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Occurrence and distribution of *Citrus tristeza virus* (CTV) in the Jordan Valley

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Summary. In a survey conducted in 2002 and 2003, *Citrus tristeza virus* (CTV) was detected in the Jordan Valley. The direct tissue blot immunoassay (DTBIA) indicated that 12.7 and 15.2% of samples tested in the central and northern Jordan Valley respectively were infected with CTV. Similar results showed that all citrus species grown in the Jordan Valley were susceptible to CTV. DAS-ELISA analysis of samples from a citrus orchard in the Dir Alla area with severe CTV symptoms indicated that 49% of samples were infected with CTV. Using a CTV specific primer pair (CTV1/CTV10), the coat protein gene of the virus was successfully amplified from leaf extracts obtained from CTV-infected trees by IC-RT-PCR. After cloning and sequencing the coat protein gene, the sequence of the amplified product was deposited in the GenBank.

Key words: citrus, DTBIA, DAS-ELISA, PCR.

Introduction

Citrus is a semi-tropical crop grown throughout the world, and is mainly propagated by grafting. Therefore many of its diseases are passed on from one generation to the next, and also from one country to another in the absence of strict and enforced phytosanitary and quarantine regulations.

In Jordan, citrus is grown mainly in the Jordan Valley. In 2002, 77,600 ha were planted with citrus crops, and in that same year 7,751 tons of citrus fruits were exported to neighboring countries (Anonymous, 2002). The most important citrus species grown in Jordan are sweet orange (*Citrus sinensis*) (cv. Washington navel, Valencia, Shamouti, French and Balady); mandarins and mandarin-like citrus (*C. reticulata*) (Mandarin, Clementine), lemon (*C. limon*) (Eureka, Shahri and Lisbon) and grapefruit (*C. paradiseii*) (Marsh, Triumph, Thompson). Approximately 98% of all these species are planted on sour orange *(C. aurantium*) rootstock.

Citrus tristeza virus (CTV) causes one of the most damaging diseases of *Citrus* spp. (Bar-Joseph and Lee, 1989). The disease destroys millions of trees throughout the world; it occurs in most citrus-producing areas and represents a very serious threat to the citrus industry of the Mediterranean Basin (Djelouah and D'Onghia, 2000). CTV is reported from almost all Mediterranean countries (Nour Eldin and Bishay, 1958; Kyriakou *et al.*,

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1993; Bové, 1995; D'Onghia *et al.*, 1998; Zemzami *et al.*, 1999; Jarrar *et al.*, 2000) and particularly severe outbreaks have occurred in Israel (Bar-Joseph and Lee, 1989) and Spain (Moreno *et al.*, 1988).

Little is known about the sanitary status of citrus in Jordan and especially about virus and virus-like diseases. Previous studies have shown the occurrence of diseases like cachexia-xyloporosis, stubborn, scaly bark psorosis, concave gum-blind pocket, and exocortis (Skaria and Batrashe, 1986; Bové, 1995), but CTV has never been reported.

In the last few years, symptoms resembling those caused by CTV, including severe tree decline, yellowing and stunting, have been observed in different orchards in the Jordan Valley. These symptoms were accompanied by low yield and poor quality. Two previous studies were carried out to investigate the presence of tristeza disease in Jordan (Skaria and Batrashe, Bové, 1995); however, they did not detect any citrus trees infected with CTV. These studies (Skaria and Batrashe, Bové, 1995) were limited by being based on (i) visual inspection and a few ELISA tests; (ii) surveys only in restricted areas where CTV was suspected; (iii) a low number of samples, which were not representative of Jordanian citrus-growing area; (iv) a sampling of orchards belonging only to the Ministry of Agriculture (supposed to be healthy) and not in commercial groves in the Jordan Valley.

CTV is a member of the genus *Closterovirus* within the family *Closteroviridae*. CTV virions are flexuous (2000×11 nm in size) and contain a non-segmented, positive-sense, single-stranded RNA genome. CTV is a phloem-limited virus transmitted by graft and in a semi-persistent manner by the aphid species *Toxoptera citricida, T. aurantii, Aphis gossypii,* and *A. spiraecola* (Bar-Joseph and Lee, 1989).

Sensitive and reliable detection of CTV is essential for suitable control measures to be taken. A number of methods have been developed for CTV detection: enzyme linked immunosorbent assay (ELISA), dot immunoblot assay, direct tissue blot immunoassay (DTBIA) and immunocapture polymerase chain reaction (IC-PCR) (Bar-Joseph *et al.*, 1979; Cambra *et al.*, 1991; Garnsey *et al.*, 1993; Nolasco *et al.*, 1993; Cambra *et al.*, 2000a).

In view of the alarming occurrence of CTV in other countries in the region (Israel, Palestine, Egypt and Lebanon), a survey was conducted to assess the possible presence of CTV in Jordan using diagnostic techniques such as DTBIA, Double-antibody sandwich-ELISA (DAS-ELISA), and IC-RT-PCR.

Materials and methods

Sample collection

Leaf samples were collected according to Mathews (1997) in the fall-winter (September–February) and spring (March–April) budding periods of 2002 and 2003. Over 3000 trees in 62 orchards, distributed in the central and northern regions of the Jordan Valley, were individually inspected for tristeza symptoms. Leaf samples were collected from citrus trees that showed CTV-like symptoms, and since lemon propagated on sour orange does not decline from CTV infection, lemon leaves were collected from trees that showed leaf yellowing. Six to eight tender shoots (20–25 cm long) with fully expanded leaves were harvested from around the entire perimeter of each tree, placed in plastic bags and kept at 4ºC. A total of 844 leaf samples with CTV-like symptoms (330 from the central and 514 from the northern regions of the Jordan Valley) from trees of six citrus species (lemon, sweet orange, grapefruit, pummelo, clementine and mandarin) were collected and used for DTBIA analysis.

For DAS-ELISA, 100 leaf samples were collected from an orchard in the Dir Alla area (*ca.* 10 ha) that showed severe yellowing, stunting and decline symptoms. Samples were kept at 4ºC until use.

DTBIA

DTBIA was performed as described in Garnsey *et al.* (1993) and Cambra *et al.* (2000a) with minor modifications. Briefly, clean cuts were made on tender shoots or leaf petioles, and freshly cut sections were pressed against a nitrocellulose membrane (0.45 μm) (Schleicher and Schuell, Dassel, Germany). Prints were air-dried and stored in a dry place until processing. The membranes were blocked in a plastic tray for 2 h at 37°C using 2% solution of bovine serum albumin (BSA) and rinsed two times with phosphate buffer saline, pH 7.2–7.4, supplemented with 0.1% Tween 20 (PBST). After that, the membranes were covered with a solution of CTV-specific polyclonal antibodies conjugated with

alkaline phosphatase (Agritest Srl, Valenzano, Italy) for 3 h at 37°C, and then the conjugate solution was discarded. The membranes were then washed two times with PBST for 3 min under continuous shaking and the alkaline phosphatase substrate solution (BCIP/NBT, Sigma, St. Louis, MO, USA) was added over the membranes. Membranes were incubated until a purple-violet color appeared (about 10 min). The reaction was stopped by washing the membranes with tap water and membranes were evaluated under low-power magnification (×40).

DAS-ELISA

Midribs and petioles from leaf samples (0.6 g) collected from trees with severe CTV symptoms were separated and chopped with a razor blade and homogenized in 6 ml of PBST containing 2% polyvinylpyrrolidone (PVP). Extracts were clarified by centrifuging at 5,000 *g* for 5 min and the supernatants were kept on ice. DAS-ELISA was performed following the general protocol of Bar-Joseph *et al.* (1979). Mean experimental readings at least three times the mean reading of the negative controls were considered positive. Citrus samples that were positive in DAS-ELISA were selected for PCR analysis.

IC-RT-PCR

Samples for IC-RT-PCR were extracted as indicated for DAS-ELISA. Sterile 0.5-ml polypropylene PCR tubes (Treff Lab, Degersheim, Switzerland) were precoated for 2 h at 37°C with 150 μl Agritest anti-CTV-IgG (4 μg ml^{-1} in 50 mM sodium carbonate buffer, pH 9.6). The precoated tubes were rinsed three times with PBST and incubated overnight at 4°C with 150 μl plant extract from CTV-infected or healthy (controls) citrus trees. After that the tubes were washed two times with PBST and once with distilled water.

PCR was performed using the Access RT-PCR System (Promega Corp., Madison, WI, USA) according to manufacturer's instructions. To amplify the coat protein (CP) gene, the primer pair CTV1 (5´-ATGGACGACGAAACAAAGAA-3´) and CTV10 (5´-ATCAACGTGTGTTGAATTTCC-3´) previously described by Sequeira and Nolasco (2002) was used. In a final volume of 50 μl, the following PCR mixture: 10 μl 5× AMV/Tfi PCR buffer; 1 μl 20mM dNTP mixture; 2 μl 25 mM $MgSO_4$; 200 nM of each primer; 33 μl sterile distilled water; 1 μl Tfl DNA polymerase (5 u μl^{-1}) and 1 μl AMV reverse transcriptase (5u μl^{-1}), was added to the PCR tubes precoated with the CTV IgG. To allow cDNA production, tubes were incubated at 48°C for 50 min and PCR was performed in a programmable thermal controller (model PTC-200, MJ Research Inc., Watertown, MA, USA) using the following parameters: one cycle at 94°C for 2 min; 35 cycles at 92°C for 30 s, 52°C for 30 s, and 72°C for 45 s; followed by one cycle at 72°C for 5 min. As a positive control, a clone of CTV containing the CP gene was obtained from G. Nolasco (Universidade do Algarve, Faro, Portugal) and included in the PCR reactions. Amplified DNA fragments were electrophoresed in 1% agarose gel in 1× TBE buffer (Tris-borate-EDTA) and visualized with UV light after staining with ethidium bromide.

cDNA cloning and sequencing of the CP gene

The PCR product was ligated to the pGEM®-T Easy Vector (Promega) and cloned according to manufacturer's instructions. Colonies containing recombinant plasmids were selected and controlled by PCR using CTV1/CTV10 primers. One clone of the CTV isolate was taken for sequencing. Both orientations of the clone were sequenced by an automatic sequencer at the Biotechnology Center, Madison, WI, USA using the Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA). Alignment analysis was carried out with BLASTN 2.0.13.

Results

DTBIA

DTBIA detected CTV in tender shoots (Fig. 1a) and in leaf petioles (Fig. 1b) from CTV-infected citrus trees. Black precipitates in the vascular region revealed the presence of CTV (Cambra *et al.*, 2000a).

Results of the survey conducted in 2002 and 2003 showed that all the areas of the Jordan Valley surveyed were CTV-infected (Table 1). The virus was identified in 12.7 and 15.2% of samples collected from the central and northern regions of the Jordan Valley respectively. The highest CTV incidence in the central Jordan Valley was in the Abu-Obida (16.6%) and Dir Alla (16.5%) areas, followed by Ma'adi (11.8%) and AL-Arda (11.3%).

However, only 7.5% of citrus trees surveyed in Dirar area were CTV-positive. In the northern Jordan Valley 15.2% of citrus samples were infected with CTV. The highest CTV incidence in the northern Jordan Valley was in the Kraima area where it reached 31%. CTV infection was 27 and 23.8% in the Al-Mashareh and Wadi Al-Rayan areas respectively. The lowest CTV incidence was in the Al-Baseleh and Waqas areas, at 1.9 and 3%, respectively.

The DTBIA showed that all citrus species were infected with CTV except mandarin in the northern Jordan Valley (Fig. 2a). The highest CTV incidence on a citrus species in the northern Jordan Valley was in pummelo (24.6%), the lowest on grapefruit at 6.7%.

Among the samples collected from central Jordan Valley, grapefruit had the highest CTV incidence (22.9%), that on lemon and orange was 18.9 and 17.4%, respectively, and that on mandarins was the lowest at 5% (Fig. 2b).

DAS-ELISA

From Table 2 it can be seen that 49% of all samples from different citrus species tested with DAS-ELISA were CTV-infected. The highest infection rate was in clementine (69.2%), followed by grapefruit (63.6%) and mandarin (46.1%). The lowest CTV infection rate was recorded in lemon, where it did not exceed 33.3%.

Amplification of the CP gene of CTV by IC-PCR and RT-PCR

The DAS-ELISA findings were confirmed by IC-RT-PCR analysis (Fig. 3). The expected size of the CTV CP gene, approximately 670 bp (Fig. 3, lanes 3 and 4), was successfully amplified only when plant extracts were from CTV-infected trees. No bands were detected in extracts from CTV-free trees. The identity of the amplicon was confirmed by cloning and sequencing.

cDNA cloning and sequencing of CP gene

Sequencing results confirm that the PCR products obtained with the primer pair CTV1 and CTV10 were indeed the CP gene of CTV. The sequence of the CP gene of the Jordanian CTV isolate (CTV-Jo) was deposited in the GenBank under accession No. AY550252. This sequence was highly homologous to the CP gene sequences of other CTV isolates. Alignment analysis of the CTV-Jo sequence revealed 98% identity with CTV-VT and

Table 1. CTV infection rate in commercial citrus orchards in the central and northern regions of the Jordan Valley.

Location		No. of positive samples / No. of tested samples						Infection rate (%)[a]
		Lemon	Sweet orange	Grapefruit	Pummelo	Clementine	Mandarin	
Central Jordan Valley	Abu-Obida	1/3	3/14	1/8	1/9	2/6	1/14	9/54 (16.6)
	Al-Arda	0/2	0/3	3/8	0/3	3/28	2/27	8/71 (11.3)
	Dir Alla	5/15	3/12	2/13	3/16	0/10	0/13	13/79 (16.5)
	Dirar	1/10	0/9	1/10	1/14	1/14	1/10	5/67 (7.5)
	Ma'adi	0/7	2/8	4/9	0/10	1/11	0/14	7/59 (11.8)
	Sub total	7/37	8/46	11/48	5/52	7/69	4/78	42/330 (12.7)
Northern Jordan Valley	Al-Baseleh	0/8	1/11	0/13	0/6	0/9	0/7	1/54 (1.9)
	Al-Harawieh	1/4	0/7	0/12	3/3	0/5	0/4	4/35 (11.4)
	Al-Mashareh	10/31	0/3	4/10	2/9	1/4	0/6	17/63 (27)
	Kraima	0/11	16/34	0/5	7/14	4/12	0/11	27/87 (31)
	North Shwneh	10/58	2/34	0/15	0/12	0/13	0/15	12/147 (8.1)
	Wadi Al-Rayan	3/11	8/25	1/4	3/8	0/7	0/8	15/63 (23.8)
	Waqas	2/10	0/8	0/15	0/9	0/11	0/12	2/65 (3)
	Sub total	26/133	27/122	5/74	15/61	5/61	0/63	78/514 (15.2)
Total		33/170	35/168	16/122	20/113	12/130	4/141	120/844 (14.2)

[a] Infection rate = No. of CTV- infected samples/No. of tested samples × 100

Table 2. CTV infection rate by DAS-ELISA in an orchard in the Dir Alla area with sever CTV symptoms.

Citrus species	No. of tested samples	No. of infected samples	Infection rate (%)
Mandarin	13	6	46.1
Clementine	26	18	69.2
Grapefruit	11	7	63.6
Sweet orange	32	12	37.5
Lemon	18	6	33.3
Total	100	49	49

CTV-28C isolates (Table 3) and 93% identity with CTV isolate TAM11 (GenBank accession No. AF342890.1) and Qaha (GenBank accession No. AY340974.1) (data not shown).

Discussion

The study showed that CTV occurs in Jordan in the regions of Jordan Valley where citrus is most grown. As can be seen from Table 1, DTBIA was successfully used to study the occurrence and distribution of CTV, showing a similar infection rate in both regions, 15.2% in the northern Jordan Valley and 12.7% in the central Jordan Valley. These findings are not surprising as leaf samples were obtained from citrus trees with CTV-like symptoms and the virus is widespread in other countries in the region. CTV thus represents a serious risk to the Jordanian citrus industry. All tested citrus species that were grafted onto sour orange and showed tristeza symptoms were CTV-positive by DTBIA and DAS-ELISA. This is to be expected since the sour orange rootstock is CTV susceptible (Bové, 1995). Although detection of CTV in lemon propagated on sour orange is generally difficult due to the low virus titer and uneven distribution in the tree, 19.4% of lemon samples from northern and central Jordan Valley (Table 1) were detected positive for CTV by DTBIA. Leaves of infected lemon trees showed severe yellowing.

In spite of the hazard posed by CTV, farmers in the Jordan Valley prefer sour orange as a rootstock over other citrus species because of its vigor, its ability to produce high fruit quality, and its tolerance to phytophthora root rot and many other citrus viruses, viroids and virus-like pathogens. In addition, sour orange is adapted to a wide range of soil conditions (Bové, 1995).

DAS-ELISA revealed a high CTV incidence (49%) in the orchard in the Dir Alla area where the trees showed severe CTV symptoms.

PCR analysis showed that the CP gene of CTV was successfully amplified from citrus trees that showed severe decline symptoms and high DAS-ELISA values. Sequencing confirmed the results with IC-RT-PCR and showed that the CP gene of CTV-Jo shared high homology with other CTV isolates. Since the level of damage caused by CTV depends mainly on the strain of CTV that prevails in an area, various CTV strains differing in the symptoms they cause on different host species, as well as in their aphid transmissibility and their capacity to interfere with other strains, have been described in many other countries (Ballester-Ol-

Table 3. Nucleotide sequence homology of the coat protein gene of the Jordanian isolate of citrus tristeza virus with CTV isolates from other countries.

Virus isolates[a]	Coat protein homology (%)	Accession No.	Origin
CTV-28C	98	AF184118	Portugal
CTV-vt”	98	U56902	Israel
CTV-SY568	96	AF001623.1	USA
CTV-13C	96	AF184113	Portugal
CTV-M2	96	AY190048	Iran
CTV-Seedling yellows	96	AB046398	Japan
CTV-M1	96	AY490208.1	Iran
CTV-M1S	96	AY490207	Iran

[a] CTV isolates in the GenBank were used for comparison with the Jordanian CTV isolate.

mos *et al.*, 1993). Identification of the prevailing CTV strains in Jordan is therefore a matter of urgency. Although nucleotide sequence analysis is considered the most accurate procedure for strain differentiation, other techniques such as single-strand conformation polymorphism (SSCP), which has been recently used to discriminate CTV strains (Rubio *et al.*, 1996; Gago-Zachert *et al.*, 1999), can also be applied to study genetic variation of CTV strains in Jordan.

The study provides clear evidence of the occurrence of CTV in Jordan. However, from an epidemiological point of view, much work still needs to be done. It is important to determine the source of the CTV infection and to ascertain how the virus is introduced into the country. Due to the lack of certification programs for fruit trees against virus diseases in Jordan, budwoods and other planting materials are currently imported without any inspection for virus and virus-like diseases. In addition, due to financial constraints, certified laboratories that apply modern techniques for disease diagnosis do not exist. These factors together may play a role in allowing destructive virus diseases like CTV to enter Jordan. In addition, studies must be made to establish whether the CTV vector *T. citricida* is really not found in Jordan. Even in the absence of *T. citricida*, *A. gossypii* is one of the most efficient CTV vectors in the Mediterranean Basin and was reported to transmit destructive strains of CTV. In Israel, the VT strain of CTV was transmitted by *A. gossypii* at a greater efficiency rate (40%) than other virus isolates (Bar-Joseph *et al.,* 1979) and in Spain, *A. gossypii* transmitted the T-300 CTV isolate at an efficiency 190% higher than the rate at which it transmitted seedling yellows isolate T-387 (Cambra *et al.*, 2000b). Thus the import of infected plant material from nearby countries to Jordan presents a real risk that severe CTV strains will be introduced into the country. Therefore the enforcement of certification and quarantine procedures to control the import of budwood materials into the country, and the implementation of a compulsory CTV control program in the framework of a certification scheme is highly recommended. This would be part of a Mediterranean-wide policy toward the harmonization of procedures for CTV control and the certification of propagating material in the region (D'Onghia *et al.*, 1998).

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