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Detection and characterization of *Citrus tristeza virus* stem pitting isolates in Jamaica

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Abstract An island wide survey for *Citrus tristeza virus* (CTV) in citrus orchards across Jamaica (13 regions) was conducted over 2 years. Trees (1, 885) showing virus-like symptoms as well as asymptomatic trees were randomly sampled for testing by ELISA and 55 samples from the 6 major citrus growing regions were graft inoculated on indicator plants. Most samples (74%) reacted to polyclonal antibodies against CTV in ELISA, while 20% were positive in tests using monoclonal antibodies specific to severe CTV strains. Samples collected from the 6 major citrus growing regions produced vein clearing and stem pitting symptoms on Mexican lime indicator plants (87%). In addition, stem pitting symptoms were induced on Duncan grapefruit, sweet orange, sour orange or sweet orange grafted on sour orange. Nucleotide sequencing of the coat protein gene sequences isolated from these samples indicated high identities (88 to 95.5%) among the Jamaican isolates and previously reported stem pitting strains from Central and North America and Eurasia (88 to 100%). The results suggest a shared ancestry with isolates from other geographical locations, rather than geographical speciation, and presumably separate CTV introductions into Jamaica.

Keywords Biological characterization · *Citrus tristeza virus* · Coat protein gene · Phylogenetic analysis

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Citrus tristeza virus (CTV), a member of the genus *Closterovirus* in the family *Closteroviridae*, is a phloem-limited virus primarily transmitted by aphids in a semi-persistent manner (Lee and Bar-Joseph 2000). The virus is geographically widespread and infects almost all citrus species of the *Rutaceae* (Rocha-Peña et al. 1995). Severe strains are the most devastating, producing three syndromes dependent on the species or scion-rootstock combination (Garnsey et al. 1987). Infections with seedling yellows strains are characterized by small chlorotic leaves, reduced root systems, and stunted sour orange (*Citrus aurantium* L.), grapefruit (*Citrus paradisi* Macf.) or lemon (*Citrus limon* (L.) Burn. f.) seedlings. Decline inducing strains (CTV-D) cause the decline and death of different citrus species, including sweet oranges

(*Citrus sinensis* (L.) Obs.), mandarins (*Citrus reticulata* Blanco), grapefruits, kumquats (*Fortunella crassifolia* Swing.) or limes (*Citrus aurantifolia* (Christm.) Swing.) propagated on rootstock species such as sour orange or lemon rootstock; while infections with stem pitting strains (CTV-SP) rarely result in scion death, but loss of vigour leads to drastic yield reductions even on CTV-D tolerant rootstocks (Moreno et al. 2008).

Citrus is an important traditional crop in Jamaica which contributes significantly to the country's gross domestic product. The domestic market is the largest market for citrus fresh fruit and accounts for approximately 85% of production (Young 2002). Fruits and juices are also exported to the United Kingdom, United States, Canada, and Barbados. Although CTV was first detected in Jamaica in the late 1950s, the pathogen currently poses a threat to the Jamaican citrus industry because of the recent establishment of the brown citrus aphid, *Toxoptera citricida* (BrCA), the most efficient vector, and serological analysis confirmed the presence of severe CTV strains in the 1990s. Further, 90% of Jamaica's citrus is cultivated on sour orange rootstock which is highly susceptible to CTV infection (Edman and Young 1999). In anticipation of losses on sour orange rootstock, efforts to develop certification schemes were initiated in 1999 and replanting with materials free of CTV on tolerant rootstock. However, surveys on the status of severe CTV-SP stains have been conducted on only two farms in one region of the island, St. Catherine. Given that these data provide the basis for the removal of CTV inoculum sources, this study evaluated the status and distribution of CTV-SP in the major citrus growing regions of Jamaica.

In 2003 and 2004, 1,885 citrus leaf and bark samples were collected in an island wide survey and tested for CTV in double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) using polyclonal and MCA-13 monoclonal antibodies (Bioreba, IN and Nokomis Corp, FL, respectively; Permar et al. 1990; Garnsey and Cambra 1991). A smaller random sample (55), collected from symptomatic and asymptomatic trees, was subsequently grafted on to Mexican lime indicator seedlings for general screening of CTV. Samples (41) that showed CTV infectivity, were later sub-inoculated onto Duncan grapefruit, sweet orange and sour orange, and sweet orange grafted onto sour orange to screen for severe CTV strains (Roistacher 1991). Two plants of each host were inoculated with each test isolate. Positive CTV controls included mild and severe strains (biotypes I and X, respectively, Lee et al. 1994) and negative controls were non-inoculated, healthy citrus seedlings. All seedlings were maintained in a temperature controlled greenhouse at 18–21°C (Roistacher 1991) and regularly fertilised. After successful CTV transmission, as evidenced by the survival of green graft inoculum after 3 weeks, symptom expression on new flush was monitored 4–12 months at which time stems were peeled and scored for stem pitting. The latter symptoms were scored on a scale of 0–5, where 0 represents no pitting, 1, 2, 3, 4 represent 1 to 15 stem pits, 16 to 30 pits, 31 to 50 pits, 51 to 80 pits, respectively, and 5 represents >80 pits and a rope-like appearance of the stem (Müller et al. 1996; Corazza-Nunes et al. 2006). The ratings were averaged for both inoculated plants of each indicator to give a consensus reading. To ensure that the mixture of CTV strains from the field isolates had not been affected by the sub-inoculations and to verify CTV infections on the indicator plants, ELISA and reverse transcription- polymerase chain reaction (RT-PCR) were carried out on the field isolates and indicator plants. An absorbance value (OD_{405nm}) higher than the negative mean (0-0.07) plus four standard deviation units (i.e. ≥0.100) was considered to be a positive in DAS-ELISA. Primers specific to severe and mild CTV were used in the RT-PCR assays (Huang et al. 2004).

The full length coat protein genes (*cp*) of five stem pitting inducing isolates were obtained with the primer pair, CTV-SF15851 (5′ATACGAAAATAAAAGGACGACCAG3′) and CTV-SR17262 (5′ACGCTAAACAAAGTGACGAGATTA3′), in RT-PCR (Life Technologies Inc, Rockville, MD, US). The forward and reverse primer sequences were derived from conserved regions of the *p27* and *p18* genes, respectively, of the T36 Florida severe CTV isolate (GenBank accession number U16304). Amplicons were purified using the QIAquick® PCR purification kit (Roche Diagnostics, Mannheim, Germany), cloned into the pGEM-T Easy vector (Promega, Madison, WI, US) and sequenced in both directions, with the universal M13 forward and reverse primers (University at Albany, Rensselaer, NY, US). Multiple sequence alignments of the Jamaican CTV *cp* and other published sequences obtained from the National Centre for Biotechnology Information (NCBI) were

performed using Clustal W (Thompson et al. 1994) and the sequences analysed with the MegAlign programme of DNASTAR to determine phylogenetic relationships. Bootstrap analysis, based on 1,000 replications, was conducted to determine the robustness of the tree topology (MegAlign, DNASTAR version 7.0).

DAS-ELISA testing of the survey samples confirmed that CTV strains are distributed across the island and that severe strains with MCA-13 reactivity are limited to 9 of 13 locations (data not shown). The locations were distributed across the six main citrus growing regions listed in Table 1 as well as in St. Ann, St. Elizabeth and St. Andrew. Of the 9 locations, only isolates from the six main citrus growing regions were biologically characterized. These isolates included MCA-13 reactive and MCA-13 negative isolates. Within 4 to 12 months, symptoms of leaf cupping, vein clearing and stem pitting or stunting were observed on 87% of the inoculated Mexican lime seedlings. Foliar symptoms associated with seedling yellows symptoms were not observed on inoculated grapefruit plants. However, stem pitting was observed with 73% of the grapefruit plants inoculated with samples from all six regions. More severe pitting (rating 4–5) on grapefruit was observed with samples

Table 1 Biological characterization of *Citrus tristeza virus* isolates from six major citrus growing regions of Jamaica

Location of origin		Biological detection							Serological and Molecular Detection		
		ML			DG	SwO	SwO/SO	SO	ELISA (%)		RT-PCR CTV (%)
		LC	VC	SP	SP	SP	SP	SP	all strains	severe strains	
Clarendon	Clarendon Park	mod	mild	2	0	0	0	nt	100	67	100
	Frankfield	sev	mod	3	nt	0	3	5	100	100	40
	Summerfield	mild	mild	5	5	1	nt	nt	60	40	100
Hanover	Burnt Ground	mild	mild	2	nt	0	nt	1	100	100	100
	Knockalva	mod	mod	4	2	nt	0	nt	100	100	100
	Montpelier	mod	sev	3	nt	0	nt	2	100	100	80
Manchester	Arscott	mod	mod	2	2	0	0	nt	80	40	80
	Campbell's Castle	sev	mod	5	nt	0	nt	1	100	50	100
	Mile Gully	sev	mild	3	nt	0	nt	2	100	67	100
St. Catherine	Linstead	mod	mod	3	4	0	0	3	100	75	100
	Bybrook	mild	mild	2	nt	0	0	0	100	0	100
	Enfield	mod	mod	5	5	0	0	2	100	100	100
	Wakefield	mod	mod	5	nt	2	nt	2	100	100	75
St. James	Blue Hole	mild	mod	2	1	0	0	0	100	67	100
	Montpelier	sev	mod	2	0	0	0	0	80	20	100
	Seven Rivers	mild	mild	2	nt	0	nt	nt	100	100	100
St. Mary	Agualta Vale	mild	mild	0	2	nt	1	0	0	0	75
	Fontabelle	mild	mild	0	nt	0	0	0	50	50	75
	Greenwood	none	none	0	nt	0	nt	nt	0	0	33
	Esher	none	none	0	nt	nt	nt	nt	100	0	0

Indicator host plants ML Mexican lime, DG Duncan grapefruit, SwO Madam vinous sweet orange, SO sour orange, SwO/SO sweet orange grafted onto sour orange rootstock. Observed symptoms, LC leaf cupping, VC vein clearing, SP stem pitting, mild symptoms, mod moderate symptoms, sev severe symptoms, nt not tested (n=55, DG n=12, SwO n=20, SO n=13, SwO/SO n=14, where n = number of samples analyzed for the respective indicator plant). SP data represent symptom severity that was rated on a scale of 0 to 5, where 0 represents no pitting, 1, 2, 3, 4 represent 1 to 15 stem pits, 16 to 30 stem pits, 31 to 50 pits, 51 to 80 stem pits, respectively and 5 represents >80 pits and severe pitting in the stem segment

Details of the DAS- ELISA assays with polyclonal and MCA13 antibodies are given in the text (n=55). RT-PCR primers were designed to amplify 672 and 320 bp amplicons of CTV- severe strains

from two regions, Clarendon and St. Catherine. The incidence of stem pitting was noticeably less (rating 1–3) in sweet orange and sweet orange grafted on to sour orange indicator plants and symptoms were obtained with samples from three regions, namely Clarendon, St. Catherine and St. Mary.

Symptoms typical of seedling yellows were not observed on inoculated sour orange seedlings (or the controls), however stem pitting (rating 1 to 5) was induced in these indicator plants by samples from four regions. Samples from locations in St. Catherine induced stem pitting symptoms in both sour orange and Duncan grapefruit, while samples from St. Catherine and St. Mary produced symptoms in sour orange and sweet orange. Moreover, not all samples eliciting stem pitting were positive in both DAS-ELISA and RT-PCR. RT-PCR using the *cp*- specific primers enabled the detection of virus in 25% of the samples that tested negative in MCA-13 DAS-ELISA and infections in 3% of the samples were only detected by DAS-ELISA. Given that the isolates have not been tested with all available CTV primers, it may be premature to assume the presence of unique strains.

RT-PCR amplicons (1.4 kb) obtained with grapefruit, sweet orange and sour orange CTV-SP samples from five regions were isolated and sequenced. Nucleotide sequence analysis showed identities of 88 to 95.5% among the Jamaican isolates and 88 to 100% identities among isolates from various geo-

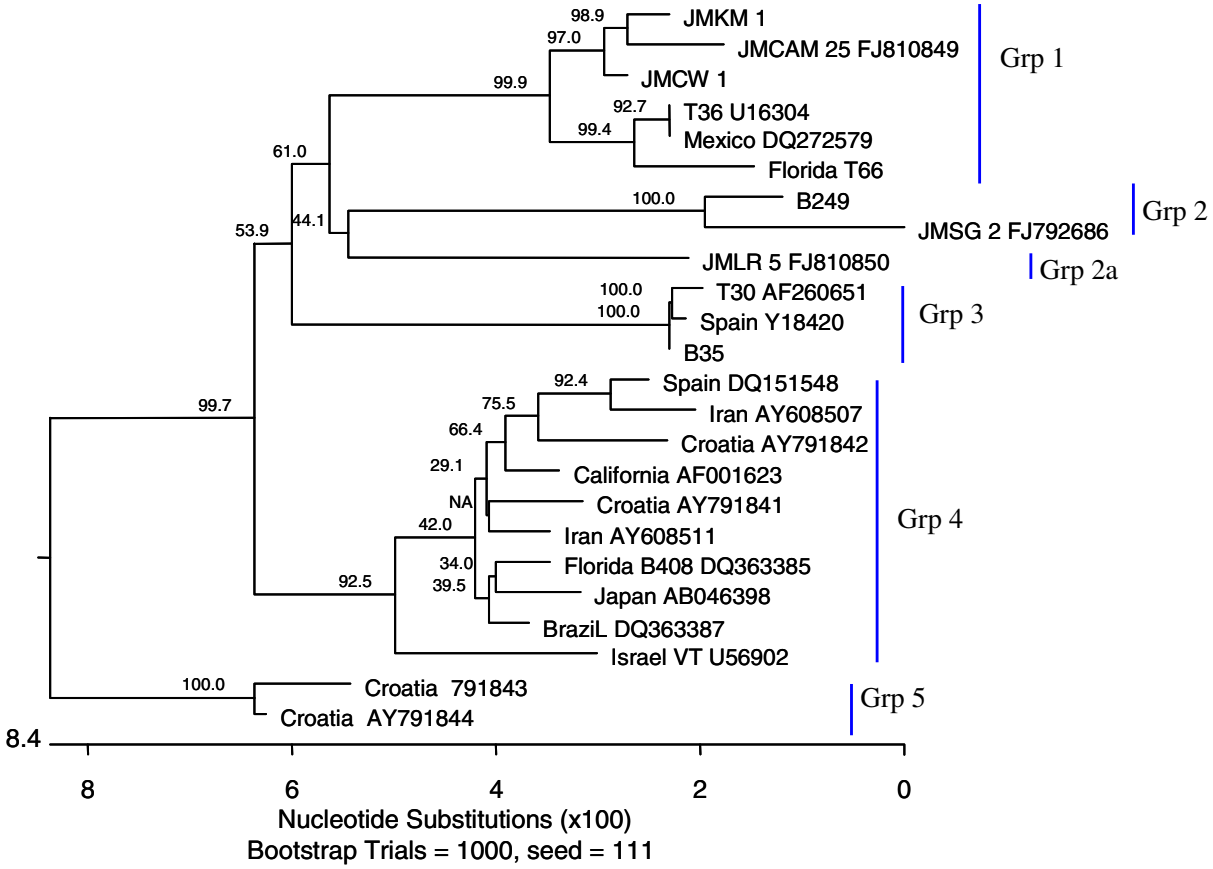

Fig. 1 Phylogenetic relationships among Jamaican stem pitting strains (JMKM1 [grapefruit CTV-SP, Knockalva, Hanover], JMCAM25 [sweet orange, Wakefield, St. Catherine], JMCW1 [grapefruit, Blue Hole, St. James], JMSG2 [sour orange, Campbell's Castle, Manchester], JMLR5 [sweet orange, Summerfield, St. Catherine]) and strains from various geographical locations. Isolate designations are followed by their GenBank accession numbers. *cp* sequences of the T66, B249, B35 isolates from Florida were provided by V.J. Febres. Construction of the tree was based on multiple alignments of derived nucleotide sequences of the entire coat protein gene coding region. Numbers close to the branches on the tree indicate bootstrap values that resulted from 1,000 replications

graphical locations. Phylogenetic analysis of severe and mild CTV strains revealed five clades with the Jamaican isolates clustering in two groups, 1 and 2 (Fig. 1). Of note, these CTV-SP strains were MCA-13 reactive. The Jamaican isolates that induced grapefruit and sweet orange stem pitting clustered with the severe quick decline and stem pitting strains from Mexico and Florida (T36), but the Jamaican sour orange stem pitting strain was most similar to and grouped separately with the Venezuelan B249, a grapefruit and sweet orange stem pitting strain (Febres et al. 2003). Other severe isolates formed two groups, 4 and 5; one with isolates from Eurasia, the US and Brazil in a related cluster, and those from Croatia in a distinct branch. Mild strains formed a unique clade (group 3). The analysis therefore suggests the presence of Jamaican CTV-SP strains with biological and molecular properties similar to those of Florida, Mexico and Venezuela.

This is the first report of the occurrence and prevalence of grapefruit and sweet orange CTV-SP in regions of Jamaica outside of St. Catherine. Previous studies by Lee et al. (2002) reported on the incidence of CTV-D, grapefruit and sweet orange CTV-SP in Enfield, St. Catherine, using strain specific CTV probes. Since then, CTV isolates from three locations of the same region in St. Catherine (Bybrook, Enfield, New Works) were subjected to similar array of strain specific probes and comparable results were obtained (Myers and Amin 2004). Although, strain specific hybridization analyses were not conducted in this study, the results of the MCA-13 DAS-ELISA and RT-PCR confirm that severe strains are located across the island. In addition, the bio-indexing tests corroborate the presence of grapefruit and sweet orange CTV-SP. Comparable findings of the rapid increase in spread of CTV shortly after the introduction of the CTV vector have been observed in countries where BrCA, which is very efficient in transmitting the severe CTV strains, is the predominant vector (Halbert et al. 2004). As in other regions, such as Colombia (Penaranda et al. 1996), Cyprus (Papayiannis et al. 2007) and Iran (Barzegar et al. 2006), geographical speciation of the Jamaican isolates was not observed. None of the groups were specific to Jamaican isolates and the isolates clustered with groups containing isolates of similar severity (e.g. group 2). In addition, clustering of the Jamaican isolates within group 1 was observed and is indicative of local virus spread. This was also recently reported with isolates in Cyprus and Argentina (Papayiannis et al. 2007; Iglesias et al. 2008). The high *cp* nucleotide similarities between the CTV isolates in Jamaica and other regions suggest a common ancestry (Rubio et al. 2001). Presumably there were different introductions of CTV into the island or an introduction from a location in which CTV is relatively diverse, generally the result of continuous exposure to environmental conditions, mixed infections and re-infections by viruliferous aphid vectors (Iglesias et al. 2008; Papayiannis et al. 2007; Martín et al. 2009). Similar assumptions have been presented on the population structure of CTV in other regions (Barzegar et al. 2006; Papayiannis et al. 2007) as well as other Closteroviruses, for example, *Grapevine leafroll-associated virus 3* (Turturo et al. 2005).

Taken together, these results have practical implications for the management of CTV in Jamaica. Although the industry is attempting to manage the spread of the virus through a certification programme, the project closed in 2007 with only one eighth of the production area replanted on certified CTV tolerant rootstock. Further work is urgently needed on RT-PCR with other primers that will allow for the detection of most if not all CTV isolates in Jamaica, the removal of severe CTV inoculum sources and alternative disease management protocols, such as mild strain cross protection and pathogen-derived resistance, to manage CTV-SP and prevent the evolution of additional aggressive biotypes.

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