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RESEARCH ARTICLE

Use of NCM based DNA extraction method for simultaneous detection of *citrus mosaic badnavirus* and *Candidatus* Liberibacter asiaticus by duplex PCR

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ABSTRACT: *Candidatus* Liberibactor asiaticus (*Ca.* Las), a phloem limited, gram negative bacterium is the causal organism of citrus greening disease (Huanglongbing/HLB) whereas citrus yellow mosaic disease is caused by *Citrus mosaic badnavirus* (CMBV). Both the diseases, singly or as mixed infection in the grove are considered as serious threat to Indian citrus industry. Early diagnosis for these systemic pathogens are essential in order to prevent the spread of these diseases and finally to devise their integrated management strategies. In the present study, a method of simultaneous detection of CMBV and '*Ca.* Las' was developed using a simple nitrocellulose membrane (NCM) based DNA isolation protocol in three commercially grown citrus cultivars in India, *viz.* mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), and acid lime (*Citrus aurantifolia*) infected with these pathogens. Citrus tissues were crushed in extraction buffer containing NaOH and EDTA. The crude extract was spotted on NCM and incubation elution was done in nuclease-free water after 30 minute of NCM spotting. Eluted products were taken for simultaneous detection of CMBV and '*Ca.* Las' using duplex PCR with newly designed primers. The developed protocol was found to be equally sensitive as multistep DNA isolation method and commercially available kit. The protocol is simple, rapid and inexpensive thus can be used for large-scale pathogen indexing of citrus plant samples in orchards and nurseries.

Keywords: Duplex PCR, nitrocellulose membrane, *Candidatus* Liberibactor asiaticus, *citrus mosaic badnavirus*

Citrus (*Citrus* spp.) is the third most important fruit crop after banana and mango in India which is grown in about 9.35 lakh hectors with an annual production of 115.1 lakh metric tons (NHB, 2015-16). The productivity of the crop is often reduced due to the infection of many virus and virus-like pathogens (Ahlawat, 2012; Baranwal *et al.,* 2003). In India there are six major diseases in citrus crop caused by these systemic pathogens. Citrus greening (Huanglongbing/HLB) and citrus yellow mosaic disease are two most important diseases which cause serious yield losses in all three commercial citrus cultivars viz. mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), and acid lime (*Citrus aurantifolia*) in India (Bhose *et al.,* 2015a).

The etiological agent of HLB that causes huge crop loss worldwide is a phloem-restricted, gram negative bacterium, '*Candidatus* Liberibacter' which belongs to á-subdivision of proteobacteria (Bhose *et al.,* 2015b). Presently three species of the bacterium are known; '*Ca.* L. asiaticus' (*Ca.* Las), '*Ca.* L. africanus' (*Ca.* Laf), and '*Ca.* L. americanus' (*Ca.* Lam), and among these '*Ca.* L. asiaticus' (*Ca.* Las) is the species reported from Indian sub-continent (Bove, 2006). '*Ca.* Las' are transmitted efficiently by a psyllid vector (*Diaphorina citri*) and through infected propagative material (Ahlawat, 2012). The typical symptoms of the disease include mottling of leaves due to which an infected plant gets a typical 'yellow shoot' appearance (Bove, 2006). HLB affected plants often produce small, lopsided, bitter tasting fruits with aborted seeds; and die within 3-8 years after infection (Rao *et al.,* 2013). All commercial citrus species and cultivar are susceptible to HLB and to date there is no known resistance cultivar.

Similarly, citrus mosaic disease, caused by *citrus yellow mosaic badnavirus* (CMBV) has been reported so far only in India and is responsible for significant yield losses (Ahlawat *et al.,* 1996a). It is predominantly present in southern states of Karnataka, Andhra Pradesh and Telengana but recent reports indicate its spread in central India as well primarily through the movement of virus infected uncertified planting material (Ghosh *et al.,* 2007). CMBV belongs to *Badnavirus* genus of family *Caulimoviridae* (Huang and Hartung, 2001). Typical symptoms of the disease are bright yellow mottling of leaves and yellow flecking along the veins but it may vary depending on the host (Ahlawat *et al.,* 1996b, Baranwal *et al.,* 2005). More variable symptoms are observed if field grown plant is co-infected with other pathogens like

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citrus tristeza virus, exocortis viroid, *Phytophthora* spp. etc (Ahlawat *et al.,* 1996a; Gopi *et al.,* 2010).

Citrus is a vegetatively propagated crop and both HLB and citrus yellow mosaic disease are graft transmissible and movement of these pathogen infected grafting material is considered as the main reason for their rapid field spread. Therefore, early diagnosis for the two systemic pathogens is essential in order to prevent further spread of these diseases. *Badnaviruses* are immunogenically weak and therefore PCR based techniques are best suitable (Bhat *et al.,* 2016; Huang and Hartung, 2001). Similarly for sensitive detection of '*Ca.* Las' different PCR based molecular techniques have been developed (Bove, 2006, Ahlawat, 2012; Ghosh *et al*., 2015 a,b). However, most of the PCR assays developed for CMBV and '*Ca.* Las', involves either multistep DNA isolation protocol or expensive commercial kit (La Notte *et al*., 1997; Baranwal *et al*., 2007). It is also not practical to rapidly index large scale field or nursery samples for pathogen testing. Therefore, a simple, sensitive and broad-spectrum assay is required for the detection of both these pathogens from large number of field samples. In the present study, we have developed nitrocellulose membrane (NCM) based nucleic acid extraction protocol for simultaneous detection of CMBV and '*Ca.* Las' using all three commercially available cultivars infected by both these pathogens.

MATERIALS AND METHODS

Sample collection

Individual pure cultures of CMBV and '*Ca.* Las' maintained separately in Mosambi sweet orange cultivar in the virology screen house of ICAR-CCRI, Nagpur was used for the experiment. Further mixed cultures of both CMBV and '*Ca.* Las' were developed by artificial graft inoculation in Mosambi sweet orange, mandarin and acid lime seedling which were used for duplex PCR experiments. The grafted plants were kept in an insect-proof greenhouse with temperatures ranging from 35 ± 02 to 28 ± 02°C (day and night, respectively). Collected samples were washed and cleaned with 70 per cent ethanol to avoid any surface contamination.

DNA extraction

DNA extraction was done using three different protocols *viz*. multistep extraction method (Cetyl trimethylammonium bromide/CTAB), commercially available kit and NCM based nucleic acid extraction taking equal amount of infected sample (100 mg) in each case. In the first set of experiment, CTAB multistep DNA extraction method was used for which midribs and petioles from infected plants were aseptically excised and grind with liquid nitrogen, and 100 milligram (mg) of the resulting powder from each sample was homogenized with 500 µl of preheated extraction buffer followed by protocol described by Cheng *et al.* (2003).

In the second set, 100 mg of grinded sample was used for DNA isolation with DNeasy mini kit (Qiagen, Germany) as per manufacturer's protocol. In the third set, 100 mg midrib and petiole tissue was directly crushed in presence of extraction solution (50 mM NaOH and 2.5 mM EDTA) using sterile mortar and pestle. No liquid nitrogen was used to grind the tissue. The extract then either incubated at room temperature for 30 minutes or centrifuged at 10,000 rpm for 10 min. Supernatant from both the treatments was spotted on NCM (pore size 0.45 µl). The membrane then incubated for 45 min at room temperature and individual spot from each sample were cut out using a small punching machine. For elution, the cut membrane was incubated at 80°C for 10 min in presence of 30 µl of sterile distilled water. The collected liquid was centrifuged at 2 min at 8000 rpm and further used for PCR detection of CMBV and '*Ca.* Las'.

Duplex PCR detection for CMBV and '*Ca.* Las'

For the PCR detection purpose, primer sets were designed for CMBV and '*Ca.* Las' based on putative coat protein and 16Sr RNA region of these pathogens respectively (Table 1). Duplex PCR reaction consists of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.3 mM of dNTP each, 0.4 mM of each primer**,** 1.25 U of GoTaq flexi DNA polymerase (Promega), nuclease free water along with template DNA which can be either of three; 5 µl of NCM-eluted DNA, 1 µl of DNA isolated by CTAB, or 1 µl DNA from commercial kit. The PCR was performed in a thermal cycler (Bio-Rad, USA) with conditions that include initial denaturation at 95°C for 4 min followed by 35 cycles of 45 sec at 95°C, 45 sec at 58°C, 1 min at 72°C, and final extension at 72°C for 10 min. The amplified DNA was visualized in UV GelDoc system (Bio-Rad, USA) using ethidium bromide (EtBr). Conditions of duplex PCR assay were optimized after using few combinations with different concentrations of dNTP (0.2 mM, 0.25 mM, 0.3 mM, 0.35 mM), $MgCl_2$ (1.5 mM, 2 mM, 2.5 mM, 3 mM) and NCM-eluted DNA (2 µl, 5 µl).

Shelf-life evaluation of spotted NCM

The spotted NCM were stored in a plastic storage box and kept at room temperature for 10, 20, 25, 30 and 40 days. Nucleic acid was eluted after respective days and used as DNA template for duplex PCR detection. PCR amplified products were separated on agarose gel and visualized under UV light. Presence or absence of specific bands for CMBV and '*Ca.* Las' were recorded on gel.

Table 1. Primer details along with its annealing temperature and reference used in this study for polymerase chain reaction

Primer set	Sequence	Annealing temp.	Amplicon size (bp)
CMBV-Df1CMBV-Dr1	GATGGTTGGGGAGGTGTCTGAGCTCGACCACTTCACACAG	58°C	811
HLB-Df3HLB-Dr3	AGCTGGTCTGAGAGGACGATCTCGCCCCCTTCGTATTACC	58°C	236

Nucleotide sequencing and analysis

Amplified PCR products were excised and eluted from the gel using the Quick Gel Extraction Kit (Invitrogen) and sequenced at automated DNA sequencing facility of Eurofins Genomics India Pvt Ltd, Bangalore. Sequence results were primarily analyzed by using Bioedit software version 7.2 and subsequently sequence similarity search of sequences was conducted using BLAST algorithm of NCBI.

RESULTS AND DISCUSSION

Optimization of duplex PCR

Optimum volume of dNTP and $MgCl_2$ was determined using various concentrations. Among different concentrations of dNTP (0.2 to 0.35 mM in increasing order), 0.3 mM found best as the concentration attuned both the primer sets amplification (Fig. 1). Similarly, in case of $MgCl_2$, four concentrations ranging from 1.5 to 3 mM were used. During initial concentration trials, CMBV amplification was fine but '*Ca.* Las' amplified with faint band, whereas at 0.25 mM of $MgCl_2$ both the pathogens showed prominent bands (Fig. 2). In order to determine optimum spot per volume, 2 and 5 µl volume of NCM eluted extract were used and both the volumes showed good intensity bands (Fig. 3). This indicates that DNA of both the pathogens adheres and retain on the membrane. Suitability of the extraction method was further verified using three commercially growing cultivars of citrus in India, *viz.* sweet orange, mandarin and acid lime. CMBV

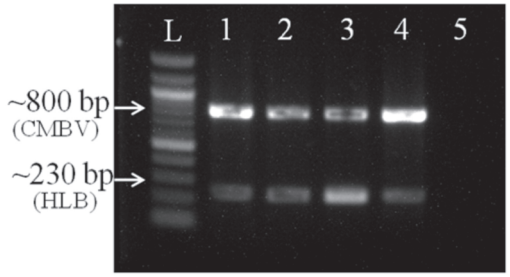

Fig. 1. Effect of dNTP on CMBV and '*Ca.* Las' amplification. Lane 1 is PCR amplification with 0.2 mM dNTP, lane 2 with 0.25 mM dNTP, lane 3 with 0.3 mM dNTP, lane 4 with 0.35 mM dNTP, lane 5 is non-template control and lane L is 100 bp DNA ladder

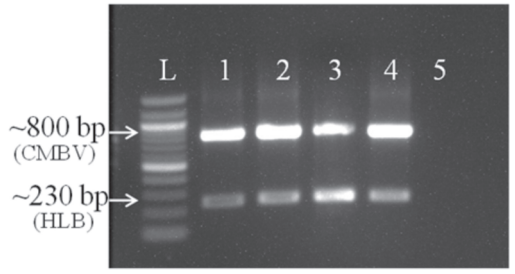

Fig. 2. Effect of $MgCl_2$ on CMBV and '*Ca.* Las' amplification. Lane 1 is PCR amplification with 1.5 mM $MgCl_2$, lane 2 with 2 mM $MgCl_2$, lane 3 with 2.5 mM $MgCl_2$, lane 4 with 3 mM $MgCl_2$, lane 5 is negative control and lane L is 100 bp DNA ladder

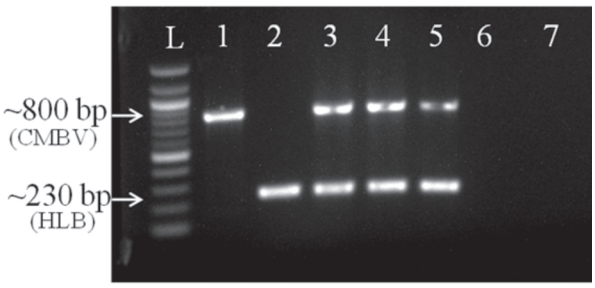

Fig. 3. Detection of CMBV and '*Ca.* Las' by simplex and duplex PCR with DNA template isolated by using NCM. Lane 1 is amplification with CMBV primers, lane 2 is amplification with '*Ca.* Las', lanes 3-4 are amplification with both CMBV and '*Ca.* Las' primers with 5 µl NCM elution, lane 5 is with 2µl NCM elution, lane 6 is healthy control and lane 7 is non-template control. Lane L is 100 bp DNA ladder

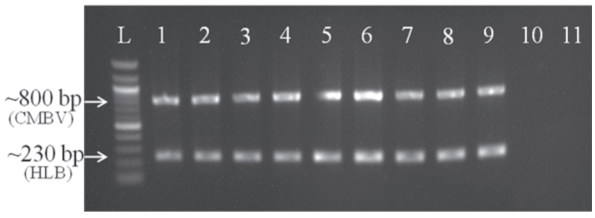

Fig. 4. Detection of CMBV and '*Ca.* Las' from three commercially grown citrus cultivars by duplex PCR using agarose gel electrophoresis. Lanes 1-3 are of sweet orange samples, lanes 4-6 are of mandarin samples, lanes 7-9 are of acid lime samples, lane 10 is healthy control and lane 11 is non-template control. Lane L is 100 bp DNA ladder

and '*Ca.* Las' specific amplifications were observed in all the three representative samples. Healthy and non-template control showed no amplification (Fig. 4).

DNA extraction protocols comparison

In the present study three DNA extraction techniques were used namely, CTAB multistep protocol, commercial kit and membrane based nucleic acid extraction. DNA isolated using all the methods were used in duplex PCR detection. Most intense bands were observed in CTAB multistep method followed by commercial kit and NCM based nucleic acid extraction method (Fig. 5). Amplification with DNA template prepared with membrane based method was found comparable with other tested techniques.

Shelf-life evaluation of spotted NCM disc

Stability of spotted NCM disc was also examined by using stored disc (at room temperature) after 10, 20, 25, 30 and 40 days. It was found that up to 20 days intensity of amplified product was good but after that the band intensity was faded (Fig. 6). NCM based technique was found good for short term storage and can be used in transportation.

Nucleotide sequence analysis

Amplicon of 170 base pair from 16S ribosomal RNA region of '*Ca.* Las' was sequenced and found to have 99

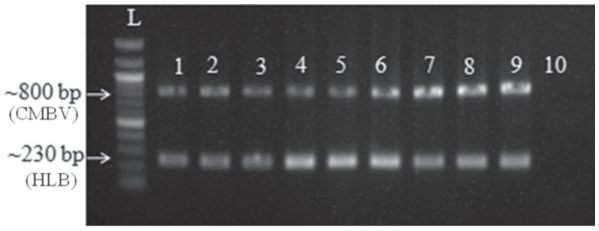

Fig. 5. Comparison of detection of CMBV and '*Ca.* Las' following three nucleic extraction techniques in duplex PCR using agarose gel electrophoresis. Lanes 1-3 using nitrocellulose membrane, lane 4-6 using commercial kit, lane 7-9 using multistep nucleic acid extraction method and lane 10 is non-template control. Lane L is 100 bp DNA ladder

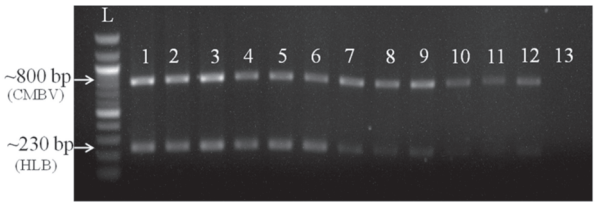

Fig. 6. Assessment of stability of nitrocellulose membrane (NCM) spots for duplex PCR using agarose gel electrophoresis. Lanes 1-2 on same day, lanes 3-4 after 10 days, lanes 5-6 after 20 days, lanes 7-8 after 25 days, lanes 9-10 after 30 days, lanes 11-12 after 40 days and lane 13 is non-template control. Lane L is100 bp DNA ladder

per cent sequence identity with other corresponding '*Ca.* Las' sequences in GenBank. The amplified product of CMBV polyprotein gene is of 697 base pairs and showed 99 per cent sequence identity with already NCBI deposited CMBV complete genomes. Nucleotide sequences of both the pathogens submitted in GenBank under accession number MF322770 and MF322771.

In the present study, we have designed the new set of primers targeting conserved regions of the pathogen genome and developed duplex PCR technique for detection of CMBV and '*Ca.* Las' pathogens responsible for citrus mosaic and greening disease respectively. Citrus greening (HLB) is present worldwide and devastating to all citrus cultivars and species. The disease is considered as major threat for citrus cultivation and industry as it causes severe damage by shortening the life span of trees. Fruits of infected plants are often not of good quality. On the other hand, citrus yellow mosaic is another serious disease mostly found in southern parts of India where Sathgudi sweet orange is grown as a commercial crop. As there is no successful treatment available for both these pathogens and therefore early diagnosis of the diseases is the best remedy.

Membrane based diagnosis is also reported for successful DNA isolation from infected plants (Baranwal *et al.,* 2007; Singh *et al.,* 2006). In some herbaceous plants crude extract of denaturized sap is also used for detection purpose but as citrus is a woody plant its crude extract may contains PCR inhibitors and therefore direct plant sap may not be used for detection purpose. There was a report of successful detection of citrus pathogens individually using membrane based technique (Baranwal *et al.,* 2007). In the present study however, we have developed a duplex PCR for simultaneous detection of CMBV and '*Ca.* Las' using NCM based template preparation method. The main advantage of the protocol is that it conjugates easy sampling with a sensitive diagnosis *i.e.*, PCR and sample preparation is so simple that it can be done on collection site itself if needed. Additionally, spotted membrane can be stored for few days before being used or can be transported to the places far away from collection site. As this method is easy, cheap and sensitive, it can be used in routine indexing program.

REFERENCES

Ahlawat YS (2012). Virus diseases of Citrus and Management. Stadium Press, Delhi.

Ahlawat YS, Pant RP, Lockhart BE, Srivastava M, Chakraborty NK and Varma A (1996a). Association of badnavirus with citrus mosaic disease in India. *Plant Dis.* 80: 590-592.

Ahlawat YS, Pant RP, Shukla A and Lockhart BE (1996b). Partial characterization of a badnavirus associated with Citrus yellow mosaic in India. In Proceedings of 13th Conference of the International Organization of Citrus Virologists, China; pp. 208-217.

Baranwal VK, Majumder S, Ahlawat YS and Singh RP (2003). Sodium sulphite yields improved DNA of higher stability for PCR detection of *Citrus yellow mosaic virus* from citrus leaves. *J. of Virol. Methods* 112: 153-156.

Baranwal VK, Singh J, Ahlawat YS, Gopal K and Charaya MU (2005). *Citrus yellow mosaic virus* is associated with mosaic disease in Rangpur lime rootstock of citrus. *Curr. Sci.* 89: 1596-1599.

Baranwal VK, Gupta KN and Singh RP (2007). Polymerase chain reaction detection of greening bacterium (*Candidatus* Liberibacter asiaticus) and *Citrus mosaic virus* in citrus tissues, by means of a simplified template-preparation protocol. *Can. J. Plant Pathol.* 29: 190-196.

Bhat AI, Hohn T and Selvarajan R (2016). Badnaviruses: The Current Global Scenario. *Viruses* 8: 177.

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

Bhose S, Misra P, Ramteke PW and Ghosh DK (2015b). Sequence analysis of ribosomal protein gene of 'Candidatus Liberibacter asiaticus' infecting major citrus cultivars in western Maharashtra of India. *Indian J. Phytopath.* 68: 334-437.

Bove J (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Plant Path.* 88: 7-37.

Cheng Y, Guo W, Yi H, Pang X and Deng X (2003). An Efficient Protocol for Genomic DNA Extraction From Citrus Species. *Plant Mol. Bio. Reporter* 21: 177a-177g.

Ghosh DK, Aglave B, Bhanare K and Baranwal VK (2007). PCR based detection of Citrus yellow mosaic disease from Vidarbha region of Maharastra. *Indian Phytopath.* 60: 520-526.

Ghosh DK, Bhose S, Mukherjee K, Aglave B, Warghane AJ, Motghare M, Baranwal VK and Dhar AK (2014). Molecular characterization of Citrus yellow mosaic badnavirus

(CMBV) isolates revealed the presence of two distinct strains infecting citrus in India. *Phytoparasitica* 42: 681-689.

Ghosh DK, Bhose S, Warghane AJ, Motghare M, Sharma AK, Dhar AK and Gowda S (2015a). 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.

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

Gopi V, Gouri Sanker T, Girish Kumar A and Gopal K (2010). Diagnosis of citrus yellow mosaic virus by PCR and ELISA in sweet orange (*Citrus sinensis* Osbeck). *J. Plant Dis. Sci.* 5: 158-162.

Huang Q and Hartung JS (2001). Cloning and sequence analysis of an infectious clone of Citrus yellow mosaic virus that can infect sweet orange via Agrobacterium mediated inoculation. *J. Gen. Virol.* 82: 2549-2558.

La Notte P, Minfra A and Saldarelli P (1997). A spot-PCR technique for the detection of phloem-limited grapevine viruses. *J. Virol. Methods* 66: 103-108.

National Horticulture Board. http://nhb.gov.in/area-pro/horst_galance_2016.

Rao M, Miyata S, Ghosh D, Irey M, Garnsey SM and Gowda S (2013). Development of rapid, sensitive and non-radioactive tissue-blot diagnostic method for the detection of citrus greening. *Mol. Cellular Probes* 27: 176-183.

Singh RP, Dilworth AD, Singh M and Babcock KM (2006). An alkaline solution simplifies nucleic acid preparation for RT-PCR and infectivity assay of viroids from crude sap and spotted membrane. *J. Virol. Methods* 132: 204-211.