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Natural Occurrence of mixed infection of Citrus Yellow Mosaic virus (CYMV) and Indian Citrus Ring Spot Virus (ICRSV) and their Detection by Duplex PCR in Sweet Orange

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A B S T R A C T

Survey of Sathgudi sweet orange was done in the Nagri village of Chittor district in the A.P. state during November, 2006. Incidence of citrus yellow mosaic disease was observed up to 50% in citrus plants. A few plants also showed unusual symptoms of vein clearing and mosaic. Bacilliform and flexous rod shaped of virus particles were seen in electron microscopy in samples of the diseased citrus plants showing vein clearing and mosaic. Immunosorbent electron microscopy indicated the baciliform particles were of *Citrus Yellow Mosaic Virus* (CYMV) while flexuous particles were of *Indian citrus ringspot virus*. Since ISEM can not be used for routine detection, a duplex PCR for detection of both the viruses was standardized, using specific primers of CYMV and ICRSV providing amplification product of 537 bp and 1121bp. The sequencing of PCR product confirmed that the amplified PCR product of ICRSV and CYMV. The duplex PCR provide a useful and rapid method for molecular detection of two viruses. The technique should prove highly useful in disease surveys, nursery certification and quarantine applications.

Introduction

Citrus is an important fruit crop grown in more than 140 countries. Its productivity is affected by number of graft transmissible viruses and virus like pathogens. Their detection in propagating material is an important requisite to ensure the production of healthy planting material in India.In a survey of sathgudi sweet orange in Andhra Pradesh during November, 2006 a few plants showed unusual symptoms of yellowing and vein banding of leaves. Electron microscopic studies of such leaf samples indicated the presence of two viruses viz., CYMV and ICRSV.

The concentration of viruses in fruit trees such as citrus is generally low and immunolgical assays may not be reliable. PCR on the other hand is a more sensitive and reliable detection assays for viruses even when they are present in lower concentration. CYMV is a badnavirus of family

caulimoviridae and affects sathgudi sweet orange and other citrus species in Southern India (Ahlawat *et. al.,* 1996; Huang and Hurtung, 2001). ICRSV is a mandsrivirus of flexiviridae and commonly affects kinnow mandarin in Punjab and Rajasthan (Ahlawat *et al.,* 2003). Standard PCR has been developed for detection of CYMV (Baranawal *et al*., 2003) and ICRSV (Hoa *et, al.,* 2004). In view of the increasing interest in plant pathology for the detection of more than one targets, such as mixed infection of viruses and viroids (Singh and Nie,2003) in single reaction, multiplex PCR protocol have been developed. However individual detection of CYMV and ICRSV by PCR or RT-PCR is not only time consuming in but it is also more expensive. Therefore a cost effective duplex PCR was standardized for simultaneous detection of CYMV and ICRSV in the present study.

Materials and Methods

Collection of CYMV and ICRSV culture

Survey of Sathgudi Sweet Orange was done in the Nagri village of Chittor district in the Andhra Pradesh State during November 2006.

Maintenance of virus culture

Bud stick plant showing unusual symptoms were wedge grafted on 1 year old healthy seedlings of sweet orange and maintained in the insect free greenhouse. After 6 months leaves from grafted plants were used for Electron microscopy and PCR detection. The infected plant material of sathgudi sweet orange collected from Nagri, Chittur District of A. P. was wedge grafted on healthy sweet orange seedling. The grafted plants were maintained in the glasshouse (Fig.1). After six months, leaf material was used for detection studies by standard and duplex PCR. Virus cultures of CYMV and ICRSV maintained on sweet orange in the glass house were used as positive control. Healthy seedlings of sweet orange were used as negative control.

E.M.

Bud stick infected collected leaves from the citrus orchard from the grafted plants were used for EM studies. A standard method of trapping and decoration procedure of ISEM (Derrick, 1973 was used to observe virus particles in the infected sweet orange leave (JEOL100)CX-11) at the Plant Virology Unit, Division of Plant Pathology, IARI, New Delhi-12 (Fig.2a and b).

Isolation of total DNA and RNA from plant leaves

Total DNA was isolated from 100 mg leaves of wedge grafted infected plants and healthy plants of sweet orange using DNeasy plant mini kit (Qiagen Gmbh, Hilden, Germany) as per manufactures instructions. Similarly total RNA was isolated from the 100mg leaves of the infected ICRSV+CYMV or only ICRSV infected sweet orange plants using RNesy plant mini kit (Qiagen Gmbh, Hilden, Germany) as per manufactures instruction. A primer pair for ICRSV was designed and synthesized from a genome sequence of ICRSV (AF 406744) the forward primer was from 5' end of ORF 2 of triple gene block while the reverse primer was from 5' end of ORF5 of coat protein. For amplification of CYMV a previously published primer pair designed from ORF 6 and 5' intergenic region was used (Hung and Hartung,2001). The details of primers sequence, and size of PCR product is given in Table1.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for ICRSV

First strand of cDNA was synthesized for ICRSV using 4 µL of total RNA and reverse transcription (RT) mixture containing reverse primers of ICRSV at a concentration of 0.2

µm, 20u M-MuLv reverse transcriptase enzyme (Fermentas, Germany), 4 µL of 5x reaction buffer and 0.3 Mm dNTPs. The total reaction mixture of 20 µL was incubated at 42 ^{o}C for 45 minutes. The enzyme was inactivated by heating at 70 ^{o}C for 10 minutes.PCR was performed using a reacting mixture containing 5 µL of RT reaction mixture, *Taq* DNA polymerase 5 U (Promega, Madison, USA), 5 µL of 10 x PCR buffer, dNTPs (Qiagen, Germany) 10 mM $MgCl_2$ and 25mM. Primer pair at a concentration of 0.2µm Table 1). The temperature profile consisted of a denaturation step at 94 ^{o}C (5 minutes), 94 ^{o}C (30s), primer annealing at 53 ^{o}C for (60 s), extension at 72 ^{o}C (60s), with a final extension of 10 min at 72 ^{o}C. 10 µL of amplified product were separated by electrophoresis in a 1.% agarose gel containing ethidium bromide at a concentration of 0.5 µg.mL^{-1} and photographed under UV illumination with an imaging system (BioRad XR documentation system).

Cloning and sequencing

Rest of the PCR product was purified using PCR purification kit (Qiagen Gmbh, Hilden, Germany). The Purified PCR product was ligated in to PGEM-T easy vector (Promega, USA) and Competent *Escherichia coli* (strain DH 5) was transformed by standard molecular biology methods (Sambrook and Russel, 2001). Recombinant clones were identified by colony PCR and Sequenced. The sequences were verified in NCBI BLAST.

Polymerase chain reaction (PCR) for CMBV

5 µL DNA isolated by commercial kit were used for PCR in a 50 µL reaction mix containing 0.2µm each of forward and reverse primer of CMBV(Table-1), *Taq* DNA polymerase 5 U (Promega, Madison, USA), 5 µL of 10 x PCR buffer, dNTPs each 10Mm and $MgCl_2$ 25Mm samples were amplified for 30 cycles, using a Mastercycler (Eppendorf, Germany). Each cycle consisted of denaturation at 94 ^{o}C (30s), primer annealing at 53-54 ^{o}C for (60 s), and extension at 72 ^{o}C (60s), with a final extension of 10 min at 72 ^{o}C. Ten microlitres of amplified product were separated by electrophoresis in a 1.% agarose gel containing ethidium bromide at a concentration of 0.5 µg.mL^{-1} and photographed under UV illumination with an imaging system (BioRad XR documentation system).

Cloning and sequencing

Rest of the PCR product was purified using PCR purification kit (Qiagen, Germany). The Purified PCR product was ligated in to PGEM-T easy vector (Promega, USA) and Competent *Escherichia coli* (strain DH 5) was transformed by standard molecular biology methods (Sambrook and Russel 2001). Recombinant clones were identified by colony PCR and Sequenced. The sequences were verified in NCBI BLAST.

Duplex-PCR (Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for IC RSV and Polymerase chain reaction (PCR) for CMBV)

For cDNA preparation were same as given PCR. cDNA was performance and 5µL of cDNA with 5µL of DNA. This mixture of cDNA and DNA was used for duplex PCR were used for PCR in a 50 µL reaction mix containing 0.2µm each of forward and reverse primer of CYMV (Table-1). We have using Qiagen multiplex kit (Qiagen Gmbh, Hilden, Germany). 25µL of multiplex PCR master mix, Q-Solution 5 µL and rest of RNAse free water. Samples were amplified for 30 cycles, using a Master cycler (Eppendorf, Germany). Each cycle consisted of denaturation at 94^{o}C (30 second) annealing at 53 ^{o}C for (60 s), extension at 72 ^{o}C (60s), with a final

extension of 10 min at 72 ^{o}C. Ten microlitres of amplified product were separated by electrophoresis in a 1.% agarose gel containing ethidium bromide at a concentration of 0.5 µg.mL^{-1} and photographed under UV illumination with an imaging system (BioRad XR documentation system).Three randam samples were collected and tested. The protocol could successfully amplify and detect both viruses in all samples the field. Out of 10 sample, duplex PCR detected CMBV and ICRSV in three samples.

Results and Discussion

Incidence of Mosaic disease was observed up to 50% f citrus planted in orchards. A few plants showed unusual symptoms of vein clearing and yellowing which may be due to infection of viruses. Two types of virus particles were seen in electron microscopy in samples of the diseased citrus plants showing vein clearing and mosaic. One particle was bacilliform and the other was flexuous. ISEM studies indicated that the baciliform virus particles of *Citrus Yellowmosaic virus* (CYMV) while flexuous particles were of *Indian citrus ring spot virus*. Pant and Ahlawat, 1996 was also observed same types of virus partial.

We found that in multiplex PCR amplification. The multiplex PCR kit (Qiagen Gmbh, Hilden, 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 extension of all primers used in the same reaction. In comparison to established methods the Qiagen multiplex PCR kit provides higher sensitivity of amplification due to the optimized Qiagen multiplex PCR buffer in combination with hot start Taq Polymerase. This allows amplification of multiple products with differing copy number in the multiplex PCR.

The duplex PCR provided the amplification product of ~1121 bp for ICRSV and ~537 bp for CMBV (Fig.3and 4). There was no amplification in healthy citrus plant. Citrus plants infected either by ICRSV or CMBV, only one PCR product was amplified. The sequencing of PCR product confirmed that the fragments amplified were of ICRSV and CMBV. The multiplux can save time and reduce the cost of PCR. The study indicated that RNA and DNA viruses can be detected simultaneously by duplex PCR.

Table.1 Primer sets used for PCR amplification of genome of CYMV sweet orange and ICRSV

Primer Name	Sequence	TM value	Fragment Size
CYMV 7011F	5’GAGCTATTAGAAGGAATCTC3’	**54.4**	~537
CYMV 18R	5’AACCAAGCTCTGATACC 3’	**55**	
ICRSVF5117	5’CTC TCC AAA CCC ATT GTC GT3’	**53.4**	~1121
ICRSVR6238	5’ATCACA GTA GTG CGG GAA GG3’	**55.4**	

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

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

Table.3 Reaction of different citrus cultivars upon grafting with mixed infected CMBV and ICRSV

Sr No.	Citrus cultivars	No of plant wedge graft	Symptoms of inoculated plants ICRSV	CMBV	ICRSV+CMBV
1	Mosambi	3	VC	YM	VC, YM
2	Rangpur lime	3	VC	YM	VC,YM
3	Rough lemon	3	VC	YM	--
4	Acid lime	3	--	YM	--

VC: Vein Clearing: YM: Yellow mosaic

Fig.1 Mixed symptoms of CYMV and ICRSV on sweet orange collected from A.P

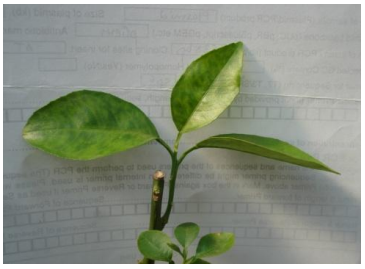
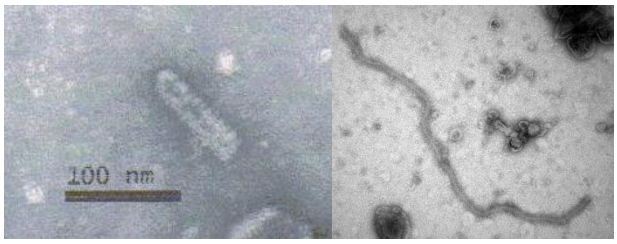


Fig.2 Electron micrograph of CYMV (a) and ICRSV (b) associated with sathgudi sweet Orange



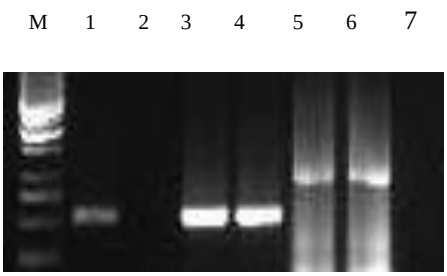


Fig.3 Detection of ICRSV and CYMV by RT-PCR and PCR from leaf of sweet orange. Lane M Marker 1 kb ladder: Lane,1 Positive control : Lane 2 Healthy control and lane 3,4 CMBV : Lane 5, 6 ICRSV, Lane 7 Healthy

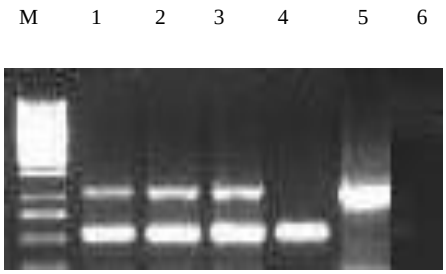


Fig.4 Detection of ICRSV and CYMV by duplex PCR using DNA template and cDNA from leaf of sweet orange. Lane M Marker 1 kb ladder: Lane,1-3 CMBV+ICRSV(Mixed Infected plant of sweet orange lane 4 CMBV Primer and Lane 5, ICRSV Primer Lane 6 Healthy Control

Mixed infection of RNA and DNA viruses such as ICRSV and can be detected by duplex PCR. The protocol could successfully amplify and detect both the viruses in few plants. The duplex PCR provide can a useful and rapid method for detection of two viruses. The technique should prove highly useful in disease surveys, nursery certification and quarantine applications. The technique should prove highly useful in disease surveys, nursery certification and quarantine applications. The multiplex PCR method developed here proved to be a sensitive and reliable method for the simultaneous detection of two different citrus viruses. The technique successfully detected both RNA and DNA viruses simultaneously using a single reaction. The method of extraction may affect the overall result s obtained using multiplex PCR (Bertolini *et al.*, 2000).

A multiplex RT-PCR was successfully used to detect four different viruses affecting strawberry (Thomson *et al.*, 2003). A multiplex polymerase chain reaction method was described for reliable sensitive and simultaneous detection of six multiple virus in citrus trees (Roy *et al*., 2005). Baranwal *et al.*, 2005 have been work out for simultaneous detection of citrus yellow mosaic virus and citrus greening bacterium. The multiplex PCR assay developed here is a single reliable rapid, sensitive, specific and cost –effective diagnostic for multiplex citrus viruses. It was successfully used for simultaneously detection of two viruses as well as multiple virus's infections in the same plant. It can also be useful for the phytosanitary assay in plant quarantine.

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