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RESEARCH ARTICLE

Diversity and characterization of *Citrus tristeza virus* and *Candidatus* Liberibacter asiaticus associated with citrus decline in India

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ABSTRACT: Tristeza and Citrus greening disease (Huanglongbing, HLB) are two most destructive diseases of citrus worldwide. Tristeza disease is caused by *Citrus tristeza virus* (CTV), a phloem limited, monopartite, and single-stranded RNA virus whereas citrus greening disease is induced by three different species of a Gram negative, non-cultured, alpha proteobacteria '*Candidatus* Liberibacter'. Among the three species, '*Candidatus* Liberibacter asiaticus' is widely distributed pathogen in Indian subcontinent. In the present study, 30 leaf and twig samples were collected during 2013-16 from citrus plants showing moderate to severe decline symptoms in field from three different states *viz.,* Maharashtra, Assam and West Bengal that are considered as important citrus growing belts of India. Molecular diagnosis by PCR (polymerase chain reaction) using pathogen-specific primers shows that out of thirty samples tested, twenty one samples were found positive with CTV, seventeen samples were infected with '*Ca.* L. asiaticus' and ten samples were infected with both the pathogens while two samples tested negative. Sequences of coat protein gene (CPG) of CTV isolates showed 97% to 100% sequence identity with CTV sequences available in the GenBank. In phylogenetic analysis, all sequenced CTV isolates were grouped into five clades indicating the presence of at least five different CTV strains that are associated with declined citrus trees of Maharashtra, Assam and West Bengal. Similarly, nucleotide sequence of 16S rRNA gene of '*Ca.* L. asiaticus' showed 97% to 100% identity with sequences of other reported isolates. Phylogenetic analysis of the 16S rRNA gene indicated that HLB isolates associated with citrus decline in the present study belong to '*Ca.* L. asiaticus' species. A robust and sensitive duplex PCR has also been developed for the detection of mixed pathogen infection in field samples.

Keywords: Citrus, Citrus greening, *Citrus tristeza virus,* phylogenetic analysis

Citrus is the third most important fruit crop in India which contributes about 8.27% of world's total citrus production. Globally, India stands as fourth largest citrus producer after China, Brazil and USA (FAOSTAT, 2014). However, per hectare production of citrus in India is considerably low compared to other citrus producing countries. Number of biotic and abiotic factors are responsible for citrus decline and low productivity in the country. Among biotic factors, two important citrus pathogens *i.e. Citrus tristeza virus* (CTV) and '*Candidatus* Liberibacter asiaticus' (*Ca*. L. asiaticus) are considered as the most destructive systemic pathogens associated with citrus decline in India (Ahlawat, 2012; Ghosh *et al*., 2009, 2013). CTV has destroyed millions of citrus trees in last 70 years (Bar-Joseph and Dawson, 2008). It is a member of *Closterovirus* genus belonging to *Closteroviridae* family. CTV, the largest reported plant virus is flexuous in morphology having a size of 2000 X 11 nm. The viral genome consists of monopartite, single-stranded positive sense RNA (ss RNA) of ~20 kb size (Albiach-Marti MR, 2012; Flores *et al.,* 2013). CTV is a phloem-limited virus and long distance spread takes place through movement and use of CTV-infected nursery propagation materials. In citrus groves, it is transmitted by several aphid species in a semi-persistent manner in which *Toxoptera citricida*, *T. aurantii, Aphis gossypi, A. spiraecola* and *Myzus persicae* are the principal vectors for CTV transmission (Ahlawat, 2012; Ghosh *et al*., 2014).

A numbers of techniques have been developed for CTV detection. Biological indexing is one of the classical methods used for detection of CTV and it consists of grafting test scion on the Mexican lime (*C. aurantifolia*) indicator plant seedlings followed by the development of characteristic vein flecking symptoms within three to four months under controlled conditions (Bhose *et al*., 2015a). A number of molecular diagnostic methods have also been used for CTV detection *viz*. enzyme-linked immunosorbant assay (ELISA), dot immunobinding assay (DIBA), cDNA probes, cRNA probes, RT-PCR,

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nested RT-PCR multiplex RT-PCR, and real time PCR (Ahlawat, 2012).

Citrus greening (Huanglongbing, HLB) is another devastating disease that has destroyed 60 million citrus trees globally (Ghosh *et al*., 2015). Greening disease is caused by a Gram negative, phloem-limited, non-culturable bacterium belonging to the á-subdivision of phylum Proteobacteria '*Ca.* Liberibacter' (Bhose *et al*., 2015b; Wang and Trivedi, 2013). There are three different species of *Candidatus* Liberibacter associated with HLB disease *viz*. '*Ca*. L. asiaticus', '*Ca*. L. africanus', and '*Ca*. L. americans'. Naturally *'Ca*. L. asiaticus' and '*Ca*. L. americanus' are heat tolerant and transmitted by *Diaphorina citri* Kuwayama while '*Ca*. L. africanus' is heat sensitive, transmitted by *Trioza erytreae* Del Glucero. Classical biological indexing method was used for the diagnosis of HLB where *Citrus sinensis* Obseck was used as an indicator plant. PCR is a laboratory based method widely used for detection of the pathogen. Recently, real time PCR, Loop-mediated isothermal amplification (LAMP), and lateral flow detection device (dipstick) etc have been developed for the fast and accurate detection of *Ca.* Liberibacter spp. (Ghosh *et al*., 2016; Rigano *et al*., 2014).

Tristeza and citrus greening are the two most economically important systemic diseases associated with the citrus decline in India. However, very limited literature is available on the molecular characterization of both these pathogens present in the country. In the present study, we have examined the diversity of both CTV and greening associated with citrus decline of different citrus cultivars in Maharashtra, West Bengal and Assam, three important states known for commercial citrus cultivation. We have characterized both the pathogens by biological indexing, sequence analysis of conserved gene, restriction digestion and phylogenetic analysis. Further, for simultaneous detection of both pathogens in field samples, a low cost, robust and sensitive duplex-PCR technique has also been developed.

MATERIALS AND METHODS

Sample collection

Samples were collected during Nov., 2013 to March, 2016 from citrus trees showing moderate to severe decline symptoms (Fig. 1A,B) under field condition in Maharashtra, Assam and West Bengal. Three to six samples (matured leaves and young twigs) from different citrus cultivars were randomly collected from various locations of each state (Table 1) and brought to ICAR-CCRI, Nagpur for further studies.

Bio-diagnosis

Biological indexing of few representative samples were done by graft inoculation (side and wedge grafting) on one-year-old acid lime (*C. aurantifolia*) and Mosambi (*C. sinensis*) seedlings which are the indicator plants for CTV and '*Ca.* L. asiaticus' respectively (Ahlawat, 2012). A minimum of 3-4 plants were graft inoculated to index a given source tree. Grafted seedlings were maintained under observation in polyhouse at 30°C temperature during daytime and 25°C at night. Time of first symptom developed on indicator plants were recorded.

Fig. 1. CTV infected declined Nagpur mandarin (*Citrus reticulata*) tree in Nagpur district of Maharashtra (A) Mosambi sweet orange (*Citrus sinensis*) field tree mixed infected with CTV and greening in Aurangabad, Maharashtra (B) Vein clearing symptoms on acid lime (*Citrus aurantifolia)* indicator plant in poly house (C)

Extraction of total DNA and RNA

Symptomatic leaves were thoroughly washed, blot dried and cleaned with 70% ethanol to reduce the surface contamination. 100 mg of midrib tissue from the leaves were ground in liquid nitrogen and used for the total DNA and RNA isolation using DNeasy plant mini kit and RNeasy® plant mini kit (Qiagen, Germany), respectively as per manufacturer's protocol.

Polymerase chain reaction (PCR)

Total RNA was used to conduct RT-PCR in which CTV specific sets of forward primer CN150 (5' ATA TAT TTA CTC TAG ATC TAC CAT GGA CGA CGA AAC AAA3') and reverse primer CN151 (5' GAA TCG GAA CGC GAA TTC TCA ACG TGT GTT AAA TTT CC 3') were used to amplify major coat protein (P_{25}) gene of CTV. RT-PCR was carried out in two steps. In the first step, cDNA was synthesized in 15 µl of master mixture with 1x first strand buffer, 0.2 mM dNTPs mix, 15.6 U of RNAsin (Promega), 0.4 µM reverse primer, 6 µl of total RNA and 120 U of MMLV reverse transcriptase (Promega). Reaction was carried out in a thermal cycler My Cycler (Bio-Rad, USA) with extension at 42°C for 50 min and denaturation at 72°C for 10 min. For second step, an aliquot (1.75 µl/15 µl) of cDNA products were used for amplification of cDNA in a 25 µl reaction mixture containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.2 µM of each primer,

Table 1. Details of samples collected from declined citrus trees, PCR detection of CTV and "*Ca*. Liberibacter asiaticus" and published GenBank accessions numbers

Sample code	Place/State of sample collection	Cultivar/Botanical name	RT-PCR/PCR results		CTV accession no.	HLB accession no.
			CTV(+/-)	HLB(+/-)		
'AK1'	Tinsukia, Assam	Khasi mandarin (*Citrus reticulata*)	+	-	KC816567	
'AK2'	Tinsukia, Assam	Khasi mandarin (*C. reticulata*)	-	+		NS
'AK3'	Tinsukia, Assam	Khasi mandarin (*C. reticulata*)	-	+		NS
'AK4'	Tinsukia, Assam	Khasi mandarin (*C. reticulata*)	-	+		KY230622
'AK9'	Tinsukia, Assam	Sweet orange (*C. reticulata*)	+	+	KJ 524841	KY230623
'AK10'	Tinsukia, Assam	Sweet orange (*C. reticulata*)	+	+	KJ524843	KY484088
'AK11'	Tinsukia, Assam	Sweet orange (*C. reticulata*)	+	+	KJ524844	NS
'AK13'	Tinsukia, Assam	Sweet orange (*C. reticulata*)	+	+	KJ524842	KY230624
'AK15'	Tinsukia, Assam	Rough Lemon (*C. jumbheri*)	+	+	KJ524846	NS
'AK16'	Tinsukia, Assam	Rough Lemon (*C. jumbheri*)	+	-	KJ524847	
'B1'	Aurangabad, Maharashtra	Mosambi (*C. sinensis*)	+	+	KY436591	KY230621
'C1'	Aurangabad, Maharashtra	Mosambi (*C. sinensis*)	+	-	NS	
'D1'	Aurangabad, Maharashtra	Mosambi (*C. sinensis*)	+	-	KY436592	
'E1'	Aurangabad, Maharashtra	Mosambi (*C. sinensis*)	+	-	NS	
'K 1'	Kalimpong , WB	Darjeeling mandarin (*C. reticulata*)	-	-		
'K 3'	Kalimpong, WB	Pomelo (*C. grandis)*	-	+		KY230619
'K 5'	Kalimpong, WB	Darjeeling mandarin (*C. reticulata*)	+	+	KY436596	KY230620
'K11'	Kalimpong, WB	Darjeeling mandarin (*C. reticulata*)	+	-	KY436597	
'MA'	Nagpur, Maharashtra	Mosambi (*C. sinensis*)	+	-		
'MD'	Nagpur, Maharashtra	Mosambi (*C. sinensis*)	+	+	KY011909	KY230616
'MG'	Nagpur, Maharashtra	Mosambi (*C. sinensis*)	+	+	KY427696	KY230617
'MG4'	Nagpur, Maharashtra	Mosambi (*C. sinensis*)	+	-	NS	
'MH'	Nagpur, Maharashtra	Mosambi (*C. sinensis*)	+	+	KY436590	KY230618
'N 1'	Nagpur, Maharashtra	Nagpur mandarin (*C. reticulata*)	-	-		
'N 24'	Nagpur, Maharashtra	Nagpur mandarin (*C. reticulata*)	-	+		KY077142
'N 49'	Nagpur, Maharashtra	Nagpur mandarin (*C. reticulata*)	-	+		KY211974
'N118'	Nagpur, Maharashtra	Nagpur mandarin (*C. reticulata*)	-	+		KY230615
'Sit 5'	Sitong, Darjeeling, WB	Darjeeling mandarin (*C. reticulata*)	+	-	KY436593	
'Sit 6'	Sitong, Darjeeling, WB	Darjeeling mandarin (*C. reticulata*)	+	-	KY436594	
'Sit 7'	Sitong, Darjeeling, WB	Darjeeling mandarin (*C. reticulata*)	+	-	KY436595	

NS: not sequenced

and 1.25 U of GoTaq flexi DNA polymerase (Promega, Madison, USA). The amplification was carried out in same thermal cycler with one cycle of 2 min at 94°C followed by 35 cycles of 0.30 min at 94°C, 0.45 min at 61°C, 1 min at 72°C and final extension for 10 min at 72°C. The resulting RT-PCR products were separated on 1% agarose gel electrophoresis and visualized by ethidium bromide staining in UV GelDoc system (Bio-Rad, USA).

Similarly, total DNA was used to conduct PCR with a set of 16S rRNA based primers OI1/OI2c for detection of *'Candidatus* Liberibacter spp' (Ghosh *et al*., 2013). PCR amplification carried out in thermal cycler (T100™ Bio-Rad, Hercules, CA, USA) with one cycle of 4 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 58°C, 1.30 min at 72°C, and final extension at 72°C for 10 min. The resulting PCR products were separated and visualized, as mentioned above.

Duplex PCR

For the simultaneous detection of CTV and '*Ca*. L. asiaticus', reverse transcription duplex PCR (RT-d-PCR) was performed. Standard coat protein gene based (CN150/CN151) and 16S rDNA based (OI1/OI2c) primers as mentioned earlier were used for the simultaneous detection of CTV and '*Ca*. L. asiaticus' respectively in duplex PCR. cDNA synthesis was performed from isolated total RNA using the same protocol mentioned previously. Subsequently, 1.75µl cDNA and 1 µl of total DNA were added in a 25 µl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.2 µM of each primer (CN150,CN151,

OI1 and OI2C) and 1.25 U of GoTaq flexi DNA polymerase (Promega, Madison, USA). The amplification was carried out in above mentioned thermal cycler with one cycle of 2 min at 94°C followed by 35 cycles of 0.30 min at 94°C, 0.45 min at 59°C and 1.30 min at 72°C and final extension for 10 min at 72°C.

Restriction digestion

Restriction digestion of the PCR product amplified by the OI1/OI2c primers (Jagoueix *et al.,* 1996; Garnier *et al.,* 2000) from '*Ca*. L. asiaticus' was performed using *Xba*I. Reaction was carried out in 20 µl volume with 10 µl of PCR product, 1X Restriction enzyme buffer, 0.5U *Xba*I, 10 ug/µl of BSA and nuclease-free water. The reaction tubes were incubated at 37°C for overnight. After digestion, restriction pattern was observed in 2% agarose gel electrophoresis.

Sequencing of PCR products

The amplified products of some selected representative samples of both CTV and '*Ca*. L. asiaticus' were eluted from the gel by using GenEluteTM Gel extraction kit, SIGMA-ALDRICH Co. St. Louies, USA as per the manufacturer's instructions. The samples were sequenced at Sanger sequencing facility from Eurofins Genomics India Pvt. Ltd., Bangalore using BigDye V3.1 technology and the instrument, Genetic Analyzer (ABI3730XL, ABI, USA). The raw sequence files are processed and converted into FASTA file by using Sequencing Analysis Software 5.3.1, ABI. To avoid the use of low quality sequencing data we selected sequences those have QV20+ Valuee" 600.

Sequence and phylogenetic analysis

Sequence similarity searches with available GenBank sequences were conducted using the BLAST (Basic local alignment search tool) algorithm of National Centre for Biotechnology Information (NCBI) and visual FASTA result tool of European Molecular Biology laboratory, European Bioinformatics Institute (EMBL-EBI). Phylogenetic analysis was conducted in MEGA5 software by Maximum Likehood (ML) method using 100 bootstrap values. The phylogenetic tree was constructed based on CP gene of CTV and 16S rRNA gene sequences of the studied isolates and compared with previously reported isolates from India and other countries.

RESULTS

Bio-diagnosis

Three to four months after graft inoculations, characteristic symptoms of CTV like vein clearing, and leaf cupping on acid lime indicator plants (Fig. 1C) was developed with samples like AK1, AK16, Sit7, D1 and MG4. Samples like AK2, K3 and N24 developed leaf mottling symptoms on Mosambi indicator seedlings specific to '*Ca.* L. asiaticus'. There was neither clear CTV nor any '*Ca.* L. asiaticus' specific symptoms in both acid lime and Mosambi seedlings respectively that were graft inoculated with samples like AK9, AK11 and B1. However these samples shown mineral deficiency and chlorotic patches followed by mild vein clearing symptoms on acid lime seedlings about 7-8 weeks after graft inoculations.

Molecular detection of CTV and '*Ca.* L. asiaticus'

CN150/CN151 primers that specifically amplify the major CP gene of CTV (p25) produced expected ~672 bp DNA band in infected samples. Out of thirty samples tested, twenty one samples *viz.* AK1, AK9, AK10, AK11, AK13, AK15, AK16, Sit5, Sit6, Sit7, K5, K11, B1, C1, D1, E1, MA, MD, MG, MG4 and MH samples were found positive since RT-PCR produced an amplicon size ~672 bp (Fig. 2). Again, OI1/ OI2C primes produced amplicon of ~1160 bp in seventeen samples *viz.* AK2, AK3, AK4, AK9, AK10, AK11, AK13, AK15, K3, K5, B1, MD, MG4, MH, N24, N49 and N118 indicating positive infection of HLB in these samples (Fig. 3). In duplex PCR assay, nine samples

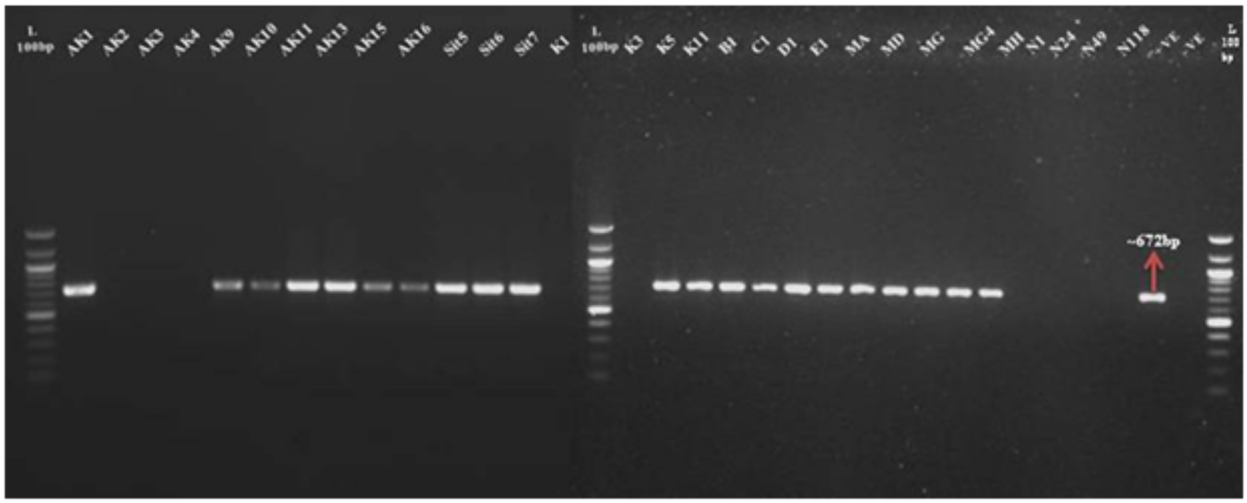

Fig. 2. Gel photograph showing the polymerase chain reaction bands, which were amplified using the *citrus tristeza virus* coat protein gene specific primers set CN150/CN151. AK1 to N118 isolates, + ve= Positive control –ve = Negative control. In lane L, 100 bp size marker was used

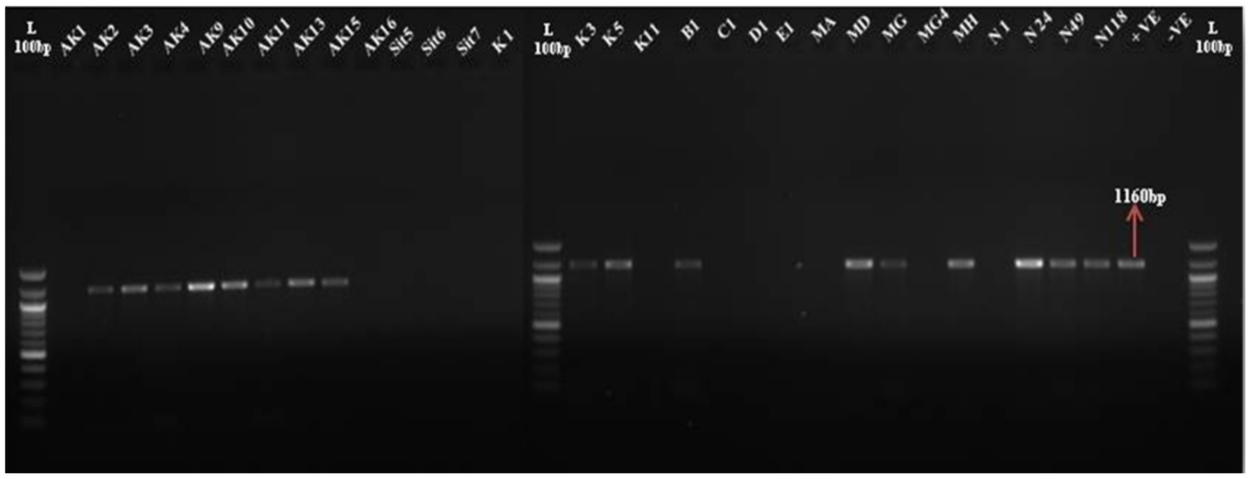

Fig. 3. Gel photograph showing the polymerase chain reaction bands, which were amplified using the '*Candidatus.* Liberibacter asiaticus' specific primers set OI1/OI2c. AK1 to N118 isolates, +ve = Positive control, -ve = Negative control. In lane L, 100bp size marker was used

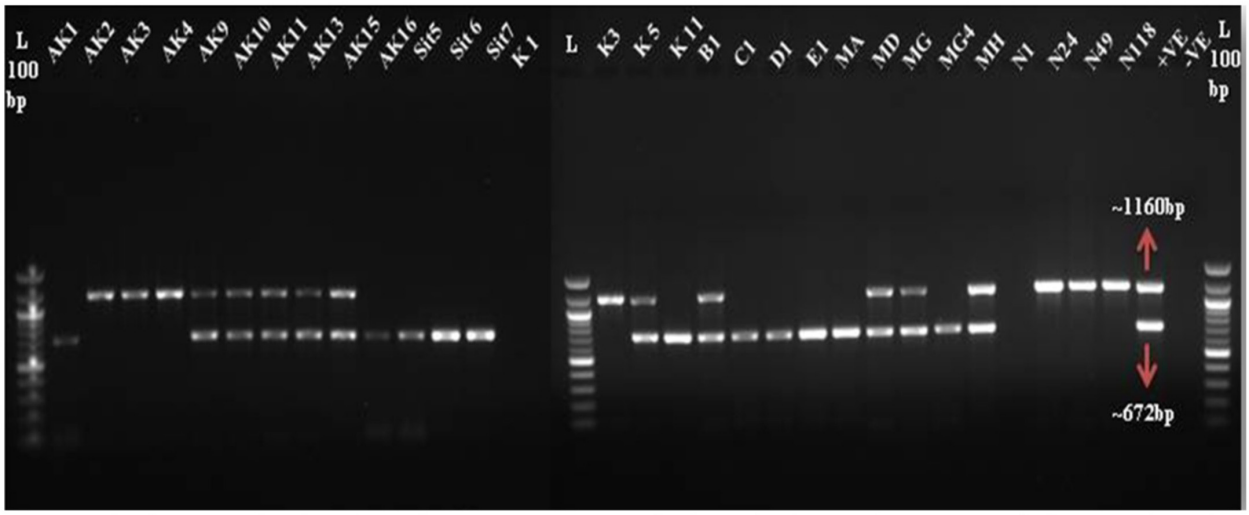

Fig. 4. Gel photograph showing the reverse transcription duplex Polymerase chain reaction (RT-d-PCR) using '*Ca.* L asiaticus' specific primers set OI1/OI2c and CTV CP gene specific primers set CN150/CN151. AK1 to N118 isolates, +Ve = Positive control, –Ve = Negative control. In lane L, 100bp size marker was used

viz. AK9, AK10, AK11, AK13, AK15, K5, B1, MD and MH were found positive with both CTV and HLB (Fig. 4). However, AK1, AK16, Sit5, Sit6, Sit7, K11, C1, D1 and E1 samples developed only CTV specific ~672 bp DNA and AK2, AK3, AK4, K3, N24, N49 and N118 samples developed only '*Ca.* L. asiaticus' specific ~1160 bp DNA band in electrophoresis (Fig. 4). The same observation was also confirmed separately by simplex PCR.

Restriction digestion

Restriction digestion of ~1160 bp PCR product (obtained by using OI1/OI2C primer) using *Xba* I restriction enzymes developed two DNA fragment *i.e.* 640 bp and 520 bp (Fig. 5).

Sequence and phylogenetic analysis

PCR amplified product of CP gene of seventeen selected CTV isolates and 16S rRNA gene of thirteen selected '*Ca.* L. asiaticus' isolates were sequenced from both orientations and the obtained sequences have been deposited in the GenBank database. These include CTV sequences under accession nos. KC816567, KJ524841, KJ524842, KJ524843, KJ524844, KJ524846, KJ524847, KY436590, KY436591, KY436592, KY436593, KY436594, KY436595, KY436596, KY436597, KY427696 and KY011909. Similarly, '*Ca.* L. asiaticus' specific deposited GenBank accession nos. Include KY230615, KY230616, KY230617, KY230618, KY230619, KY230620, KY230621, KY230622, KY230623, KY230624, KY211974 and KY077142, KY484088 (Table 1).

Homology search performed using BLAST protocol of NCBI database shows 97% to 100% sequence similarity with the previously reported CTV and '*Ca.* L. asiaticus' isolates. Three CTV isolates *viz.*, MD, MG and MH (KY011909, KY427696 and KY436590 respectively) collected from declined Mosambi plant from Vidarbha

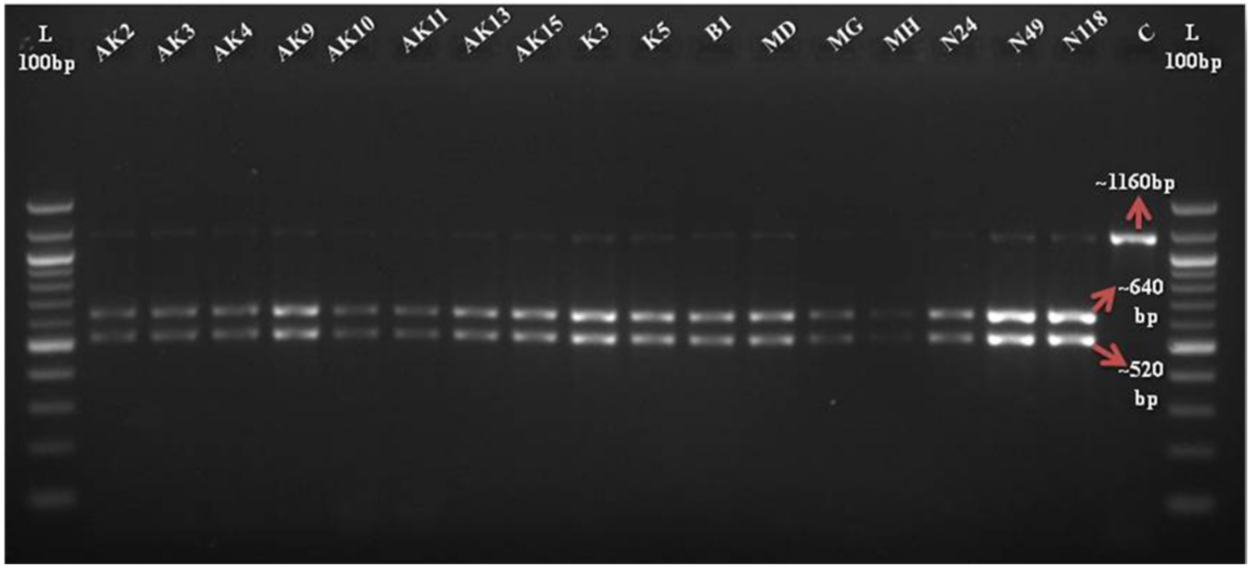

Fig. 5. Gel photograph showing the restriction digestion of PCR products of '*Candidatus* Liberibacter asiaticus' specific OI1/OI2C primer using XbaI restriction enzymes. AK1 to N118 are isolates, +ve= Positive control –ve = Negative control. In lane L, 100bp size marker was used

region of Maharashtra under present study showed 99% sequence identity with earlier reported CTV isolate ARP1 (LN997804) from Arunachal Pradesh. While CTV isolate B1 from Marathwada region of Maharashtra (KY436591) shows 100% sequence identity with CTV D13 (FJ001829) isolate from Delhi. CTV isolates collected from declined Darjeeling mandarin (WB) trees *viz*., Sit5 (KY436593), Sit6 (KY436594) and Sit7 (KY436595) shows 99% sequence identity with earlier reported Darjeeling isolate K18 (GQ475549) and Bangalore isolate B5 (HQ912023).CTV isolate K5 from Kalimpong (WB) shows 98% sequence similarity with Thailand isolate A18 (JQ798289), while CTV isolate K11 (Darjeeling mandarin, Kalimpong) shows 99% sequence similarity with earlier reported CTV isolate from Darjeeling K18 (GQ475549). Assam CTV isolates (AK1, AK9, AK11 and AK15) shows 99% sequence identity with Rolandia isolate (KC202897) of Brazil while other Assam CTV isolates (AK10, AK13, AK14 and AK16) shows 99% sequence similarity with the CA-RB-AT35 (KU358530) isolate of USA.

HLB isolate 'N24' collected from the Vidarbha region of Maharashtra showed 99% sequence identity with '*Ca.* L. asiaticus' Hyplotype H34Y isolate (JQ867427), collected from the Mexico. While HLB isolates collected from Maharashtra (N49, N118, 128D, MG, B1), Assam HLB (AK4, AK9 and AK10) and West Bengal (K3 and K5) shows 97 to 100% sequence identity with '*Ca.* L. asiaticus' A4 strain (CP010804) reported from China. Assam isolate (AK13) shows 99% sequence identity with '*Ca.* L. asiaticus' Hyplotype H35Y isolate.

The phylogenetic studies were performed using Maximum Likelihood (ML) analysis of tree building approach of the selected full length coat protein gene sequences of seventeen CTV isolates in the present study (Table 1) and other reported CTV isolates from different parts of the world retrieved from GenBank database. All CTV isolates were grouped into six clades (Fig. 6). CTV isolates of Vidharbha region of Maharashtra (MD, MG, MH) along with earlier reported USA isolate T3 (KC525952) and Kalimpong isolate K10 (KC694145) were grouped in Clade I. CTV isolates from WB (Sit5, Sit6, Sit7 and K11) along with previously reported isolate K18 (GQ475549) from Darjeeling Hills (WB) were grouped in Clade II. Assam CTV isolates (AK14, AK16, AK1, AK10, AK13, AK9, AK11 and AK15) and CTV (B1) isolate of Marathwada region (Maharashtra) were grouped along with HA16-5 (GQ454870) strain from USA in Clade VI. While CTV D1 isolate of Aurangabad, Marathwada (KY436592) was grouped in Clade V along with T30 (AF260651), NZRB-G90 (FJ525432), and T36 (U16304) strains collected from different parts of the world. Previously identified CTV isolate GFA-MH (KT981894) from Nagpur, Vidarbha region of Maharashtra and Linsted3 (HM160501) isolate from Jamaica were also grouped into Clade V. West Bengal CTV isolate K5 was not grouped with previously identified strain hence it is considered as Clade III.

Similarly, ML tree building approach was performed for phylogenetic studies of the 16S rRNA gene sequences of selected HLB isolates under present study and other "*Ca*. Liberibacter" sequences retrieved from the GenBank database. The ML analysis suggests that all 12 HLB isolates associated with citrus decline in Maharashtra, Assam and WB under study are grouped into '*Ca*. L. asiaticus' (Fig. 7). HLB isolates used in the present study (N49, N118, MD, MG, B1, AK4, AK9, AK10, AK13, K3 and K5) are closely related to the previously characterized Poona strain of '*Ca* L asiaticus' from the India (L22532) and with the other '*Ca*. L. asiaticus' strains reported from USA, Japan, Iran and Indonesia.

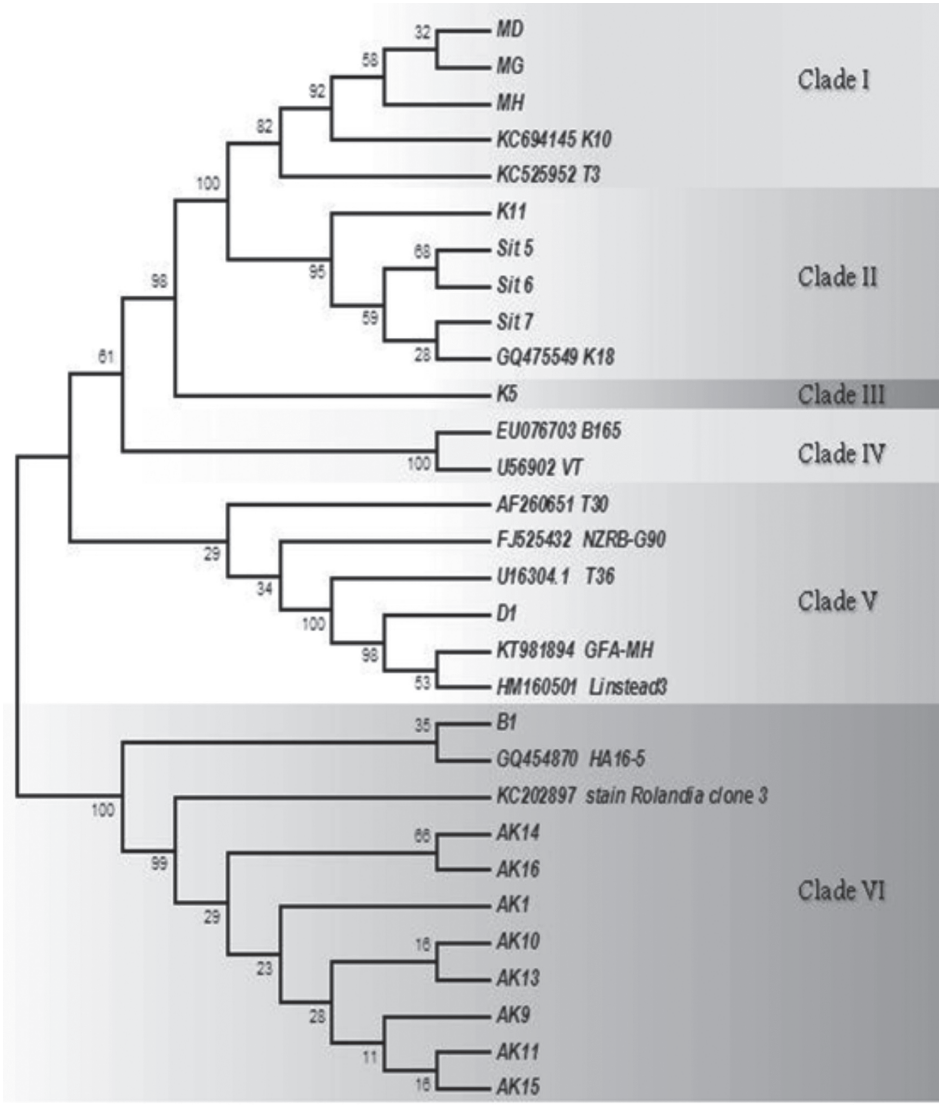

Fig. 6. Phylogenetic tree constructed with coat protein gene sequences of Maharashtra, Assam and West Bengal isolate of India and other reported *Citrus tristeza virus* from the world. The value under nodes refers to Bootstrap values

DISCUSSION

The areas under study of present investigation are the major citrus growing belts in India. We observed that declined citrus plants in general develop small upright leaves that are frequently associated with Zn-deficiency-like symptoms, green veins, corking of veins, and chlorotic interveinal areas. Declined citrus trees were sparsely foliated due to extensive die-back of twigs. This decline syndrome is very destructive and lethal and prevalent in all citrus cultivars and regions. Although there are few reports of association of CTV with citrus decline in these areas, exact role of citrus greening disease either alone or in association with CTV in citrus decline has long been questionable and contentious. Therefore, present investigation was carried out to know the exact molecular identity and extent of involvement of both CTV and '*Ca.* L. asiaticus' (greening pathogen) with citrus decline in these areas.

Results of bio-diagnosis and PCR based diagnosis indicate wide spread presence of CTV and '*Ca.* L. asiaticus' either individually or as mixed infection in thirty out of thirty two samples tested (Table 1). Interestingly, if a field sample is infected with CTV and '*Ca.* L. asiaticus' both, initial symptoms of mineral deficiency and chlorotic patches followed by mild vein clearing symptoms on acid lime seedlings were developed about 7-8 weeks after graft inoculations. Such type of symptom appearance due to mixed infection of both CTV and '*Ca.* L. asiaticus' on acid lime indicator host was earlier reported by Narayani and Raychaudhari (1971).

Diagnosis of these pathogens in conventional biological assay, light or electron microscopy, and ELISA is sometimes difficult as it fails to consistently detect the pathogens due to their lower concentration and uneven distribution in a woody plant host like citrus. In addition, non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other diseases. In recent past, several PCR-based molecular approaches have been developed to detect CTV and '*Ca.* L. asiaticus' using specific primers targeting conserved

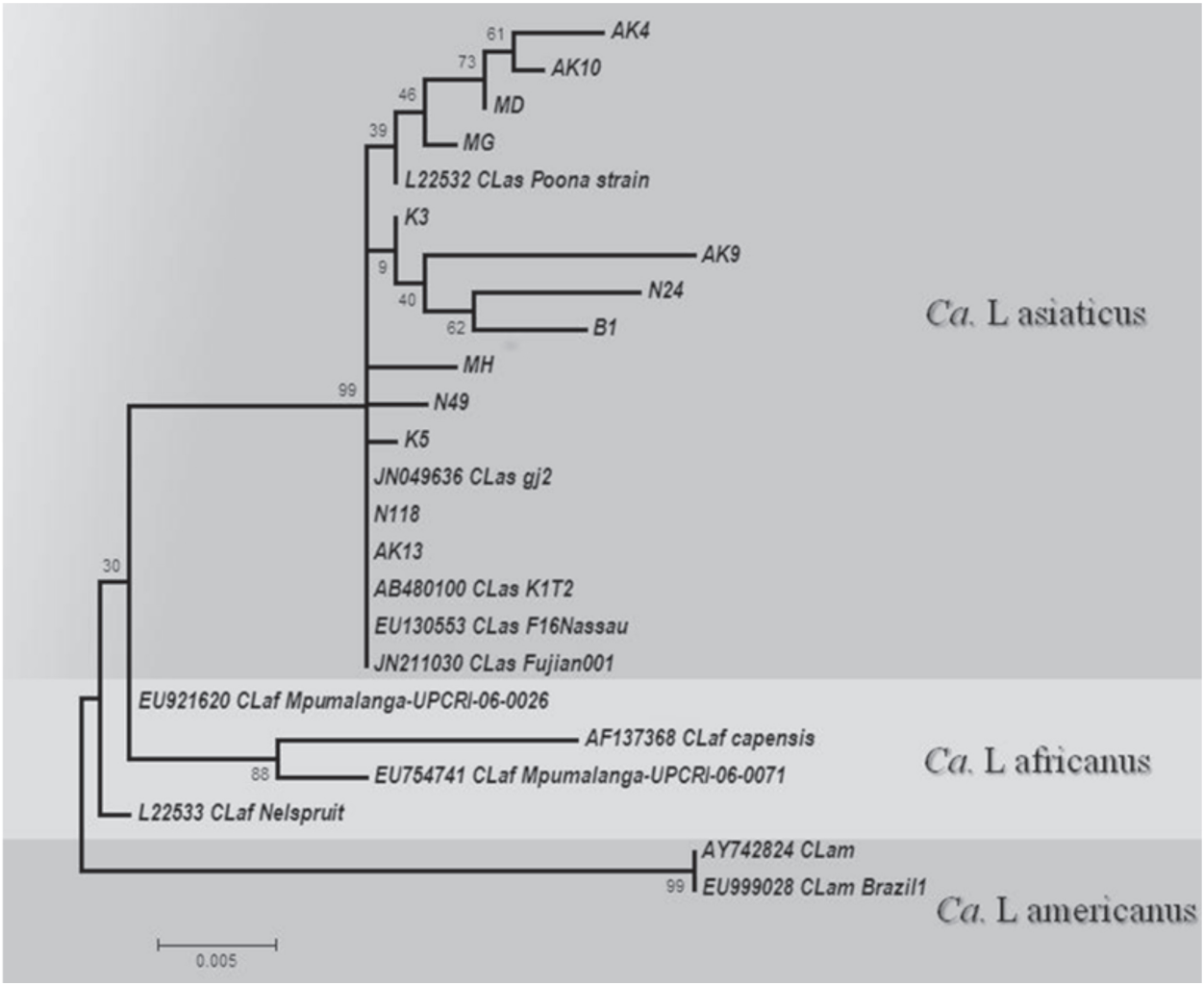

Fig. 7. Phylogenetic tree constructed with 16S rDNA sequences of Maharashtra, Assam and West Bengal isolate of India and other reported *Ca.* Liberibacter species. The value under nodes refers to Bootstrap values

regions of the pathogens, *viz*. CP gene of CTV and 16S rDNA and other regions of bacterial genome. In the present studies, we have developed PCR based detection of both pathogens targeting CP gene of CTV and 16S rRNA gene of '*Ca.* L. asiaticus' in infected citrus samples. Again, a duplex PCR assay has been standardized to detect co-infection of CTV and HLB. Duplex PCR, developed and validated in the present study for simultaneous detection of both pathogens in field infected samples will reduce the time and cost required to detect mixed infection of field samples.

Analysis of the molecular diversity of CTV and '*Ca.* L. asiaticus', two pathogens associated with citrus decline was done to understand population structure of these pathogens. Seventeen CTV isolates analyzed in the present study were from three different states and classified into five different clades, suggesting that there are at least five different CTV strains present in India. Again Phylogenetic tree shows that all twelve HLB isolates belong to the '*Ca*. L. asiaticus' group. Moreover our HLB isolates are closely related to previously characterized Poona strain (L22532) of '*Ca* L asiaticus' from the India along with other '*Ca*. L. asiaticus' strains reported from USA, Japan, Iran and Indonesia. Further amplified ~1160 bp DNA was digested by *Xba* I into two fragments of about 640 bp and 520 bp as reported only for '*Ca.* L. asiaticus' since it has only one restriction site for *Xba* I unlike that of '*Ca.* L. africanus' which has two *Xba* I restriction sites (Jagoueix *et al*., 1996). This further confirmed the identity of all HLB isolates belongs to '*Ca*. L. asiaticus'.

Ghosh *et al.* (2015) suggested that the Asian greening bacterium might include a number of several strains and the disease syndrome differs depending on the variety of citrus. It is considered that 16S rDNA fragment is best characterized regions of '*Candidatus* Liberibacter spp.' for better understanding and knowledge about biology, diagnosis and epidemiology of noncultivable bacteria, however its resolution is not always sufficient to differentiate between closely related species (Ding *et al*., 2009). According to previous reports, 16S/23S intergenic region failed to distinguish among the different strains of '*Ca*. L. asiaticus' and therefore may not be an excellent target for differentiation among different HLB isolates (Ghosh *et al*., 2013). Therefore for further strainal variation study, other regions like 'variable

tandom nucleotide repeats' (VNTRs) needs to be exploited (Chen *et al*., 2010). This could further help to understand biological traits associated with geographical distribution of '*Ca*. L. asiaticus'.

The present findings report the molecular identification and characterization of CTV and '*Ca*. L. asiaticus' associated with citrus decline in Maharashtra, Assam and WB. It once again confirmed the claim made earlier by different workers that tristeza and greening are the principal cause of citrus decline in India. Citrus is a vegetatively propagated crop and diseases like tristeza and greening spreads mainly through uncertified/ unchecked budwood. We observed that farmers in these areas use uncertified nursery plants developed locally by private nursery owners. Mother plants from which buds were taken for large scale propagation are often infected with tristeza and greening which results only in devolopment of diseased plants with poor yield potential and which eventually leads to development of declined citrus orchards. Therefore until and unless growers have access to tristeza and greening-free planting material, it would be very difficult to establish a profitable citrus industy in these parts of India.

REFERENCES

Ahlawat YS (2012). Virus diseases of citrus and management. Stadium Press (India) Pvt. Ltd., New Delhi. pp. 25-40.

Albiach-Marti MR (2012). Molecular virology and Pathogenicity of *Citrus tristeza virus*, Viral Genomes-Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions, Prof. Maria Garcia (Ed.), ISBN: 978-953-51-0098-0, *In Tech.*

Bar-Joseph M and Dawson WO (2008). *Citrus tristeza virus*. *Encyclo of Virol*. 1: 520-525.

Bhose S, Misra P, Ramteke PW and Ghosh D (2015a). Sequence analysis of ribosomal protein gene of '*Candidatus* Liberibacter asiaticus' infecting major citrus cultivars in western Maharashtra of India. *Indian Phytopath*. 68: 334-341.

Bhose S, Misra P, Shukla PK and Ghosh D. (2015b). Comparative analysis of 16S/23S intergenic region of *Candidatus* Liberibacter asiaticus associated with citrus greening disease in different citrus cultivars of Assam. *Indian Phytopath*. 68: 432-437.

Chen J, Deng X, Sun X, Jones D, Irey M and Civerolo E (2010). Guangdong and Florida populations of '*Candidatus* Liberibacter asiaticus' distinguished by a genomic locus with short tandem repeats. *Phytopathology* 100: 567-572.

Ding F, Deng X, Hong N, Zhong Y, Wang G and Yi G (2009). Phylogenetic analysis of the citrus Huanglongbing (HLB) bacterium based on the sequences of 16S rDNA and 16S/23S rDNA intergenic regions among isolates in China. *European J. Plant Pathol*. 124: 495-503.

FAOSTAT (2014). http://faostat.fao.org/site/567/desktopdefault.aspx#ancor

Flores R, Ruiz-Ruiz S, Nuria Soler, Navarro JS, Fagoaga C, Lopez C, Navarro L, Moreno P and Pena L (2013). *Citrus tristeza virus* p23: a unique protein mediating key virus-host interactions. *Fron. Microbiol*. 3: 98.

Garnier M, Jagoueix-Eveillard S, Cronje PR, Roux HF and Bove JM (2000).Genomic characterization of a Liberibacter present in an ornamental rutaceous tree, Calodendrum capense, in the Western Cape Province of South Africa. Proposal of '*Candidatus* Liberibacter africanus subsp. capensis'. *Int. J. Evol. Microbiol*. 50: 2119- 2125.

Ghosh A, Das A, Pun KB, Kumar R, Meena R and Baranwal K (2014). Present status of *Citrus tristeza virus* infecting citrus spp. in Darjeeling hills and its detection in different plant parts. *Phytoparasitica* 42: 381-386.

Ghosh DK, Aglave B, Roy A and Ahlawat YS (2009). Molecular cloning, sequencing and phylogenetic analysis of coat protein gene of a biologically distinct *Citrus tristeza virus* isolate occurring in central India. *J. Plant Biochem. Biotech*. 18: 105-108.

Ghosh D, Bhose S, Mukherjee K and Baranwal VK (2013). Sequence and evolutionary analysis of ribosomal DNA from Huanglongbing (HLB) isolates of Western India. *Phytoparasitica* 41:295-305.

Ghosh DK, Bhose S, Motghare M, Warghane A, Mukherjee K, Ghosh DK Sr, Sharma AK, Ladaniya MS and Gowda S (2015). Genetic diversity of the Indian populations of '*Candidatus* Liberibacter asiaticus' based on the tandem repeat variability in a genomic locus. *Phytopathology* 105: 1043-1049.

Ghosh DK, Bhose S, Warghane A, Motghare M, Sharma AK, Dhar AK and Gowda S (2016). Loop-mediated isothermal amplification (LAMP) based method for rapid and sensitive detection of '*Candidatus* Liberibacter asiaticus' in citrus and the psyllid vector, *Diaphorina citri* Kuwayama. *J. Plant Biochem. Biotech* 25: 219-223.

Jagoueix S, Bove JM and Grainer M (1996). PCR detection of the two '*Candidatus*' liberobacter species associated with greening disease of citrus. *Mol. Cell. Probes* 10: 43-50.

Narianiri TK and Raichaudhuri SP (1971). Kagzi lime common indicator plants for tristeza and greening viruses. *Calif. Citrograph* 56: 85.

Rigano LA, Malamud F, Orce IG, Filippone MP, Marano MR and Amaral AM (2014). Rapid and sensitive detection of *Candidatus* Liberibacter asiaticus by loop mediated isothermal amplification combined with a lateral flow dipstick. *BMC Microbiol*. 14: 86.

Wang N and Trivedi P (2013). Citrus Huanglongbing: A newly relevant disease presents unprecedented challenges. *Phytopathology* 103: 652-665.