A novel approach for simultaneous detection of *Citrus yellow mosaic virus* and *Citrus greening bacterium* by multiplex polymerase chain reaction

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A method of multiplex polymerase chain reaction (PCR) was developed for the simultaneous detection of *Citrus yellow mosaic virus* (CYMV) and fastidious *Citrus greening bacterium, Candidatus Liberibacter asiaticus* (CLa) in sweet orange trees. Initially total DNA from individual CLa and CYMV infected citrus plants were mixed and both pathogens were detected simultaneously by multiplex PCR. Subsequently, both pathogens were detected from the total DNA obtained after mixing midribs of CLa-infected and CYMV-infected leaf lamina of the sweet oranges. The finally adopted multiplex PCR protocol simultaneously detected CLa and CYMV from the total DNA extracted from the midrib of leaf of citrus plants infected by both the pathogens. Thus, the present protocol demonstrated the presence of mixed infections of CLa and CYMV in citrus orchards trees. The technique would also prove highly useful in disease survey, nursery certification and quarantine applications.

**Keywords**: Citrus greening bacterium, *Citrus yellow mosaic virus*, multiplex polymerase chain reaction, simultaneous detection

**IPC Code**: Int. Cl.7 C12N15/10

**Introduction**

Citrus is an important fruit crop in India. It is grown in about 0.49 million ha with an annual production of 4.39 million tons. Citrus decline is a serious problem and has been attributed to many causes, e.g. *Citrus tristeza virus*, *Indian Citrus ring spot virus*, *Citrus yellow mosaic virus* (CYMV), *Citrus exocortis viroid* and the greening bacterium\(^1\). The symptoms in citrus caused by CLa is non-specific and is often confused with nutritional deficiency, root diseases and other stress related factors. CYMD is caused by a non-enveloped bacilliform virus (CYMV), which was previously referred as *Citrus mosaic virus* in the genus *badnavirus* of family *Caulimoviridae*\(^6,7,8\). The virus particles measure approximately 130×30 nm\(^3\) and contain a circular double stranded genome of 7559 base pairs\(^8\).

CGD is caused by a fastidious, phloem restricted, gram negative bacterium, *Candidatus Liberibacter asiaticus* (CLa)\(^4,5\). The symptoms in citrus caused by CLa is non-specific and is often confused with nutritional deficiency, root diseases and other stress related factors. CYMD is caused by a non-enveloped bacilliform virus (CYMV), which was previously referred as *Citrus mosaic virus* in the genus *badnavirus* of family *Caulimoviridae*\(^6,7,8\). The virus particles measure approximately 130×30 nm\(^3\) and contain a circular double stranded genome of 7559 base pairs\(^8\).

The citrus pathogens are inadvertently disseminated through budwood as it is the main source of vegetative material for propagation. 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 CYMV are moderately immunogenic\(^9\) and production of antibodies involves unusually complex virus purification and immunization steps. The detection of the bacterium by ultrathin electron microscopy is a satisfactory method but due to erratic distribution of

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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. Although, the use of monoclonal antibodies for field diagnosis has proven unsatisfactory because of strain specificity\textsuperscript{10,11}, the use of specific DNA probes has proven to be more reliable and as sensitive as the electron microscopy\textsuperscript{12} but it is time consuming.

Recently, both the pathogens have been detected by a standard PCR, using the DNA extracted from leaf for CYMV\textsuperscript{7} and from midrib and petiole for CLa\textsuperscript{5,13}. In view of the increasing interest in plant pathology for the detection of more than one targets, such as mixed infection of viruses and bacteria\textsuperscript{14}, and viroids and viruses\textsuperscript{15} in single reaction, multiplex PCR protocols have been developed. We describe for the first time, a multiplex PCR for the detection of a bacterium and a DNA virus frequently infecting sweet orange trees.

Materials and Methods

Plant Materials

The isolate of CYMV, originally collected from sweet orange and maintained on its seedlings, was used in the studies. CLa-infected material obtained from a local mandarin orange tree was wedge-grafted on five sweet orange seedlings. The same infected material was also grafted on five seedlings previously infected with CYMV to generate doubly infected material by both pathogens. The samples from grafted plants were collected three months post-grafting. All the plants were maintained in a greenhouse at the Indian Agricultural Research Institute, New Delhi.

Field Samples

Survey was conducted in five citrus orchards of Kadapa district of Andhra Pradesh State, covering a wide area (10 km \times 5 km). Samples of plants apparently showing the symptoms of only CGD or CYMD were collected for individual analysis in a PCR system. Samples of plants showing apparent symptoms of both CGD as well as CYMD from these orchards were also collected and subjected to a multiplex PCR for the determination of the presence of CLa and CYMV.

DNA Isolation

In one set of experiments, total DNA from 200 mg leaves of CYMV infected sweet orange seedling was isolated with DNeasy Plant Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) or using a previously described sodium sulphite based extraction buffer\textsuperscript{5}. Similarly, total DNA was also isolated from 200 mg of midrib 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:bacterium). In another set of experiments for DNA isolation, infected CYMV leaf lamina and CLa infected midrib 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 midrib of sweet orange plants infected by both pathogens. For study of occurrence of mixed infection by both pathogens, DNA was isolated from midrib and petiole of leaves from trees of orchards. Healthy seedlings of sweet orange were used for DNA isolation as negative control. The final DNA was eluted in 150 \(\mu\)L of elution buffer.

Standard and Multiplex Polymerase Chain Reaction

A primer pair from the ribosomal protein genes of \(\beta\) operon specific for CLa and a primer pair designed manually from the genomic sequence of CYMV (Table 1) were used. A standard PCR with each of the primer pairs was carried out. The final conditions of standard PCR assay in a 50 \(\mu\)L of PCR mixture were as follows: 1 \(\mu\)M of each primer, 200 \(\mu\)M each of dNTPs, 0.05 U/\(\mu\)L of Taq DNA polymerase (Fermentas, Lithuania), 1 X reaction buffer, 1.5 mM of MgCl\(_2\) and 10 \(\mu\)L of DNA template. The PCR was performed in a thermal cycler (Biometra, Germany).

<table>
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<th>Name</th>
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<td>CLa</td>
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<td>703</td>
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<td></td>
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<td>AY262011\textsuperscript{8}</td>
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with 30 cycles of denaturation at 94°C for 30 second, primer annealing at 58°C for 30 second and primer extension at 72°C for 45 second with the first denaturation at 94°C for 5 min and the final extension at 72°C for 10 min.

The conditions of multiplex PCR assay for mixed DNA were adopted after extensive trials with primers ratio, dNTPs, MgCl₂ concentrations and number of PCR cycles. These were as follows: DNA template in 2:1 ratio of CLa and CYMV affected plant in 15 μL, MgCl₂ 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 midrib of leaves of CLa-infected and leaf lamina of CYMV-infected sweet orange in 1:1 and 3:1 were adopted. Multiplex PCR conditions for DNA isolated from the midrib of leaves as well as the leaf lamina of sweet orange plant infected by both the pathogens were also same, except that a 10 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 MgCl₂. 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.

Sequencing
The DNA fragments amplified from CLa and CYMV were excised and eluted from the gel using Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The purified PCR product was ligated into pGEM-T Easy Vector (Promega, Madison, USA). Competent Escherichia coli (strain DH 5 α) were transformed by standard molecular biology procedures. Recombinant clones were identified by colony PCR and selected clones were sequenced at the automated sequencing facility, Department of Biochemistry, South Campus, Delhi University, Delhi.

Results
Effect of Template Ratio and MgCl₂ Concentration
When DNA of CYMV-infected plants and CLa-infected plants were mixed in equal amounts and the conditions of standard PCR were used for the amplification, only CYMV was amplified (Fig. 1, lanes 1-2). The increase in primer concentration of CLa (from 1 to 1.5 μM) or decrease in primer concentration of CYMV (from 1 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 CYMV. Increase in DNA template from CLa-infected plant (1:1 to 2:1) coupled with the increase in MgCl₂ from 1.5 to 3.0 mM provided amplification of both pathogens (Fig. 1, lane 4). The increased MgCl₂ of 3.0 mM provided some amplification (faint band) of CLa in addition to the amplification of CYMV when template DNA was mixed in 1:1 ratio (Fig. 1, lane 3). Specific amplification products (CLa, 703 bp and CYMV, 498 bp) were obtained from the respective positive bacterial and viral controls in standard PCR. Cloning and sequencing of amplified products revealed that the amplicon for CLa was 703 bp and matched exactly with the sequence available in GenBank accession number M94319. Similarly the amplicon of CYMV was found to be 498 bp and matched with sequence of CYMV GenBank accession number AY262011. No amplification products were obtained from the healthy plant extracts (not shown).

Evaluation of Different Tissue Types for Both Pathogens in Multiplex PCR
In the multiplex PCR, when concentration of MgCl₂ was increased from 1.5 to 3.0 mM, both targets were successfully detected from the DNA
preparations of combined tissues of CLa-infected midrib of leaf and CYMV-infected leaf lamina in a ratio of 3:1 (Fig. 2, lane 4). However, the intensity of CYMV amplicon was higher in 1:1 ratio of tissues but both pathogens produced amplicon of almost same intensity in tissues mixed in 3:1 ratio (Fig. 2, lanes 3 & 4). Increase in concentration of each dNTPs from 200 to 400 μM reduced the amplification of CLa (not shown). The increase in number of PCR cycle from 30 to 40 improved the amplification of both the pathogens only marginally (not shown).

Use of One Type of Tissue for Both Pathogens

The mixed infections of CYMV 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 midrib as well as leaf lamina (Fig. 3, lanes 1 & 2), while CLa was detectable only from midrib (Fig. 3, lanes 4 & 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 CYMV (Fig. 3, lane 3). The multiplex PCR fragment for each of the samples corresponded perfectly with the result of standard PCR. Sequencing of the fragment of multiplex also matched with that of standard PCR fragment of individual pathogens.

Evaluation of 31 symptomatic samples from five citrus orchards (each orchard has 115 to 165 trees) in Andhra Pradesh showed that 5 samples from one orchard showed the presence of only CLa out of 8 symptomatic samples. Of 23 samples of other 4 orchards, 16 trees showed the amplification of CYMV only, while 5 trees showed the amplification of CLa and CYMV both, indicating the presence of mixed infection in the field. Amplification of no pathogens was observed in 3 citrus trees having the symptom of citrus greening (Table 2). The detection of greening

| Table 2—Test results of the multiplex PCR detection assays from midrib of sweet orange leaves of orchard samples |
|---------------------------------|-----------------|-----------------|-----------------|
| Samples                        | Number of plants with visible symptom/Total number of plants | Number of sample positive in PCR/Total number of plants |
|                                | CYMV | CLa | CYMV+CLa | CYMV | CLa | CYMV+CLa |
| Orchard 1                      | 0/10 | 8/10 | 0/10     | 0/10 | 5/10 | 0/10     |
| Orchard 2                      | 4/8  | 1/8  | 1/8      | 4/8  | 0/8  | 1/8      |
| Orchard 3                      | 3/7  | 0/7  | 2/7      | 4/7  | 0/7  | 1/7      |
| Orchard 4                      | 4/9  | 0/9  | 2/9      | 5/9  | 0/9  | 1/9      |
| Orchard 5                      | 4/10 | 0/10 | 2/10     | 3/10 | 0/10 | 2/10     |
| Total                          | 15/44| 9/44 | 7/44     | 16/44| 5/44 | 5/44     |

| Fig. 2—Detection of CLa and CYMV by multiplex PCR from the mixed tissues of midribs of leaves of sweet orange infected by citrus greening bacterium and leaf lamina of sweet orange infected by CYMV (3:1 ratio). M, 1 Kb DNA Ladder; 1, CYMV primers only; 2, CLa primers only; 3, Both the primers and MgCl₂ 1.5 mM; 4, Both the primers and MgCl₂ 3.0 mM. |
| Fig. 3—Detection of CLa and CYMV by multiplex PCR using DNA template either from leaf lamina or midrib of sweet orange infected by both the pathogens. M, 1 Kb DNA ladder; 1, leaf lamina and CYMV primers; 2, midrib of leaves and CYMV primers; 3, leaf lamina and primers of CLa and CYMV; 4, midrib of leaves and primers of CLa and CYMV; 5, midrib of leaves and primers of CLa; 6, lamina and primers of CLa. |
bacterium is some times erratic because of their uneven distribution in plants. Nevertheless, multiplex PCR is a sensitive technique for detection of CYMV and CLa as both could be detected in 5 samples out of 7 samples showing apparent symptoms of both pathogens.

Discussion

The multiplex PCR can save time and energy 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 leaves but a protocol has been used for the detection of a bacterium and viruses in olive tree. In this study, detection of bacterium from the naturally infected olive tissues was not possible unless the bacterium was enriched on semi selective medium for 72 h for bacterial DNA isolation. However, detection of greening bacterium directly from infected tissues is important. The use of DNA from midrib of leaves of infected trees demonstrated that, in case of mixed infection of 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 CYMV either singly or together if present 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 mixed-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 MgCl₂ concentration and other parameters used in the standard PCR. Using the standard PCR conditions, we could identify only CYMV targets from mixed DNA templates even after increasing the DNA two-fold from citrus infected by CLa. Similar observation was made when DNA isolation was done from mixed tissues. However, with increased concentrations of MgCl₂ and increased DNA template from CLa-infected leaf, both targets were successfully amplified. In contrast, only increased concentrations of MgCl₂ were required to amplify both the bacterial and viral DNA targets from the citrus trees infected by both pathogens. Consequently, the multiplex PCR, which can detect and identify simultaneously greening bacterium and CYMV in citrus trees appears 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.

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