sia and dhaincha plants also showed sustainable growth, but at a much reduced rate.

Stomatal response is undoubtedly a sensitive indicator of plant growth under stressful situations. The greater number of stomata per leaf with stomatal components of reduced size clearly indicate that cassia and dhaincha are potential candidates for rehabilitation of areas under typical coalmine tailings.

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Simultaneous detection of one RNA and one DNA virus from naturally infected citrus plants using duplex PCR technique

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Citrus tristeza clostero virus (CTV), an aphid-transmitted RNA virus having a genome size of about 19.3 kb, singly or as a mixed infection with Citrus mosaic badna virus (CMBV), a non-enveloped bacilliform DNA virus having genome of 7.5 kb, plays a significant role in causing citrus decline, particularly in sweet orange in India. Rapid detection techniques are important in the prevention of spread of these two diseases in field conditions. Since CMBV is weakly immunogenic, sero-diagnosis is not the preferred diagnostic method. Similarly, for detection of CTV though serological techniques like ELISA are being widely used, production of polyclonal antibodies to various isolates of CTV is often limited by various factors, namely low yields of the virus in plant tissues, uneven distribution, difficulties in production of sufficient quantities of infected tissue, contamination by host proteins in purified preparation, international quarantine, etc. As an alternative, a rapid and reliable PCR based detection protocol has been standardized. Sets of primers were designed based on the respective virus isolate sequence data available in GenBank, to obtain anticipated products of calculated size.

Keywords: Citrus plants, detection protocol, duplex PCR technique, RNA and DNA virus.

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CITRUS (*Citrus* sp.) being the third most important fruit crop after mango and banana, occupies an important place in the horticultural wealth and economy of India. However, the crop suffers from extensive damage by diseases caused by virus and virus-like pathogens. Among these pathogens, Citrus tristeza clostero virus (CTV), a member of the genus *Closterovirus*, family Closteroviridae, having a positive single-stranded RNA genome of approximately 19.3 kb size and citrus mosaic badna virus (CMBV), a bacilliform double-stranded circular DNA virus having genome of 7.5 kb, play a significant role in causing citrus decline, particularly in sweet orange in India. CTV is a phloem-limited virus having a size of 2000 × 11 nm and can be transmitted by grafting, budding and by aphid vectors, namely *Toxoptera citricida* (Kirk.), *T. aurantii* Fonse, *Aphis gossypii* Glover, *A. spiraecola* Parch, etc. in a semi-persistent manner. The virus is genetically and biologically diverse and can cause field symptoms ranging from stunting, slow decline, quick decline, stem pitting and yellowing (Figure 1), or no symptoms depending on virus isolate, time of infection, rootstocks, citrus cultivars and environmental conditions[1]. Citrus yellow mosaic disease is a common, widely distributed and severe disease in India and is reported to occur especially in satgudi sweet orange [*Citrus sinensis* (L.) Osbeck] in the southern states, mosambi sweet orange [*Citrus sinensis* (L.) Osbeck], acid lime [*Citrus aurantifolia* (Chirstm.) Swing] and Nagpur mandarin (*Citrus reticulata* Blanco) in central India and Khasi mandarins (*Citrus reticulata* Blanco) in the northeastern states[2–4]. The characteristic symptoms due to CMBV vary from general chlorosis to uniformly distributed leaf mosaic in field-infected mosambi and Nagpur mandarin and yellow mottling to yellow patches on acid lime. Mosambi sweet orange leaf sometimes develop yellow flecking along the veins (Figure 2). Infected plants often develop symptoms on part of the canopy and are moderately stunted with leathery texture of mature leaves. More variable symptoms may be observed if the field-grown plant is co-infected with other graft-transmissible or soil-borne pathogens[5]. CMBV measures 130 × 30 nm in size and is transmitted by grafting, dodder and on few citrus species by mechanical means[6]. Reports of experimental transmission by mealybug, *Planococcus citri* Risso and aphid species, *Myzus persicae* Sulzer and *Aphis craccivora* Koch is available in the literature[3,6]. However, the role of insect vectors for natural spread of the disease needs further tests and confirmation. CMBV is serologically related to Banana streak virus (BSV), Cocoa swollen shoot virus (CSSV), Sugarcane bacilliform virus (SBV) and Commelina yellow mosaic virus (ComYMV), and belongs to the member of *Badnavirus* genus of the family Caulimoviridae[6,7].

These two pathogens are associated with citrus decline in our country, particularly in sweet orange cultivars. Conventional ELISA technique is sensitive to detect CTV in plant material only during favourable period of the year, but not when the virus concentration in the host is low[8]. Moreover, poorly immunogenic CMBV can hardly be detected by using technique. The difficulty is overcome using nucleo-diagnostic techniques like polymerase chain reaction (PCR). There are reports of multiplex PCR systems that have been developed for the detection of two/three or even more plant viruses and viroids infecting crop plants, but few examples are available on citrus[9–15]. However, in citrus mixed infection different RNA (CTV, ICRSV, CEVd) and DNA (CMBV, greening *Liberobacter*) viruses are common in field conditions. This necessitated conducting the present experiments for simultaneous detection of more than one virus in citrus samples.

CTV and CMBV cultures used in this study were earlier collected from different citrus orchards and nurseries in Maharashtra and maintained as pure cultures inside the

Figure 1. CTV-infected mosambi showing chlorosis and yellowing of leaves.

Figure 2. CMBV-infected mosambi leaves showing mosaic symptom along the veins.

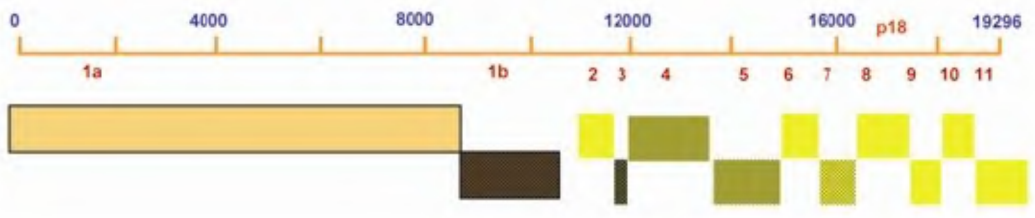

Figure 3. Schematic presentation of 19.3 kb single-stranded RNA of CTV genome that consists of 12 open reading frames (ORFs), 105–108 nucleotide long 5′-NTR (non-translated region) and 270 nucleotide long 3′-NTR. Out of 12 ORFs (that potentially encode at least 19 proteins), two are at the 5′-end and remaining ten ORFs are at the 3′-end, which are expressed through 3′-co-terminal sub-genomic mRNAs. ORF-8 encodes for 18 kDa poly protein.

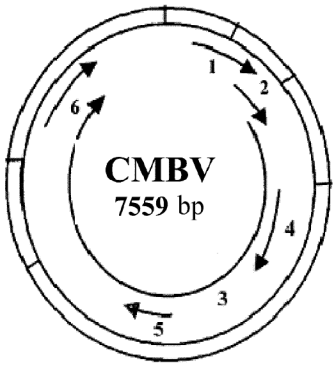

Figure 4. Schematic presentation of circular double-stranded DNA of CMBV genome that contains six putative ORFs, all on plus stand and each capable of encoding proteins with molecular weight more than 10 kDa. ORF-3, the largest ORF contains domains that are conserved among other badna viruses and encodes for putative polyprotein for functions involved in virus movement and assembly.

insect-proof greenhouse at the National Research Centre for Citrus, Nagpur. The CTV culture was maintained in acid lime and mosambi seedlings, and CMBV culture in mosambi seedlings. Five mosambi seedlings were also graft-inoculated with cultures of both CTV and CMBV to obtain mixed infection of both viruses, which was used as known positive control in duplex PCR experiments. Two sets of primer pairs were synthesized based on specificity, stability and compatibility for simplex and duplex PCR amplification (IDT). These are forward primer 5′AATGTCAGGCAGCTTGGGAAAT3′ and reverse primer 5′TTCGTGTCTAAGTCR CGCTAAAC3′ for CTV ORF-8, and forward primer 5′AACTTGAGGAGG-TTAAAGGGCTTAG3′ and reverse primer 5′CATGCATCCATCCGTTTCGATGA3′ for CMBV ORF-3 (Figures 3 and 4). Total RNA from CTV-infected plants was extracted using RNeasy plant mini kit (Qiagen), according to the manufacturer's protocol and also by the method described by Hung *et al.*[16]. Similarly, for extraction of total DNA from CMBV-infected plants, the method described by Baranwal *et al.*[17] and the commercially available DNeasy plant mini kit (Qiagen) were used. In both the cases, 100 mg of healthy and virus-infected citrus tissue was used for nucleic acid extraction.

To standardize simplex PCR for CTV detection, first the cDNA was synthesized in a PCR tube using 10–15 µl of total RNA and 2 µl of 10 µM of the antisense primer, which was microcentrifuged and incubated at 72°C for 10 min. A master mix was prepared separately using 5× first strand buffer, 0.1 M DTT, 10 mM dNTP (Ambion), incubated for 2–3 min at 42°C and returned to room temperature. Twenty units of MMLV RT (Promega) and 40 units of RNasin (Ambion) were added and microcentrifuged for 10 s. This master mix was then transferred into the PCR tube containing template RNA and mixed gently. The PCR tube was incubated at 42°C for 50 min, followed by 72°C for 10 min in a thermal cycler (Bio-rad), which gives the cDNA. Three to 5 µl of amplified cDNA (for CTV) or DNA (for CMBV) was used in 50 µl of reaction mixture separately, containing 5 units of *Taq* DNA polymerase (Ambion), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Fermentas) and 10 µM of each of the sense and antisense primers. PCR was performed using the following parameters: one cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 55–60°C for 30 s and 72°C for 45 s, followed by one cycle at 72°C for 10 min. The PCR product was analysed by electrophoresis using 1% agarose gel containing 500 ng ethidium bromide per ml.

In order to standardize the duplex PCR, two sets of primers were mixed in the PCR tube and a 10X primer mix containing 2 µM of each of the forward and reverse primers was prepared. cDNA synthesis was performed using the same protocol followed for simplex PCR. Four to 5 µl of cDNA and 1.5 µl of CMBV DNA were added in a 50 µl PCR reaction mixture. Qiagen multiplex PCR kit was used for duplex PCR amplification. The mPCR mix was prepared containing 25 µl of mPCR master mix, 5 µl of 10X primer mix (2 µM of each primer) and the final volume made up with distilled water. Gradient PCR was performed using the following parameters: one cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 55–60°C for 30 s and 72°C for 45 s followed by 72°C extension for 10 min to determine the annealing temperature for the two viruses. PCR products were analysed on 1% agarose gel. Simplex and duplex PCR techniques were carried out simultaneously for comparison. Once the duplex PCR

technique was standardized using known greenhouse-maintained positive virus cultures, it was subsequently utilized for validation purpose using field-infected citrus plants. During a survey of citrus orchards located in central India, suspected field samples were collected and analysed by both simplex and duplex PCR techniques. For further confirmation, leaf samples were also examined under an electron microscope either using leaf-dip method or partially purified preparations, as described by Ahlawat *et al.*[6]. Electron microscopic preparations were negatively stained using 2% sodium phosphotungstate, pH 7.0 or 2% aqueous uranyl acetate, both containing 100 μg/ml bacitracin and observed under a JEOL-100 CX-11 transmission electron microscope.

In the present investigation, using two different sets of primer pairs specific for ORF 8 of CTV genome and ORF 3 of CMBV genome, amplified products of expected size, i.e. 510 and 245 bp respectively, were successfully obtained from CTV- and CMBV-infected citrus leaf samples by simplex PCR, while no amplification products were obtained in healthy plant samples (Figures 5 and 6). Primers were designed using a computer program after alignment of conserved areas of both viruses obtained from the GenBank sequences of a particular virus. A set of two primer pairs was designed, which displayed similar melting temperature, G + C content and nucleotide length, that enables simultaneous detection of two citrus viruses in duplex PCR (Figure 7). No amplification was obtained from extracts of healthy samples. Both the methods of RNA extraction produced a good yield of high-quality RNA. For simplex PCR reaction, both the methods produced consistent amplification products, but for duplex PCR reaction, the RNeasy kit produced more consistent amplification compared to the method of Hung *et al.*[16]. Between the two methods of DNA extraction, both DNeasy plant mini kit and the method described by Baranwal *et al.*[17] produced a good yield of high-quality DNA. For simplex and duplex PCR reactions both the methods produced consistent amplification, but the second method was more economical than the DNeasy plant mini kit.

Various parameters such as primer concentration of forward and reverse primers (0.1–2 μM), annealing temperature (50–65°C), annealing time (30–90 s), number of cycles (25–40) and type of *Taq* DNA polymerase (standard and HotStar *Taq*) used were taken into consideration to standardize the simplex and duplex PCR reactions for CTV and CMBV detection. In the case of simplex PCR, better amplification was obtained at 0.4 μM of each primer and 58°C annealing temperature for 30 s and 30 cycles. For duplex PCR, optimum results were obtained with 0.2 μM of each primer and 59–60°C annealing temperature for 90 s using 35 cycles. HotStar *Taq* DNA polymerase was found more efficient than standard *Taq*

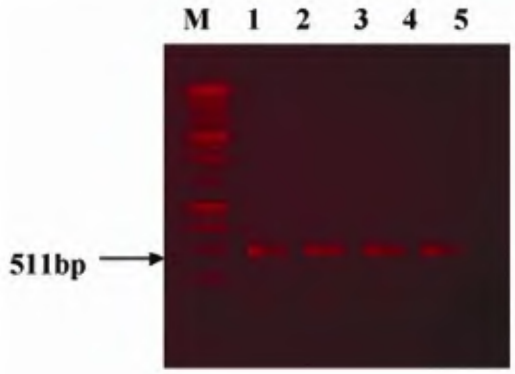

Figure 5. Detection of CTV by simplex PCR using virus-specific primer pairs. Lanes 1–4, CTV-specific amplified products of 511 bp. Lanes 1 and 2, Glasshouse-maintained pure cultures of CTV; lanes 3 and 4, Field-infected CTV samples and lane 5, Healthy control. M, 1 kb marker.

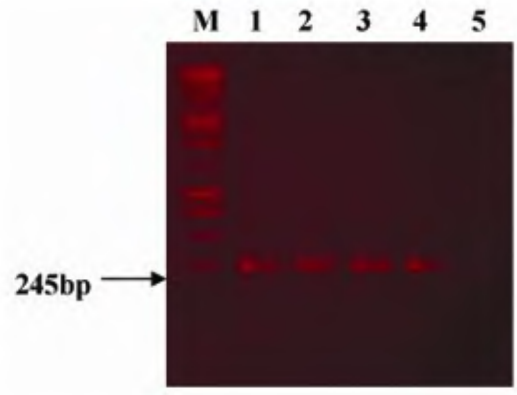

Figure 6. Detection of CMBV by simplex PCR using virus-specific primer pairs. Lanes 1–4, CMBV-specific amplified products of 245 bp. Lanes 1 and 2, Glasshouse-maintained pure cultures of CMBV; lanes 3 and 4, Field-infected CMBV sample and lane 5, Healthy control. M, 1 kb marker.

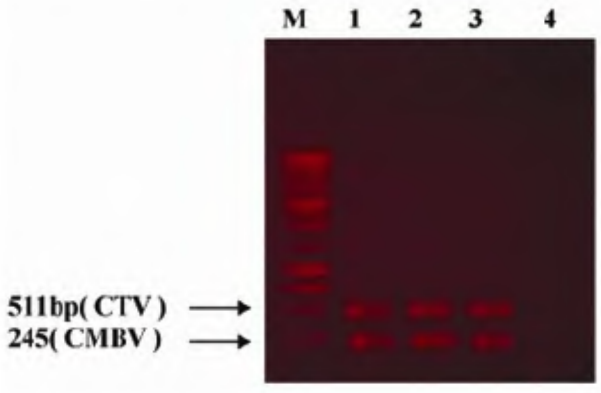

Figure 7. Agarose gel electrophoresis of duplex PCR products obtained from mosambi sweet orange plant mixed-infected by CTV and CMBV. Lanes 1 and 2, Field infected samples from Rohona and Narkhed area respectively; lane 3, Green maintained known positive culture mixed infected with CTV and CMBV and lane 4, Healthy virus-free mosambi plant. M, 1 kb marker.

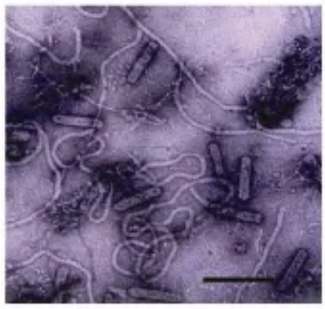

Figure 8. Electron photomicrograph of field samples of mosambi mixed infected with CTV (flexuous paraticle) and CMBV (bacilliform particle) in partially purified preparation. Bar represents 200 nm.

polymerase. Initially the reaction was carried out using conventional PCR mixture, but failed to amplify the two viruses. When the Qiagen multiplex PCR kit was used, both viruses amplified in a single reaction. The most consistent results were obtained at annealing temperature of 60°C. According to the manufacturer's literature, multiplex PCR buffer contains a balanced mixture of salts and additives that increase the efficiency for annealing and extension of all the primers used in the reaction. To compare the sensitivity level of duplex PCR and simplex PCR, ten fold serial dilution in nuclease-free water containing one cDNA from CTV- and one DNA from CMBV-infected samples was tested simultaneously by simplex and duplex PCR. For the ten fold dilution, 10^{-6} and 10^{-5} were the highest dilutions at which duplex PCR showed positive results for CTV and CMBV respectively. While in case of simplex PCR, positive results were obtained at 10^{-6} dilution for CTV and 10^{-4} dilution for CMBV. The duplex PCR method developed is a sensitive, reliable and rapid method for simultaneous detection of CTV and CMBV.

During our attempt to validate the standardized protocols to index field-infected samples, it was observed that all doubly infected field samples tested produced two bands in the gel corresponding to the expected sizes of CTV and CMBV. Healthy plants did not produce any amplification product. Plants infected with only one of the viruses resulted in amplification with the primer pair designed only to the homologous virus. These results indicate that it is possible to detect the two most important viruses infecting citrus in India in a single reaction. Presence of both viruses in tissues does not affect their detection by duplex PCR. The PCR-based detection is also confirmed by directly observing virus particles under electron microscope in leaf-dip as well as partially purified preparation, which showed 130 × 30 nm non-envelop bacilliform CMBV particle and 2000 × 11 nm long CTV particle in doubly infected citrus samples (Figure 8). Thus, in this communication we report the simultaneous detection of one RNA and one DNA virus infecting citrus using duplex PCR from India. This sensitive and simultaneous detection decreases the risk of contamination, saves time and reduces cost compared to other conventional methods for citrus virus detection. The standardized technique provides a useful and rapid method for detecting multiple viruses in citrus plants, that will aid in any citrus budwood certification programme to produce virus-free planting materials.

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