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One Step RT-PCR Method for Quick and Reliable Detection of *Citrus tristeza* virus (CTV) in Mid-Hills of Meghalaya, India

T. Ganesh[1*], T. Rajesh[1], A. Banerjee[2] and H. Rymbai[2]

[1]College of Post Graduate Studies, CAU, Umiam-793103, Meghalaya, India
[2]ICAR RC for NEH Region, Umiam-793103, Meghalaya, India

**Corresponding author*

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A B S T R A C T

Citrus tristeza disease (CTD) is the most dangerous and lethal viral disease among all the viral diseases that attack to the citrus. It is caused by *Citrus tristeza virus* (CTV) belongs to the genus Closterovirus in the family Closteroviridae. For quick and reliable detection of CTV a one-step RT-PCR technique was standardised. The total RNA from the leaf sample was extracted by using the Quiazen RNAeasy plant mini kit. The RT-PCR kit was used for the RT-PCR reaction and the primers specific to the coat protein component of the virus is used for detection. The PCR products were subjected to the gel electrophoresis on the 1% agarose gel containing 2.5 ug of ethidium bromide (Edbr). After gel running these products are visualized under UV transilluminator. Where the CTV positive samples yield an amplicon of 672 bps but where as in negative samples there was no such amplicon. Therefore the standardized RT-PCR is found to be most sensitive and reliable method for quick detection of CTV in the infected samples.

Introduction

Citrus tristeza virus (CTV) is the most important viral pathogen under the genus *Closterovirus* in family *Closteroviridae* and the members of this family are characterized by the long, flexuous and thread like particles with 10-12nm x 2000nm, having a single stranded positive sense RNA as genome (monopartite) which comprises about 19,256 nucleotides (Karasev *et al.*, 1995) with 12 ORF'S encoding at least 19 proteins (Bar-Joseph and Dawson, 2008) and about 95% of the genome of CTV is encapsidated by the 25 kDa, considered as major CP (Febres *et al.*, 1996; Ahlawat and Pant, 2003). CTV is the longest plant virus among all plant viruses (Yokomi, 2011) and it next to genome of animal *Coronaviruses*, the largest known genome worldwide (Albiach-Marti, 2013). CTV is not a seed (McClean, 1957) or pollen borne virus (Moreno *et al.*, 2008). It is a phloem limited virus, mostly transmitted by grafting and insect vector citrus brown aphid (*Toxoptera citricida*; Kirkaldy), (Michaud, 1998*;* Moreno *et al.*, 2008; Gottwald, 2010) and black aphid (*Toxoptera auranti*) (Yokomi and Garnsey, 1987; Hermoso *et al.*, 1984) in a semi-persistent manner (McClean, 1957).

Both nymphs and adults of the aphids were found to capable transmission of virus

(Yokomi and Garnsey, 1987). While the transmission efficiency can vary from one isolate to the other isolates of virus. In general host range of CTV is restricted to plant species of the genera *Citrus, Poncirus* (a citrus relative which is widely used as a rootstock) and *Fortunella* (subfamily Aurantioidae, family Rutaceae) (Moreno *et al.*, 2008). The virus shows greatest diversity in terms of its genetic and biological properties and it causes various kinds of symptoms on host plant like stunting, seedling yellowing or chlorosis, vein clearing, vein flecking, vein corcking, stem pitting, slow decline, quick decline all associated with the disruption of the phloem tissue and its functions (Lee and Blansky, 1989: Roistacher and Moreno, 1991; Kashyap *et al.*, 2013) or no symptoms depending on the virus strain, citrus cultivar, root stock, time of infection and environmental conditions (Lee and Rocha- Pena, 1992).

Materials and Methods

The CTV suspected leaf samples were collected from the citrus orchards in mid-hills of Meghalaya and these field collected samples were brought to the laboratory for further confirmation of the virus by using the molecular tools like RT-PCR. The presence of the CTV was confirmed through Reverse Transcription- Polymerase chain reaction (RT-PCR) using primers specific to Coat protein gene (CPg) component of the virus. First the total RNA from the leaf tissue was extracted by using the QIAGEN RNeasy Plant Mini Kit by following the manufactures protocol. The RT-PCR reaction was carried out by using the total RNA as template and one-step RT-PCR kit, where it contains the different components like one step RT-PCR buffer containing 12.5 mM $Mgcl_2$, 10 mM dNTP-mix, One step RT-PCR enzyme mix 1 µl/reaction and RNase free water. The RT-PCR amplifications were carried out in thermal cycler (AB applied Biosystems, Life technologies, Singapore). The reaction mixture contained 1 µl of total RNA, 1 µl each of 10 µM forward and reverse primers, 5 µl of 5X one step RT-PCR buffer, containing 2.5 mM $MgCl_2$,1 µl of 10 mM dNTPs,1 µl of one-step RT-PCR enzyme mix and RNase free water to make up volume up to 25 µl. RT-PCR cycles were composed of an initial Reveres transcription step at 50 °C for 30 min followed by an initial PCR activation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 1 min and extension for 1 min at 72 °C. A final extension was allowed for 10 min at 72 °C. The amplified products were subjected to electrophoresis on 1% agarose gel containing 2.5 µl of Ethidium bromide. Then the amplified products were loaded into the wells by mixing with loading dye (final concentration 1X) along with the 1kb DNA ladder as a marker and the gel was run for 1hr at a fixed voltage (7 V/cm). The gel was examined in a UV trans-illuminator (GelDoc XR, Biorad, Germany) and photographed. The presence of CTV was confirmed with the presence of 672 bp amplicon, but where as in healthy samples there was no such amplicon.

Results and Discussion

The yellowing, vein clearing, vein corking symptoms were observed on the plants in the orchards during the sample collection (Fig. 1). In some plants they did not show any such type of symptoms, even though they were infected with the CTV *i.e.*, latent infection. But during this study, any aphid vectors (*Toxoptera citricida*) as well as the stem pitting symptom related to CTV infection on khasi mandarin plants, were not noticed in the plants. Detection of CTV was carried out through (RT-PCR) by using primers specific to the coat protein gene of the virus. The extracted total leaf RNA (template) from each sample was assayed. In CTV positive samples, the RT-PCR amplicon of ~672 bp corresponding to the full length of coat protein

was amplified, where as in healthy samples such amplification was absent (Fig. 2). The results from RT- PCR assay confirmed the occurrence of CTV in field collected samples. During the survey, *Citrus tristeza virus* incidence in affected orchards was recorded as 30-70 % based on symptomatology. At least 6-7 plants with yellowing, vein clearing like symptom related to CTV was observed in during sample collection.

The presence of CTV was confirmed with the presence of 672 bp amplicon

Primers	Sequence (5'-3')	Working Concentration	Annealing temperature (Tm)
CP- gene			
Fp	5' CTCTAGATCTTTTGAATTATGGACGAC 3'	10 µM	
Rp	5' CGCGAATTCAACAGATCAACGTGTGT 3'	10 µM	60 °C

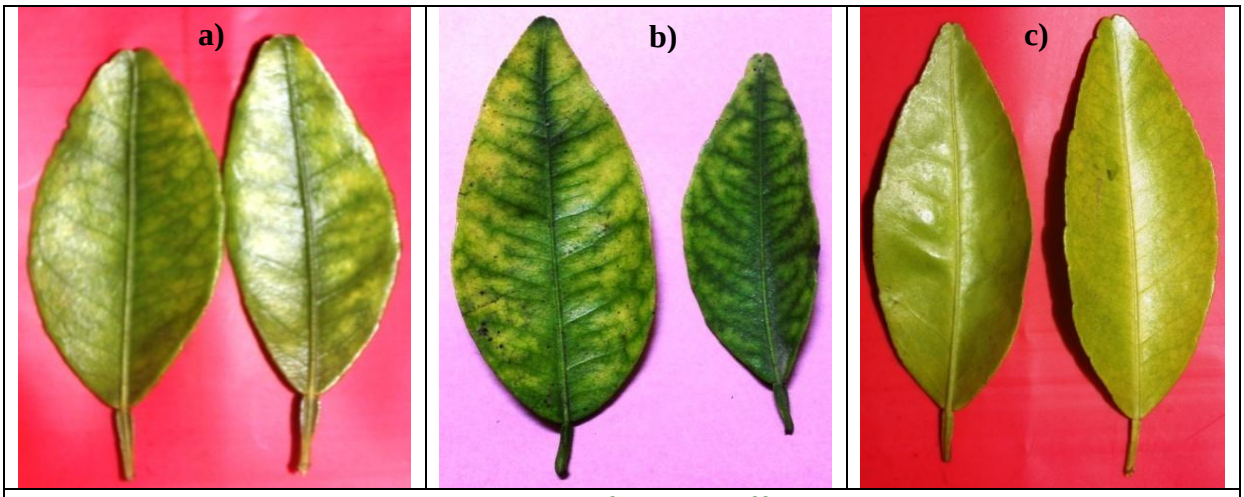

Fig.1 CTV Suspected samples collected from the different orchards (a. Amlarem; b. Bawrywmbong; c. Umiam) in the Mid hills of Meghalaya

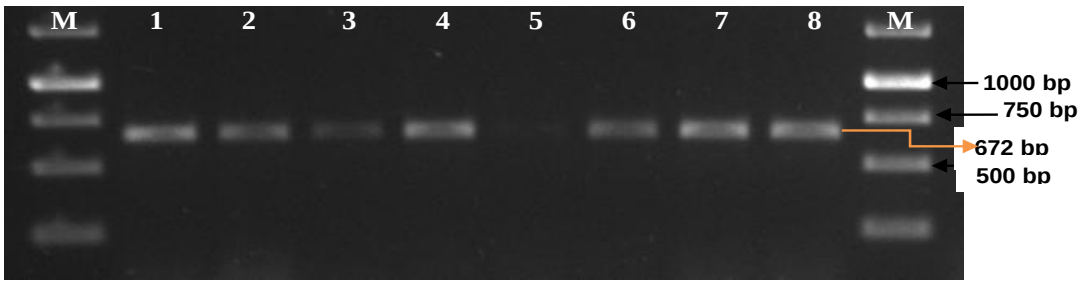

Fig.2 RT- PCR based detection of CTV using the Coat protein (CP) gene specific primers. Presence of CTV was confirmed by an amplication of 672 bp. M= 1 kb ladder as a marker; Lane 1-4 & 7, 8 field collected samples. Lane 5- Negative control; Lane-6 Positive control.

In the present study the one step RT-PCR method detected the CTV in the field collected samples from the Mid-hills of Meghalaya. The RT- PCR also detected the CTV in latent infected samples where these samples do not show the any kind of CTV symptoms in the orchards. The earlier researchers Hung *et al.*, (2000), also used the rapid and sensitive method, one-step RT-PCR technique for detection of CTV this method combines the reverse transcription (RT) and polymerase chain reaction (PCR) in one tube using an optimized buffer condition and efficient enzyme mix. Similarly Latanya *et al.*, (2010) detected the CTV in grape fruit, sweet orange and sour orange orchards in Jamaica by using the RT-PCR assay.

Nikolaeva *et al.,* (1995) and Kim *et al.,* (2000) were detected the severe isolates of CTV in California and Satsuma mandarin (*Citrus unshiu*) in Korea and in mandarin from Darjeeling hills in India. Hung *et al.,* (2000) also used the RT-PCR for differentiating CTV isolates into decline inducing or non- decline inducing in CTV infected field trees in Florida. In comparison with the two-step RT-PCR and ELISA tests the one step RT-PCR method has high sensitivity in CTV detection especially when CTV concentration is low in its host. Harper *et al.,* (2009) also used both one step and two-step reverse transcription-polymerase chain reaction (RT-PCR) for the detection of CTV in New Zealand. Gillings *et al.,* (1993) used the RT–PCR by using a one-step RT–PCR kit (QIAGEN) by following the manufacturers protocol. RT–PCR thermo cycling conditions followed were one cycle at 50°C for 30min, one cycle at 94°C for 2min, followed by 35 cycles at 94°C for 30s, 52°C for 30s and 72°C for 60 s and a final extension at 72°C for 10min. Amplified products were detected in a 1% agarosegel in 1xTBE buffer, stained with Ethidium bromide and visualised on a ultraviolet transilluminator. The one step RT-PCR standardized in this study can be useful for the reliable and sensitive tool for quick detection of CTV in latent infections and as well as in CTV infected samples where the virus concentration is low. This RT-PCR was found to be good molecular based tool for quick detection of CTV in the infected samples.

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