

Original Research Article

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PCR Detection of Citrus Yellow Mosaic Virus (CYMV) and Citrus Greening Bacterium in Different Tissue of Infected Citrus Plant

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ABSTRACT

Keywords

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Citrus Yellow Mosaic Virus (CYMV) disease and citrus greening bacterium are two important diseases in citrus. Citrus yellow mosaic virus (CYMV) disease is caused by bacilliform DNA virus while citrus greening disease caused by a fastidious bacterium (*Candidatus liberibacter asiaticus*) (Cla). Both the pathogens are infected citrus plants may detected by PCR using leaf tissues midrib tissue incase of greening. Both the disease are graft transmissible, and their relative distribution in tissues other than leaves are not known, we have demonstrated that CYMV and Cla can be detected by PCR in bark, and bud in addition to leaf tissue of infected citrus plants. These pathogens were absent in root tissue. It barks tissue can be a better tissue for DNA template preparation for PCR detection of CYMV. However, greening was amplified better in from leaf mid rib in comparison to bark tissue.

Introduction

Citrus yellow mosaic and greening disease are two common diseases in citrus orchard of Southern India. Both the disease are vegetatively propagated Citrus mosaic virus (CMBV) disease is caused by a bacilliform DNA virus, a Badnavirus of family Caulimoviridae. (Ahlawat, 1996; Huang and Hartung, 2001) The virus produces mosaic symptoms in Sathgudi Sweet Orange and Rangpur lime, while distinct golden mosaic symptoms are observed in Pummello (Ahlawat, 1996; Baranwal *et al.*, 2005). Citrus greening disease is widely distributed in India and other citrus growing countries. It is

transmitted the psyllid vector (*Diaphorina citri*). It is caused by a fastidious bacterium (*Candidatus liberibacter asiaticus*) (Cla). The bacterium is restricted in the sieve tube of host plants and causes symptoms such as interveinal chlorosis and mottling on leaves of citrus and similar to symptoms caused by Zn deficiency. Both the disease may occur in mixed infection also (Varma *et al.*, 1993; Baranwal *et al.*, 2005). CYMV and Cla is poor immunogenic. Hence serodiagnosis is not a preferred method. As an alternative quick and reliable detection by PCR is a preferred method for both the pathogens.

Their detection by PCR has been developed using whole leaf tissues for CMBV and midrib for Cla (Jagouiex *et al.*, 1996; Baranwal *et al.*, 2003; Ahlawat *et al.*, 2003) and from midrib and petiole for Cla (Hocquellet *et al.*, 2000). However not much is known about the distribution of CMBV and Cla in other tissues of is infected citrus plants. Therefore, an attempt was made to study the distribution of there two pathogens is leaf, bark, bud and roots using PCR. This will also facilitate in determining the suitability of tissues from infected citrus plants for preparation of DNA template for PCR detection of CMBV and Cla.

Materials and Methods

The cultures of CMBV were maintained on sweet orange Pummelo and Rangpur lime in the glasshouse conditions. PCR detection was used to determine its presence and relative distribution of CMBV leaf, bark, bud and root tissues of infected sweet orange, Pummelo and Rangpur lime (Fig 1). For determining the distribution of Cla, only sweet orange infected with Cla was used (Fig 2). The DNA template was prepared by simplified NCM based protocol and commercial kit and was used in standardized PCR reaction. 100 mg of plant tissue from leaf, bark, bud, and root of CMBV infected plants and 100 mg of midrib of leaves tissue, bark and bud tissue from Cla infected plants were homogenized in 1 mL of an alkaline solution (50 mmol·L⁻¹ NaOH, 2.5 mmol·L⁻¹ EDTA) using a sterilized mortar and pestle. No liquid nitrogen was used for the grinding of tissues. The resulting extract was incubated at room temperature (24–32 °C) for 15 min or centrifuged at 12,000 x g for 10 min and 5 µL of supernatant was spotted on untreated nitrocellulose membranes (NCM, Fig.4) (BAS 85, pore size 0.45 µm, Schleicher and Schuell, Keene, NH) that were then dried for 30 min at (24–32 °C). Individual spots (4.0 mm) for each sample were cut out using a paper hole-punch (Kangaro Industries,

Ludhiana, India) and eluted in 30 µL of sterile distilled water by incubating at 80 °C for 10 min on a heat block. The liquid was collected by centrifugation (termed 'NCM-eluted' extract). 20, µL were used for detection of Cla and CMBV DNA using PCR. Total DNA template was also obtained using commercial DNA isolation kit (Plant DNeasy mini kit, Qiagen, Gmbh, Hilden, Germany) from CMBV infected and Cla infected citrus plant. The protocol used as per manufacturer's protocol Here liquid nitrogen was used to grind the tissue.

Polymerase chain reaction (PCR) for detection of CMBV and Cla

20 µL of NCM eluted extract was used in PCR for detection of Cla. In case of CMBV, only 10 µL of NCM eluted extracted was used as DNA template 5 µL of DNA obtained by commercial kit was used for PCR detection of either CMBV or Cla. PCR was performed in a 50 µL reaction mix containing 0.1 µg each of forward and reverse primer of CMBV (5'GAGCTATTAGAAGGAATCTC, 5' AAC CAAGCTCTGATACCA), or Cla (5'TGG GTGGTTTACCATTTCAGTG, 5'CGCGACT TCGCAACCCATTG), *Taq* DNA polymerase 5 U (Promega, Madison, USA), 5 µL of 10 x PCR buffer, dNTPs (Qiagen, Germany) each 200 µmol·L⁻¹, and MgCl₂ 1.5 mmol·L⁻¹. Samples were amplified for 30 cycles, using a Mastercycler (Eppendorf, Germany). Each cycle consisted of denaturation at 94 °C (30s), primer annealing at 54 °C for CMBV and 58 °C for Cla (60 s), extension at 72 °C (60s), with a final extension of 10 min at 72 °C. 10 µL of amplified product were separated by electrophoresis in a 1.5% 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). All the experiments were repeated at least twice.

Results and Discussion

Electro micrograph of CMBV associated with sweet orange is presented in Fig 4. Amplification of CYMV was observed in bud, leaf and bark tissue of all three citrus varieties wise sweet orange, Rangpur lime and Pummelo. However, the PCR products was

more instance in comparison to leaves and bud in bark DNA obtained (Fig. 5) of CYMV irrespective of the DNA by method of commercial kit or membrane (Fig. 6). Amplification of CLa was also observed in bud, leaf and bark of sweet orange but intensity of PCR product was more in comparison to bark and buds (Fig. 7 and 8).

Table.1 Distribution on pattern detection of citrus mosaic virus (CMBV) in different parts of symptomatic sweet orange citrus trees

Sr. No	Citrus Cultivar	Leaf			Bark			Bud		
		R1	R2	R3	R1	R2	R3	R1	R2	R3
1	Sweet Orange	+	+	+	++	++	++	+	+	+
2	Rangpur Lime	+	+	+	++	++	+	+	+	+
3	Sweet Orange	+	+	+	++	++	+	+	+	+

Table.2 Dilution pattern of detection of citrus mosaic in different parts of symptomatic Pummelo of citrus

Dilution	Mid rib			Bark			Bud		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
0.1	--	---	----	--	---	---	----	----	---
1	+	+	+	+	+	+	----	---	--
5	++	++	++	++	++	++	+	+	+
10	++	++	++	++	++	++	+	+	+

Table.3 Distribution on pattern detection of greening bacterium in different parts of symptomatic sweet orange citrus trees

Tree no.	Complete greening healthy parts		Healthy shoots in infected twig		Complete infected twig		
	Midrib	Bark	Midrib	Bark	Mid rib	Bark	Bud
1	---	----	+	+	+	+	+
2	---	----	+	---	+	+	+
3	---	----	----	---	+	+	--
4	---	----	---	---	+	+	_
5	---	----	----	---	+	-	---

Table.4 Dilution pattern of detection of citrus greening bacterium in different parts of symptomatic sweet orange of citrus

Dilution	Mid rib			Bark			Bud		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
0.1	--	---	---	---	---	---	---	---	--
1	--	--	--	--	--	---	---	---	--
5	+	+	+	---	--	--	--	---	---
10	++	++	++	+	+	+	--	---	+



Fig.1 Symptoms of Citrus greening



Fig.2 Symptoms of Citrus mosaic Virus

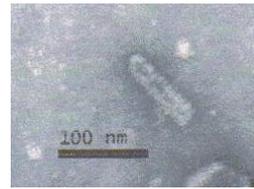


Fig.3 Electromicrograph of CMBV associated with sweet orange

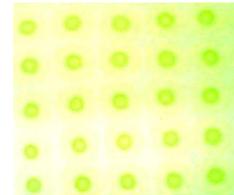


Fig.4 Supernatant spot on NCM of CYMV

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

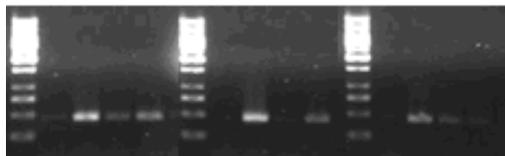


Fig.7 Lane 1,6,11 Marker 1 kb ladder; Lane 3,4,5 midrib; Lane ,8, 9,10, bark; lane 13,14,15 bud and lane2, 7, 12,,Healthy midrib, bark and bud tissue of citrus plant

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



Fig.8 Lane 1,8,15 Marker 1 kb: Lane 2,3,4,5,6 midrib: Lane , 9,10,11,12,13 bark: Lane 16,17,18,19,20 bud and lane 7, 14,,Healthy tissue of citrus

The study indicated that NCM based DNA extraction would be useful for PCR detection of CYMV in bud, bark and leaves but bark can be better test material for PCR detection of CYMV. However for Cla seems to be a better tissue than bark and bud. In most studies on PCR detection of Cla, midrib is commonly used for DNA template on our studies also confirm the same (Table 1 to 4). It also indicated that the concentration of Cla may be more in phloem tissue of midrib than bark. Detection of CYMV in bark, leaf and bud indicates that will spread of CYMV in

citrus plants and all the tissues can be used for PCR detection of CYMV. PCR amplification of CYMV and Cla was observed when DNA isolated by simplified membrane based method or by commercial kit, though the intensity of PCR product was slightly more when DNA from commercial kit was used. It is concluded that bark can better source material for PCR detection of CYMV leaves for Cla in infected citrus plant. NCM membrane based DNA isolation method is alternative to commercial kit as reported by Singh *et al.*, 2004. This study provides a

convenient reproducible and rapid method for the NCM based DNA Isolation method. It can also be useful for the phytosanitary assay in plant quarantine.

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