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MOLECULAR DETECTION OF *CANDIDATUS* LIBERIBACTER ASIATICUS, THE CAUSAL ORGANISM OF HUANGLONGBING (CITRUS GREENING) IN FAISALABAD, PAKISTAN FOR HUANGLONGBING MANAGEMENT

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Huanglongbing is an extremely dangerous disease of citrus. It is caused by different species of *Candidatus* Liberibacter bacterium: *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter americanus and *Candidatus* Liberibacter africanus. The bacterium is transmitted from one plant to other by psyllid vector and through budding/grafting of infected plant material. For the diagnosis of huanglongbing disease citrus orchards: Square 9, citrus research area Plant Pathology and Post Graduate Agriculture Research Station of the University of Agriculture, Faisalabad, Pakistan were surveyed in 2010-11. Conventional PCR was performed by using 16S rDNA primer OI1/OI2c and Liberibacter asiaticus gene (β operon) specific primer A2/J5. Leaves were used from *Citrus sinensis* cultivar succari for the detection of huanglongbing pathogen. Out of 30 samples collected from Post graduate Agriculture Research Station, Faisalabad, 21 (70%) belonging from seven symptomatic trees were amplified and produced amplicons of 1160bp and 703 bp from OI1/OI2c and A2/J5 primers respectively in 2011, that confirmed the presence of *Candidatus* Liberibacter asiaticus in the samples. The diagnosis of disease will be helpful for future huanglongbing management experiments. According to our knowledge this is the first report of huanglongbing detection at molecular level from the University of Agriculture, Faisalabad, Punjab, Pakistan.
Keywords: A2/J5, β operon , Blotchy mottling, HLB, OI1/OI2c, PARS, Polymerase Chain Reaction, Succari

INTRODUCTION

Citrus is an important fruit crop of the world with production of 122976 thousand tones. China ranks first in the list of citrus producing countries with an area of 2118 thousand hectare and production of 23977 thousand tones, Brazil has the 2nd position and USA is the 3rd largest producer of the citrus in the world. Pakistan stands at the 13th position with an area of 198 thousand hectare and production 2150 thousand tones . Yield of citrus per acre in Pakistan is lower as compared to the majority of the rest of the world. Kinnow is an important citrus fruit of Pakistan. World's best kinnow mandarin is produced in Pakistan. Pakistan is the world's number one producer of kinnow mandarin (Memon, 2014).

Candidatus Liberibacter, a Gram negative alphaproteobacterium is the causal organism of the huanglongbing (HLB) disease (Jagoueix *et al*., 1994; Li *et al*., 2012). In citrus plants, HLB bacterium multiplies in phloem. This bacterium was defined and named by Murray and Schleifer (1994) as *Candidatus* (uncultured bacteria) Liberibacter. The citrus HLB bacterium is about 2µm long and 0.2 µm in diameter (Bove, 2006). There are three isolate types of the bacterium: *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter africanus (Laf) (Da Graca, 1991) and *Candidatus* Liberibacter americanus (Lam) (Teixeira *et al*., 2005b). *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) and *Trioza erytreae* (Del Guercio) (Hemiptera: Sternorrynca: Triozidae) are the natural vectors of citrus greening disease pathogen (Aubert, 1987). *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) transmits both *Candidatus* Liberibacter asiaticus (Las) and *Candidatus* Liberibacter americanus (Lam) while *Trioza erytreae* (Del Guercio) (Hemiptera: Sternorrynca: Triozidae) transmits only *Candidatus* Liberibacter africanus (Laf). Liberibacter asiaticus is the most abundant species among HLB infected trees (Capoor *et al.*, 1967; Jagoueix *et al*., 1994; Bove, 2006). The disease can also be transmitted through budding/grafting (Lin, 1956). In genus *Diaphorina*, there are also six other species found on citrus and their relatives but, of these six species only one species *Diaphorina communis* Mathur (Hemiptera: Sternorrynca: Liviidae) and one more psyllid: *Cacopsylla citrisuga* (Hemiptera: Psyllidae) also reported as *Candidatus* Liberibacter asiaticus vectors (Donovan *et al*., 2011; Cen *et al.*, 2012).

Among biotic stresses to citrus industry, HLB is a dangerous disease (Teixeira *et al.*, 2005a; Hall *et al*., 2012). It has caused extreme loss (about 60 million) of citrus plants in African and Asian countries (Timmer *et al*., 2003). Huanglongbing was present in china in the 1800s. The report of the disease in China in 1800s was based on interviews with technicians and

farmers from 1947 to 1955 (Lin, 1956). Huanglongbing was present in north-eastern and north-western India in the 1800s and early 1900s (Husain and Nath, 1927; Gottwald *et al*., 2007). Husain and Nath (1927) described severe damage caused by populations of *Diaphorina citri* at Sargodha from 1915 to 1920. It means that this disease has been present in Pakistan from hundred years because Sargodha is the part of Punjab, Pakistan. In USA the 3rd largest citrus producer of the world, HLB was first reported in Florida in 2005 and after eight years the whole state came under quarantine, revenue of $ 4.5 billion, that is 30-40 % of the total production of the state, was lost by this disease and also more than 8000 people lost their jobs (Chin *et al.,* 2014).

In Pakistan occurrence of greening was reported by Cochran (1976). He noted disease symptoms in the citrus collection of Peshawar experimental station. Akhtar and Ahmad (1999) used symptomtology for the diagnosis of HLB. On symptom basis, they suggested that HLB is present in Punjab and Peshawar, Pakistan. Huanglongbing presence in the citrus orchards of Khyber Pakhtoon khawa province (formerly NWFP) Pakistan was confirmed first time at molecular level (Chohan *et al*., 2007). For population dynamic studies of psyllid vector, psyllid samples were collected from different areas of Punjab, Pakistan and molecular study for detection of *Candidatus* Liberibacter asiaticus in plants and *Diaphorina citri* Kuwayama was conducted at USDA-ARS, Riverside, CA. This study showed that Las was detectable from trees at average maximum temperatures of 42°C (Razi *et al*., 2014). Diagnosis of the disease is not easy due to the uneven distribution of pathogen in the affected citrus plants (McClean, 1970; Hartung *et al*., 1993). HLB symptoms on leaves are just like zinc deficiency and may cause confusion that it is due to nutrition deficiency (Timmer *et al*., 2003; Saifullah *et al*., 2015). Psyllid control is not an easy option because of alternate hosts (Westbrook *et al*., 2011) and disease symptoms do not appear in the plant immediately after infestation. For early diagnosis, PCR technique is found to be more sensitive and reliable than electron microscopy, ELISA and DNA hybridization for the detection of greening bacterium (Mahajan *et al*., 2013). Hocquellet *et al*. (1999) developed the conventional PCR primer set A2/J5 that was based on the β-operon ribosomal protein gene. This primer set directly provides amplicon of 703bp for Liberibacter asiaticus and 669bp for Liberibacter africanus.

For the management of HLB to save the citrus industry of Pakistan, detection of HLB pathogen in the host plants was necessary. In the present study, objectives were to detect at molecular level the causal organism of citrus greening disease *Candidatus* Liberibacter asiaticus in suspected to be HLB positive citrus plants by polymerase chain reaction method in the University of Agriculture, Faisalabad (UAF), Punjab, Pakistan; and to find HLB positive plants for use in future antibiotic treatment, thermotherapy and other experiments for HLB management. The findings are important because an infrastructure has been developed for HLB diagnostics in UAF. To our knowledge, this is the first report of HLB pathogen detection at molecular level from the University of Agriculture, Faisalabad, Punjab, Pakistan.

MATERIALS AND METHODS

For the diagnosis of HLB disease, citrus orchards: Square 9, citrus research area Plant Pathology and Post Graduate Agriculture Research Station (PARS) of the University of Agriculture, Faisalabad, Pakistan were surveyed in 2010-11. During survey of orchards special emphasis was given on the presence of *Diaphorina citri* adults or nymphs and typical symptoms of HLB especially blotchy mottling and vein yellowing. Kinnow (*Citrus reticulata* Blanco), Feutrell's early (*Citrus reticulata*), sweet orange (*Citrus sinensis* (L) Osbeck.) and grapefruit (*Citrus paradisi* Macf.) fields were observed. Sweet orange succari trees were selected for sample collection because of prominent symptoms and *D. citri* presence. Total ten symptomatic trees from PARS (Fig.1A and1B) were selected for HLB diagnosis and three seedlings from insect free greenhouse, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan as healthy/negative controls.

Three samples were collected from each tree. In the month of November 2011, for each replicate, 5-8 mature leaves from a tree with blotchy mottling and vein yellowing symptoms as well as from healthy controls were collected all around the canopy.

Leaves collected from HLB suspected sweet orange trees and healthy/negative controls were kept in zip lock bags. Bags were labelled and placed in box with ice. Samples were transported to the laboratory as soon as possible. Samples were then kept at 4°C and used for DNA extraction next day.

***Molecular studies*:** Molecular studies for the diagnosis of HLB were conducted in the Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan.

***DNA extraction*:** DNA was isolated from mature leaf midribs and petioles of symptomatic and healthy sweet orange plants by the cetyltrimethylammonium bromide (CTAB) method modified from protocol 3 of Ruangwong and Akarapisan (2006). Leaf midribs and petioles (0.5g) were cut in to small pieces with sterile scissors. Chopped midribs and petioles were pulverized in liquid nitrogen by using sterile pestle mortars. Added 4mL CTAB buffer (2% CTAB, 1% Lauroyl sarcosine, 100mM Tris HCl, 1.4mM NaCl and 20mM EDTA) to that powder and incubate at 55°C for 1 hour and centrifuged at 4000g for 5 minutes. To supernatant, added 0.125 vol. of 5M NaCl and 10% CTAB in 0.7M NaCl and incubated at 65°C for 10 minutes. Added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), shaked the mix gently and centrifuged at 12000g for 10 minutes. The supernatant taken carefully in properly labelled separate 1.5 mL

microfuge tubes and added 1/10 volume of 3M sodium acetate. Then added equal volume of chilled isopropanol and placed at -20^{O}C overnight. DNA pellet was formed by centrifugation at 13000 rpm for 15 minutes. Then DNA pellets were washed with 70% ethanol, dried for 30 minutes at room temperature and dissolved in 100µL of 1x TE buffer pH 8.

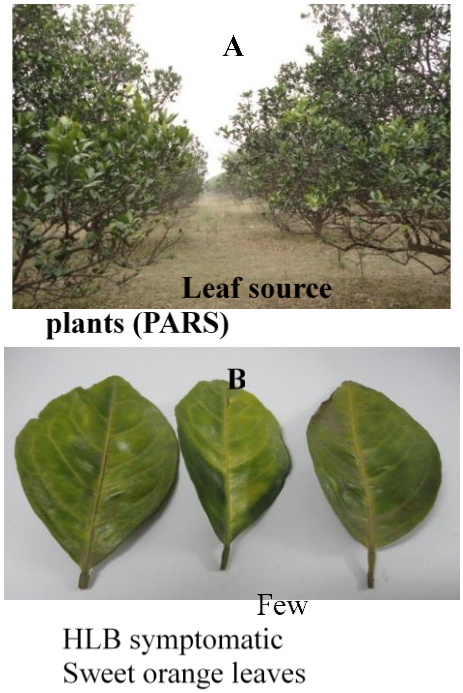

Figure 1. Source trees and leaves for huanglongbing detection: A, Huanglongbing affected symptomatic sweet orange (*Citrus sinensis* (L.) Osbeck) trees; B, Huanglongbing affected sweet orange leaves showing vein yellowing and blotchy mottle symptoms.

***DNA quantification*:** Genomic DNA extracted from suspected to be HLB positive as well as healthy sweet orange leaf midrib and petiole was quantified by gel electrophoresis technique. Agarose gel (0.8 %) prepared in 0.5X TBE buffer (Tris base, boric acid, EDTA) stained with 0.5mg/mL ethidium bromide was used for DNA quantification. Gel was visualized in the gel documentation system (BioRad) by using software quantity one.

***Polymerase Chain Reactions for HLB Detection*:** Singleplex and multiplex conventional PCR performed after DNA extraction and quantification. 16S rDNA primer OI1/OI2c (Jagoueix *et al*., 1996; Ruangwong and Akarapisan, 2006) and ribosomal protein gene of the rplKAJL-rpoBC operon (β operon) primer A2/J5 (Hocquellet *et al*., 1999) were used for the detection of HLB bacterium in suspected positive samples and healthy controls (Table 1). A total volume of 25 µL was used in the PCR reaction mix. Thin walled, flat capped, 0.2 mL, nuclease free, individual PCR tubes were used for PCR reaction mix. Amplification was carried out in peqSTAR 96 universal gradient thermocycler with the following thermal profile: one cycle for initial denaturation at 94°C for 2 minutes; followed by 35 cycles at 94°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute; one cycle for final extension at 72°C for 10 minute. The PCR products were also analyzed by gel electrophoresis using 0.8 % agarose in 0.5X TBE buffer.

RESULTS

In total, 39 citrus leaf samples (5-8 mature leaves per sample) collected from 10 trees of sweet orange orchard from PARS and 3 seedlings reared in insect free greenhouse, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan were tested for the presence of *Candidatus* Liberibacter asiaticus. Highly intact genomic DNA with smear in few samples obtained was used for the amplification (Figure 2A). When conventional PCR was performed by using 16S rDNA primer pair OI1/OI2c and *rplKAJL - rpoBC* operon (β operon) primers A2/J5, out of 30 DNA samples suspected to be HLB positive, 21 (70%) were amplified belonging from seven symptomatic trees (tree No.1, 3, 4, 5, 6, 7 and 10) and produced amplicons of ≈1160bp as observed by Jagoueix *et al.*(1996) and ≈703 bp as reported by Hocquellet *et al*.(1999) that confirmed the presence of *Candidatus* Liberibacter asiaticus in the samples (Fig.2B and C). Amplification percentage of the samples was calculated according to the method of Smith (1958). One sample

Table 1. Primers used in conventional PCR studies to amplify genomic regions of *Candidatus* Liberibacter asiaticus

Primer	Sequences	Target DNA	Orientation	Region of amplification	Comments
OI1	GCG CGT ATG CAA TAC GAG CGG CA	Las	Forward	16s ribosomal RNA	Primer described by Jagoueix *et al*., 1996
OI2c	GCC TCG CGA CTT CGC AAC CCA T	Las	Reverse	16s ribosomal RNA	Primer described by Jagoueix *et al*., 1996
A2	TAT AAA GGT TGA CCT TTC GAG TTT	Las	Forward	rplKAJL-rpoBC(β operon)	Primer described by Hocquellet *et al*.,1999
J5	ACA AAA GCA GAA ATA GCA CGA ACA A	Las	Reverse	rplKAJL-rpoBC(β operon)	Primer described by Hocquellet *et al*.,1999

(33.3%) from tree number 2, zero samples from tree number 8 and two samples (66.67%) from tree number 9 were amplified, whereas no amplification observed in all (100%) samples collected from the insect free greenhouse (Fig.3) confirmed them healthy. Positive results by conventional PCR also confirmed that the symptoms on the leaves were because of the presence of HLB in the plants. Remaining three trees from which samples showed variable results of amplification (33.3%, zero and 66.67% HLB positive) may be due to uneven distribution of pathogen in the host plants (McClean, 1970; Hartung *et al*., 1993).

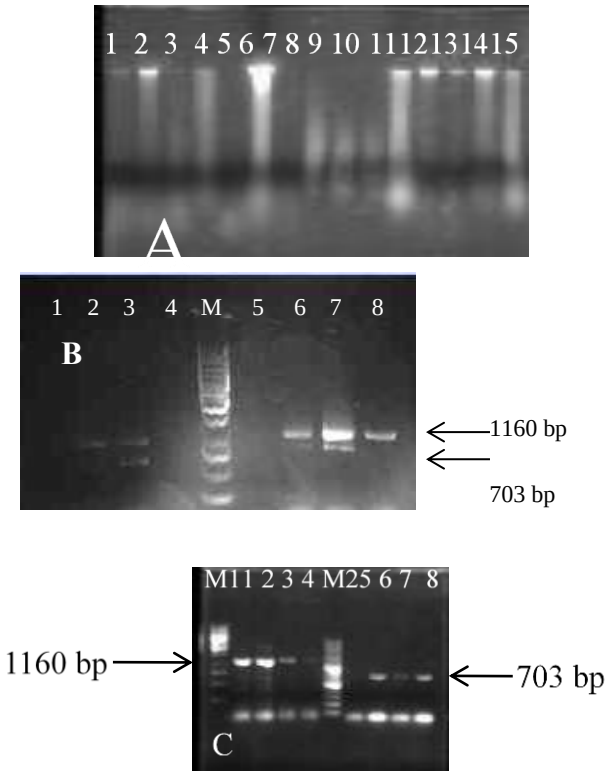

Figure 2.Gel pictures of DNA and PCR for molecular detection of *Candidatus* liberibacter asiaticus: A, Genomic DNA from sweet orange leaf samples; **B,** PCR amplification showing Lane 1,4 and 5= healthy control, Lane 2,3,6,7 and 8= bands of 1160bp from OI1/OI2c primer pair, M= 1kb DNA ladder (Fermentas), Lane 3,6 and 7= bands of 703bp from A2/J5 primer pair for *Candidatus* Liberibacter asiaticus **;** and **C,** PCR amplification showing M1= 1Kb DNA ladder, Lane 1= Positive control , Lane 2,3,4 = amplified with primers OI1/OI2c , M2= 100bp plus DNA ladder (Fermentas), Lane 5= healthy control and Lane 6,7 and 8= amplified with primers A2/J5.

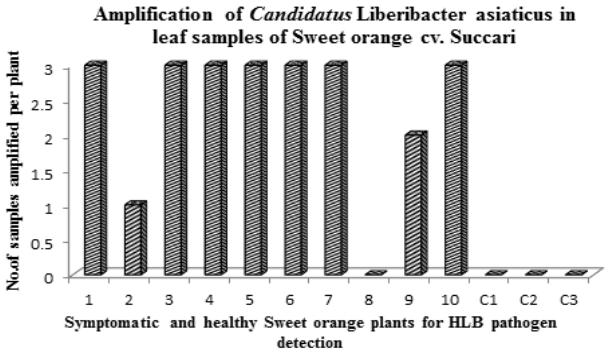

Figure 3. Graph representing number of samples amplified from each sweet orange plant

DISCUSSION

Huanglongbing has become a serious threat to citrus industry of Pakistan. In Pakistan, population of *D. citri* is prevalent in almost all citrus growing areas that co exists with citrus HLB. According to earlier Indian records, greening symptoms in citrus trees were reported in the mid 1700s in India by Roghoji, the Bhonsla Raja of Nagpur (Capoor, 1963). Other writings proved that huanglongbing was present in north-eastern and north-western India in the 1800s and early 1900s (Husain and Nath, 1927; Gottwald *et al*., 2007). Husain and Nath (1927) described severe damage caused by populations of *Diaphorina citri* at Sargodha from 1915 to 1920. It means that this disease is present in Pakistan from hundred years because Sargodha is the part of Punjab, Pakistan. Citrus is grown in all provinces of Pakistan but over 95% citrus is produced in Punjab province of Pakistan (Nawaz *et al*., 2007). Citrus industry survival of Pakistan is not less than a miracle in the presence of psyllid vector and HLB pathogen.

The present study was the most important and initial step towards the management of HLB in Pakistan. We used primer pairs OI1/OI2c and A2/J5 for the detection of *Ca.* L. asiaticus in sweet orange succari leaf samples. After amplification by conventional PCR, discrete bands of ≈1160bp and ≈703bp were obtained in 70% samples from OI1/OI2c and A2/J5 respectively as described by Jagoueix *et al.* (1996) and Hocquellet *et al*. (1999). Efforts are being made to control HLB all around the world but, complete control has not yet discovered. One of the main reasons may be the non cultureable nature of the bacterium (Davis *et al*., 2008; Sechler *et al*., 2009; Parker *et al*., 2014). Huanglongbing management may be difficult in older orchards due to severe damage caused by *Ca*. L. asiaticus and its insect host *D. citri*. However, the development of infrastructure for molecular studies proved helpful for HLB management experiments by early diagnosis of disease in the budwood and nursery plants raised in the greenhouse used as healthy control were confirmed disease free.

Conclusion: We conclude that the bacterial species which causes the HLB disease in Pakistan is *Candidatus* Liberibacter asiaticus as β operon region of bacterium amplified to produce amplicon of 703bp by using primer pair A2/J5. The distribution of bacterium in the host sweet orange cultivar succari plants was found uneven. Proper sampling is very important for molecular detection of the pathogen. Pathogen detection in host plants at molecular level is the most important step for huanglongbing management.

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