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COMPARISON OF A CONVENTIONAL PCR AND QUANTITATIVE REAL TIME PCR (qPCR) ASSAY FOR THE DETECTION OF HUANGLONGBING DISEASE ASSOCIATED WITH '*CANDIDATUS* LIBERIBACTER ASIATICUS' IN CITRUS VARIETIES

A. Zafarullah[1], S. Naz[1,3] and F. Osman[2]

[1]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan
[2]Department of Plant Pathology, University of California, Davis, CA 95616, USA.
Corresponding Author's E-mail: drsnaz31@hotmail.com

ABSTRACT

Citrus greening or Huanglongbing (HLB) is one of the main devastating citrus diseases worldwide. HLB causing infectious bacterium "*Candidatus* Liberibacter" is a phloem specific and fastidious agent, having three different species. A comparison of conventional PCR and real-time quantitative PCR (qPCR) was done by universal 16S rDNA marker. In the current study conventional PCR was used to early screening of *Candidatus* Liberibacter spp and real-time quantitative PCR used for the quantification of HLB causing agent in citrus trees. Total 35 citrus cultivars were selected from mandarins and sweet oranges groups from an orchard "Citrus Research Institute, Sargodha," Pakistan. The DNA was extracted from HLB asymptomatic and symptomatic leaves. By conventional PCR, the HLB infectious bacterium has identified in 11 sweet oranges samples (31%) in which an amplicon of 1174 bp was generated. While qPCR subsequently reported to quantify the titer of *Ca.* L.asiaticus in host plant. In the present study qPCR results showed the pathogen could be detected with a Cq value getting in ranged of 26.58to 38.17. Samples with higher Cq ratio showed lower copy number of targeted gene. There was no amplification detected with healthy citrus samples and water control. A robust and sensitive qPCR assay can be used for the screening and quantify the *Ca.* L. asiaticus strain to confirm the HLB infection in Pakistan. This diagnostic test will be very helpful for implementing a wide range of diagnostics research programs aiming at ultimately developing strategies for the control of HLB in Pakistan.

Keywords: Citrus greening, Conventional PCR, *Candidatus* Liberibacter asiaticus, Huanglongbing, cytochrome oxidase, real-time qPCR.

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INTRODUCTION

Huanglongbing (HLB) previously known as Citrus greening is mainly caustic disease in the citrus producing countries and significant economic loss worldwide (Gottwald *et al.,* 2010). A fastidious bacteria cause HLB disease; *Candidatus* Liberibacter spp. occurring in the phloem cells of HLB infected citrus plants. Grafting as well as two insect vectors (sap sucking) transmits *Candidatus* Liberibacter: *Diaphorina citri* for *Ca.* L. asiaticus and *Ca.* L. americanus strains and *Triozaerytreae* vector for *Ca.* L. africanus strain transmission (Bove, 2006). HLB causing pathogen Las (*Candidatus* Liberibacter asiaticus) and Lam (*Candidatus* Liberibacter americanus) are spread by vectored *Diaphorina citri* that is heat-tolerant while Laf (*Candidatus* Liberibacter africanus) by *Triozaerytreae* that is a heat-sensitive form. These HLB associated bacteria widely occur in North America, Southern Africa and Asia (Teixeira *et al.,*2005).

HLB infected citrus tree symptoms are blotchy mottled leaves with yellowing veins and shoots having stunted growth that makes the disease detection in the field complicated that not easy to distinguish from mineral deficiencies symptoms (Jagoueix *et al.,*1996). HLB infected trees are short in length, stunt growth and produce lopsided and small-sized fruits that mature poorly with green color in their stylarend. All major citrus varieties such as mandarin and sweet oranges are susceptible to HLB which are commercially most important (Tatineni *et al.,* 2008).

The HLB associated pathogens are difficult to detect due to the long incubation time for showing symptom due to presence of low titer and uneven distribution in the host tissues. These symptomless trees had proven harmful since they are serving as constant sources of inoculums that spread through vectors (Nageswara *et al.,* 2013). There is no other treatment for HLB infected trees and the disease management in the field includes a multilevel approach that includes elimination of HLB infected trees, control the spread of vector populations and replanting with HLB-negative citrus nursery stock. The success of such approach depends on rapid, reliable and accurate identification of the HLB associated bacteria (Morgan *et al.,*2012).

In the current study, early detection of HLB associated bacteria in samples collected from Citrus Research Institute, Sargodha, a traditional or

conventional PCR and quantitative PCR (qPCR) assays were established. For decades, conventional PCR method with targeted specific primer has used to amplify a specific sequence in the 16S rRNA region of *Candidatus* Liberibacter spp. qPCR detection of HLB bacteria in citrus trees has been successful, even if bacteria occur in low titers inside the phloem cells of citrus infected trees (Hung *et al.* 1999; Teixeira *et al.*, 2008).

The main purpose of this research was to establish a fast, cost-effective and sensitive qPCR-based assay for the identification and quantification of the HLB associated bacteria *Candidatus* Liberibacter in citrus. Such detection methods are simple and more effective for the screening of the HLB causing pathogen. In addition, this method may be used to screen the citrus germplasm and assist in the development of disease management approaches to reduce the spreading of Huanglongbing disease causing pathogen in citrus orchards.

MATERIALS AND METHODS

Plant material: Citrus greening or Huanglongbing (HLB) associated diseased leaves both symptomatic and asymptomatic were collected from all around the tree canopy of citrus trees (Fig. 1). The HLB suspected plants showed overall chlorosis, corky veins, nutrient deficiencies and yellowish veins symptoms on orchard of the Citrus Research Institute, Sargodha, Pakistan. Total 50 leaves of symptomatic or asymptomatic of each sample were collected and placed in labeled polythene bags and kept in icebox till shifted to the Plant Diagnostic laboratory in the Department of Biotechnology, LCWU Lahore, Pakistan.

Fig. 1. (a) & (b) Nutrient deficiency symptoms (c) corky veins of citrus plants

(d) Tree with yellow shoots (e) yellowing veins of HLB infected plants (f) HLB mottling on citrus

DNA isolation: Total nucleic acid of HLB symptomatic and asymptomatic citrus leaves was extracted using the procedure illustrated by Murray and Thompson (1980) with a few modifications (Naz *et al.,* 2013). About 1-2 grams of fresh symptomatic or asymptomatic leaves midribs were taken and ground in ice chilled pestle and mortar. Then added 1ml of CTAB extraction buffer in centrifuge tube and incubated for 30 min at 65°C in water bath. All the samples were then centrifuged at 6000 rpm for 10 min and the resulted supernatant was shifted into new vial. Added CO (chloroform/isoamylalcohol) in ratio 24:1 in each vial to the equal volume of supernatant and then centrifuged at above-mentioned condition. The total nucleic acid was precipitated by adding chilled propanol and after centrifugation. The pellet of DNA was washed with ethanol (70%) and re-suspended in TE buffer (Table 1). The isolated DNA was quantified by spectrophotometer and stored at -20°C for more use.

Conventional PCR: The occurrence of HLB associated bacteria in citrus was firstly screened with conventional PCR by amplification of gene targeted 16SrDNA region. PCR reaction mixture was taken in total volume of 25µl having Taq buffer (10X), $MgCl_2$ (25mM), dNTPs (0.2 mM),0.4 µM of forward and reverse primer (sequence given in Table 2), DNA template (25ng) and Taq DNA polymerase (0.75 units). The PCR (Primus-96 Thermal cycler) cycling condition for DNA amplification was

(showed in Fig. 2) initial denaturation at 95°C, followed by 35 cycles at 95°C for 40 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min followed by final extension at 72°C for 10 min (Zafarullah *et al.,* 2016). The resulted PCR fragment was excised from agarose gel; gene cleaned and then sequenced the PCR amplicon from both forward and reverse strands and the resulted consensus sequence was submitted under accession number LN835770 in EMBL DNA database.

Table 1. Chemicals and buffers required for the DNA extraction and Gel electrophoresis.

Sr. No.	Solutions	Chemicals	Final concentration
1	CTAB buffer	TrisHCl	100mM
		Na_2. EDTA	20mM
		NaCl	1.4M
		CTAB	2%
		b-mercapto ethanol	2%
2	50X TAE (pH 8.0)	Tris-HCl	1M
		Acetic acid	57ml
		Na_2-EDTA	0.5M
3	TE Buffer	Na_2-EDTA	0.5 mM
		Tris-HCl	10 mM

Table 2. Nucleotide sequence and product size of 16S r DNA primer pair.

Primer Name	Nucleotide sequence	Product Size (bp)
16S r DNA	OI1:5'-GCGCGTATGCAATACGAGCGGCA-3' OI2c:5'-GCGTCGCGACTTCGCAACCCAT-3'	1174 bp

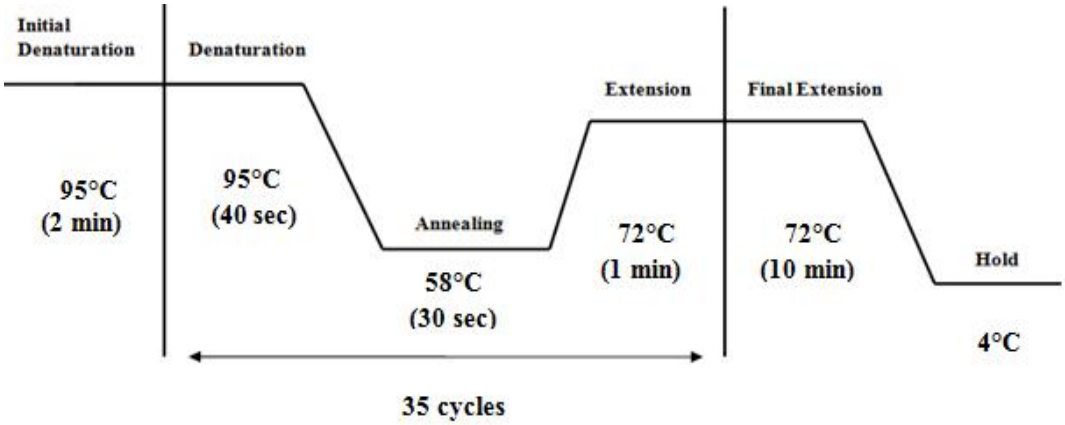

Fig.2. PCR cycling condition for 16S r DNA marker.

qPCR Primer and probe design: Complete GenBank sequence alignment of *Ca.* L. asiaticus (Las) was generated by Sequence Analysis and Molecular Biology Data Management Software Vector NTI Advance™ 11 (Invitrogen; Life technologies, Carlsbad, CA) and then BLAST the resulted sequence. For the qPCR detection of pathogen, sequences were aligned for designing primers and probes (Table 3). The fluorophore selected for *Ca.* Liberibacter asiaticus was 6-carboxyfluorescein FAM and 3' quencher was Black Hole Quencher (BHQ)-1 (Table.3). The plant internal positive control was designed as primer-probe set from citrus plant cytochrome oxidase (COX) conserved gene based on sequence (under accession number CX297817, GenBank) in the EST database. Use of qPCR, cytochrome oxidase assay analyzed to check the quality of the total DNA. The qPCR assay was designed by using the primer express software (Applied Biosystems, Foster City, CA, USA) using following procedure suggested for multiplex qPCR analysis. The favorable primers' annealing Tm was 58°C and the probes Tm used in qPCR were 10°C higher to avoid the primer dimmers formation. The qPCR analysis was performed by Life Technologies, Carlsbad, CA, USA. The homology of qPCR primers and probes was checked by BLAST software adjacent to nucleotide sequences taken in GenBank databases (NCBI). The preparation of qPCR primer/probe reaction by adding 20 µl of each primer (100pmol/µl), and 4 µl volumes of the

probes (80pmol/µl) then added sterile water to made final volume of reaction upto 240 µl.

Quantitative Real Time PCR: The reaction mixture of qPCR having water (0.42 µl), 0.58 µl of primers and probe (final conc. of each primer and probes was 400 nM and 80 nM respectively), 6 µl of PCR master mix (TaqMan® Genotyping Master mix, Applied Biosystems) and DNA (5 µl) in 12 µl of PCR reaction. The prepared samples were amplified by placing in a 7900HT FAST Real-time PCR system (Applied Biosystems, Foster City, CA, USA) in a 384 well plate of PCR. Then followed the manufacturer's instructions for PCR condition (for initial denaturation of DNA template set the PCR for 2 min at 50 °C, denaturation at 95 °C for 10 min, then followed 40 cycles for 15 s at 95 °C and for 1 min at 60 °C). For qPCR analysis took 1µl aliquot of each diseased sample using the COX as positive control qPCR assay to observe the background signal and evaluate the negative RT control value in comparison. At annealing temperature fluorescent signals were composed and the Cq value was noted and exported with a threshold of 0.06 and a baseline of 3-12 for the targets of interest. The quantitative cycle value was measured by an algorithm with a set range of cycles of qPCR machine. Thus, a Cq value less than 40 showed positive result and ranged 40 or above showed negative result or no amplification occurred.

Assay Validation: To check the efficiency of the qPCR assay, for the detection of the pathogen a series of *Ca.* L. asiaticus infected varieties were taken from the orchard and kept in the greenhouse for dilution endpoint analysis of undiluted DNA. A set of serial dilutions (10-fold) up to $1:10^{5-6}$ of three different samples in triplicate were made. The HLB positive citrus samples DNA were earlier detected by qPCR analysis. For all set of serial dilutions of *Ca.* L. asiaticus (Las) infected samples standard curves were generated by plotting the Cq value adjacent to the concentration of logarithm for the exponential phase and draw a straight line to these resulted data by linear regression equation (Bustin *et al.,*2009). The slope of standard curve generated on serial dilutions in triplicate of cDNA samples help to calculate the amplification efficiency (E) by using this formula $E = 10^{(-1/slope)}$ (Bustin *et al.,*2009). At a slope of –3.32 the standard qPCR efficiency was obtained. A blast search was performed to check validation of the assay.

Candidatus Liberibacter asiaticus qPCR assay

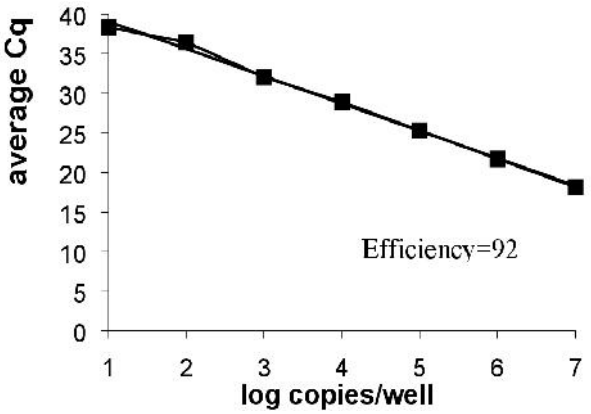

Fig. 3. Standard curve showed sensitivity of qPCR. The log copies/well displayed at X-axis and the Cq value showed at Y-axis. By using 10-fold serial dilutions of total DNA samples determined the qPCR efficiency.

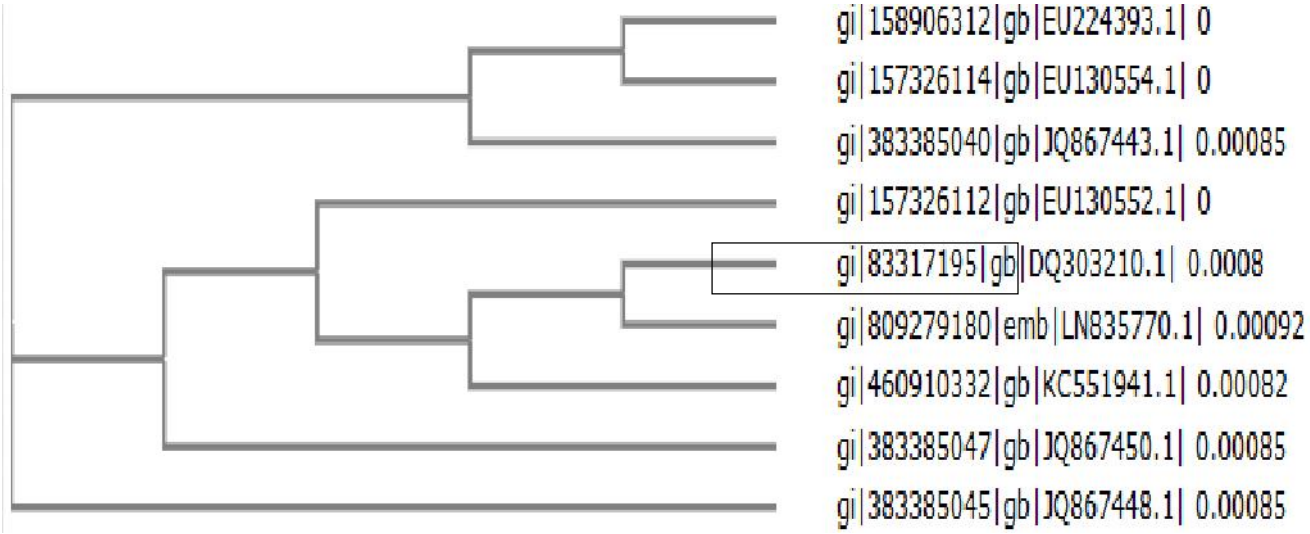

Fig. 4. On the bases of 16S rDNA sequence phylogenetic tree was constructed by ClustalW software, showing the location of Kinnow isolate infected by *Candidatus* Liberibacter spp. selected the sequence from NCBI Genbank.

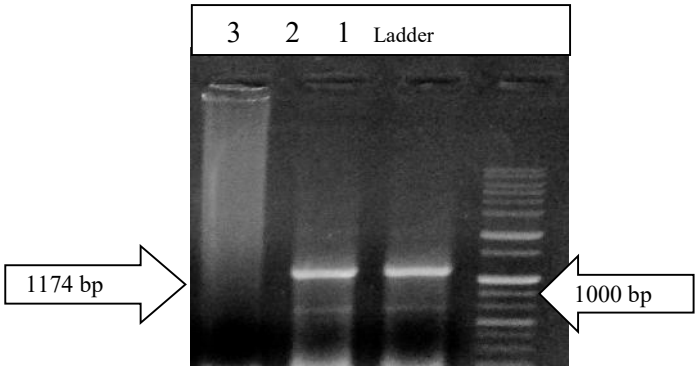

Fig.4. Visualization of amplified DNA with 16S rDNA marker yielded 1174 bp (Lane 1 and Lane 2) on 1% Agarose gel

Table 3. PCR primers and qPCR probes used for HLB detection.

Primers/probes	Sequence 5'- 3'	Nucleotide Position[1]	Amplicon size (bp)
COX multi-32 F	AATCTGACCTTCTTTCCCATGC	32-53	162
COX multi-194 R	AAGTGATTGTTACGACCACGAAGA	194-171	
COX multi-96 **p**	ATCCAGATGCTTACGCTGG	96-114	
HLBas.2	CGAGCGCGTATGCAATACG	113067-113086	
HLBas.3	CGAGCGCGTATGCGAATAC	113067-113086	113
HLB3 r 2014	AGGGCGTATACGGTATTAGCACA	113180-113155	
HLB **p**	AGACGGGTGAGTAACG	113093-113109	

Table 4. Screening of *Candidatus* Liberibacter in citrus varieties by conventional PCR and qPCR using 16S rDNA region.

Sr. No.	Varieties Name	Conventional PCR	qPCR	
			Cox	HLB assay
1.	Ruby sweet	+	14.97	27.16
2.	Pera rio	+	14.91	33.34
3.	Hamlin	+	15.30	29.19
4.	Netal	+	14.02	31.52
5.	Jaffa	+	16.29	30.48
6.	Kozan	+	14.37	27.43
7.	Blood red	-	15.32	30.32
8.	Ruby blood	-	15.21	27.48
9.	Jaffa 2	-	15.28	26.28
10.	Tracco 3	-	15.10	28.41
11.	Hinckley	-	14.78	27.88
12.	Cambelvalancia	-	15.74	31.57
13.	Salustiana	-	15.46	33.55
14.	Casa grande	-	18.32	32.00
15.	Valancia Late	-	17.00	30.22
16.	New Hall	-	15.44	30.12
17.	Frost red	-	14.89	2878
18.	Sangenello	-	16.18	33.25
19.	Washington navel	-	16.45	33.21
20.	Mars early	+	16.15	30.18
21.	Tracco rose	-	14.85	29.86
22.	Salustiana 2	-	14.73	26.79
23.	Spring navel	-	15.64	30.74
24.	Glane navel	-	14.59	29.88
25.	Washington navel	-	16.45	33.21

26.	Emby gold	+	14.33	29.39
27.	Cambel valancia	-	13.89	28.65
28.	Tracco	-	15.30	38.17
29.	Tracco Nucllar	-	13.87	32.40
30.	Musambi	-	14.87	26.58
31.	Lane Navel	-	14.31	34.81
32.	Kinnow 1	+	20.04	34.21
33.	Kinnow2	-	14.45	36.53
34.	Kinnow3	-	19.02	32.66
35.	Kinnow4	-	14.21	33.97

RESULTS

Conventional PCR assay: The early identification and molecular characterization of HLB causing infectious agent *Candidatus* Liberibacter was taken from citrus cultivars. PCR was used with gene specific primer of 16S rDNA region for screening of *Candidatus* Liberibacter from Citrus research institute (CRI), Sargodha. The amplicon of size 1174 bp was generated by targeted primer 16S rDNA in HLB symptomatic leaves of expected citrus varieties. Total 35 different citrus cultivars (having sweet oranges and mandarin) of HLB causing diseased plants were examined (Table 4). The plants showed typical HLB caused disease symptoms such as blotchy mottled leaves and yellowish midribs on the first PCR analysis using the gene targeted 16S rDNA primer, the bacterium was detected in total 11 samples (31%) by amplifying 1174 bp fragment of 16S rDNA (Fig. 5).The PCR generated amplicon was sequenced from both strands by ABI sequencer (Macrogen, Korea) and consensus sequence was submitted under accession number LN835770 to EMBL DNA database. The resulted 16SrDNA sequence was detected as "*Candidatus* Liberibacter asiaticus" is an infecting agent, showing 99% sequence similarity to Malaysian isolate of a *Ca.* Liberibacter asiaticus isolate with sequence of the same genome region (Accession No. EU224393).

By using ClustalW software, phylogenetic tree was generated that displayed the 16S rDNA targeting sequence of *Candidatus* Liberibacter asiaticus in Pakistani Kinnow isolate, bearing 99% nucleotide sequence similarity with Florida and Malaysian strains (Fig.4). The given sequence amplified by 16S rDNA suggested that Kinnow isolate was infected by HLB associated *Ca.* Liberibacter asiaticus.

Real-time qPCR assay: In singleplex real-time PCR, primer-probe combination specificity assay was evaluated in with the internal control COX assay (Table. 4). The DNA was isolated from 35 citrus varieties (having mandarin and sweet oranges) infected with HLB causing infectious agent called *Ca.* Liberibacter. The HLB screening qPCR analysis produced positive results from those DNA samples that were affected with *Ca.* Liberibacter asiaticus derived from sweet oranges. HLB could not be detecting in other citrus samples (Table 4). These results clearly showed that the combination of primer–probe was specific to *Ca.* Liberibacter asiaticus and do not overlap with other infectious agent that might be co- infected in the same citrus cultivars.

The Real time qPCR assay (primers and probe) was designed from the conserved region of *Ca.* Liberibacter asiaticus. By ABI primer express software, the designed primer sequence showed similarity (100%) with *Ca.* Liberibacter asiaticus (Fig. 4). No other visible band could be amplified for HLB infected citrus samples.

In this research, conventional and quantitative Real time (qPCR) assays were used to avoid the false positive and false negative results of *Ca.* Liberibacter spp. in asymptomatic and symptomatic citrus trees.

qPCR assay Validation: The targeted gene sequence for *Ca.* Liberibacter asiaticus was search blast and known the pathogen of particular sequence, verifying the designed primers's specificity for multiplex qPCR assay. Then calculated the primer/probe qPCR assay's average amplification efficiencies of *Ca.* Liberibacter asiaticus that was 92.3%, fall within the series set for quantitative real-time PCR by following the MIQE guidelines (Bustin *et al.,* 2009).

Single plex qPCR assay: The *Ca.* Liberibacter asiaticus qPCR assay was evaluated to screen all tested samples. The results noted in Table 4, showed that the targeted *Candidatus* strain could be detected with a quantitative cycle (Cq) ratio ranged from 26.58to 38.17. Sample with higher Cq showed less copy number. Hence; no amplification was observed in uninfected citrus samples or water control.

DISCUSSION

The comparison of a conventional PCR and qPCR assay was done for detecting HLB associated '*Candidatus* Liberibacter asiaticus' in citrus varieties from Pakistan. This study perceived the particular screening of HLB causing pathogen *Ca.* Liberibacter spp. in Citrus Research Institute, Sargodha, Pakistan. The infectious agent of *Candidatus* Liberibacter asiaticus was identified total 11 citrus varieties having typical HLB-

like symptoms by traditional PCR. The infecting HLB causing agent have been reported in Asia as *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter africanus strain in Africa was detected by conventional PCR with gene targeted primers of 16S rDNA (Manjunath *et al.,*2008). HLB causing disease in Malaysia was confirmed by identifying and sequencing of specific 16S rDNA targeting region of *Ca.* Liberibacter asiaticus.

Real-time qPCR has quantitative assay for identification and quantification of *Ca.* Liberibacter asiaticus. The HLB associated infection has been more complex to identify due asymptomatic plants, with low titer of pathogen and random distribution in leaves midribs (Weinert *et al.,* 2004). The qPCR analysis showed the occurrence of infecting bacterium "*Ca.* Liberibacter asiaticus*"* in various citrus infested varieties due to irregular infected bacterium distribution within the plants. HLB susceptible plants showed nutrient deficiency symptoms and infected bacterium has long incubation period in certain plants (Ukuda *et al.,* 2015). The real-time qPCR method targets had limited identification of one to ten copies per gene and low copy genes. The identification of low titer levels can be variable when the target sample is very dilute that caused inability to get reproducible results (Angel *et al.,* 2015). Therefore, the targeted gene amplification by PCR of a recently revealed highly repetitive conserve region of *Ca.* Liberibacter asiaticus was used in this study for PCR analysis. The PCR assay sensitivity is quantified by Cq value, as giving a lower Cq for a targeted sample as highly reproducible and more sensitive using low DNA template copy number. Li *et al.,* (2006) studied that qPCR using species-specific primer-probe set in qPCR assay to different the *Candidatus* Liberibacter spp. To estimate the quality of the DNA extracts in qPCR, Plant cytochrome oxidase (COX) was used. The traditional PCR method was used for HLB disease analysis in China (Wang *et al.,* 2006). Primers and probes of species-specific for qPCR were designed from the conserve region *rplJ/rplL* ribosomal gene. Conventional PCR and RT-PCR assay were used to amplify the conserved region sequence of *Candidatus* Liberibacter asiaticus (Li *et al.,* 2006). Quantification study of the HLB bacterium showed that infecting pathogen was unevenly distributed in plant tissues (Kunta *et al.,* 2014).

Conclusion: The HLB causing agent has been complicated to detect in asymptomatic plants due to irregular distribution with low titer in midribs tissues. The present analyses recommend the early detection of *Candidatus* Liberibacter asiaticus in citrus by conventional PCR. While real time qPCR assay using 16S rDNA-based primers/probe is most sensitive diagnostic methods for quantitative analysis of HLB associated bacterium. The most significant aspect of this study is to offer researchers proficient PCR based assays to confirm the presence of the HLB associated infectious agent in citrus.

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