International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (2), pp. 001-006, February, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Status of *Citrus tristeza* virus (CTV) in Peninsular Malaysia

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Accepted 08 January, 2019

***Citrus tristeza* virus (CTV) is the most important viral disease of *Citrus* spp. and has a worldwide distribution. Results of ELISA and PCR showed that all Citrus varieties including *Fortunella* sp., *Citrofortunella microcarpa* and Citromelo in major citrus growing areas of Malaysia had a high infection rate with CTV. In most areas, pomelo however was free of infection, but in Cameron Highlands, we found some strains of CTV that were severe to Citromelo and pomelo. Phylogeny studies revealed that these strains were similar to CTV isolates from China and Japan and were very different from CTV isolates from USA and New Zealand.**

Key words: *Citrus tristeza* virus, phylogeny analysis, strain, CP gene, Malaysia.

INTRODUCTION

Citrus (namely species and hybrids of genera *Citrus*, *Fortunella and Poncirus*) is an important commercial fruit crop worldwide with approximately 7 million hectare cultivated surface (Fagoaga et al., 2006). Most of the citrus cultivars are propagated by grafting on rootstocks. The use of infected grafting materials for nursery propagation is primarily responsible for the distribution of virus and virus-like diseases in citrus (Abbas et al., 2006). Viral pathogens are transmitted by insect vectors as well as grafting, and cause large economic loses (Nolasco et al., 2009; Roy et al., 2005a).

Citrus tristeza virus (CTV) is a member of the genus *Closterovirus* (Family, Closteroviridae), distributed worldwide and causes one of the most economically important diseases of *Citrus* spp. (Bar-Joseph et al., 1989; Che et al., 2001; Gowda et al., 2009; Lair et al., 1994; Narvaez et al., 2000; Satyanarayana et al., 2001). CTV particles are flexuous, threadlike and 2000 nm in length (Huang et al., 2004; Jiang et al., 2008; Ruiz-Ruiz et al., 2007). They contain a positive-sense, single-stranded genomic RNA about 20 kb (Che et al., 2002; Hilf et al., 1995; Huang et al., 2004; Ruiz-Ruiz et al., 2007) with a molecular weight of 6.3 to 6.9 × 106 (Bar-Joseph et al., 1985) . The RNA contains 12 open reading frames (Che et al., 2002; Fagoaga et al., 2005; Narvaez et al., 2000; Satyanarayana et al., 2001) and encodes at least 19 proteins (Che et al., 2002, 2001; Huang et al., 2004; Satyanarayana et al., 2001). Two of these are capsid proteins of 25 and 27 kDa, which coat respectively about 95 and 5% of the virus length (Bar-Joseph et al., 1989; Jiang et al., 2008; Roy et al., 2005b; Ruiz-Ruiz et al., 2007). Members of Closteroviridae are unusual in their size, genomic composition and have a complex replication strategy (Che et al., 2002). CTV is phloem-limited and is transmitted in a semi-persistent manner by aphids such as *Aphis gossypii* (Bar-Joseph et al., 1989; Brown et al., 1988; Genc, 2005; Gottwald et al., 1999), *Toxoptera citricida* (Bar - Joseph et al., 1989; Broadbent et al., 1996; Brown et al., 1988; Gottwald et al., 1999; Roy et al., 2005b), *Aphis spiraecola* (Gottwald et al., 1999) and *Toxoptera aurantii* (Brown et al., 1988; Gottwald et al., 1999). *T. citricida* and *A. gossypii* are the most efficient vectors of CTV (Bar-Joseph et al., 1989; Brlansky et al., 2003; Brown et al., 1988; Gottwald et al., 1999; Roy et al., 2005b).

The virus is genetically and biologically diverse. Virus isolate, citrus cultivar, rootstocks, time of infection, and environmental conditions can affect the symptoms (Huang et al., 2004; Satyanarayana et al., 2001). A complex range of symptoms is produced under field conditions. There are three economically devastating field

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symptoms caused by CTV. The first is decline and death of trees grafted onto sour orange rootstock. The second is stem pitting of scions, regardless of rootstock (Broadbent et al., 1996; Garnsey et al., 1987; Genc, 2005; Gmitter et al., 1996; Satyanarayana et al., 2001). Trees affected with CTV stem pitting strains, have reduced fruit production and quality. A third type of symptoms can cause losses in tree nurseries termed 'seedling yellows'. Symptoms of seedling yellows are leaf chlorosis and stunting of sour orange, grapefruit and lemon seedlings (Ruiz-Ruiz et al., 2007).

Diagnosis of CTV infection was been performed indexing on sensitive indicator species for many years (Garnsey et al., 1987; Genc, 2005; Ruiz-Ruiz et al., 2007). Double stranded RNA (dsRNA) analysis can discriminate between some CTV isolates in infected plants (Pappu et al., 1993), but dsRNA pattern and pathogenicity are not necessary correlated (Moreno et al., 1990). Reaction with monoclonal (Albiach-Marti et al., 2000a; Bar-Joseph et al., 1997; Genc, 2005; Permar et al., 1990; Peroni et al., 2009; Rocha- Pena and Lee, 1991; Ruiz-Ruiz et al., 2007; Vela et al., 1986) and polyclonal antibodies (Albiach-Marti et al., 2000a; Bar-Joseph et al., 1997; Lair et al., 1994; Rocha-Pena and Lee, 1991; Ruiz-Ruiz et al., 2007), dsRNA (Dodds and Bar-Joseph, 1983; Korkmaz, 2002; Yang et al., 1999) molecular hybridization with complementary DNA (Rosner and Bar -Joseph, 1984) or RNA probes (Narvaez et al., 2000), or reverse transcription followed by PCR amplification (RT-PCR) have been successfully used for quick and sensitive detection and characterization of CTV (Ruiz-Ruiz et al., 2007).

Decline and death of most *Citrus* spp. grafted on sour orange (*Citrus aurantium L.)* can be avoided by using rootstocks resistant or tolerant to CTV (Dominguez et al., 2000). Some species, such as Mexican lime (*Citrus aurantifolia),* are very sensitive to CTV infection and show disease symptoms with most CTV strains, whereas others, such as grapefruit and sweet orange (Citrus sinensis), are affected only by severe strains. Although sour orange is sensitive as a rootstock, seedlings accumulate virus at low titer with most CTV isolates. General resistance to CTV had been observed in trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and resistance to some isolates occurs in Pomelo (*C. grandis*) and kumquat (*Fortunella crassifolia*) (Fagoaga et al., 2005). In Citrus growing areas where severe isolates of CTV are common, cross protection with mild strains (Lin et al., 2002) and coat protein (CP) mediated-resistance (Dominguez et al., 2002; Febres et al., 2008) can reduce yield losses (Dominguez et al., 2000).

According to our observations and discussion with Citrus growers in Malaysia, the Citrus trees are propagated by air layering method in this country. There were some reports on occurrences of CTV in Peninsular Malaysia that needs attention, as information on CTV in Malaysia is limited; hence, this research was carried out to determine the distribution and host range of CTV in Peninsular Malaysia.

Figure 1. Outline map of Peninsular Malaysia showing the locations of samplings performed in the present work.

MATERIALS AND METHODS

Plant materials

Field samples were obtained randomly from 340 citrus trees, including *C.aurantifolia*, *C. sinensis*, *C. maxima*, *C. reticulata*, *C. hystrix, Citrofortunella microcarpa, Fortunella* sp., seven *Poncirus* sp. and 10 weeds including *Passiflora foetida, Melothria pendula* and *Mikania micrantha* growing in Selangor, Pahang, Johor, Terengganu, Perak and Kedah states in Malaysia (Figure 1). Mature shoots and leaves of *Citrus* plants were gathered from all sides of the trees pointing east, west, south and northward and then samples mixed for the test. Petioles, midrib of leaves and bark of shoots were used to prepare extractions for ELISA and extraction of total RNA for RT-PCR.

ELISA determination

To diagnose *Citrus* trees infected by CTV, direct double antibody sandwich (DAS) ELISA was performed (Rocha-Pena et al., 1991). In this study one polyclonal antiserum (Bioreba) was used. Extractions were prepared from 0.5 g of shoot barks, midribs and petioles in 5 ml of 1 × PBST buffer (0.15 M NaCl; 0.015 M NaH_2PO_4; 0.05% Tween 20, pH 7.0). Positive reactions were defined as an OD_{405nm} two times higher than negative control.

1000 bp

500 bp

Figure 2. PCR product profile of some Malaysian *Citrus tristeza* virus isolates in 1.2% agarose gel. M, molecular marker; line 1 to 17, infected plants; line 18, healthy plant.

Nucleic acid extraction from citrus tissues

Total RNA (tRNA) was extracted from 0.2 g of shoot barks, midribs and petioles. First tissues were pulverized with liquid nitrogen by pestle and mortar and then collected in 1.5 ml sterile eppendorf tube. Each sample was suspended in 400 µl TES buffer (100 mM Tris-HCl pH 8.0; 2 mM EDTA; 2% w/v SDS) and 400 µl phenol/chloroform/isopropanol (25/24/1) and shook vigorously for 10 min. After centrifugation (14000 rpm) for 10 min, the supernatant (400 µl) was treated with 200 µl ethanol (99.8%) and used in RNeasy mini kit and tRNA extracted according to the manufacturer's instructions and was used as template for the amplification of the coat protein (CP) gene of CTV.

Primers

For amplification of the complete CP cistron (672 bp) of CTV, two primers were used based on Jiang et al. (2008) report. The sense primer was CP1: 5 -ATG-GAC-GAC-GAA-ACA-AAG-AA-3 and the anti sense primer was CP2: 5 -TCA-ACG-TGT-GTT-GAA-TTT-CC-3

cDNA synthesis and polymerase chain reaction amplification

By using tRNAs extracted from citrus tissues as templates and CP2 as primer cDNA was synthesized. The total reaction volume was 40 µl, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 10 mM DTT, 0.2 mM each of the four dNTPs, 1 µm CP2, 20 U reverse transcriptase, and 18.75 µl extracted RNA. First tRNA and primer were mixed gently, heated for 10 min at 65°C, and then immediately cooled on ice. After this, other materials were added and the contents were mixed gently and incubated at 25°C for 10 min, 42°C for 60 min and 72°C for 10 min., respectively. The PCR amplification was performed in 25 µl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.05 mM each of the four dNTPs, 2 mM MgCl2, 0.3 µM of each primer (CP1, CP2), 1.25 U Taq DNA polymerase (iNtRON Biotechnology) and 1 to 4 µl of RT mixture. The PCR cycling profile consisted of one cycle at 94°C for 5 min., followed by 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR amplified fragments were separated in 1.2% agarose gels in Tris-borate (TBE) buffer (89 mM Tris; 89 mM boric acid; two mM EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and analyzed using BIO imaging system (Syngene). A 100 bp DNA Ladder (Fermentas) was used as a nucleic acid marker.

Table 1- Percentage CTV infection from survey of plants in Peninsular using ELISA and PCR tests.

Variety	Total sample	No. infected with CTV	% infection
C. sinensis	21	17	80.95
Lemons	7	4	57.14
Citrumelo	3	2	66.67
C. limonia	2	2	100
C. hystrix	22	21	95.45
C. aurantifolia	62	59	95.16
C. reticulata	66	44	66.67
Fortunella sp.	15	15	100
C. microcarpa	50	50	100
Poncirus	7	0	0
Mikania micrantha	3	0	0
Melothria pendula	3	0	0
Passiflora foetida	4	0	0
C. maxima	92	2	2.02

RESULTS

PCR results showed that primers CP1 and CP2 amplified a part of the genome of all isolates of CTV and produced a band of about 672 bp in agarose gel electrophoresis (Figure 2). The results of CTV infection survey by using ELISA and PCR methods showed that the virus was present in all samples from different varieties of *Citrus* and related genera collected from Peninsular Malaysia (Table 1). This survey showed that in almost areas, all pomelo trees were free of virus infection, except for Cameron Highland areas where CTV was detected in both pomelo and citromelo trees. The sequences of the coat protein gene of these isolates were compared with some other isolates in the world. Phylogeny tree of these isolates in comparison with 10 isolates from other countries (Table 2) showed that AMC2 and AMC7 are very close to CT-W1, isolated from *Poncirus* in China and are close to NUagA isolate from Japan and ML12 isolate from China. They were grouped in one cluster. AMC6 and Bangalore isolates from India were placed in another cluster. Resistant break isolates (NZRB-M17, NZRB-G90 and NZRB-TH28) from New Zealand produced a completely separate cluster. These strains had been isolated from *P. trifoliata*. T36, T30 and T385 were grouped in another cluster and were the furthest from the others (Figure 3).

Table 2. *Citrus tristeza* virus isolates used in this study for drawing phylogenic tree.

Isolate	Pathogenicity or host	Accession number	Source	Reference
T385	Mild	Y18420	Spain	(Vives et al., 1999)
T30	Mild	AF260651	Florida, USA	(Albiach-Marti et al., 2000b)
T36	Quick decline	U16304	Florida, USA	(Karasev et al., 1995)
NUagA	Seedling yellows and stem pitting	AB046398	Japan	(Suastika et al., 2001)
Bangalore	Stem pitting	AF501867	India	(Roy et al., 2003)
ML-12	Stem pitting	EF028325	China	(Jiang et al., 2008)
CT-W1	*Poncirus*	FJ998191	China	
NZRB-G90	*Poncirus*	FJ525432	New Zealand	(Harper et al., 2010)
NZRB-TH28	*Poncirus*	FJ525433	New Zealand	(Harper et al., 2010)
NZRB-M17	*Poncirus*	FJ525435	New Zealand	(Harper et al., 2010)
AMC2	Citromelo	HQ012375	Malaysia	
AMC6	Pomelo	HQ012376	Malaysia	
AMC7	Pomelo	HQ012377	Malaysia	

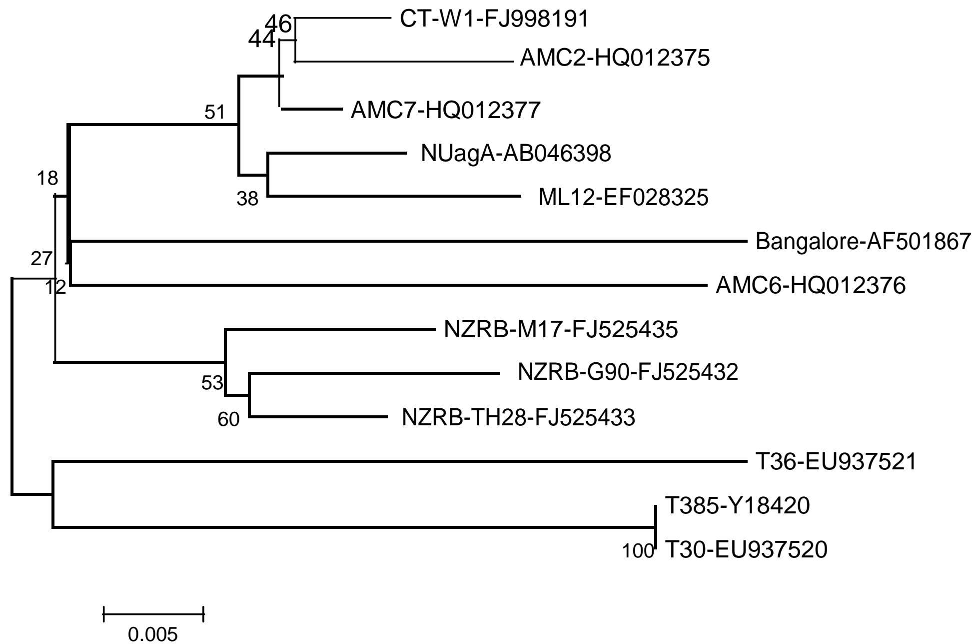

Figure 3. The bootstrap phylogenic tree of pomelo and *Poncirus* isolates of *Citrus tristeza* virus from Malaysia in comparison to 10 isolates from around the world using MEGA software version 4 (Tamura et al., 2007).

DISCUSSION

Results showed that the percentage of CTV infection in *Citrus* and relatives genera in Malaysia is very high but these plants were almost symptomless. No stem pitting and no vein clearing was observed. According to the statements of Satyanarayana et al. (2001) and Huang et al. (2004), it might be due to *Citrus tristeza* Virus strains or environmental conditions of Malaysia. Also, results of Garnsey et al. (1987) and Broadbent et al. (1996) confirm that propagation method of citrus in Malaysia can affects on appearance of CTV symptoms. However, further verification of the virulence status of this strains need using molecular characterization.

In Keluang area, virus infection was observed in three years old *C. hystrix*, *C. aurantifolia* and *C. microcarpa* trees which may indicate that these trees were propagated from CTV infected plant materials and infection was started from the off- farm source. Results of Table 1 showed that pomelo is free of CTV in the most

areas of Malaysia that indicates resistance of pomelo to CTV which is confirmed by Fagoaga et al. (2005), but infected pomelos can be found in Cameron Highlands. We suppose that some aggressive strains have been present in Cameron highlands imported from other countries, since these samples were collected from the citrus collection of Malaysian Agricultural Research and Development Institute (MARDI) of Cameron Highlands. This area is quarantined and separated by natural conditions from the other citrus groves. So, inhibition of citrus budwoods, cuttings and seedlings transfer from Cameron Highlands to the other states of Malaysia is recommended.

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