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SEROLOGICAL AND MOLECULAR BASED DETECTION OF GRAFT-TRANSMISSIBLE PATHOGENS ASSOCIATED WITH CITRUS FROM NON-CORE AREAS OF PAKISTAN

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To assess prevalence of virus and virus-like diseases of citrus, surveys were conducted in citrus groves of Southern Punjab during 2013-15. Based on serological and PCR detection methods, citrus orchards and nurseries were tested for prevalence of *Citrus tristeza virus* (CTV), *Citrus psrosis virus* (CPsV), *Citrus variegated chlorosis* (CVC) caused by *Xyellela fastidiosa*, huanglongbing (HLB, citrus greening) caused by '*Candidatus* Liberibacter asiaticus' and citrus stubborn disease (CSD) caused by *Spiroplasma citri*. Citrus leaves and twigs samples of 'Kinnow' and'Feutrell's Early' mandarins (*Citrus reticulate* Blanco), 'Mosambi' sweet oranges (*Citrus aurantium* L.), 'Shamber' grapefruit (*Citrus aurantium*L.) were collected from five districts of southern Punjab viz. Multan, Khanewal, Lodhran, Layyah and Bhakkar. Direct antibody sandwich enzyme linked immuno sorbent assay (DAS-ELISA) was employed to detect CTV, CPsV, CVC and CSD, where-as HLB was detected by PCR using specific primers from two regions 16S rDNA intergenic region and ribosomal protein gene of rpl*KAJL*-rpo*BC*operon (*β*-operon) of '*C.* Librebacter asiaticus'. Sequence of 16S rDNA revealed nucleotide identity level upto 98% among different isolates. Disease incidence of CTV, CVC, HLB and CSD was 36.7, 10, 40 and 36.7%, respectively, where-as CPsV was not detected. All 'citrus species and relatives' *viz.*, Kinnow, sweet oranges, Feutrell's early and grapefruit (shamber) were susceptible to these diseases. To our understanding this is first investigation of virus and virus-like diseases in citrus from non-core areas of Punjab, Pakistan.

Keywords: HLB, CTV, CSD, CVC, ELISA, DNA, PCR, electrophoresis

INTRODUCTION

Citrus is considered the top ranked fruit crop in tropics and subtropics (Arif *et al.*, 2005). The quality and production of Pakistani oranges has placed Pakistan among top ten most promising countries of world (Chohan *et al.*, 2007). The taste of Pakistani oranges has also been acknowledged at world level. Citrus is cultivated on 0.198 million hectares and Pakistan produces 2.47 million tonnes oranges each year (Anonymous, 2014). In Pakistan, like other crops, oranges production is hampered due to many pathogens viz., fungal, bacterial, viral and virus-like diseases of citrus that have been documented since 1920 (Arif *et al*, 2005; Khan *et al.*, 2006; Naz *et al*., 2007; Naseem *et al.*, 2016). Currently in Pakistan, many crops such as mango, guava and pomegranate are facing problems of decline. Similarly, citrus plantations are also facing decline attributable to many devastating diseases. Virus and virus-like diseases of citrus crop are considered a major citrus production constraint (Yaqub *et al*., 2017; Fateh *et al*., 2017; Satir *et al*., 2016). Reports showed that area under citrus cultivation and its production has been increased incredibly in southern Punjab in last two decades (Table 1, 2).

The devastating virus and virus-like pathogens of citrus reported in Pakistan are *Citrus tristeza virus* (CTV), infectious citrus variegation, exocortis, cachexia-xyloporosis, huanglongbing (HLB, citrus greening) and stubborn disease (Catara, 1987; Catara *et al*., 1988). It is believed that these fastidious phloem degradation pathogens not only prevent the movement of photoassimilates to the root system but also promote the defoliation and deformation of fruit along with seed abortion. During 1980's, survey reports of foreign and local scientists found citrus crop in Punjab and North Western Frontier Province (NWFP) to be infected with various virus and virus-like pathogens (Catara *et al*., 1988). In Pakistan, tristeza was first time observed and detected by electron microscopy as well as by enzyme linked immunesorbent assay (ELISA) in samples from different locations (Catara *et al*., 1988). Now, 15 years after the previous reports, situation for citrus crop has worsened, as all citrus orchards found to be near collapse in Punjab and NWFP and 100% of citrus plantations are infected with virus and virus-like problems in

Table 1. Summary of cultivation area and production of citrus in Southern Punjab.

District	Cultivation (Area in hectares)	Production (Tonnes)	Varieties grown
Khanewal	7077	80394.72	Grapefruit, Kinnow, Musambi
Lodhran	1504	17085.44	Kinnow, Musambi, Sweet lime
Multan	5849	66444.64	Kinnow, Musambi, Sweet lime
Layyah	4476	50847.36	Kinnow, Musambi
Bhakkar	1409	16006.24	Kinnow, Musambi
MuzaffarGarh	1004	11405.44	Kinnow

Table 2. Cultivation area and production of citrus species and relatives in Southern Punjab

Varieties	Cultivation (Area in hectares)	Overall Production (tonnes)
Kinnow	19250	218680.00
Feutrell's early	3727	42338.72
Grapefruit (shamber)	10	113.60
Sweet orange/ Musambi	2318	26332.48
Other citrus cultivars	297	3373.92

Pakistan (Arif *et al*., 2005). CTV, the causal agent of the disease, belongs to family *Closteroviridae* is transmitted by grafting and in nature by several aphidspecies. CTV mild strains are generally considered not to induce infection nor other symptoms like stem pitting while moderate and severe strains of CTV are responsible for symptom development (BarJosephet *al*., 1989; Garnesy *et al*., 1991; Rocha Pena *et al*., 1995). The management of CTV is solely dependent on replacement of rootstock (sour orange) with rough lemon and some erstwhile species of citrus which will help to reduce the infestation of CTV. In 1919, Huanglongbing was reported in China by Renking and is caused by a phloem-limited, fastidious bacterium ('*C.* Liberibacter asiaticus') usually transmitted by *Diaphorina citri* (Asian citrus psyllid) and in Pakistan previously united with India (Nath *et al*., 1927; Garnsey, 1991).

Trees affected are generally stunted have dwarfed foliage, misshape fruit, yellowing of the leaves and aborted seed (Husain and Nath, 1927; Coehran, 1976; Gottwald, 2010). *Spiroplamacitri* is also a phloem-inhibiting fastidious pathogen normally vectored by leaf hoppers, causes CSD (Citrus stubborn disease). The bacterium has a wide range of hosts including many citrus species and broader range of non-host plants. Disease symptoms consist of upright leaves, stunted growth of the plant, variable chlorotic patterns on leaves resembling the nutritional deficiencies, flowering in off seasons resulting misshapen fruit of various sizes (Bove *et al*., 1988). The citrus plantations are also badly attacked by a fastidious bacterium, *Xyllela fastidiosa*, which causes CVC (Citrus variegated cholorosis) vectored by numerous species of sharpshooters. Characteristic symptoms of disease include small, yellow spots resembling zinc deficiency on the upper sides of leaves while gummy lesions and dark brown spots appear on underside of leaves, plant also bear smaller size fruit which ripen early. Disease appears in patches on newly affected plants while severely infected plants show uniformity of symptoms (Catara, 1987). Like other pathogens of citrus, (CPsV) *Citrus psorosis virus* is also considered one of the serious issue for citrus orchards. The virus is reported to be transmitted by grafting and may also be spread by an *Olpidium*-like fungus in the field (Palle *et al*., 2005). Characteristic symptoms of disease are small chlorotic flecks irregularly present on leaves and mottling of leaves (Catara, 1987). Many scientists have also worked on citrus diseases in

Table 3.A brief description of core areas of citrus in which various pathogens have been detected in earlier studies.

Year	Location	Disease	Detection Technique	Reference
1916	Sargodha	Dieback	Visual symptoms	Husain and Nath (1927)
1927	Sargodha, Layallpur	Citrus greening	Visual symptoms	Husain and Nath (1927)
1988	NWFP, Sargodha	CTV	ELISA, PAGE	Catara*et al*. (1988)
1988	Faisalabad, Okara	Citrus Greening	Electron microscopy,	Catara*et al*. (1988)
1989	Faisalabad	CTV	Electron microscopy	Bove, 1989
2003	Faisalabad	CTV	ELISA	Mughal (2003)
2005	NWFP	CTV	ELISA	Arif*et al*. (2005)
2007	NWFP	Citrus greening	PCR	Chohan*et al*. (2007)
2013	Sargodha	HLB	qPCR	Razi*et al*. (2013)
2013	Islamabad	CTV	ELISA, PCR	Ammar *et al*. (2013)

Pakistan at different locations yet this area was remained unexposed (Table 3).

In southern Punjab too, situation is very devastating. Diseases are prevalent in all citrus cultivars grown in this area, but there is no proper identification of the diseases and their management. The spatial and temporal increases of symptomatic trees have shown the alarming situation of citrus in these groves. Surveys of diseases in crops are required for many purposes: to determine general levels of crop health; or the presence of particular diseases of quarantine significance; to identify the problems and enable proper allocation of crop protection resources; and to assess the losses caused by crop disease. This diagnostic survey combines identification of diseases with a measure of their intensity. The current study was conducted with the firm objective to identify and confirm the pathogens causing disease in citrus in this area.

MATERIALS AND METHODS

***Study site*:** The current research work was carried out at the "Department of Plant Pathology", Faculty of Agricultural Sciences and Technology, BahauddinZakariya University, Multan, Pakistan (30.268° N and 71.495° E, 122 m) where the climatic conditions are extreme with temperature ranging from 48°C in summer to −1°C in winter with an average annual rainfall approximately of 127mm. Citrus leaf samples were collected from the different districts of southern Punjab (Fig. 1.) viz., Multan (30.1984° N, 71.4687° E), Khanewal (30.3039°N, 71.9299°E), Lodhran (29.5363°N, 71.6317°E), Layyah (30.9648° N, 70.9399° E) and Bhakkar (31.6230°N 71.0626°E) during 2013-2015 considered as the non-core areas of citrus cultivation.

***Sample collection of various citrus cultivars*:** Samples were collected from diseased plants during 2014-2016 showing the typical symptoms of virus and virus like diseases. Samples were collected in plastic bags, kept in an ice box and brought to the laboratory for the identification of diseases. A total of 750 samples were collected (500 for CTV, 300 for HLB, 90 for CVC, 90 for CSD and 30 for CPsV).

***CTV, CVC, CSD and CPsV detection through ELISA*:** For the detection of CTV, CVC, CSD and CPsV, DAS-ELISA technique (Clark and Adams, 1977) was performed. The antibodies were provided by Agdia Inc. USA. Ploystyrene microtiter plates were coated with 100ul of capture antibody and antigen was extracted from 0.5g leaf tissues in 5ml extraction buffer and loaded in each well of the plates. After incubating over night, the plates were washed 5-7 times with PBST buffer. In next step plates were coated with enzyme conjugate and incubated at room temperature for 2 hours. After washing, plate wells were loaded with substrate buffer containing pNpp. The colour development in the wells showed the positive reaction. The virus titter was measured on a plate reader (Bio Tek, Model ELx 800) at 405 nm (Clark and Adam, 1977).

Figure 1. Map of Punjab, Pakistan showing the survey sites for citrus pathogens.

***DNA extraction*:** For the molecular detection of HLB using PCR technique, genomic DNA was extracted from the mid ribs of citrus leaves using modified CTAB method (Murray and Thompson, 1980). Leaf midribs (250 mg) were ground in liquid nitrogen to a fine powder and transferred to 1.5 ml of eppendorf tube followed by adding pre-heated 1 ml of 2% CTAB extraction buffer (100mM Tris-HCl, 50mM EDTA, 1.4M NaCl with 1% PVP and 0.1% β-mercaptethanol) and incubated for 1hour at 65°C. The suspension was treated with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 10 minutes. The upper phase was collected in new Eppendorf tubes, DNA was precipitated by mixing an equal volume of isopropanol and kept for an hour at -20ºC. DNA pellets were obtained by centrifugation at 12,000 rpm for 15 minutes and washed with 70% ethanol, dried overnight and re-suspended in 100 µl of TE buffer (Dellaporta*et al*., 1983, Hung *et al*., 1999). The extracted DNA was used as template for PCR with '*Ca*. L. *asiaticus*'-specific primers for 16S rDNA intergenic region and ribosomal protein gene of rplKAJL-rpoBC operon (β-operon).

***PCR detection of HLB*:** The PCR reactions were carried out in 25 µl of reaction mixture containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 2 µM of each primer, 0.25mM of each four dNTPs, 0.5 units *Taq*DNA polymerase (Fermantas) and 100 ng genomic DNA as template. Specific primers from 16S rDNA intergenic region (Forward primer OI1 5′-GCGCGTATGCAAGAGCGGCA-3′ and reverse primer OI2c 5′-GCCTCGCGACTTCGCAACCCAT-3′ (Jagoueix*et al*. 1994) were used for the amplification in a thermal cycler (Model Mycycler, Biorad, Inc. USA). The conditions for OI1 and OI2cprimers were: one cycle at 94°C for 3 min, 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec, followed by 72°C extension for 10 min. Sequence from the ribosomal protein gene of rplKAJL-rpoBC operon (β-operon) was also amplified using forward primer A2- 5′-TATAAAGGTTGACCTTTCGAGTTT-3′ and reverse primer J5 5′-ACAAAAGCAGAAATAGCA CGAACAA-3′ (Jagouex*et al*., 1996) for further confirmation of '*Ca.* Librebacterasiaticus' strain. The optimized conditions for A2 and J5primers were: one cycle at 94°C for 2 min, 35 cycles at 92°C for 30 sec, 50°C for 45 sec, and 72°C for 45 sec, followed by 72°C extension for 10 min.

***Gel electrophoresis*:** Gel electrophoresis was performed for the separation of PCR products in 1.5% agarose in TBE buffer using ethidium bromide as a stain. The visuals of the gel were analyzed in a Gel Doc. system, (Bio RAD, USA). The sequence identity was analyzed by searching in NCBI Blast.

***DNA Sequencing*:** The amplified PCR product was sequenced by using automated fluorescent DNA sequencing/ senger (DNTCT) method.

***Statistical analysis*:** The ELISA data were subjected to ANOVA with means being separated by LSD tests at P≤0.05 using SAS version 8.0; Carry Inc, USA).

RESULTS

***Prevalence of citrus pathosystems in various Districts*:** During the course of surveys and three years studies on citrus graft-transmissible diseases it is calculated that Citrus groves are badly affected with HLB having maximum disease incidence 32.67% in all the surveyed Districts followed by CTV 23.09%, CSD 19.56% and CVC 4.44% (Fig. 2).

CTV, CVC, CSD and HLB were present in all the surveyed areas of the non-core regions of citrus in Punjab. The highest incidence of CTV was observed in the Multan district (36.7%) and the lowest (12%) in the Bakhar district (Fig. 3.). The highest incidence (40%) of HLB was recorded in the Layyah district followed by 38.2%, 32% and 20% in the Multan, Khanewal, Bakhar and Lodhran districts, respectively. CVC incidences of 10% and 6.8% were recorded from the Multan and Khanewal districts, while rest of the districts were found free from the CVC infection. During the survey, citrus trees were also found to be infected with CSD. The highest incidence was in the citrus groves of Layyah District with 36.7% followed by 26.7% Multan and 20% in Bakhar district with respect to positive (Fig. 3).

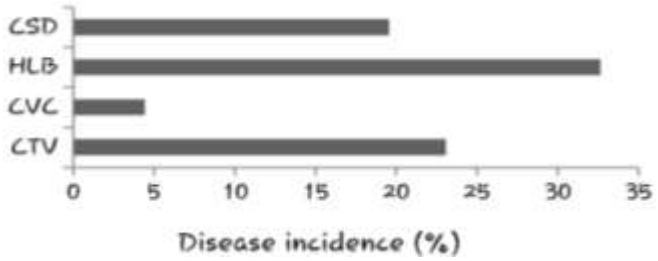

Figure 2. Overall percentage disease incidence of graft-transmissible citrus diseases in non-core region of the Punjab.

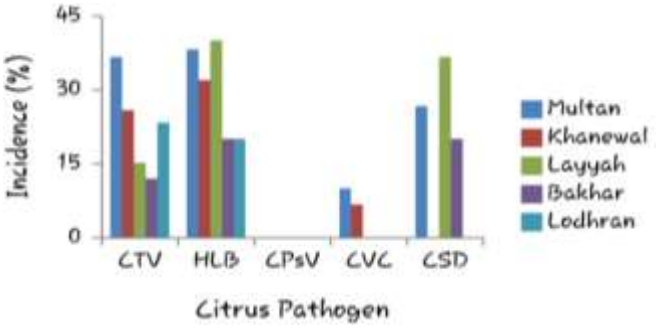

Figure 3. Incidence of various diseases in some non-core areas of citrus in the Southern Punjab.

***Disease incidence in different citrus types*:** The incidence of the graft-transmissible diseases in the different citrus species and hybrids showed that grape-fruit and sweet oranges had the highest frequency of infection by CTV. Survey results showed a 42.5% disease incidence was for grape-fruit followed by 36% for sweet orange. Kinnow and Feutrell'sEarly showed the lowest incidence of CTV. The highest incidence of HLB was found in Kinnow with 51.7% followed by 26.2, 22.2, and 10% for sweet orange, grape-fruit and Feutrell's Early, respectively. CVC infection was only found in Kinnow (10% incidence) and Feutrell's Early (5% incidence). CSD infection was only observed in sweet orange with incidence of 34.6% (Fig. 4).

***Detection and sequencing of genomic region of 'Ca.* L. asiaticus' *isolate*:** The confirmation of '*Ca.* L. asiaticus' in the samples was done by PCR followed by comparing the sequences of existing 16s rDNA regions. The results of PCR detection of '*Ca.* L. asiaticus' clearly showed that primers OI1/OI2c amplified the 16s rDNA region with expected product size of 1160 bp (Fig. 5). Whereas A2/J5 primers amplified 703 bp band (Fig. 6). The deposited GenBank accession No. KX434453 of '*Ca.* L. asiaticus' isolate showed 98% similarity with previously submitted isolates.

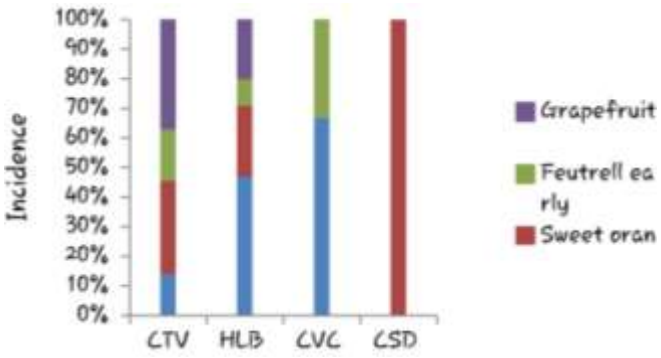

Figure 4. Cultivar susceptibility to various fastidious citrus pathogens, the colours indicative are the representatives of the concerned fruit showing the incidence of the disease.

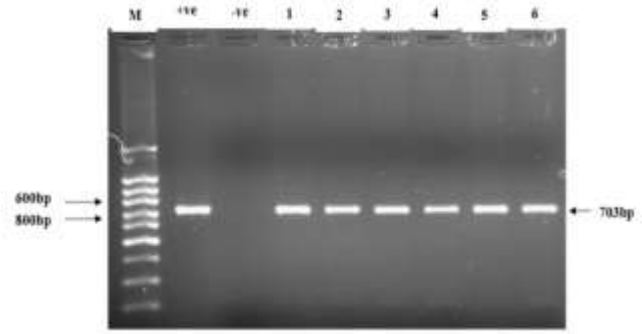

Figure 5. Electrophoresis in 1.5% agarose gel of DNA amplified with primers OI1/OI2 from positive control (+ive), negative control (-ive), Lane 1-to-6 from infected Kinnow leaves; Lane 7-to-10 from infected sweet orange leaves; Lane 11 from Feutrells Early while lane 12 with grape fruit; Lane M (100 bp of DNA ladder.

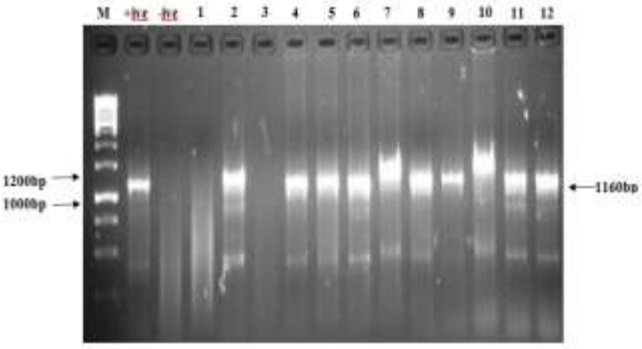

Figure 6. Electrophoresis in 1.5% agarose gels of DNA amplified with primers A2/J5 from positive control (+ive), negative control (-ive), Lane 1 infected Kinnow leaves; Lane 2 infected sweet orange; Lane 3 from Feutrells Early and Lane 4 with grape fruit; Lane M (100 bp DNA ladder.

DISCUSSION

Oranges and other citrus are nourishing and important crops of the regions due to their dietary and medicinal values. Citrus plantations are suffering from many biotic and abiotic problems due to a variety of pathogens prevailing in citrus orchards. In Pakistan, some districts of the NWFP and upper Punjab *viz*., Sargodha, Faisalabad, and Sahiwal are considered citrus plantations hubs and research has been focused in these core areas of Pakistan. However, no specific research spotlight has been given to the non-core areas of the Pakistan viz., Khanewal, Multan, MuzaffarGarh, Layyah, Bhakkar, Lodhran and Bahawalpur regarding the pathogens of citrus. The soil of these districts is favourable for citrus orchards and environmental factors are also facilitating the development of this citrus in this zone. Apart from the survey of the Catara in the 1980's and works in the core areas of the citrus cultivation, this is first and comprehensive report of graft-transmissible citrus pathogens prevailing in non-core areas of the Punjab. Roistacher (1988) reported that graft-transmissible pathogens are key limiting factor of citrus production. Our survey results during the previous three years' work done showed increasing incidence of virus and virus like diseases in the non-core vicinities of the Southern Punjab which is leading factor for hampering the citrus production. We also observed that even no citrus plant was present free from any type of infection in any orchard of this region. Our results are also supported by Roistacher (1988) who reported that graft transmissible pathogens are key limiting factor for production of citrus. Khan (1992) reported that in Pakistan more than 30 virus and virus like diseases of citrus are known to exist. However, in Pakistan, only limited numbers of surveys have been made to test the presence of disease in citrus orchards (Atta *et al*., 2012). Our survey results during 2014-2016 documents the incidence of graft-transmissible pathogens in the non-core vicinities of the Southern Punjab that are leading factors hampering citrus production. Our studies also confirmed the presence of several graft-transmissible pathogens in the southern part of Punjab including HLB, CTV, CVC, and CSD. We found that no citrus plant was free from any type of infection in any of the orchards of this region. Among viral disease of citrus, CTV is the most serious problem worldwide. Once a tree becomes infected with CTV, it always remains infectious and there are no management tactics available to free the plant from the virus. CTV causes degeneration of cambium layers with results that infected citrus trees start to decline. CTV and HLB were found to be most common in citrus groves of southern Punjab and nurseries with maximum average incidence of 40 and 36.8% respectively. Our results are in line with the findings of other studies. Catara (1987) and Catara*et al*. (1988) reported CTV infestations in some vicinities of the NWFP, and Catara*et al*. (1988) forecasted that HLB would be a real threat for citrus production in Pakistan after CTV. Arif*et al*. (2005) reported that CTV was

the most prevalent disease in citrus orchards of all the surveyed areas of NWFP with an average incidence of 27% likely to be increased in the coming years. Our observations suggest that if the trend for grafting sweet orange on sour orange continues, then every management tactic will fail to control CTV infestations. Similar findings were reported by Arif*et al*. (2005) that sour orange is susceptible to many pathogens viz., viruses, viroids and prokaryotes, hence the practice of using infected scion may be cause of increasing incidence of CTV, resulting in decline of citrus tree in 8-10 years. Atta *et al*. (2012) reported that mild strain of CTV may be used for the biological indexing of the host crop and in the management programs.

HLB is not only the threat for Pakistan but also for all citrus growing areas of the world and the incidence of HLB is increasing day by day in Pakistan. In our opinion, CTV, CVC, HLB and CSD are all major causes of decline of citrus plantations and the presence of these diseases makes it difficult to differentiate between the symptoms of the disease and the nutritional imbalances. The introduction of virus, viroid and prokaryotic organisms through exchange of and movement of infected nursery plants/ bud-wood inside and outside the country is another reason for the establishment of these diseases. The detection of HLB disease in young citrus plants is important to prevent a wide- spread outbreak of this disease. The sensitivity of PCR assay is an excellent method for detecting fastidious bacteria in its host (Hung *et al*., 1999). DNA extraction from leaf mid rib and PCR with specific primers OI1/OI2c and A2/J5 can be utilized for the detection of '*Ca.* L. asiaticus' strain. When amplification was performed on these DNA extractions, a band of 1160 bp and 703 bp were observed from primer OI1/OI2c and A2/J5, respectively. The size of the amplified DNA with primers A2/J5 is smaller than OI1/OI2c, thus the target degradation is less critical (Hocquellet*et al*., 1999). Therefore, the sensitivity of primers A2/J5 was the same or slightly better compared with primers OI1/OI2c.

***Conclusion*:** The study demonstrates the incidence of the graft-transmissible diseases of citrus in the citrus groves of the Punjab, which is a real threat not only to the citrus industry in Pakistan but also to the citrus growers in the country. Management of graft-transmissible diseases of citrus in Pakistan is quite challenging due to the absence of certified nurseries and a lack of regulations regarding the use of disease-free planting material. Now there is a dire need of time to focus our attention to the citrus pathogens because "Unless we find a cure for these disease, there will not be citrus in this state."

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