Turkish Journal of Agriculture and Forestry

Volume 32 | Number 5 Article 3

1-1-2008

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SAVAŞ KORKMAZ

BAYRAM ÇEVİK

SERKAN ÖNDER

N. KEMAL KOÇ

Turk J Agric For
32 (2008) 369-379

Biological, Serological, and Molecular Characterization of Citrus tristeza virus Isolates from Different Citrus Cultivation Regions of Turkey

Savaş KORKMAZ[1,*], Bayram ÇEVİK[2], Serkan ÖNDER[1], N. Kemal KOÇ[3]

[1]Department of Plant Protection, Faculty of Agriculture, Çanakkale Onsekiz Mart University, 17020 Çanakkale - TURKEY

[2]Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University, 32260 Isparta - TURKEY

[3]Subtropical Fruits Research and Experimental Center, Çukurova University, 01330, Adana - TURKEY

Received: 14.02.2008

Abstract: Field surveys were carried out in 5 different citrus cultivation regions of Turkey in 2005 and 2006, and 201 samples were collected from different citrus species. Samples were tested for the presence of Citrus tristeza virus (CTV) by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). While DAS-ELISA showed that 41 trees were infected with CTV, an additional 13 trees were found to be positive based on RT-PCR. When CTV-positive samples were tested with the Western blot method using the monoclonal antibody MCA13, which is specific to severe isolates of CTV, 32 isolates, mostly from satsuma, were found to be positive. These isolates were then verified by bidirectional/PCR (BD/PCR), allowing differentiation of the MCA13 positive and negative isolates, and detection of mixed infections. The BD/PCR results were generally in agreement with the results of the Western blot assay with MCA13. In total, 28 isolates representing different geographic locations and hosts were selected for biological indexing. Although none of these 28 isolates induced any symptoms in sour orange, grapefruit, or sweet orange, all isolates induced the vein clearing symptom in Mexican lime. Additionally, all the tested satsuma isolates and 1 kumquat isolate produced stem pitting in Mexican lime. The results revealed that potentially severe isolates of CTV are present in different citrus cultivation regions of Turkey.

Key Words: Citrus tristeza virus, RT-PCR, Western blot, biological indexing, ELISA, Turkey

Ülkemizin Farklı Turunçgil Üretim Bölgelerinden Elde Edilen Turunçgil Tristeza Virüsü İzolatlarının Biyolojik, Serolojik ve Moleküler Karakterizasyonu

Özet: Ülkemizin beş farklı turunçgil üretim bölgesinde 2005–2006 yılları arasında arazi çalışmaları yürütülmüş ve farklı turunçgil çeşitlerinden toplam 201 örnek toplanmıştır. Örnekler turunçgil tristeza virüsünün varlığını belirlemek amacıyla DAS-ELISA ve RT-PCR yöntemleriyle test edilmiştir. ELISA testi sonucunda 41 örnek tristeza ile infekteli bulunurken, PCR çalışmalarında ilaveten 13 örnek daha tristeza ile infekteli bulunmuştur. Tristezanın şiddetli ırklarının belirlenmesi için geliştirilmiş olan MCA13 monoklonal antibadisi ile yapılan Western blot analizi çalışmalarında ise çoğunluğu Satsuma çeşitlerine ait olan 32 örnek pozitif sonuç vermiştir. MCA13 monoklonal antibadi sonuçlarını desteklemek ve aynı zamanda MCA 13 pozitif, negatif ya da karışık infeksiyonlarını belirlemek için geliştirilmiş olan iki yönlü PCR çalışmalarında ise MCA13 bulgularını destekler nitelikte sonuçlar elde edilmiştir. Ayrıca elde edildiği coğrafik bölgeler ve çeşitler göz önünde bulundurularak seçilen toplam 28 tristeza izolatının biyolojik özellikleri çalışılmıştır. Biyolojik indekslemeler sonucunda izolatların hiçbiri inokule edildiği portakal, turunç ve altıntop bitkilerinde simptom oluşturmazken, Meksika laymı bitkisinde ise damar açılması simptomu oluşturmuşlardır. İlaveten tüm Satsuma izolatları ve bir kamkat izolatı Meksika laymı bitkisinde gövde çukurlaşması simptomu oluşturmuştur. Bu sonuçlar farklı turunçgil üretim bölgelerinde turunçgil tristeza virüsünün şiddetli ırklarının bulunduğunu göstermiştir.

Anahtar Sözcükler: Turuçgil tristeza virüsü, RT-PCR, Western blot analizi, Biyolojik indeksleme, ELISA, Türkiye

* Correspondence to: skorkmaz@comu.edu.tr

Introduction

Tristeza disease caused by *Citrus tristeza virus* (CTV) is one of the most destructive and economically important viral diseases of commercial citrus worldwide (Bar-Joseph et al., 1989; Lee and Rocha-Pena, 1992; Rocha-Pena et al., 1995). The disease is distributed in most citrus growing regions of the world, including Turkey. CTV belongs to the genus *Closterovirus* in the family *Closteroviridae* (Bar-Joseph et al., 1979; Bar-Joseph and Lee, 1990). It has long thread-like, flexuous, filamentous particles that are about 2000 nm by 11 nm (Bar-Joseph et al., 1979; Bar-Joseph and Lee, 1990). Virions consist of 1 single-stranded, positive-sense RNA molecule encapsidated with 2 capsid proteins (CP). The 25 kDa major CP encapsidates about 95% of the genome, and the remaining portion of the genome is encapsidated by the 27 kDa minor CP on one end of the virion (Febres et al., 1996).

CTV isolates cause a variety of symptoms in citrus, depending on the scion and rootstock combination. Mild vein clearing (M), seedling yellows (SY), quick decline (QD), and stem pitting in grapefruit (SP-G) and in sweet orange (SP-O) are several symptoms caused by different isolates of CTV. A standardized set of citrus indicator plants has been established to determine the biological characteristics of CTV isolates (Garnsey et al., 1987b). CTV strains are traditionally characterized based on symptoms induced in differential indicator hosts (McClean, 1977; Garnsey et al., 1987a). The common indicator is Mexican lime (*C. aurantifolia* {Christm.} Swingle) in which vein clearing, leaf cupping, and stem pitting symptoms were induced in accordance with the severity of CTV isolates (Garnsey et al., 1987b).

Although biological indexing is a reliable method and provides important information, it is costly and time-consuming, requiring 12-15 months under ideal conditions; therefore, a number of serological and molecular assays, including direct tissue blot immunoassay (DTBIA) (Garnsey et al., 1993), enzyme-linked immunosorbent assay (ELISA) (Bar-Joseph et al., 1979; Cambra et al., 1991; Rocha-Pena and Lee, 1991), Western blot (Lee et al., 1988; Rocha-Pena and Lee, 1991), and reverse transcription-polymerase chain reaction (RT-PCR) (Nolasco et al., 1993) have been used to rapidly detect CTV. In addition, a monoclonal antibody, MCA13, which reacts with severe CTV isolates was developed (Permar et al., 1990) and used to differentiate mild and severe isolates, mainly in Florida and the Caribbean Basin. It was demonstrated by site-directed mutagenesis that the specificity of MCA13 is due to a single nucleotide change (A to T) at position 371 of the CP gene of severe and mild CTV isolates (Pappu et al., 1993). Based on this knowledge a more sensitive PCR-based method, bidirectional PCR (BD/PCR), was developed and used to differentiate CTV isolates (Çevik et al., 1996).

In Turkey, symptoms associated with CTV infection were first detected near Adana in the eastern Mediterranean region (Norman, 1963) and İzmir in the Aegean region (Özalp and Azeri, 1967). The presence of CTV was confirmed by serological and biological assays (Baloğlu, 1988). Recently, CTV was also detected in the eastern Black Sea region by ELISA and RT-PCR (Korkmaz et al., 2006). Although serological and biological characterization of a limited number of CTV isolates from the eastern Mediterranean region indicated that only mild isolates were present in Turkey (Baloğlu, 1988; Çınar et al., 1993; Korkmaz, 2002), no comprehensive surveys or characterization studies covering all the citrus growing regions of Turkey have been conducted. Consequently, the distribution of CTV isolates in different citrus growing regions and their characteristics are largely unknown.

The present study surveyed different citrus growing regions of Turkey, and the presence of CTV was detected in different commercial citrus species by ELISA and RT-PCR. Selected CTV isolates representative of different regions were further characterized by biological, serological, and molecular methods.

Materials and Methods

Disease Survey and Sample Collection

Different citrus producing areas of Turkey, including the eastern Mediterranean (Adana, İçel, and Hatay provinces), western Mediterranean (Antalya Province), western Aegean (İzmir and Aydın provinces), northern Aegean (Edremit, Havran, and Burhaniye sub-provinces), and eastern Black Sea regions (Rize Province) were surveyed during the cool seasons (November-April) in 2005 and 2006. Citrus plants were visually examined and samples were collected from symptomatic trees in randomly selected commercial citrus groves in all 5 regions. In addition, a small number of samples were taken from a few home gardens in Rize Province. Several

young flushes were collected from the north-, east-, south-, and west-facing sides of trees that showed tristeza-like symptoms, such as stunting, leaf cupping, and malformation.

Enzyme Linked Immunosorbent Assay (ELISA)

The direct double antibody sandwich ELISA (DAS-ELISA) procedure, as previously described by Garnsey et al. (1993), was used with commercially available CTV-specific coating and alkaline phosphatase conjugated antibodies (Loewe, Germany). Absorbance values at 405 nm (OD 405) were measured with a Stat Fax-2100 microtiter plate reader (Awareness Tech. Ins., USA) and OD values 2.5 times greater then the negative control were recorded as positive.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was isolated from 100 mg of bark tissue using the one-step RNA isolation solution according to the manufacturer's instructions (BioBasic, Canada). CTV was detected from 1 µl of total RNA sample by RT-PCR using the SuperScript III One-Step RT-PCR system with a platinum Taq DNA polymerase kit (Invitrogen, USA), 1 mM of $MgSO_4$, and 20 pmol of primers (BC24 5' ATGGACGACGAAACAAAG 3' and BC25 5' CGCTCAACGTGTGTT AAATTTCC 3') specific to the 5' and 3' ends of the coat protein gene, respectively. The MJ Mini PTC-1148 thermal cycler (Bio-Rad, USA) was programmed for cDNA synthesis at 42 °C for 60 min, followed by initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, primer extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and analyzed with a Doc-It gel imaging system (UVP, England).

Western Blot Analysis

About 100 mg of bark tissue was homogenized in 0.5 ml of 2 Western extraction buffer (0.125 M of Tris-Cl at pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and incubated at 95 ºC for 5 min. A 10-µl aliquot of the extract was loaded into a 12% SDS polyacrylamide gel, separated by electrophoresis, and transferred to a nitrocellulose membrane (Stratagene, USA) using a Mini Transblot (Bio-Rad, USA). Samples were tested for the presence of 25 kDa major CP of CTV using the MCA13 monoclonal antibody (Permar et al., 1990) (kindly provided by Richard F. Lee), followed by goat anti-rabbit or rabbit anti-mouse IgG conjugated with alkaline phosphatase (Promega, USA), or CTV-specific polyclonal antibody conjugated with alkaline phosphatase (Loewe, Germany). The reaction was detected colorimetrically with a p-nitrophenol phosphate substrate (Loewe, Germany).

Biological Indexing

In total, 28 CTV isolates were recovered from different citrus species in 5 different regions in order to determine their biological properties. These isolates were graft-inoculated by 3-4 buds or leaf pieces to 4 indicator plants: *Citrus aurantifolia* (Christm.) Swing. (Mexican lime), *C. aurantium* L. (sour orange), *C. paradisi* L. 'Rio-Red' (grapefruit), and *C. sinensis* (L.) Obs. 'Madam Vinous' (sweet orange). Grafted plants were maintained at an average temperature of 18-25 °C in a greenhouse at the Subtropical Fruits Research and Experimental Center, University of Çukurova, Adana. Graft-inoculated indicator plants were examined periodically and leaf symptoms, including vein clearing, leaf cupping, and chlorosis, were recorded after major growth flushes.

Bidirectional-Polymerase Chain Reaction (BD-PCR) Assay

Bidirectional polymerase chain reaction (BD-PCR) enables the amplification of about 300 bp, 400 bp, or both 300 and 400 bp CP fragments from samples infected with isolates carrying the MCA13 epitope, isolates without the MCA13 epitope, or a mixture infected with both types of isolates, respectively. This was accomplished by using 2 internal primers, BC 26 (5' TTTGGACTGACGTCGTGTT 3') and BC27 (5' TTACCAATACCCTTAGAATTAT 3'), along with external primers BC24 and BC25 in the same reaction (Çevik et al., 1996). PCR amplification was performed in 25 µl of reaction mixture that contained 20 mM of Tris-HCl (pH 8.4), 50 mM of KCl, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 10 pmol of external primers BC24 and BC25, 20 pmol of internal primers BC26 and BC27, 1.25 units of Taq DNA polymerase (Bioron, Germany), and 0.5 µl of diluted PCR products. The MJ Mini PTC1148 thermal cycler (Bio-Rad, USA) was programmed for initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, primer extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. Along with DNA, size maker PCR products were analyzed, as above, in 1.5% agarose gel.

Results

Field Surveys

No specific CTV symptoms, such as stem pitting or quick-decline were present in the citrus orchards inspected during field surveys in 2005 and 2006, whereas stunting, leaf cupping, and malformation were common in the research areas. In all, 201 samples were collected from trees showing these symptoms in commercial citrus orchards and from a few home gardens. The number of samples collected from each region is summarized in Table 1, and the distribution of these samples to different scion and rootstock combinations is shown in Table 2. While sweet orange grafted on sour orange was the prevalent scion-rootstock combination in the Mediterranean region, satsuma mandarins grafted on *Poncirus trifolita* were grown in the Aegean Coast, Edremit Gulf, and eastern Black Sea region; therefore, the majority of isolates collected from the Mediterranean region were from sweet orange trees, while satsuma mandarins constituted the majority of samples collected from other regions.

Detection of CTV by Enzyme Linked Immunosorbent Assay (ELISA)

Results from DAS-ELISA using a polyclonal antibody that recognizes all known CTV isolates showed that about 20% (41 of 201) of the samples were infected (Table 1). CTV was detected in all citrus growing regions of Turkey and the infection rate ranged from 11.5% to 36%. The eastern Mediterranean region had the highest level of CTV infection (36%), whereas the lowest infection rate (11.5%) was observed in the western Mediterranean region. Between the different rootstock/scion combinations, CTV was most commonly detected in satsuma/sour orange samples from the eastern Mediterranean region, with an infection rate of 50%, followed by satsuma/*P. trifoliate* plants from Edremit Gulf, with an infection rate of 20.8%. None of the sweet orange samples grafted on *P. trifoliata* from the eastern Black Sea region were infected with CTV; however, 19.4% of sweet orange samples grafted on sour orange rootstock from the eastern Mediterranean region were infected with CTV. One kumquat sample tested positive with ELISA, but all 3 clementine mandarin samples were free of CTV.

Detection of CTV by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The incidence of CTV was verified by RT-PCR. Along with field samples, a healthy sweet orange sample and CTV Iğdır isolate were used as negative and positive controls, respectively. A 672-bp DNA fragment corresponding to the CPG of CTV was amplified from the positive control Iğdır isolate, but not from the healthy control (Figure 1). When the field samples were tested, the 672-bp CPG was amplified from 41 ELISA-positive samples and 13 ELISA-negative samples. RT-PCR results of samples from the eastern Mediterranean region are shown in Figure 1. Results of RT-PCR showed that 54 of the 201 samples (26.8%) tested were infected with CTV (Table 1). A higher incidence of CTV was detected with

Table 1. Detection of CTV in 5 different citrus growing regions of Turkey.

Region	Number of Samples Tested	ELISA		RT-PCR	
		Number of CTV-Positive Samples	% CTV Infection*	Number of CTV-Positive Samples	% CTV Infection*
Eastern Mediterranean	25	9	36.0	13	52.0
Western Mediterranean	26	3	11.5	3	11.5
Aegean coast	43	9	20.9	12	27.9
Edremit Gulf	66	15	22.7	18	27.2
Eastern Black Sea	41	5	12.1	8	19.5
Total	201	41	20.3	54	26.8

*As only trees showing virus-like symptoms were tested, infection rates do not reflect the actual incidence of disease in a specific region.
ELISA: Enzyme-linked immunosorbent assay; RT-PCR: reverse transcription polymerase chain reaction.

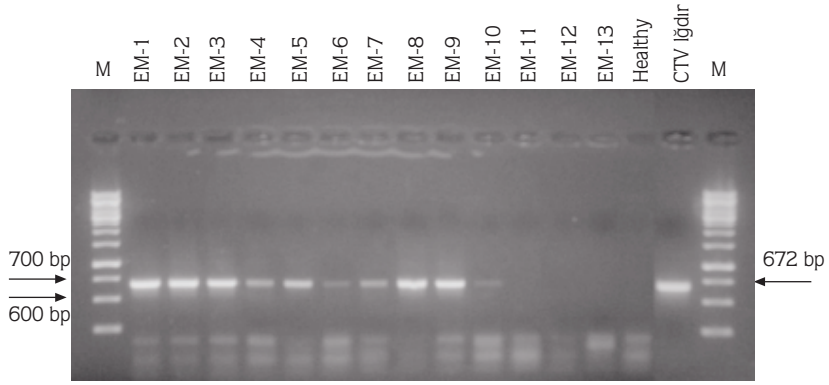

Figure 1. Detection of CTV from different citrus species grown in the eastern Mediterranean (EM) region based on RT-PCR using CPG-specific primers. M: 1 kb DNA ladder; Healthy: uninfected Mexican lime; CTV Iğdır: a previously identified CTV isolate maintained in Mexican lime.

RT-PCR (11.5%-52%) than with ELISA. With an infection rate of 52% the eastern Mediterranean region had the highest level of CTV infection, whereas the lowest infection rate (11.5%) was observed in the western Mediterranean region.

When the distribution of CTV in different scion/rootstock combinations were evaluated (Table 2), CTV was most commonly detected in satsuma/sour orange samples from the eastern Mediterranean region (75% infection rate), followed by satsuma/*P. trifoliate* plants from Edremit Gulf (27.3% infection rate). According to ELISA results, none of the sweet orange samples grafted on *P. trifoliata* from the eastern Black Sea region were infected with CTV; however, 22.2% of sweet orange samples grafted on sour orange rootstock from the eastern Mediterranean region were infected with CTV. One kumquat sample was found to be positive with ELISA, but all 3 clementine mandarin samples were free of CTV according to ELISA.

Differentiation of CTV Isolates by Western Blot Assay

To confirm the ELISA results, and to differentiate mild and severe CTV isolates, ELISA-positive samples were tested with Western blot using a polyclonal antibody that recognizes all known CTV isolates and the MCA13 monoclonal antibody that predominantly reacts with

Table 2. Detection of CTV based on ELISA and RT-PCR from various citrus species grown in 5 different citrus growing regions of Turkey.

Citrus species Scion / Rootstock	Number of Samples Tested	ELISA		RT-PCR	
		Number of CTV-Positive Samples	% CTV Infection*	Number of CTV-Positive Samples	% CTV Infection*
Satsuma/*Poncirus trifoliata*	139	29	20.8	38	27.3
Satsuma/Sour Orange	8	4	50	6	75
Sweet Orange/*Poncirus trifoliata*	11	0	0	0	0
Sweet Orange/Sour Orange	36	7	19.4	8	22.2
Clementine/Sour Orange	3	0	0	0	0
Kumquat/Sour Orange	1	1	100	1	100
Total	201	41	20.3	54	26.8

*As only trees showing virus-like symptoms were tested and only a few samples of some species were tested, infection rates do not reflect the actual incidence of disease in a specific host.

severe isolates. In total, 39 of 41 ELISA-positive samples from different regions of Turkey were tested along with T30 and T36 (mild and severe strains) as controls, respectively (kindly provided by Richard F. Lee). The 25 kDa major CP was detected from all field samples as well as from T30 and T36 controls (data not shown). When the same set of samples was tested using the MCA13 antibody, no reaction was observed with the mild isolate T30, but the 25 kDa major CP was detected from the severe isolate T36 (Figure 2). Among the 39 CTV field isolates tested, a majority of them (30) gave a positive reaction with MCA13, indicating that they are possibly severe isolates of CTV; however, no CP was detected by MCA13 from 9 of the 39 samples tested. The results of Western blot analysis of the samples from the Aegean coast and eastern Black Sea region are shown in Figure 2. The results of Western blot assays showed that while all

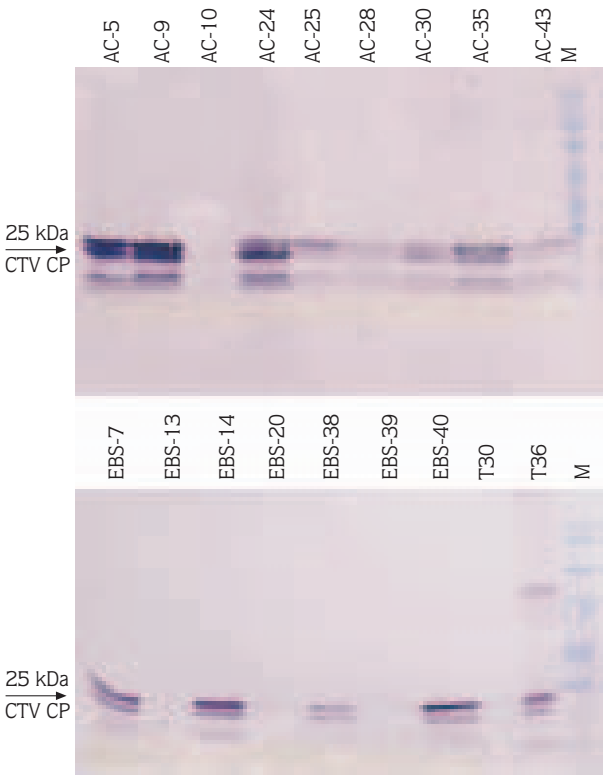

Figure 2. CTV isolates from different citrus species grown in the Aegean coast (AC) (top) and eastern Black Sea (EBS) (bottom) region based on Western blot assay using the MCA13 antibody, which predominantly reacts with severe isolates of CTV. M: Pre-stained protein size marker; T30: a mild isolate of CTV from Florida not detected by the MCA13 antibody; T36: a decline-inducing severe isolate of CTV from Florida, specifically detected by the MCA13 antibody.

CTV isolates tested from Edremit Gulf and the western Mediterranean gave a positive reaction with PCA and MCA13, CP was not detected in 1, 4, and 4 isolates from the Aegean coast, eastern Black Sea, and eastern Mediterranean regions, respectively.

Biological Indexing

Twenty-eight isolates that tested positive both with ELISA and RT-PCR, and representing different citrus growing regions were graft-inoculated to a set of indicator plants. These isolates did not show any symptoms on grapefruit or sweet orange plants, nor did they cause any stunting when compared to healthy controls that were not inoculated. Nonetheless, all isolates showed vein clearing symptoms in Mexican lime within 6 to 9 months of graft inoculation. In addition, 3 isolates from the Aegean coast (AC-5, AC-9, and AC-35), 2 from the eastern Black Sea region (EBS-7 and EBS-20), 1 from Edremit Gulf (EG-1), and 1 from the eastern Mediterranean (EM-15) induced severe leaf cupping and chlorosis symptoms (Table 3). In all, 10 isolates from the Aegean coast, Edremit Gulf, and eastern Black Sea region caused leaf cupping in addition to vein clearing within 6 to 9 months of graft inoculation. Although none of the CTV isolates evaluated in this study caused stem pitting in Mexican lime within 6 to 9 months of graft inoculation, all the tested isolates, except for 5 isolates from the Mediterranean region (WM-22, WM-23, WM24, EM-1, and EM-3) caused stem pitting in Mexican lime within 15 months of inoculation (Table 3). Stem pitting symptoms induced by 3 isolates from different regions are shown in Figure 3. The severity of stem pitting was generally mild with few visible pits, but a few isolates caused moderate stem pitting with many visible pits, indicating that these may have been more severe isolates of CTV.

Bidirectional-Polymerase Chain Reaction (BD-PCR) Assay

Isolates selected for biological indexing were further tested by BD-PCR to confirm the Western blot with MCA13 results and the possible presence of mixed infections of MCA13-positive and -negative isolates. A 300-bp DNA fragment specific to the MCA13 positive isolates was amplified from 24 of the 28 isolates tested, indicating that majority of isolates were severe isolates of CTV (Figure 4); however, an approximately 400-bp fragment specific to MCA13-negative isolates was amplified from only 3 isolates, 1 each from the Aegean coast, eastern Black Sea region, and Edremit Gulf,

Table 3. Biological and molecular characteristics of CTV isolates collected from various citrus species in different regions of Turkey.

Isolate name and region[1]	Scion/Rootstock	Biological Indexing	Serological Assays			Molecular assays		
		Symptoms on Mexican lime[2]	ELISA	Western Blot[3]		RT-PCR[4]	BD/PCR[5]	
			PCA	PCA	MCA13	672 bp	400 bp	300 bp
EG-5 (EK-1)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (mild)	+	+	+	+	-	+
EG-11 (EK-2)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
EG-16 (EK-3)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
EG-24 (EK-4)	Satsuma/*P. trifoliate*	vc, sp (moderate)	+	+	+	+	-	+
EG-28 (EK-5)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	-	+	+	-
EG-34 (EK-6)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
WM-22 (BA-1)	W. Navel/*C. aurantium*	vc,	+	+	+	+	-	+
WM-23 (BA-2)	W. Navel/*C. aurantium*	vc	+	+	+	+	-	+
WM-24 (BA-3)	W. Navel/*C. aurantium*	vc,	+	+	+	+	-	+
AC-5 (KE-1)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (moderate)	+	+	+	+	-	+
AC-9 (KE-2)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (mild)	+	+	+	+	-	+
AC-10 (KE-3)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	-	+	+	-
AC-24 (KE-4)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
AC-25 (KE-5)	Satsuma/*P. trifoliate*	vc, sp (mild)	+	+	+	+	-	+
AC-28 (KE-6)	Satsuma/*P. trifoliate*	vc, sp (mild)	+	+	+	+	-	+
AC-30 (KE-7)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
AC-35 (KE-8)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (mild)	+	+	+	+	-	+
AC-43 (KE-9)	Satsuma/*P. trifoliate*	vc, sp (mild)	+	+	+	+	-	+
EBS-7 (DK-1)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (mild)	+	+	+	+	-	+
EBS-14 (DK-2)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	-	+
EBS-20 (DK-3)	Satsuma/*P. trifoliate*	vc, lc, ch, sp (mild)	+	+	-	+	+	-
EBS-38 (DK-4)	Satsuma/*P. trifoliate*	vc, lc, sp (mild)	+	+	+	+	+	+
EBS-40 (DK-5)	Satsuma/*P. trifoliate*	vc, sp (mild)	+	+	+	+	-	+
EM-1 (DA-1)	Shamouti/*C. aurantium*	vc	+	+	+	+	-	+
EM-3 (DA-2)	Shamouti/*C. aurantium*	vc	+	+	+	+	-	+
EM-7 (DA-3)	Satsuma/*C. aurantium*	vc, sp (mild)	+	+	+	+	-	+
EM-9 (DA-4)	Satsuma/*C. aurantium*	vc, sp (mild)	+	+	+	+	-	+
EM-15 (DA-5)	Kumquat/*C. aurantium*	vc, lc, ch, sp (mild)	+	+	+	+	-	+

[1]EG: Edremit Gulf; WM: western Mediterranean; AC: Aegean coast; EBS: eastern Black Sea; EM: eastern Mediterranean.
[2]vc: Vein clearing; lc: leaf cupping; ch: chlorosis; sp: stem pitting; mild: very weak stem pitting.
[3]PCA: Polyclonal antibody; MCA13: monoclonal antibody 13.
[4]RT-PCR: Reverse transcription-polymerase chain reaction, 672 bp is the size of the DNA fragment corresponding to the CP gene of CTV.
[5]BD-PCR: Bidirectional-polymerase chain reaction, 300 bp and 400 bp are the approximate sizes of the DNA fragments amplified by BD-PCR that correspond to the MCA13-positive and -negative CTV CP, respectively.

suggesting that they were mild isolates of CTV. In addition, both 300-bp and 400-bp fragments were detected in 1 isolate (EBS-38) from the eastern Black Sea (Figure 4, Table 3), demonstrating that this sample was doubly infected with mild and severe isolates of CTV.

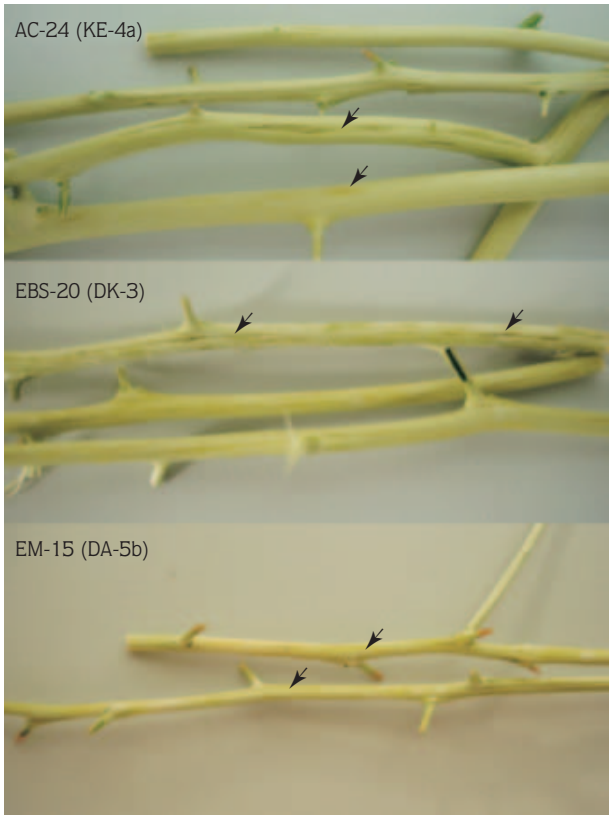

Figure 3. Stem pitting symptom induced in Mexican lime (indicator plant) 15 months after graft inoculation of CTV isolates recovered from different citrus species. Some of the stem pitting induced by CTV is indicated by arrows. AC: Aegean coast; EBS: eastern Black Sea; EM: eastern Mediterranean.

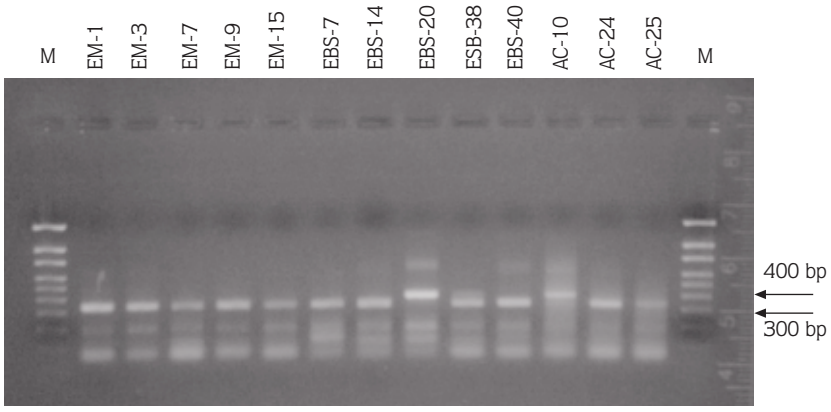

Figure 4. Differentiation of CTV isolates from different citrus growing regions of Turkey based on BD-PCR. M: 100-bp DNA ladder; EM: eastern Mediterranean; EBS: eastern Black Sea; AC: Aegean coast.

Discussion

This study conducted the first comprehensive national survey for the detection of CTV in Turkey. The presence of CTV in all 5 of the citrus growing regions of Turkey was determined with ELISA and confirmed with RT-PCR. Although the presence of CTV was known in some citrus growing regions, such as the eastern Mediterranean (Norman, 1963; Baloğlu, 1988; Çınar et al., 1993; Korkmaz, 2002) and Aegean regions (Özalp and Azeri, 1967) for many years, it was only recently reported in the eastern Black Sea region (Korkmaz et al., 2006). This study reports for the first time CTV in satsuma grown in the Edremit Gulf region and orange grown in the western Mediterranean region.

The infection rate ranged from 20.3% to 26.8% based on ELISA and RT-PCR, respectively, and an infection rate as high as 50% was observed in some regions. Since samples were collected only from trees that showed virus-like symptoms, the average infection rate determined with ELISA and RT-PCR does not reflect the actual infection rate in the field. In the eastern Mediterranean region, where more than 50% of the samples tested were infected, some of the samples were collected from orchards previously known to be infected with CTV (Korkmaz, 2002); therefore, the actual infection rate in the field is probably lower than the rates reported in this study. Nonetheless, it became clear from this study that CTV is present in all citrus growing regions of Turkey. When different citrus hosts were compared, a higher rate of CTV infection was observed in satsuma mandarin. While an infection rate of 27% was observed in satsuma mandarin grafted on CTV-resistant *P. trifoliate*, as much as 75% of satsuma mandarins grafted on CTV-susceptible sour orange were infected with CTV. Although a limited number of satsumas grafted on sour orange were tested and samples were collected from old orchards previously known to be infected with CTV, the infection rate was significantly higher in satsuma mandarin-grafted CTV-susceptible rootstock. Since CTV was not detected in newly established satsuma orchards in the eastern Mediterranean region, where the majority of older satsuma orchards were infected, we think CTV was introduced and possibly spread by grafting imported infected budwood in the older orchards. Although CTV was detected in old satsuma mandarin and Washington navel orange orchards in this region, even newly established orchards located next to the long-established orchards in the same region were free of CTV. This suggested that CTV isolates were not transmitted by the *A. gossypii* vector present in the region. We have not conducted any transmission studies as yet, but it has been documented in other regions that CTV isolates were transmitted by different vectors and that some CTV isolates are not transmitted by *A. gossypii* (Raccha et al., 1976; Roistacher et al., 1980; Roistacher and Bar-Joseph, 1984, 1987). Yet, the possible introduction of *T. citricida* to the region could drastically change this situation and may initiate new epidemics in the region.

Different methods were used for the detection and identification of CTV isolates in Turkey. Comparison of ELISA and RT-PCR for CTV detection showed that more citrus trees were found to be infected using RT-PCR than by using ELISA. This was not surprising because RT-PCR is more sensitive than ELISA and the results were consistent with comparative studies of ELISA and RT-PCR for CTV detection (Nolasco et al., 1993; Çevik et al., 1996; Olmos et al., 1999; Cambra et al., 2002). Consequently, when necessary, ELISA results should be confirmed with RT-PCR and samples with ELISA values closer to the positive threshold should be verified with RT-PCR.

Serological assays using the monoclonal antibody MCA13 (Permar et al., 1990), which reacts predominantly with severe isolates of CTV, but not with mild isolates, has been useful for distinguishing mild and severe isolates of CTV in Florida and the Caribbean Basin (Çevik et al., 1996; Ochoa et al., 2000; Powell et al., 2003; Huang et al., 2004). A majority of the Turkish CTV isolates showed a positive reaction with MCA13, indicating that CTV isolates from Turkey may be severe isolates.

Evaluation of CTV isolates with BD-PCR confirmed that, except for a few isolates, CTV isolates from Turkey have the MCA13 epitope, suggesting that they may be severe isolates of CTV. In addition, some trees had mixed infections of MCA13-reactive and non-reactive isolates of CTV. Mixed infections of MCA13-reactive and non-reactive isolates of CTV were commonly observed in Florida (Çevik et al., 1996; Ochoa et al., 2000; Huang et al., 2004) and other regions (Cambra et al., 1993). Serological assays with MCA13 and the BD-PCR method have routinely been used for the detection and identification of severe, in particular, decline-inducing CTV isolates in Florida. However, in other citrus growing

regions it has been reported that some severe isolates of CTV that cause decline or stem pitting, in the field or in indicator plants, were not recognized by MCA13 (Cambra et al., 1993), which suggests that some severe isolates of CTV may not be recognized by MCA13. As there are no reports of mild isolates reacting with MCA13 in Turkey, the MCA13-positive isolates identified in this study are likely to be severe isolates of CTV.

Although serological and molecular tests conducted in this study indicated that MCA13-reactive isolates with the potential to cause severe symptoms are present in Turkey, no severe symptoms, such as quick decline or stem pitting, have been observed in the field. As a result, to better understand the biological nature of CTV, isolates representing different regions were inoculated into a series of indicator plants. All CTV isolates induced symptoms such as vein clearing and leaf cupping, and small leaf in the universal indicator host Mexican lime, suggesting that they may be mild isolates. Yet, 15 months after inoculation some of the isolates evaluated by biological indexing induced mild to moderate stem pitting in Mexican lime seedlings. While many Turkish CTV isolates induced stem pitting in Mexican lime, no stem pitting was observed in the other indicator plants, including Madam Vinous sweet orange and Rio-red grapefruit, suggesting that the isolates identified in this study were mild isolates of CTV. On the other hand, well-characterized mild CTV isolates, such as T30 from Florida and T318 from Spain, cause only vein clearing in Mexican lime and do not induce any stem pitting; therefore, CTV isolates that induce mild to moderate stem pitting in Mexican lime are more severe than the typical mild isolates. Considering the MCA13 reactivity in serological and molecular assays, the degree of growth retardation on citrus species grafted on CTV-resistant *P. trifoliate* caused by these isolates suggests that they may be moderate isolates of CTV; therefore, characterization of CTV isolates should not be based on a single assay, but the results should be supported by multiple assays, including biological indexing, and serological and molecular assays.

In summary, this study presents the first national survey of CTV in Turkey and the most comprehensive and detailed characterization of CTV isolates from different citrus growing regions based on different methods. The MCA13 reactivity of the majority of isolates suggested that severe isolates of CTV may be present in Turkey. In addition, biological assays revealed that, in addition to vein clearing, these isolates were able to cause mild to moderate stem pitting in Mexican lime, suggesting that these isolates were different from the typical mild isolates of CTV. Sequencing the CP gene of CTV isolates from different regions of Turkey will provide more information on the molecular properties of these isolates.

Acknowledgments

This study was supported by a grant (number TÜBİTAK-TOVAG 1040129) from the Scientific and Technological Research Council of Turkey. The authors thank H.R. Pappu for critical review of the manuscript.

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