

Original Research Article

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## Molecular Detection of Citrus Yellow Mosaic Virus (CYMV) and Citrus Greening Bacterium (CGB) in Sathgudi Sweet Orange by Duplex Polymerase Chain Reaction (dPCR)

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### ABSTRACT

Citrus is one of the most economically important fruit crop in India. Commercially grown citrus group in India includes sweet orange, acid lime and mandarin. Various factors constraint fruit production globally but the major factor is diseases caused by fungal, bacterial and viral pathogens. Citrus yellow mosaic virus (CYMV), a viral disease and Citrus greening bacterium (CGB), a bacterial disease are the two most important diseases that are impending fruit production throughout the world. The combined incidence of the diseases in Andhra Pradesh was found to be 32 to 68%. A rapid and reliable duplex PCR is developed for the simultaneous detection of CYMV and CGB in Sathgudi sweet orange plants. Initially total DNA was isolated from mixed infected field samples for both pathogens were detected simultaneously through duplex PCR. Using duplex PCR two different fragments of 726 bp and 451 bp specific to CYMV and CGB respectively were simultaneously amplified. The consistent result of duplex PCR was compared with simplex PCR for detection of each pathogen. The duplex PCR method developed in the present investigation proved to be highly sensitive, economic and reliable method for detection of both CYMV and CGB in citrus plants. The technique would also prove highly useful in disease survey, nursery certification and quarantine applications.

### Keywords

Citrus Yellow Mosaic Virus (CYMV), Citrus Greening Bacterium (CGB), Duplex PCR, Simultaneous Detection.

### Article Info

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### Introduction

Citrus is one of the important fruit crops grown throughout the world. It is grown in more than 50 countries of the world and is one of the choicest fruits having high consumer's preference both as fresh fruit as well as for its refreshing processed juice. Citrus cultivation is facing plethora of production constraints viz., Non availability

of disease free planting materials, bud wood transmissible diseases, scarcity of water for irrigation, cultivation in unsuitable soils, etc. Citrus species is prone to 150 types of diseases & disorders by fungal, viral, bacterial infections. Some of these diseases are Greening, Tristeza, Ring spot, Mosaic, Ganoderma Root Rot, Powdered Mildew,

and Anthracnose or whither tip, Sooty mould, Blue mould.

*Citrus yellow mosaic virus* (CYMV) disease is common disease where ever Citrus is grown as a commercial crop. In India CYMV was first described by Murthi and Reddy (1975). It was later studied in detail by Ahlawat *et al.*, (1985, 1996a and 1996b). Main symptoms in field trees were mosaic pattern with irregular yellow or light green patches alternating with normal green leaf area irregularly distributed all over the leaf without any definitive pattern (Fig. 1A). The losses caused by the mosaic disease were apparent in Sathgudi sweet orange orchards in Andhra Pradesh and Karnataka because several orchards with trees 4 to 10 years old were abandoned since they were no longer productive. The reduction in fruit yield was 77% in 10 years old trees and fruit from affected trees had 10% less juice and ascorbic acid (Reddy and Murti, 1985). It was described as a new graft transmissible disorder in sweet orange characterized by yellow mottling of leaves and yellow flecking along the veins.

Citrus Huanglongbing (HLB), earlier known as citrus greening disease (CGD), is one of the most destructive diseases of citrus and responsible for decline in AP and elsewhere in the country (Ahlawat *et al.*, 1995; Bove *et al.*, 1996; Gopal *et al.*, 1999, 2001). Symptoms caused by HLB were characterised by yellowing of veins and adjacent tissues, followed by premature defoliation, dieback of twigs, decay of feeder rootlets and lateral roots, decline in vigour, and ultimately the death of the trees (Fig. 1B). Diseased leaves become hardened and turn outward, while young leaves, which develop after premature defoliation, are small and slender with symptoms resembling those of zinc deficiency. Trees affected with greening become stunted, bear

multiple off season flowers, most of which fall off, and produce small misshapen fruit with thick, pale green peel.

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 (Lockhart and Olszewski, 1993) 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 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 (Korsten *et al.*, 1993; Varma *et al.*, 1993) the use of specific DNA probes has proven to be more reliable and as sensitive as the electron microscopy (Jagouiex *et al.*, 1996) but it is time consuming.

Recently, both the pathogens have been detected by a standard PCR, using the DNA extracted from leaf for CYMV (Baranwal *et al.*, 2003) and from midrib and petiole for CLa (Hocquellet *et al.*, 2000; Ahlawat *et al.*, 2003). In view of the increasing interest in plant pathology for the detection of more than one targets, such as mixed infection (Fig. 3) of viruses and bacteria (Bertolini *et*

*al.*, 2003), and viroids and viruses (Singh and Nie, 2003) in single reaction, multiplex PCR protocols have been developed. We describe a duplex PCR for the detection of a bacterium and a DNA virus frequently infecting sweet orange trees.

## Materials and Methods

### Plant Materials

Mixed infected samples (CGB and CYMV) were collected from Sathgudi sweet orange trees at Citrus Research Station, Tirupati.

### DNA Isolation

DNA isolation and PCR detection of HLB and CYMV in sweet orange. The addition of sodium sulphite to Tris-EDTA to reduce the degradation of DNA (Gopal *et al.*, 2007) was followed for extraction of DNA. One ml of extraction buffer (0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 1M KCl, 0.65% sodium sulphite) was added to the ground tissue in an Eppendorf tube and incubated at 95<sup>0</sup>C for 10 min with occasional agitation.

The homogenate was placed on ice for 2 min and centrifuged for 10 min at 12 000 rpm. The supernatant was treated with Rnase (100 mg/ml) and the DNA was precipitated with 0.6 vol of ice-cold isopropanol. After centrifugation, sterile distilled water was added to the precipitate and heated briefly to 65<sup>0</sup>C to completely dissolve DNA. DNA was re-precipitated with 2 vol of ethanol and 0.1 vol of sodium acetate (pH 5.2) at -20<sup>0</sup>C for overnight. After incubation the tubes were centrifuged at 12 000 rpm for 15 min. The pellet obtained was washed with 70% ethanol (100 µl) and centrifuged at 12 000 rpm for 5 min. The pellet obtained was air dried, dissolved in TE buffer and stored at -20<sup>0</sup>C for further use.

### PCR amplification

PCR was performed in 50 µl of reaction mixtures, using 1 µM of each primer (Table 1), 200 mM each of dNTPs, 0.05 U/µl of Taq DNA polymerase, 1X PCR reaction buffer, 2.0 mM of MgCl<sub>2</sub> and 6 µl of DNA template. The amplification was performed in a thermal cycler (Carbett Research, Australia). PCR conditions used were one cycle of 95<sup>0</sup>C for 2 min, 30 cycles of 94<sup>0</sup>C for 1 min, 58<sup>0</sup>C for 1 min, 72<sup>0</sup>C for 1 min and one cycle of 72<sup>0</sup>C for 10 min. PCR products were analysed in 1% agarose gel electrophoresis in 1X TBE buffer containing ethidium bromide and the gel was observed under (UV) transilluminator and photographed.

### Results and Discussion

The mixed infections of CYMV and CGB from citrus were detected simultaneously from the midrib of leaves from sweet orange trees infected by both pathogens (Fig. 1C). CYMV could be detected from midrib as well as leaf lamina, while CGB was detectable only from midrib and not from leaf lamina of citrus trees infected by both pathogens.

In the present investigation amplified product of expected sizes i.e. 726 bp and 451 bp respectively were successfully obtained from CYMV and greening infected citrus leaf samples by duplex PCR by using two different set of primer pairs specific for ORF-III region in CYMV and 16 S rDNA region in CGB, while no amplicon was obtained in healthy plant samples (Fig. 2).

Evaluation of 23 field samples from citrus orchards in Citrus Research Station, Tirupati, 10 samples showed the presence of only CGB. Of 23 samples, 10 trees showed the amplification CYMV of and CGB both,

indicating the presence of mixed infection in the field. Amplification of no pathogens was observed in 3 samples. The detection of greening bacterium is sometimes erratic because of their uneven distribution in

plants. Nevertheless, duplex PCR is a sensitive technique for detection of CYMV and CGB as both could be detected in 10 samples out of samples showing apparent symptoms of both pathogens.

**Fig.1a** Citrus Yellow Mosaic virus symptoms on sweet orange leaves



**Fig.1b** Citrus Greening Bacterium symptoms on sweet orange leaves

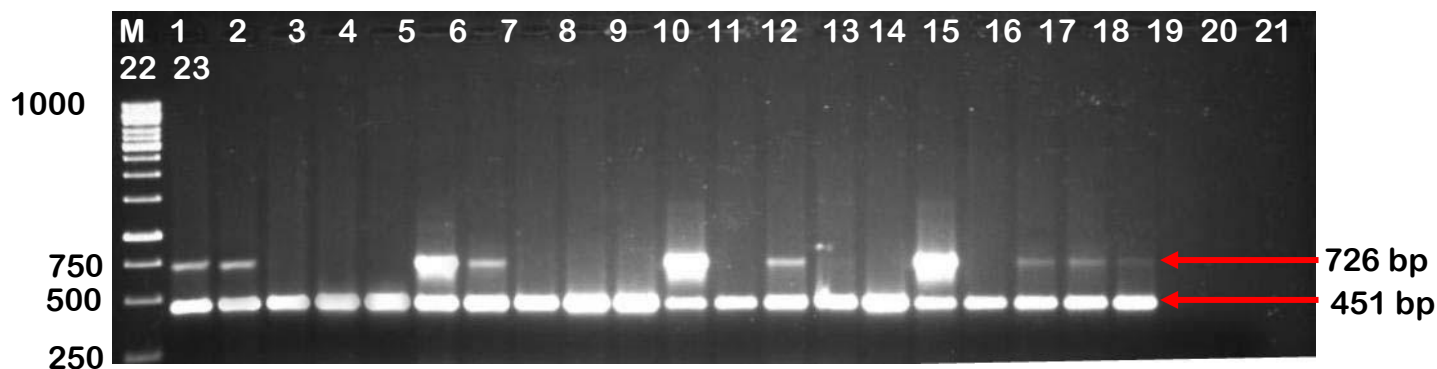


**Fig.1c** Mixed infections of CYMV and CGB on sweet orange tree





Fig.2 Simultaneous detection of CYMV and CGB from naturally infected field samples by duplex PCR



The duplex 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 (Bertolini *et al.*, 2003).

In this study, 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 duplex PCR could detect the infection of CGB and CYMV either singly or together if present in the tree. Performance of a duplex 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 duplex PCR reaction needs adjustments in the amount of primers, dNTPs and MgCl<sub>2</sub> concentration and other parameters used in the standard PCR

(Chamberlain and Chamberlain, 1994). In the present study, we optimised the duplex PCR conditions and amplified product of expected sizes i.e. 726 bp obtained from CYMV and 451 bp from CGB. Consequently, the duplex 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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