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Novel set of real-time PCR primers for simultaneous detection of Liberibacter species associated with citrus Huanglongbing

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ABSTRACT: Huanglongbing (HLB), a devastating citrus disease caused by the bacterium "*Candidatus* Liberibacter spp.", is now responsible for significant economic losses worldwide. Yet, no effective disease control has been found, and the non-cultivability of the bacterium has severely hampered studies on the pathogen. The 16S rDNA gene is a well-characterized sequence, essential for cell survival, and is used for bacterial identification or assignment of close relationships at the genus and species levels. Quantitative Real-Time PCR (qPCR) assays based on 16S rDNA genes are widely used in the detection of "*Ca.* Liberibacter spp." in multiplex reactions. We have developed for the first time a set of qPCR primers based on the conserved 16S rDNA gene, which specifically and simultaneously detects in a singleplex reaction, all three bacterial species associated with HLB, and can differentiate *Ca.* Liberibacter asiaticus or africanus from americanus by their characteristic melting curves. The assay is very sensitive, and it was possible to amplify expected DNA fragments with an efficiency of 98 % using the Syber Green system and a Ct value lower than tested methods for HLB diagnosis. The application of this fast, simple and efficient detection methodology could also be important in the detection of all species of HLB-associated Liberibacters and could contribute to early pathogen detection, a crucial step in the development of preventive strategies aimed at avoiding the dissemination of this devastating disease in HLB-free areas.

Keywords: *Candidatus* Liberibacter spp., Syber Green qPCR, Argentina, greening, pathogen detection

Introduction

Huanglongbing (HLB) is the most serious citrus disease that gives rise to global economic losses (Gottwald et al., 2007). Its causal agent is a Gram negative and phloem-inhabiting bacterium, "*Candidatus* Liberibacter spp." (Jagoueix et al., 1994). For many years, two species have been associated with HLB: "*Ca*. Liberibacter asiaticus" (Las), first known in Asia and later found in South, North and Central America, and "*Ca*. Liberibacter africanus" (Laf), only reported in Africa (Gottwald, 2010). However, in 2004, a third bacterial species was detected in Brazil, "*Ca.* Liberibacter americanus" (Lam), (Teixeira et al., 2005). Liberibacter can be transmitted by grafting, dodder or two insect vectors: *Diaphorina citri* for Las and Lam, and *Trioza erytreae* for Laf (Bové, 2006).

HLB is a significant threat to all regions not yet infested. In Argentina, *D. citri* was first reported in the province of Entre Ríos (32°02'52" S and 60°16'52" O) (Vaccaro, 1994) and later found in Salta (23°21'28" S and 64º09'63" W) and Jujuy (23°45' S and 65°30' O) (Augier et al., 2006). In June 2012, positive detection of HLB was confirmed in backyard trees in Misiones (26°55' S and 54°31' O), located in the northeast of Argentina, but no HLB has been detected yet in commercial citrus groves in those regions (Dr. Outi, personal communication, 2013). No direct disease control is available, and the little knowledge we have of the disease, combined with the aggressive infection behavior and difficulties to control the bacteria, makes tree protection the most important management measure.

HLB diagnosis based on disease symptoms is difficult because of similarities to nutritional deficiencies (Bové, 2006). Microscopy, serology and DNA–DNA hybridization used to detect the pathogens (Bové, 2006; Villechanoux et al., 1990) are either time consuming or lacking in sensitivity. Methods based on PCR have been widely used to detect HLB-associated Liberibacter, mainly based on the 16S rDNA and the beta operon sequences (Hocquellet et al., 1999; Hung et al., 1999; Jagoueix et al., 1996; Teixeira et al., 2005), and several real-time quantitative PCR assays (qPCR) have been designed to detect and quantify low Liberibacter titers, both in plants (Li et al., 2006; Teixeira et al., 2008) and insects (Manjunath et al., 2008).

With the aim of detecting simultaneously the three Liberibacter species associated with HLB, we have designed a qPCR primer set that amplifies a region of the 16S rDNA gene, considering the high degree of conservation of this gene for its importance in the functioning of cells (Clarridge, 2004). This primer set is as sensitive or even more sensitive than other reported primers, which makes this methodology a simple and efficient tool for the early detection of the pathogen and the development of management strategies to avoid the rapid dissemination of HLB.

Materials and Methods

DNA sample and plasmid preparation

The pGEM-T Easy vector containing the 16S rDNA sequence from Las, Lam and Laf was used to construct a standard curve for the different sets of qPCR-primers. Quantification of the plasmid DNA based on the copy number (CN) of the target gene was determined as previously described by Wang et al. (2006). The plasmid solution was serially diluted from 10^8 CN to 1 CN.

DNA samples of symptomatic leaves from 65 citrus trees and 40 insects were collected as microbial-free, non-infectious DNA samples. The Las/Lam-infected leaves were from Sao Paulo, Brazil (23°30' S and 46°37' O), and the Laf-infected leaves were from Pretoria, South Africa (25°43'32" S and 28°14'38" E).

As a negative control for the PCR and qPCR assays, total DNA was extracted from the midribs (about 250 mg) of healthy greenhouse-grown sweet orange (*Citrus sinensis* L.) trees (about five years old) by using the CTAB method (Murray and Thompson, 1980) and suspended in 100 μL of water. As a non-template control (NTC), water was included.

Primer design and validation of primer-specificity

A new set of qPCR primer pair, named p3G, was designed based on 16S rDNA sequences of "*Ca*. Liberibacter spp." obtained from the DNA database GenBank (accession numbers: NC_020549 of Las, EU921621 of Laf and AY742824 of Lam). Multiple sequence alignments were performed using DNAMAN software and from these analyses primers were designed for a specific sequence region in order to amplify the DNA from all three species of different sizes. The p3G primers were empirically designed in this region using the software Primer3 (http://frodo.wi.mit.edu/). The sequences of forward primer f-p3G and reverse primer r-p3G were 5′-CTTATCACCGGCAGTCCCTATAAAG-3′ and 5′-CAGCTCGTGTCGTGAGATGTTG-3′,respectively, and led to an amplicon of 122 bp for Las, 121 bp for Laf and 105 bp for Lam.

Analyses for the detection of *Ca.* L. solanacearum (Lso, accession numbers EU834130.1) were carried out by *in silico* PCR using the program http://insilico.ehu.es/PCR/.

To make sure that the designed primers were unique to "*Ca*. Liberibacter spp.", a search was performed against available microbial sequences in the GenBank and *in silico* PCR (http://insilico.ehu.es/PCR/). The specificity of primer pairs were also PCR-evaluated with DNA prepared from a number of citrus-related pathogens, including *Xanthomonas citri* subsp. *citri,* the causal agent of citrus canker; *Xylella fastidiosa* strain 9a5c, the causal agent of citrus variegated chlorosis, and *Guirnardia citricarpa,* the causal agent of black spot. Genomic DNA from pure cultures of other bacterial species like *Enterococcus* and citrus endophytes, *Burkholderia* sp. and *Methylobacterium* sp., were also included. Twenty samples of citrus leaves (4 of *C*. sinensis and 16 of *C*. limon) collected in non-infected areas in Tucumán (27°0'0" S, 65°30'0" W), Argentina, were tested with the p3G primers.

Conventional PCR and Real-time PCR for the new p3G primers

To determine optimal annealing temperatures, a conventional PCR experiment was conducted by using a MyCycler thermal-cycler with a temperature gradient heating block. Primer annealing temperatures were set from 58 °C to 72 °C. The PCR reaction was performed in a total volume of 25 uL containing 1 × master mix, 0.4 uM of each primer (f-p3G and r-p3G) and 500 ng of DNA template.

For real-time PCR, SYBR Green (SG) systems were used (SG qPCR). Real-time PCR assays with the new sets of primers were performed by using a Miniopticom Real-time PCR. All reactions were generated in triplicate and each run contained one negative (-DNA, HLB-free citrus DNA) and one positive DNA control (plasmid DNA). PCR amplification conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and, 70 °C for 45 s. The reactions were generated using a total volume of 25 uL containing 1 × master mix (iQ SYBR Green, BioRad), 0.4 uM of each primer (f-p3G and r-p3G) and the appropriate amount of DNA template. Data were analyzed using CFX Manager Software Version 2.1 from BioRad.

Sensitivity assay of the qPCR method

To compare the assay sensitivity of the qPCR, different HLB-detection PCR reactions were chosen. For a SYBR Green real-time PCR comparison, the primer pair f-*rpl*LAs/r-*rpl*LAs for "*Ca*. Liberibacter asiaticus", previously published by Teixeira et al. (2008) was used. The standard curve for *rpl*Las was constructed using ten-fold serial dilutions of plasmid DNA containing the sequence of the *rplKAJL-rpoBC* gene cluster (β-operon) from Las. A Taqman based real-time PCR assay was performed using an HLBasp 16S rDNA primer-probe set as previously described (Li et al., 2006). To compare the different qPCR systems, optimization and standardization were carried out with ten-fold dilutions of total DNA samples obtained from field-grown plants infected with Las.

Results

Novel qPCR primers designed to detect HLB Liberibacter species

The new set of primers p3G was empirically designed based on the 16S rDNA sequences of "*Ca*. Liberibacter spp." (GenBank accession numbers: NC_020549 of Las, EU921621 of Laf and AY742824 of Lam). A specific sequence region was retrieved by aligning and comparing the various bacterial 16S rDNA sequences and a new set of primers was designed. Primer pair p3G is specific to Liberibacter and recognizes all three known HLB-associated species. Amplification products expected for Las, Laf and Lam are 122 bp, 121 bp and 105 bp, respectively. A BLAST search against all available microbial sequence da-

tabases at NCBI and an *in silico* PCR amplification experiment did not identify any additional homologous DNA sequences, suggesting high specificity of the designed primers (Figure 1). Even though these primers were specifically designed for HLB "*Ca*. Liberibacters spp.", p3G was able to amplify "*Ca*. Liberibacter solanacearum". *In silico* PCR performed with this bacterium gave the expected fragment (121 bp) with one mismatch at the beginning of the sequence, thus indicating the potential broad application of the p3G primers.

Optimizations of SG qPCR conditions and standard curve

To attain an efficient and solid SG qPCR, the optimization of the primer's annealing temperature is important for determining high endpoint fluorescence and low Ct values. To determine optimal annealing temperatures, conventional PCR experiments were conducted by using plasmid DNA containing the 16S rDNA gene sequence from Las. Annealing test temperatures were set from 62 °C to 72 °C. Distinct and single DNA bands were amplified at annealing temperatures between 65 °C and 70 °C (Figure 2). The higher temperature of 70 °C was considered to be the optimal annealing temperature for the qPCR primers, since it allowed for higher stringency of the PCR reaction.

The SG qPCR yielded positive amplification results only for plasmid DNA corresponding to Las, Lam or Laf with Ct values between 12 and 14. In the absence of DNA (NTC) or with DNA from healthy sweet orange, no Ct values were obtained. Melt-curve analysis showed that samples detected by SG qPCR were Liberibacter-specific amplicons with a melting peak at 82 °C for Lam and 83 °C for Las and Laf (Figure 3).

Ten-fold serial dilution series of the plasmid pGEM-T Easy vector containing the 16S rDNA gene sequence from either Las, Lam or Laf ranging from 10^8 to 1 CN uL^{-1}, were used to make a standard curve with the p3G primers. DNA amplification was carried out using three replicates for each dilution tested. The standard curve obtained for Las had an average slope value of 3.370, indicating an efficiency of the PCR of 98 %. Similar results were obtained when standard curves were made for Lam and Laf. The detection limit for all three Liberibacters was equivalent to ten copies of 16S rDNA. A linear relationship was observed between Ct values and the log concentrations of recombinant plasmid DNA (Figure 4).

Quantification of Liberibacter species in plant and insect samples by using p3G primers

Positive DNA microbial-free, non-infectious DNA samples of leaves from 65 citrus trees and 40 insects were checked and species were identified by conventional PCR (data not shown) by using established protocols (Jagoueix et al., 1996; Teixeira et al., 2005). Next, the population of each Liberibacter was quantified by SG qPCR using the p3G primer set. Quantification of Liberibacter in target samples was performed as an indirect measurement of CN of the 16S rDNA inserts deduced from the standard curve.

Out of 65 vegetal samples tested positive by qPCR for Liberibacter, 30 were positive for Las, 20 were positive for Lam, 13 samples tested positive for both species and only two were positive for Laf, which corresponded to the Laf positive control used in the assay. All Ct values of positive DNA leaf samples were between 14 and 24 (Table 1). Where a mix of Las and Lam was present in DNA samples, Ct values between 14 and 20 were observed (Table 1). When DNA samples extracted from insects were evaluated, 27 samples were positive for Las and 13 were positive for Lam. The Ct value was between 20 and 26 for both, Las and Lam pathogens. No discrepancy was observed between the results from real-time PCR with those obtained with conventional PCR (data not shown). The copy number of 16S rDNA obtained with these Ct values in DNA samples varied from 10^2 to 10^6 CN (Table 1).

Specificity of SG qPCR assay for the detection of "*Ca*. Liberibacter spp."

The p3G set of primers amplified the specific target from DNA extracts of HLB infected plants with an optimal temperature of 70 °C, without any amplification of negative bacterial controls and NTC. The primer pair p3G was specific to Las, Lam and Laf, and did not cross-react with endophytes commonly resident in citrus or other citrus pathogens including *Xylella fastidiosa*, *Xanthomonas citri* subsp. *citri* or the fungus *Guirnardia citricarpa*. Additionally, as expected these primers did not cross-react with other bacterial species like *Enterococcus* sp., isolated from the soil of sugarcane fields (Table 1), nor amplified samples of citrus leaves collected in non-infected growth areas in Tucumán, Argentina.

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Las CTTATCACCGGCAGTCCCTATAAAGTNCCCAACATCTAGGTAAAAACCTAAACTTGATGGCAACTAGAGGCAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
Lam CTTATCACCGGCAGTCCCTATAAAGTTCCCAACTT........AA.........TGATGGCAAATATAGGCAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
Laf CTTATCACCGGCAGTCCCTATAAAGTACCCAACATCTAGATAAAA.TCTAAACTTGATGGCAACTAGAGGTAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
Lso ATTATCACCGGCAGTCCCTATAAAGTACCCAACATCTAGATAAAA.TCTAAACTTGATGGCAACTAGAGGTAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
     ************************* ****** *        **         ********* ** *** ****************************************************
p3G CTTATCACCGGCAGTCCCTATAAAG                                                                           CAACATCTCACGACACGAGCTG
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Figure 1 – Sequences of p3G qPCR primers for amplification of 16S rDNA of "*Ca*. Liberibacter spp." associated with citrus Huanglongbing and "*Ca*. Liberibacter solanacearum", the causal agent of diseases in *Solanaceous* plants. Las: "*Ca*. L. asiaticus", Lam: "*Ca*. L. americanus", Laf: "*Ca*. L. africanus", Lso: *Ca*. L. solanacearum, p3G: primer sequence, left: p3G-f, right: p3G-r.

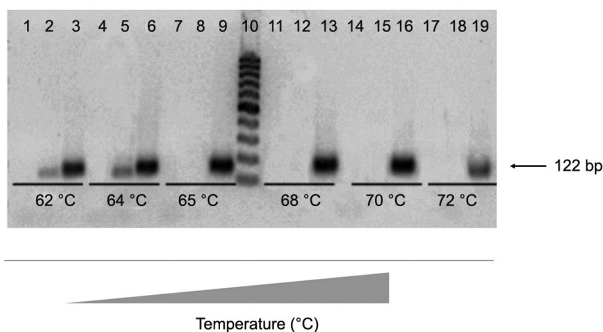

Figure 2 – Optimization of annealing temperature for p3G primers setting from 62 ºC to 72 °C. Line 10: Cien Marker (Biodynamics). Lines 1, 4, 7, 11, 14 and 17: non-template control (NTC). Lines 2, 5, 8, 12, 15 and 18: DNA from healthy sweet orange. Lines 3, 6, 9, 13, 16 and 19: positive control for "*Ca*. L. asiaticus".

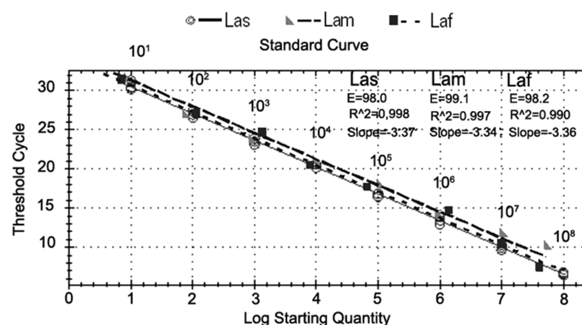

Figure 4 – Standard curve obtained with p3G primers using tenfold serial dilution ranging from 10^8 to 1 copy number (CN) for the pGEM-T Easy vector containing 16S rDNA sequence from "*Ca*. L. asiaticus" (Las), "*Ca*. L. americanus" (Lam) and "*Ca*. L. africanus" (Laf).

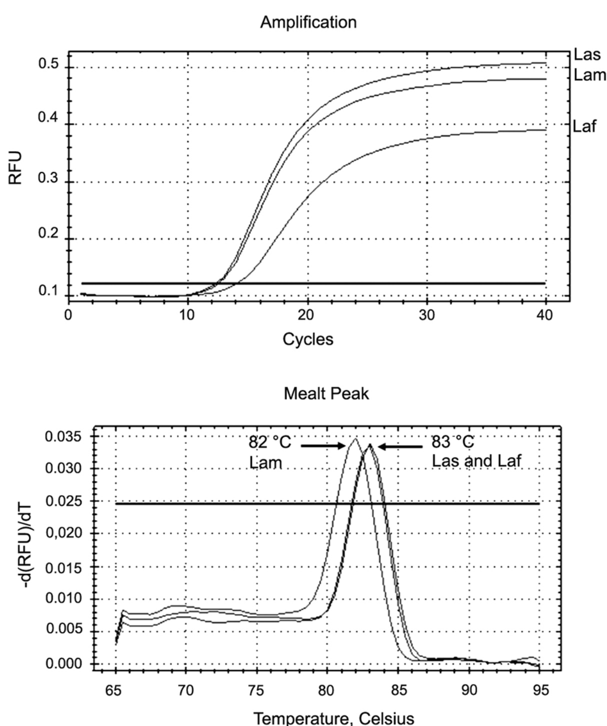

Figure 3 – SYBR Green quantitative PCR amplification curves and melting curves with primers p3G for detection of "*Ca*. Liberibacter spp." in DNA samples of Liberibacters (Las: "*Ca*. L. asiaticus", Lam: "*Ca*. L. americanus" or Laf: "*Ca*. L. africanus"). Melting curve peak observed was 82 °C for Lam and 83 °C for Las and Laf.

Comparison of sensitivity between PCR diagnostic methods

The sensitivity of the SG qPCR assay developed in this study was compared to a certain number of previously established protocols. For this purpose, the β-operon primer set f-*rpl*LAs/r-*rpl*LAs was selected for Las as described by Teixeira et al. (2008). The standard curve for *rpl*Las was made by using ten-fold serial dilutions of plasmid DNA containing the sequence of the *rplKAJL-rpoBC* gene cluster (β-operon) from Las. The efficiency of the PCR reaction was 110 % for Las with a detection limit of 10 CN (Figure 5). When comparing both primer sets in SG qPCR assays, *rpl*Las and p3G had the same detection limit. Both primer sets succeeded in detecting 10 CN with an efficiency of 98 % and 110 % for p3G and *rpl*Las, respectively. However, the lowest Ct was observed for the p3G primers when the same copy number of targeted DNA was compared (Figure 5). For 10^8 CN the Ct value for p3G was 7, whereas the Ct value for the *rpl*Las primers was 13.

In vegetal samples, the detection limit was 10 CN for both, p3G and *rpl*Las primers with a Ct value of 33 and 35, respectively. The PCR efficiency was 103.3 % for *rpl*Las primers and 98 %, for p3G primers (Figure 6). The Ct values obtained with vegetal samples correlates with the Ct value observed for plasmid DNA. To further evaluate the p3G assay, another comparison was made against a detection method based on 16S rDNA amplification; this time the TaqMan system was applied by using a primer-probe set previously reported by Li et al. (2006). Vegetal samples containing DNA templates ranging from 10^5 to 1 CN had Ct values between 23 and 38. The efficiency of the PCR reaction was 98.2 % for Las and the detection limit of TaqMan PCR for HLB pathogens was therefore equal to 10 copies. Figure 6 shows curves obtained with one of the plant samples, named AS13.

Discussion

To prevent the outbreak and spreading of HLB-associated Liberibacter species in unaffected citrus production areas, it is necessary to have sensitive and reliable diagnostic methods for the early detection of the pathogen. This is particularly critical in areas where

Table 1 – Specificity of SYBR Green quantitative PCR assays for "*Candidatus* Liberibacter spp." in 21 of 105 analyzed DNA samples.

Samples	Host	Origin	Ct Values with p3G primers:			Copy Number of target 16S rDNA uL^{-1}
			Las	Lam	Laf	
Ca. L. asiaticus 101	Insect	Brazil	20	-	-	10^4
Ca. L. asiaticus 105	Insect	Brazil	24	-	-	10^3
Ca. L. americanus 120	Insect	Brazil	-	26	-	10^2
Ca. L. americanus 121	Insect	Brazil	-	24	-	10^3
Ca. L. asiaticus 330	*Citrus* sp.	Brazil	21	-	-	10^4
Ca. L. asiaticus 331	*Citrus* sp.	Brazil	18	-	-	10^5
Ca. L. asiaticus 332	*Citrus* sp.	Brazil	20	-	-	10^4
Ca. L. asiaticus 333	*Citrus* sp.	Brazil	14	-	-	10^6
Ca. L. asiaticus 334	*Citrus* sp.	Brazil	17	-	-	10^5
Ca. L. americanus 430	*Citrus* sp.	Brazil	-	18	-	10^5
Ca. L. americanus 431	*Citrus* sp.	Brazil	-	17	-	10^5
Ca. L. americanus 432	*Citrus* sp.	Brazil	-	21	-	10^4
Ca. L. americanus 433	*Citrus* sp.	Brazil	-	18	-	10^5
Ca. L. americanus 434	*Citrus* sp.	Brazil	-	20	-	10^4
Ca. L. americanus/*Ca.* L. asiaticus 530	*Citrus* sp.	Brazil	20	20	-	10^4
Ca. L. americanus/ *Ca.* L. asiaticus 531	*Citrus* sp.	Brazil	18	18	-	10^5
Ca. L. americanus/ *Ca.* L. asiaticus 532	*Citrus* sp.	Brazil	17	17	-	10^5
Ca. L. americanus/ *Ca.* L. asiaticus 533	*Citrus* sp.	Brazil	14	14	-	10^6
Ca. L. americanus/ *Ca.* L. asiaticus 534	*Citrus* sp.	Brazil	15	15	-	10^6
Ca. L. africanus 701	*Citrus* sp.	Africa		-	23	10^3
Ca. L. africanus 705	*Citrus* sp.	Africa	-	-	24	10^3
Xanthomonas citri subsp. citri	*Citrus* sp.	Argentina	-	-	-	-
Xylella fastidiosa	*Citrus* sp.	Argentina	-	-	-	-
Guignardia citricarpa	*Citrus* sp.	Argentina	-	-	-	-
Enteroccocus		Argentina	-	-	-	-
Endophytes citrus bacterial	*Citrus* sp.	Argentina				
Burkholderia sp.			-	-	-	-
Methylobacterium sp.			-	-	-	-

Las: "*Ca.* L. asiaticus"; Lam: "*Ca.* L. americanus"; Laf: "*Ca.* L. africanus".

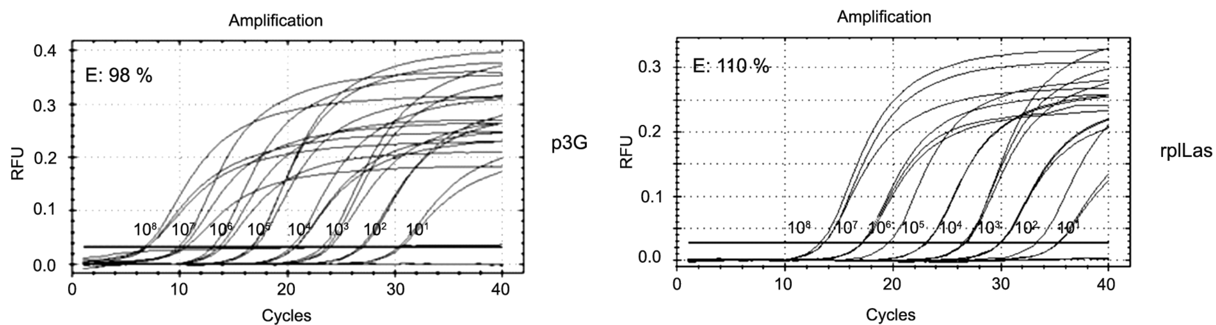

Figure 5 – Comparing sensitivity of SYBR Green quantitative PCR assays with p3G primers (left) and *rpl*L as primers (right) for detection of "*Ca.* L. asiaticus" using ten-fold serial dilutions of plasmid DNA.

psyllids are present and HLB has not yet been reported, as in the northwest of Argentina.

The rapid expansion of HLB in Brazil and Florida are examples where the confirmation of the disease occurred much later than the infection by the pathogen (Coletta-Filho et al., 2004; Manjunath et al., 2008), which clearly attests that the plant had been infected for a long period of time when disease symptoms appeared. It is rather difficult to diagnose HLB on the basis of disease symptoms, and when it happens, it is too late to prevent it from spreading. In areas where the HLB pathogen is unknown or undetected, a strategy that allows for both early detection of any Liberibacter species and low inoculum loads is necessary in order to detect the disease in time. Thus, it is imperative to be able to confirm the presence of

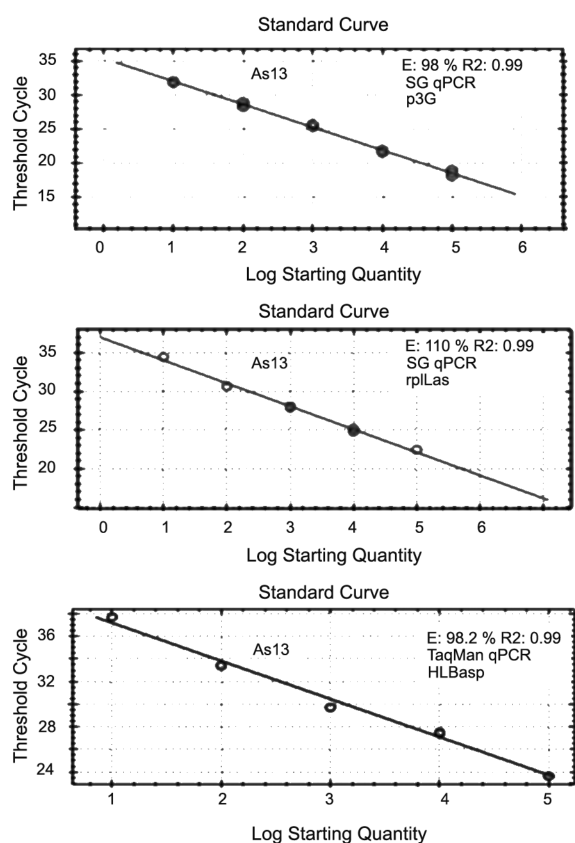

Figure 6 – Comparison of qPCR systems for HLB diagnostic. The standard curves for SYBR Green (p3G and *rpl*Las, upper and middle, respectively) and TaqMan qPCR assays (HLBasp, down) were obtained by using as a template a ten-fold serial dilution from a quantified DNA extracted from a vegetal sample infected with "*Ca*. L. asiaticus", (named AS13), ranging from 10^5 to 1 copy number (CN).

the pathogen in order to act as quickly as possible, and then additional measures can be taken to identify the species present.

Recently, new diagnosis systems such as biosensors and optical sensing techniques have been developed. However, the most sensitive and rapid method for detecting the pathogen is to use various PCR-based molecular techniques. This method is currently being used in most laboratories for HLB diagnosis in different parts of the world. Continuous improvements have been introduced in these diagnostic strategies, particularly to make possible detection of more than one Liberibacter species. For this purpose, Li et al. (2006) have developed a multiplex real-time PCR (TaqMan) assay using a probe-primer set for detection of the three known species of Liberibacter causing HLB. This test uses a 16S rDNA-based TaqMan probe and a set of three different forward primers for the detection of the three different species of Liberibacter and one common reverse primer. This set has been successfully applied in the detection, identification and quantification of Las in host plants of citrus (Li et al., 2009; Tatineni et al., 2008) and in the psyllid vector (Manjunath et al., 2008).

In order to simplify methods currently used in the detection of HLB-associated pathogens, a region of the 16S rDNA gene has been used to design a new set of primers; they hybridize perfectly to the genome of all three species of Liberibacter causing HLB in a singleplex real-time PCR reaction. The 16S rDNA sequences are highly conserved among the species of Liberibacter but variation between them is sufficient to prompt designing primers capable of detecting and identifying the bacterium in PCR assays (Coletta-Filho et al., 2005; Jagoueix et al., 1996; Teixeira et al., 2005). The gene is large enough to provide distinctly and statistically valid measurements as it has sufficient interspecific polymorphisms of the 16S rDNA gene.

The sensitivity of the qPCR assay development in this study was tested against a standard qPCR methodology and the Ct values of the p3G primers were compared with PCR-primers targeting the 16S rDNA gene (Li et al., 2006) or the β-operon region (Teixeira et al., 2005) of "*Ca*. Liberibacter spp.". qPCR protocols tested showed the same detection limit, ten copies, but the Ct value obtained with the p3G primers was lower than that obtained by the other methods tested. Kim and Wang (2009) indicate that *Ca.* Liberibacter asiaticus contains three copies of 16S rDNA and, compared to qPCR assays targeting β-operon, a 16S rDNA based qPCR assay which is likely to be more sensitive, due to its higher copy number per genome. This could help to define diagnostic situations where a very high Ct is obtained, close to the safety limit.

Ananthakrishnan et al. (2013) have reported the design of a single pair of degenerate primer-probes targeting the *rpo*B gene of Liberibacter enabling the detection of "*Ca*. Liberibacter spp." at the genus level in one PCR reaction. In a similar way, like the method developed for the *rpo*B gene, the p3G primers described in this analysis detect all three Liberibacter species associated with HLB with high sensibility and specificity. Both methods can confirm whether Libericabter is present in a plant or insect sample; however, with the p3G primers Las/Laf from Lam samples can be differentiated by their characteristic melting curves. This is especially interesting in the continents of North and South America where two forms of HLB have been reported, Las and Lam (Teixeira et al., 2005; Teixeira et al., 2008).

If necessary, the Liberibacter species present in a sample could be determined by an electrophoresis by using a DNA analyzer system like a sequencer; the amplification produces three different sizes of DNA for each Liberibacter species. In contrast, this is not possible for the *rpo* assay because the size of the amplified fragment is the same for all three species of Liberibacter. Further-

more, the comparison of the 16S rDNA gene sequences allows for differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including at specie and subspecie level (Clarridge, 2004). The degree of conservation is assumed to result from the importance of the 16S rDNA as a critical component of cell function, a fact which contrasts with other genes which can usually tolerate mutations with more frequency since they may affect structures not as unique and essential as rDNA (Clarridge, 2004).

Although it is possible to design three fluorescent probes to detect the three species of Liberibacter in the same reaction tube using the p3G assay, our main goal was to design a method that is sensitive, simple and low-cost for diagnosis. The addition of more probes to the reaction tube could not only interfere and hamper the efficiency of the test but would also increase the cost of reaction per sample. To avoid this, a representative region of the 16S rDNA gene was chosen; this area allowed to design of highly specific primers, without decreasing the sensitivity and specificity, as well as producing a new cost-effective tool for HLB diagnosis.

Reaction *in silico* was performed using the 16S rDNA sequence from "*Ca*. Liberibacter solanacearum", which is the causal agent of diseases of high economic impact on solanaceous crops (mainly potatoes and tomatoes) and carrots (Munyaneza et al., 2012). Thus, the p3G primers designed for HLB Liberibacter detection were expected to amplify the expected fragment (121 bp) with one mismatch at the 5′ end, showing the possible broad application of these primers.

The singleplex assay was designed using the p3G primers hybridizing to a very conserved sequence of the Liberibacter genomes associated with HLB. This strategy could be of importance for the detection of new species which cause the diseases, especially after two HLB causing species were identified in Brazil, one of which had never been reported previously. The present analysis offers an efficient technique for the diagnosis of HLB which, combined with a thoroughly designed sample testing program, would help to prevent the outbreak and spread of this devastating disease in major citrus production areas not yet affected.

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