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Molecular detection of *Citrus yellow mosaic virus* (CYMV) and citrus greening bacterium (CGB) in sweet orange by multiplex polymerase chain reaction

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Abstract
Citrus is one of the most economically important fruit crop in India. Commercially grown citrus includes sweet orange, acid lime and mandarin. Citrus Yellow Mosaic (CYMV), a viral disease and citrus greening bacterium (CGB), a bacterial disease are the two most important diseases that are impending fruit production through the world. A method of multiplex polymerase chain reaction (PCR) was developed for the simultaneous detection of *Citrus yellow mosaic virus* (CYMV) and citrus greening bacterium, *Candidatus Liberibacter asiaticus* (CLa) from sweet orange trees. Initially total DNA from individual CLa and CMBV infected citrus plants were mixed infected field sample for both pathogens were detected by Multiplex PCR. Using multiplex PCR two different fragments of 1024 bp and 451 bp specific to CYMV and CGB respectively were simultaneously amplified. The consistent result of multiplex PCR was compared with Simplex PCR for detection of each pathogen. The Muliplex PCR method developed in the present investigation proved to be highly sensitive, economic and reliable methods for detection of citrus greening bacterium (CGB) and the *Citrus yellow mosaic virus* (CYMV) in citrus trees from the orchards. The technique should prove highly useful in disease surveys, nursery certification and quarantine applications.

Keywords: multiplex PCR CGB, CYMV detection

1. Introduction
Citrus is an important fruit crop in India and grown in about 0.49 million ha with an annual production of 4.39 million tones. Citrus decline is a serious problem and has been attributed to foot rot, *Citrus tristeza virus*, *Indian ring spot virus*, *Citrus mosaic badnavirus* (also called as *Citrus yellow mosaic virus*), *Citrus exocortis viroid* and the Citrus greening disease (CGD) (Ahlawat, 1997) [2]. Gade, 2012 [9] was tested three root stocks against foot rot and found that Cleopatra and rough lemon more susceptible than Rangpur lime. Dhakad *et al.*, 2014 [8] was tested eleven root stocks against the foot rot pathogen and found that Cleopatra and Rough lemon showed maximum reduction in number of leaves. Citrus greening disease (CGD) is caused by a fastidious; phloem restricted a gram negative bacterium *Candidatus* Liberibacter asiaticus (CLa) (Hocquellet *et al*. 2000, Garnier and Bove, 1993) [11, 6]. The symptom in citrus caused by CLa is non specific and is often confused with nutritional deficiency, root diseases and other stress related factors. Citrus yellow mosaic Virus (CYMV) is caused by a bacilliform non-enveloped virus, *Citrus mosaic badnavirus* (CMBV) which has also been referred as Citrus Yellow mosaic virus in the genus *Badnavirus* of family *Caulimoviridae* (Ahlawat *et al*., 1996) [3]. The virus particles measure approximately 130 x 30 nm (Ahlawat *et al*., 1996) [3] and contain a circular double stranded genome of 7559 base pairs (Huang and Hartung, 2001). Therefore, a reliable and sensitive detection technique is needed, which can detect the bacterial and virus pathogens, preferably simultaneously. Such a technique may help in the prevention and spread of these pathogens. Serological methods for the detection of both pathogens are not preferred as badnaviruses including CMBV are moderately immunogenic (Lockhart and Olszewski, 1993) [15] and production of antibodies involve unusually complex virus purification and immunization steps. The detection of the bacterium by ultrathin electron microscopy is a good method but due to erratic distribution of the bacterium and non availability of electron microscope in most laboratories, this method has limited application.

However, several indirect approaches such as monoclonal antibodies and DNA probes have been used for diagnosis. The use of monoclonal antibodies for field diagnosis has proven unsatisfactory because of strain specificity (Korsten *et al*., 1993, Varma *et al*., 1993) [14, 6, 18]. Although, the use of specific DNA probes has proven to be more reliable and as sensitive as electron microscopy (Bove *et al*., 1993, Jagouiex *et al*., 1996) [6, 13] but it is time consuming. However, both these pathogens have been detected by a uniplex PCR using the DNA extracted from leaf for CMBV (Baranwal *et al*., 2003) [1, 4] and from midrib and petiole for CLa (Hocquellet *et al*., 2000, Ahlawat *et al*., 2003) [11, 4, 1]. In view of the increasing interest in plant pathology for the detection of more than one targets such as mixed infection of viruses and bacterium (Bertolini *et al*., 2003) [5], viroids and viruses (Singh and Nie, 2003) [4, 17] in single reaction, multiplex PCR protocols have been developed. A multiplex PCR for the detection of a fastidious greening bacterium and a DNA virus frequently infecting sweet orange trees have been discuss with in the paper.

2. Materials and methods

2.1 Field Samples

Surveys were conducted from wide areas in five citrus orchards of Southern India in Nagri (A.P.) during 2007. Samples of plants apparently showing the symptoms of CGD, CMBV or both were collected for individual analysis in a PCR system. Samples of plants showing apparent symptoms of CGD as well as CMBV from each orchard were also collected and subjected to a multiplex PCR for the determination of the presence of CLa and CYMV.

2.2 DNA Isolation

Initially total DNA from 100 mg leaves of CYMV infected sweet orange seedling was isolated with DNeasy Plant Mini Kit according to the manufacturer's protocol (Qiagen, Germany). Sodium Sulphite, NCM Protocol and Huge protocol. Similarly total DNA was also isolated from 100 mg of mid rib tissues of CLa - infected plant. DNA isolated from CYMV infected plant was mixed with DNA isolated from CLa - infected plant in 1: 1 or 1:2 ratio (virus: bacteria). In another set of experiments for DNA isolation, infected CYMV leaf lamina and CLa infected mid rib tissues were mixed together in 1:1, and 1:3 to a total of 200 mg of tissue. For the distribution of individual pathogens, the DNA was also isolated from leaf lamina as well as mid rib of sweet orange plants infected by both pathogens. Healthy seedlings of sweet orange were used for DNA isolation as negative control. The final DNA was eluted in 100 µl of elution buffer.

2.3 Standard and Multiplex Polymerase chain reaction

Primer pair from the 16s-rDNA ribosomal genes of specific for CLa and a primer pair designed manually from the genomic sequence of CMBV (Table 1) were used. Initially a PCR with each of the primer pairs was carried out. The final conditions of standard PCR assay in a 50 µl of PCR mixture were as follows: 1 µM of each primer, 200 µM each of dNTPs, 0.05 U / µl of Taq DNA polymerase, 1 x reaction buffer, 1.5 mM of $MgCl_2$ and 10 µl of DNA template. The PCR was performed in a thermal cycler (Eppendr of) with 30 cycles of denaturation at 94 ^{0}C for 30 s, primer annealing at 58 ^{0}C for 30 s and primer extension at 72 ^{0}C for 45 s with the first denaturation at 94 ^{0}C for 5 min and the final extension at 72 ^{0}C for 10 min. The final conditions of multiplex PCR assay for mixed DNA were adopted after extensive trials with primers ratio, dNTPs, $MgCl_2$ concentrations and number of PCR cycle. These were as follows: DNA template in 2: 1 ratio of CLa and CYMV affected plant in 15 µl, $MgCl_2$ 3.0 mM and 1 µM of each primer. The remaining conditions for a 50 µl PCR reaction mixture were same as used for the standard PCR. Similar PCR conditions using 15 µl of DNA isolated from combined tissues of mid rib of leaves of CLa infected and leaf lamina of CMBV infected sweet orange in 2:1 were adopted. Multiplex PCR conditions for DNA isolated from the mid rib of leaves as well or the leaf lamina of sweet orange plant infected by both the pathogens, were also same except that a10 µl of template DNA sample was used. For the orchard samples, 10 µl DNA isolated from midrib of leaves was used and multiplex PCR was done using 3.0 mM $MgCl_2$. Amplified products were detected by 1% agarose gel electrophoresis in Tris - acetate - EDTA buffer, stained with ethidium bromide, and visualized under UV light. Each experiment was repeated at least twice.

2.4 Sequencing

The amplified DNAs of CLa and CMBV were excised and eluted from the gel using Qiaquick gel extraction kit (Quiagen Gmbh, Hilden, Germany). The purified PCR product was ligated into pGEM-T Easy Vector (Promega, Madison, USA). Competent *Escherichia coli* (strain DH 5α) were transformed by standard molecular biology procedures (Sambrook and Russel 2001) [16]. Recombinant clones were identified by colony PCR and selected clones were sequenced at the automated sequencing facility, Department of Biochemistry, Delhi University, South Campus, Delhi, India.

3. Results & Discussion

3.1 Effect of template ratio and $MgCl_2$ concentration

When DNA of CMBV infected plant and CLa infected plants were mixed in equal amount and the conditions of uniplex PCR were used for the amplification, only CMBV was amplified (Fig. 1, lane 3). The increase in primer concentration of CLa (from 1 µM to 1.5 µM) or decrease in primer concentration of CMBV (from 1 µM to 0.7 µM) did not improve the amplification of CLa. Variation in terms of increase in template DNA concentration from CLa infected plants from 1:1 to 2:1 ratio also resulted in the amplification of only CMBV. Increase in DNA template from CLa infected plant (1:1 to 2:1) coupled with the increase in $MgCl_2$ from 1.5 mM to 3.0 mM provided amplification of both pathogens (Fig. 1, lane 4). The increased $MgCl_2$ of 3.0 mM provided some amplification (faint band) of CLa in addition to the amplification of CMBV when template DNA was mixed in 1:1 ratio. (Fig. 1., lane 5). Specific amplification products (CLa, 451 bp and CMBV, 1024 bp) were obtained from the respective positive bacterial and viral controls in uniplex PCR. The multiplex PCR kit from Qiagen, Germany produced better results than a conventional PCR mixture. According to the quiagen multiplex PCR hand book the quiagen multiplex buffer contains a balanced mix of salts and additives that ensures comparable efficiencies for annealing and extraction of all primers used in the same reaction.

3.2 Evaluation of different tissue types for both pathogens in the multiplex PCR

In the multiplex PCR, when concentration of $MgCl_2$ was increased from 1.5 mM to 3.0 mM, mixed targets were successfully detected from the DNA preparations of combined tissues in a ratio of 3:1 CLa infected mid rib of leaf and CMBV infected leaf lamina (Fig 2., lane 4). However, the

intensity of CMBV amplicon was higher in 1:1 ratio of tissues (lane 1 vs 2), but both pathogens produced amplicon of almost same intensity in tissues mixed in 3:1 ratio (Fig 3. Lane1& 3) Increase in concentration of each dNTPs from 200 µM to 400 µM reduced the amplification of CLa (Fig 3. Lane 2 & 4).

3.4 Use of one type of tissue for both pathogens

The mixed infections of CMBV and Cla from citrus were detected simultaneously from the midrib of leaves from sweet orange trees infected by both pathogens (Fig. 3, lane, 4). CYMV could be detected from mid rib as well as leaf lamina (Fig. 3, lane, 1&2) while Cla was detectable only from mid rib (Fig. 3, lane 5) and not from leaf lamina (Fig. 3 lane 6) of citrus trees infected by both pathogens. The multiplex reaction of DNA isolated from leaf lamina of tree with mixed infection amplified only CMBV (Fig. 3 lane 3). The multiplex PCR fragment for each of the samples corresponded perfectly with the result of uniplex PCR. Sequencing of the fragment of multiplex also matched with that of uniplex PCR fragment of individual pathogens.

3.5 Validation of Technique

The results data in presented in Table 2 and Figure I to 4. Data indicated that 12 samples from Three citrus orchard (each orchard has 100 to 150 trees) in South India showed that 17 out of 36 samples from three orchard showed the presence of only CYMV. Of the 36 samples of other 3 orchards, 8 trees showed the amplification of CGB and eight trees showed the presence of CGB and CYMV. Amplification of no pathogens was observed in 13 citrus trees. It is also evident from the multiplex PCR is a sensitive technique for detection of these pathogens as CYMV, CLa or both could be detected in 15 samples while visible symptoms were present only in 5 samples.

4. Conclusions

The multiplex PCR is time and energy saver because it can be performed in a single reaction. Although, there is no such reports for simultaneous detection of bacterial and viral pathogens from citrus, but it has been used for the detection of a bacterium and viruses in olive tree (Bertolini *et al*., 2003) [5]. In this study, detection of greening bacterium directly from infected tissue is important. The use of DNA from mid rib of leaves of infected trees indicated that in case of mixed infection in citrus trees, template DNA can be isolated from midrib alone for both bacterial and viral pathogens. This was confirmed in our field evaluation studies where multiplex PCR could detect the infection of CLa and CMBV either singly or both if present together in the tree. Performance of a multiplex PCR with both pairs of primer, could provide information with regards to the individual infection of each pathogen as well as the double- infections in sweet orange trees where both pathogens are found frequently. Optimization of multiplex PCR reaction needs adjustments in the amount of primers, dNTPs and $MgCl_2$ concentration and other parameters used in the uniplex PCR (Chamberlain and Chamberlain, 1994). In this present study, we optimized the duplex PCR condition and amplified product of expected sized i.e.1024 bp obtained from CYMV and 451 bp from CGB consequently. The multiplex PCR, which can detect and identified simultaneously greening bacterium and CYMV in citrus trees and suitable for large scale indexing. This study provides a convenient reproducible and rapid method for the detection of mixed infections as well as single infection of two pathogens in citrus and determines their extent of mixed infection. It can also be useful for the phytosanitary assay in plant quarantine.

Table 1: Primer Set for PCR amplification of Citrus greening bacterium and citrus yellow mosaic virus.

Pr. Set N.	Primer	Primer sequence	Tm ^{0}C	Annealing Tem	Fragment size bp
1	Forward Primer CG B	5'TGG GTG GTT TAC CAT TCA GTG3'	63	58	~451
	Reverse Primer CGB	5'CGC GAC TTC GCA ACC CAT TG 3'	65		
2	Forward Primer CYMV	5' AGT GGC TTT CAT CAG GTA GC 3'	57..5	58	~1024
	Reverse Primer CMBV	5' ATC TGG ACA GAG CAT CAG CC 3'	55.4		

Table 2: Test Result of the multiplex PCR detection from sweet orange leaves of orchard samples.

Samples	Total no of plant	CMBV	CG	CMBV+CG
Orchard 1	12	4/12	5/12	5/12
Orchard2	12	5/12	0/12	0/12
Orchard3	12	8/12	3/12	3/12

Figures: Multiplex PCR detection

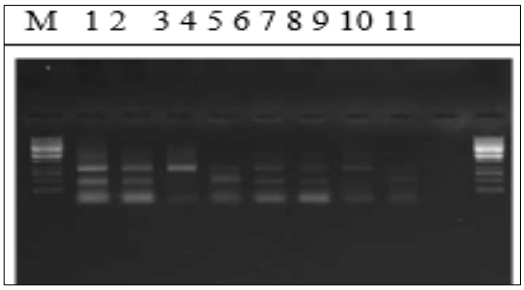

Fig 1: Effect of $MgCl_2$ on the amplification of CLa and CYMV in mixed DNA of CLa and CYMV in different ratio. M, 1 kb DNA ladder; 1, DNA 1:1 and $MgCl_2$ 1.5 mM, 2, DNA 2:1 and $MgCl_2$ 1.5 mM; 3, DNA 1:1 and $MgCl_2$ 3.0 mM; and 20mMdNTPS 4, DNA 2:1 and $MgCl_2$ 3.0 mM20mMdNTPS

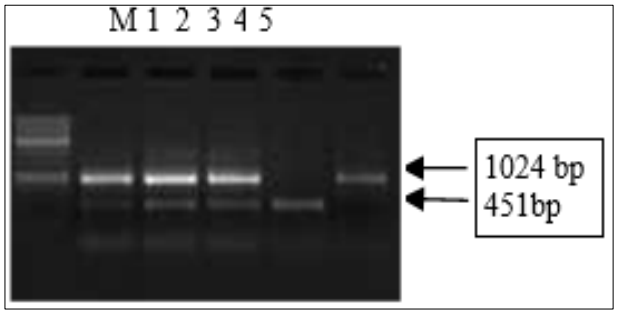

Fig 2: Detection of CLa and CYMV by the multiplex PCR from the mixed tissues of mid ribs of leaves of sweet orange infected by citrus greening bacterium and leaf lamina of sweet orange infected by CYMV (2:1 ratio). M, 1 Kb DNA Ladder; 6, CYMV primers only; 5, Citrus greening bacterium primers only; 2, 3, 4, Both the primers

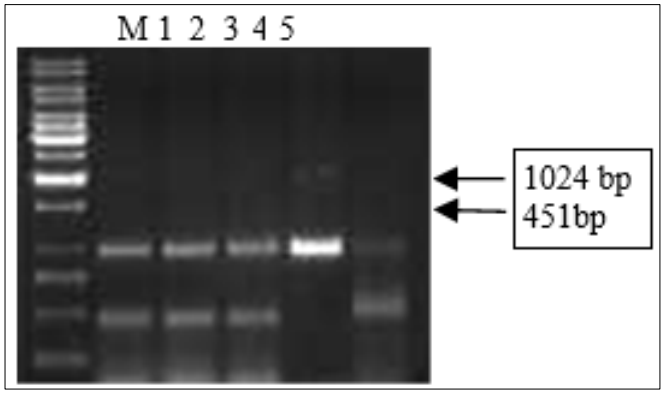

Fig 3: Lane M; 1kb DNA Ladder: Lane 1, 2, 3 multiplex (CYMV and CG):lane4, 4 Cg Lane 5: CYMV 10 Mm dntp: 25mM Mgcl2.

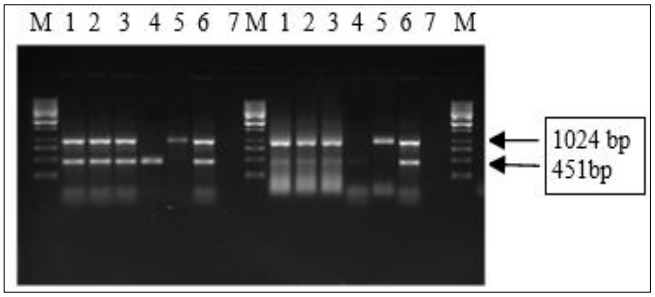

Fig 4: A. DNA from kit & Qiagen Multiplex PCR Kit B. NCM eluted DNA & Qiagen Multiplex PCR Kit C. DNA from kit & normal PCR reaction mixture. M- 1 kb ladder, 1,2 & 3 mixed DNA of CYMV and CG, Lane 4- CG, Lane 5- CMBV lane 6 positive control and lane 7 negative control.

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