] The threshold cycle (C_t) values are shown and (–) indicates that presence of *Ca*. L. asiaticus was not determined, C_t value >37.

mottle symptom expressed by HLB-infected trees is a later stage and several leaves without classic HLB symptoms gave a positive PCR result (unpublished data). Moreover, it requires expertise to differentiate the disease symptom from abiotic stress or other biotic stress, which is further complicated by uneven distribution of *Ca*. L. asiaticus in the infected tree, variations in disease symptomology, and only relatively small portion of leaves showing symptoms. This was evidenced by the qPCR results (HLB positive) obtained when DNA from S leaves from infected trees (collection 2) were used as template, compared to those obtained (HLB negative) from DNA extracted from random leaf samples (collection 3) from the same trees, clearly indicating that even though a tree may be HLB positive, use of asymptomatic leaves for diagnosis may lead to false negatives. Hence, in this study, we evaluated the efficacy of different DNA extraction and PCR methods in early detection of HLB from root samples before the tree show symptomatic leaves.

It is a general opinion that root sample collection is laborious and difficult task compared to leaf sample collection. According to

Table 6
Conventional PCR on fibrous feeder root samples collected from the periphery of the tree canopy of sweet orange trees on sour orange rootstock with symptomatic leaves and asymptomatic leaves using A2/J5 and Las606/LSS primers amplifying β-operon gene and 16S rDNA and quantitative PCR using LJ900fpr and * CQULA (CQULA04F/CQULA04R primers, and CQULAP10 probe) targeting *Candidatus* L. asiaticus prophage *hyv*I and *hyv*II multiple tandem repeats and *rplJ*/*rplL* ribosomal protein gene, respectively.

Symptomatic trees					Asymptomatic trees				
Sample ID[a]	cPCR[b]		qPCR[c]		Sample ID	cPCR[b]		qPCR[c]	
	A2/J5	Las606/LSS	LJ900 fpr	CQULA		A2/J5	Las606/LSS	LJ900fpr	CQULA
R12-T1-1	+	+	27.80	32.47	R12-T8-1	–	–	–	–
R12-T1-2	+	+	26.70	32.71	R12-T8-2	+	+	27.70	34.30
R12-T1-3	+	+	28.70	34.85	R12-T8-3	–	+	25.02	34.55
R12-T1-4	+	+	26.60	32.43	R12-T8-4	+	+	24.03	30.08
R12-T7-1	+	+	25.10	29.29	R12-T10-1	+	+	25.31	30.23
R12-T7-2	+	+	24.40	27.95	R12-T10-2	+	+	24.40	28.61
R12-T7-3	+	+	27.40	27.00	R12-T10-3	+	+	23.11	26.69
R12-T7-4	+	+	29.20	31.86	R12-T10-4	–	+	27.79	35.22
R12-T11-1	+	+	26.00	33.34	R12-T12-1	–	–	–	–
R12-T11-2	+	+	24.10	28.23	R12-T12-2	–	+	–	–
R12-T11-3	+	+	28.80	33.48	R12-T12-3	–	+	34.29	36.28
R12-T11-4	+	+	24.70	28.59	R12-T12-4	–	–	29.81	36.38
R12-T15-1	+	+	24.09	27.04	R12-T13-1	+	+	29.00	32.44
R12-T15-2	+	+	35.10	30.81	R12-T13-2	+	+	25.33	31.15
R12-T15-3	+	+	25.26	28.42	R12-T13-3	–	–	–	–
R12-T15-4	+	+	25.27	30.41	R12-T13-4	+	+	24.12	26.22
R13-T2-1	+	+	29.29	31.51	R12-T19-1	–	–	–	–
R13-T2-2	+	+	32.27	34.14	R12-T19-2	–	–	–	–
R13-T2-3	+	+	25.23	27.90	R12-T19-3	–	–	–	–
R13-T2-4	+	+	27.45	30.07	R12-T19-4	–	–	–	–

[a] Sample identification where R—row number, T—tree number.
[b] cPCR result where (+) expected amplification product obtained and (–) no amplification product was obtained.
[c] The threshold cycle (C_t) values are shown and (–) indicates that presence of *Ca*. L. asiaticus was not determined, C_t value >37.

Johnson et al. (2014), root sampling is labor intensive and impractical for area-wide field survey. To resolve this issue, we found that Fiskars garden knife serves as an excellent tool in citrus root collection which will dramatically reduce the difficulty associated with root collection using soil probes and it makes sample collection a fast procedure.

We were puzzled in seeing the initial HLBaspr qPCR positive results with all the 206 root samples collected from citrus trees lacking typical leaf symptoms from three geographically separated citrus orchards in the Lower Rio Grande Valley, and those were without any typical leaf symptoms and were HLBaspr qPCR negative in the canopy. Further analysis showed that the results were due to false positive produced by non-specific binding of HLBaspr qPCR primers and probes to DNA from other organisms. It was noteworthy that all these samples produced non-specific amplification products in cPCR using OI1/OI2c primers (Fig. 2) while none yielded any PCR amplicons using A2/J5 primers and were negative by qPCR using LJ 900fpr. Both HLBaspr primer used in qPCR and OI1/OI2c primer used in cPCR were missing a nucleotide base G that is present in target genomic sequence of *Ca.* L. asiaticus (Parker et al., 2014). New HLBaspr primers including the G nucleotide was used, but the results did not change (unpublished data). The nucleotide sequence of 728 bp non-specific PCR fragment showed similarity to beta-glucosidase from several proteobacteria at the protein database of NCBI. It was recently reported that 16S rDNA-based primers OI1/OI2c sequences show high similarity to 16S rDNA gene from many non-specific bacteria, therefore, a new set of 16S rDNA-based Las606/LSS primers were designed to specifically detect *Ca.* L. asiaticus (Fujikawa and Iwanami, 2012). They also demonstrated that Las606/LSS primers are more sensitive compared to common primer sets including OI1/OI2c, A2/J5, and MHO353/MHO354 and interfusion of non-specific genomic DNAs did not affect the ability of Las606/LSS primers to sensitively detect *Ca.* L. asiaticus. Interestingly, we observed in our comparative assays that Las606/LSS primers could detect *Ca.* L. asiaticus in trees where A2/J5 primers failed and the sensitivity of these primers was comparable to the LJ900fpr and CQULA qPCR assays (Tables 5 and 6).

Throughout this study, HLBaspr qPCR detection of *Ca.* L. asiaticus worked well for leaf samples, and it is the one that we routinely use in HLB surveys in Texas and elsewhere, but we showed herein that it produces false positive results for root samples assay, however, we do not know the source organism for this unexpected qPCR results. Citrus plant roots harbor enormous number of endophytic bacteria (Trivedi et al., 2010) and it is out of scope of our study to find the exact endophytic bacterium from which the false positive HLBaspr qPCR amplifications were produced. The fact that all soil samples shown amplification using HLBaspr qPCR, and that *in vitro* samples showed no amplification, demonstrates that the false positives in root samples is indeed due to soil borne microorganisms. The primers Las606/LSS and A2/J5 for cPCR and LJ 900fpr and CQULA for qPCR produced reliable test results for the presence of *Ca.* L. asiaticus in the root samples. Some of the AS trees with HLBaspr qPCR negative leaves in the canopy turned out to be *Ca.* L. asiaticus positive when root samples were used in the detection. Moreover, root samples collected either near the tree trunk or 1.8 m away from the trunk produced consistent results. However, if the roots are collected far away from the tree trunk, it will be difficult to determine whether the roots were associated with the expected tree or from the adjacent tree. In Texas, where flood irrigation is the main system used by growers, superficial roots can reach more than 9 m and entangle to roots of adjacent trees, so for accurate root diagnosis, roots should be collected no more than 30 cm around the tree. Based on our results we conclude that the roots will serve as a better diagnostic sample compared to the leaves for detection of *Ca.* L. asiaticus in citrus trees where leaf symptoms are not present. Furthermore, at any time where leaf samples show any dubious positive results, root samples should be collected and properly evaluated. Roots collected in all 5 S trees analyzed did not show any *Phytophthora* damage, but in cases where *Phytophthora* is present, root collection may be a difficult task.

Ca. L. asiaticus is a member of Rhizobiaceae based on a recent phylogenetic analysis (Duan et al., 2009). The Rhizobiaceae family constitutes soil bacteria and several genera in this family infect the roots either resulting in plant-microbe nitrogen fixing symbiosis or diseases. Early detection of *Ca.* L. asiaticus in the roots prior to leaf symptom development could be very instrumental in planning *Phytophthora* prevention measures as it was reported that prior infection of *Ca.* L. asiaticus promotes *Phytophthora* growth and subsequent damage to fibrous roots causing root decline (Graham et al., 2013); this is very important in Texas where *Phytophthora* inoculum is widespread (Timmer, 1972). Moreover, Trivedi et al. (2010, 2012) reported that the relative composition of the rhizosphere microbial community is affected and changed due to HLB infection. Furthermore, it was speculated that microbial community composition may play a significant role in the progression of the HLB (Zhang et al., 2013). For root analysis in our lab, we are currently using qPCR with CQULA primers and probe, and when necessary, verification is performed by cPCR using Las606/LSS.

5. Conclusion

In summary, HLBaspr primers and probe produced unreliable qPCR results for detection of *Ca.* L. asiaticus in citrus root samples from Texas. cPCR usingLas606/LSS primers could detect *Ca.* L. asiaticus in trees where A2/J5 primers failed and the sensitivity of these primers was comparable to the LJ900fpr and CQULA qPCR assays. All the primers and probes used in this study could detect *Ca.* L. asiaticus from leaf samples without any problem. Nevertheless, fibrous root samples collected either close to the trunk or 1.8 m away from the trunk produced consistent detection of *Ca.* L. asiaticus.

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