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RESEARCH PAPERS

Molecular and biological evidence for a severe seedling yellows strain of *Citrus tristeza virus* spreading in southern Italy

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Summary. *Citrus tristeza virus* (CTV) outbreaks have been reported in the main citrus-growing regions of Italy in the past 10 years. In some areas where eradication efforts failed to suppress spread, high CTV incidence is now observed. Recently, potentially severe CTV strains were detected in Calabria (southern Italy), one of the major citrus-growing area. As a result, investigations of the virulence and molecular features of CTV populations spreading in this region were undertaken. Virus was detected by enzyme-linked immunosorbent assay (ELISA) using a broad spectrum polyclonal antiserum, and was differentiated into potential virulent categories with the severe-strain discriminating monoclonal antibody MCA13. Isolate genotyping was conducted using reverse-transcription polymerase chain reaction (RT-PCR) with multiple molecular markers (MMM), single-strand conformation polymorphism (SSCP) analysis of the amplicons from the genes coding for the coat protein (CP) p25 and the non-structural p20 protein as well as sequence analysis. Based on the serological reactivity, the isolates were differentiated in two distinct serogroups: MCA13-reactive and MCA13 non-reactive. Similarly, based on the molecular profile, the isolates were grouped in two genetically distinct phylogenetic clusters, and associated either with a T30-like or with a T3-like genotype. These data were related to the results of biological indexing on standard indicator plants, which distinguished isolates causing mild or severe seedling yellow reactions. The study has demonstrated the presence of MCA13-reactive isolates associated with a T3-like genotype and causing severe seedling yellows in sour orange, grapefruit and lemon seedlings, and stem pitting in Mexican lime.

Key words: CTV, citrus, MCA13.

Introduction

Citrus tristeza virus (CTV), genus *Closterovirus* (family *Closteroviridae*), is the causal agent of one of the most destructive and economically important viral diseases affecting citrus worldwide. CTV virions are flexuous filaments of about 2000 × 11 nm (Bar-Joseph *et al.*, 1972), with bipolar morphology and containing two coat proteins of 25 kDa (CP) and 27 kDa (CPm) encapsidating, respectively, about 97% and 3% of their length (Febres *et al.*, 1996; Satyanarayana *et al.*, 2004). The ≈19.3 kb genomic RNA (gRNA) of CTV is single-stranded and positive sense, and is organized into 12 open reading frames (ORFs) encoding at least 19 proteins (Moreno *et al.*, 2008).

Long distance spread of CTV occurs by introduction of infected plants or propagation material, while local virus spread is mediated semipersistently by several aphid species (Duran-Vila and Moreno, 2000;

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Timmer *et al.*, 2000). Although asymptomatic infections have been reported (Moreno *et al*., 1993), CTV isolates can cause various symptoms depending on the strain virulence, citrus cultivars and scion/rootstock combinations, and environmental conditions. There are two major economically devastating syndromes caused by CTV. The first is a quick decline and death of trees grafted on sour orange (*Citrus aurantium* L.) rootstock. The second is stem pitting (SP) of scions causing reduced fruit production, regardless of rootstock. A third syndrome, consisting in plant dwarfing and leaf yellowing, termed seedling yellows (SY), is observed only on sour orange, lemon and grapefruit seedlings. Mild and severe isolates have been identified based on the symptoms induced on indicator plants (Garnsey *et al.*, 2005). In field conditions, mild CTV strains are generally symptomless on commercial citrus varieties grafted on tolerant rootstocks, whereas severe strains can induce SP regardless of the rootstock.

The first significant CTV foci in Calabria (southern Italy) were identified on varieties introduced from countries where infections are endemic (Davino *et al.*, 1983). Other CTV outbreaks were discovered subsequently (Davino *et al.*, 1988). Despite efforts to prevent the spread, new and severe CTV outbreaks were reported in this region (Caruso *et al.*, 2006; Albanese *et al.*, 2010), as well as in Sicily (Davino *et al.*, 2003, 2005) and Apulia (Saponari *et al.*, 2009). Preliminary serological detection using the strain-discriminating monoclonal antibody (MAb) MCA13 (Permar *et al.*, 1990) on newly infected trees from Calabria revealed the presence both of MCA13-non reactive and reactive CTV isolates (Ferretti *et al.*, 2009), with reactive isolates typically being virulent. However, MCA13 does not distinguish isolates that cause only decline, generally harbouring a T36 genotype, from isolates that may also cause stem pitting (i.e. VT and T3 strains). Furthermore, the existence of isolates harbouring T30 genotypes which react with MCA13 has also been reported (Hilf and Garnsey, 2002). Serological differentiation was thus implemented using different molecular approaches.

Molecular characterization by single-strand conformation polymorphism (SSCP) of RT-PCR amplified products of the genes coding for p25 and p20 from selected CTV isolates tested previously showed different SSCP profiles for both genes. RT-PCR analyses using a panel of multiple molecular markers (MMM) and sequencing of these two genes provided molecular evidence for the presence of CTV genotypes genetically related to the reference T3 and T30 strains (Ferretti *et al.*, 2009, 2010). Transmission by local aphid populations was also demonstrated (Albanese *et al.*, 2010). The risk connected with the identification of potential severe strains transmitted efficiently in fields prompted further investigations to assess the virulence and genetic diversity of the CTV isolates spreading in Calabria.

Here we report results of serological and molecular analyses accomplished on 35 CTV isolates recovered from 11 contaminated fields. Additional sequencing and biological assays were done on seven out of 35 CTV isolates and comparing these different diagnostic approaches, T30- and T3-like genotypes associated, respectively, with mild and virulent isolates were effectively identified in the monitored area.

Materials and methods

Source of CTV isolates

A total of 35 CTV-infected samples were collected from trees previously found to be infected by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) tests using a polyclonal CTV antiserum (Agritest Srl). Infected mature trees (20–25 years old) were selected from 11 commercial citrus orchards located in two districts of Reggio Calabria Province, where recent CTV outbreaks were reported. The presence/absence of decline symptoms was recorded during the sampling (Table 1).

The following CTV reference strains were used as controls in the molecular assays: DS1-SR (mild) and DS2-CT (severe), provided by S. Davino (University of Palermo, Italy); T385 (mild), provided by P. Moreno (IVIA, Spain); DkVT (severe), provided by R.K. Yokomi (ARS-USDA, Parlier, California, USA); IT-08 (mild) provided by V. Savino (University of Bari). Negative controls consisted of leaves taken from virus-free sour orange seedlings maintained in the greenhouse.

Serological characterization

All CTV-infected trees were tested with the MAb MCA13. Plant sap was extracted from ≈ 0.5 g of fresh leaves as described by Garnsey and Cambra (1991). The homogenates were loaded in microtiter plates

Table 1. *Citrus tristeza virus* isolates detected in two districts of Reggio Calabria province (Italy) from symptomatic or symptomless trees and their serological characterization by ELISA using the monoclonal antibody MCA13.

Origin (district)	Field	Host (scion/rootstock)	Isolate ID	Symptoms of decline	MCA13* reaction
San Ferdinando	1	Washington	1A	Mild	-
		Navel/Sour orange (SO)	1B	Mild	-
			1C	Mild	-
	2	W. Navel/SO	2A	Severe	-
			2B	Mild	-
			2C	Severe	-
	3	Navelina/SO	3A	Mild	-
			3B	Severe	-
			3D	Mild	-
	4	Navelina/SO	5B	Mild	-
			5C	Severe	-
			5D	Mild	-
Candidoni	5	Satsuma/SO	6B	Severe	+
	6	Tarocco/SO	12A	Severe	-
			12B	Severe	-
			12C	Severe	-
	7	Ovale/SO	13A	Severe	+
			13C	Severe	+
			13D	Severe	+
	8	Navelate/SO	14A	Severe	+
			14B	Severe	+
		Navelate/citrange Troyer	14C	Absent	+
			14D	Absent	+
			14E	Absent	+
	9	Satsuma/SO	15C	Severe	-
	10	Hernandina/SO	16A	Absent	-
			16B	Mild	+
	11	Navelina/SO	17A	Severe	+
			17B	Mild	+
			17C	Severe	+
			17D	Mild	-
			17F	Mild	+
			17G	Severe	+
			17H	Mild	+
			17I	Mild	+

*Non-reactive (-) and reactive (+)

pre-coated with the CTV polyclonal antiserum and then incubated at 4°C overnight. After washing, the plates were treated with MCA13 (1:10000 dilution) (Nokomis, Intercession City, FL, USA) and incubated at 37°C for 2 h. The goat anti-mouse (Sigma-Aldrich, A3562) alkaline phosphatase conjugate was added and incubated for 2 h at 37°C. Plates were washed and treated with p-nitrophenyl phosphate at room temperature and the reaction was measured at 405 nm with a spectrophotometric plate reader. Samples were considered MCA13-positive when their absorbance resulted three times greater than that measured in the negative controls.

Molecular characterization

RT-PCR and single strand conformation polymorphism (SSCP) analysis

Total nucleic acids (TNA) were extracted from young leaves collected from the 35 selected CTV-infected samples using the Qiagen RNeasy plant mini Kit (Qiagen), following the manufacturer's instructions.

Purified TNA were adjusted to a concentration of approximately 100 ng/μL and subjected to one-step RT-PCR using the primer pairs P20-f/r (Rubio *et al.*, 2001), amplifying a fragment of 557 bp of gene *p20*, and T36CP-F/R (Hilf *et al.*, 2005) amplifying gene p25 (672 bp).

One-step RT-PCR reactions were set up using 2 μL of TNA of each sample in 48 μL of a reaction mixture containing: 1× *GoTaq* DNA Polymerase buffer (Promega), 4 mM DTT (Invitrogen), 0.2 mM each dNTPs, 0.2 mM of each primer, 8 U of RNaseOUT (Invitrogen), 40 U of SuperScript II (Invitrogen), and 1 U of *GoTaq* DNA Polymerase (5 U μL^{-1}) (Promega). The amplification conditions were: 42°C for 45 min, 94°C for 90 s, followed by 40 cycles of 94°C for 30 s, 50°C (primers P20-f/r) or 56**°C (pri**mers T36CP-F/R) for 30 s and 72**°C for** 60 s, with a final elongation step of 5 min at 72°C. The RT-PCR products **were separa**ted by electrophoresis in a 1.5% agarose gel and DNA bands visualized by staining with SYBR SAFE DNA (Invitrogen).

For SSCP analysis, the PCR products were mixed (1:10) with a denaturing buffer (95% deionized formamide, 20 mM EDTA pH 8, 0.05 % bromophenol blue and 0.05 % xylene-cyanol), incubated at 97°C for 15 min and immediately chilled. Electrophoresis of the *p20* amplicons was performed in non-denaturing 8% polyacrylamide gel (PAGE) at 200 V for 4 h at 4°C (Rubio *et al.*, 1996), and for the CP amplicons in 10% PAGE at 200 V for 4 h at room temperature. SSCP profiles were visualized by staining the gels with silver nitrate.

Multiple molecular markers analysis (MMM)

The above mentioned CTV isolates were also examined by MMM analysis using primer pairs (VT-POL, T3K17, T30POL, T36POL) and the conditions described by Hilf *et al*. (2005). The amplification profiles obtained using these key strain-specific primers served to categorize the Calabrian isolates with respect to the reference genotypes VT, T36, T3 or T30.

Cloning and sequence analysis

Based on the MCA13 reactivity and the SSCP and MMM profiles, the isolates 5B, 6B, 12C, 13A, 14A, 17A and 17D were selected, and further examined by cloning and sequence analysis. Specifically, for each selected isolate the PCR amplicons obtained using primer pairs P20-f/r, T36CP-F/R, VTPOL, T3K17 and T30POL were cloned in pGEM-T Easy vector (Promega). Twenty to 30 recombinant clones were then screened by SSCP analysis, and at least three clones representative of each SSCP pattern subjected to sequence analyses. Multiple sequence alignments were produced for the genes coding for *p25* and *p20* using ClustalW and phylogenetic trees generated by MEGA 5 software. The sequences retrieved from GenBank used as strain references were: VT (Florida, USA), SY568 (California, USA), NUagA (Japan), DS2-CT (Sicily, Italy), SP (New Zealand), T385 (Spain), T30 (Florida, USA), DS1-SR (Sicily, Italy), CTV-DS4CZ (Calabria, Italy), T36 (Florida, USA), NZRB-M17 (New Zealand) and 81P (California, USA). In addition, the sequences from the following isolates, selected by searching for high homologous sequences in NCBI database, were included: 159 (Pakistan), AN0-1 (Egypt), K1-76 (Tunisia), C315-16 (Argentina), C8 (Egypt), and B6 (Egypt).

Biological characterization

Bark pieces from cuttings collected from the seven infected field trees, selected for cloning and sequencing, were used to graft-inoculate three replicates of the following indicator plants: seedlings of sour orange (SO), Duncan grapefruit (DGF), Mexican lime (ML), Madam Vinous (MV), Eureka lemon (EL) and plants of MV grafted on SO (Garnsey *et al.*, 2005).

One month after the graft-inoculations, all indicators were tested for CTV by ELISA, and those that tested negative were re-inoculated with bark from the same source tree. Plants were examined for symptoms 6, 12 and 18 months post-inoculation (pi). Composite scores were made for: i) stunting, foliar symptoms and SP symptoms in ML and MV, ii) stunting, SY reaction and woody alterations (SP, cheesy bark, wood bristles) in DGF, sour orange, and EL, and iii) stunting and woody alterations (inverse pitting, woody bristles) in SO grafted with MV. In all cases, the scores ranged from zero (no symptoms) to 3 (severe symptoms). Presented score for each indicator was the average of three replications, and overall severity was rated as the summation of all averages obtained for each isolate/indicator combination.

Results

Serological and molecular characterization

Positive reactions with the MAb MCA13 were obtained in 17 out of 35 tested samples. Isolates collected within the same plot usually gave consistent results, testing either all MCA13-positive or all negative, except in two plots (10 and 11) where both MCA13 reactive and non-reactive isolates were found (Table 1).

SSCP analysis of RT-PCR products from gene *p20* revealed two different profiles (denoted as haplotype I and II). Haplotype I was distinct from those observed in the reference isolates, while haplotype II showed a pattern similar to that observed in the sample DS1-SR (Figure 1). Eleven isolates had haplotype I and 18 had haplotype II, whereas the remaining six contained a mixture of haplotypes I and II (Table 2), in which haplotype I consistently predominated and was associated with much stronger DNA bands (Figure 1).

SSCP analysis of the RT-PCR products from gene *p25* also showed two different SSCP patterns recorded as haplotype A (in 11 samples) and B (in 18 samples); with six samples showing the coexistence of both (Table 2, Figure 2). Haplotype A was distinct from those observed in the reference isolates, whereas patterns similar to haplotype B were observed in sample IT-08 and DS1-SR (Figure 1).

MMM analysis indicated that 17 isolates were positive with the primer pairs VTPOL and T3K17, whereas 24 isolates reacted with the T30POL primers (Table 2). None of the samples yielded RT-PCR products with the T36POL primers. Based on these results, 11 isolates were characterized as having a T3-like genotype (positive reaction with VTPOL and T3K17 primers), 18 as having a T30-like genotype (positive with T30POL primers) and six having a mixture of the two genotypes (positive with T30POL, VTPOL and T3K17 primers).

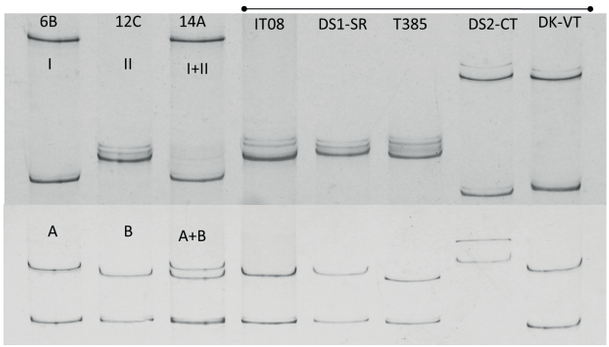

Figure 1. Representative electrophoretic SSCP patterns of RT-PCR products amplified from the CTV Calabrian isolates 6B, 12C and 14A, in comparison with those obtained for reference isolates. (Top) Haplotypes I and II recovered for gene *p20*. (Bottom) Haplotypes A and B recovered for the gene *p25*. Reference isolates IT-08, DS1-SR and T385 have been characterized as mild T30 isolates. Isolates DS2-CT and DK-VT have been associated with VT genotypes and severe symptoms on indicator plants.

The different serological and molecular approaches produced results that were consistent for each isolate. MCA13-reactive isolates were found to be strictly associated to the SSCP haplotypes I for gene *p20* and A for gene *p25,* and gave consistent positive reactions with the T3K17 and VTPOL primers. Conversely, all MCA13-non reactive isolates were associated with SSCP haplotypes II for gene *p20* and B for gene *p25,* and they consistently tested positive with the marker T30POL (Table 2). Six MCA13-reactive isolates were associated to composite SSCP patterns (I+II and A+B) and gave positive reactions with both T3 (T3K17 and VTPOL) and T30 (T30POL) markers.

When the VTPOL-, T3K17- and T30POL- recombinant clones were screened by SSCP, for each sample a common and simple pattern was recovered. Similarly, for genes *p25* and *p20*, the recombinant clones recovered from the isolates 5B, 12C and 17D, all showed SSCP pattern B (*p25*) and II (*p20*); whereas for the isolates 6B and 17A all showed SSCP pattern A (*p25*) and I (*p20*). For the isolates 13A and 14A, the

Table 2. Serological and molecular features of *Citrus tristeza virus* isolates recovered in Calabria.

Isolate[a]	MCA13 reaction[b]	SSCP haplotype (p20 gene)	SSCP haplotype (p25 gene)	MMM generating RT-PCR amplicons
6B, 14B, 14C, 16B, **17A**, 17B, 17C, 17F, 17G, 17H, 17I	+	I	A	VTPOL, T3K17
13A, 13C, 13D, **14A**, 14D, 14E	+	I+II	A+B	VTPOL, T3K17, T30POL
1A, 1B , 1C, 2A, 2B, 2C, 3A, 3B, 3D, **5B**, 5C, 5D, 12A, 12B, **12C**, 15C, 16A, **17D**	-	II	B	T30POL

[a] Isolates in bold indicates those selected for sequencing and biological indexing.
[b] Non-reactive (-) and reactive (+).

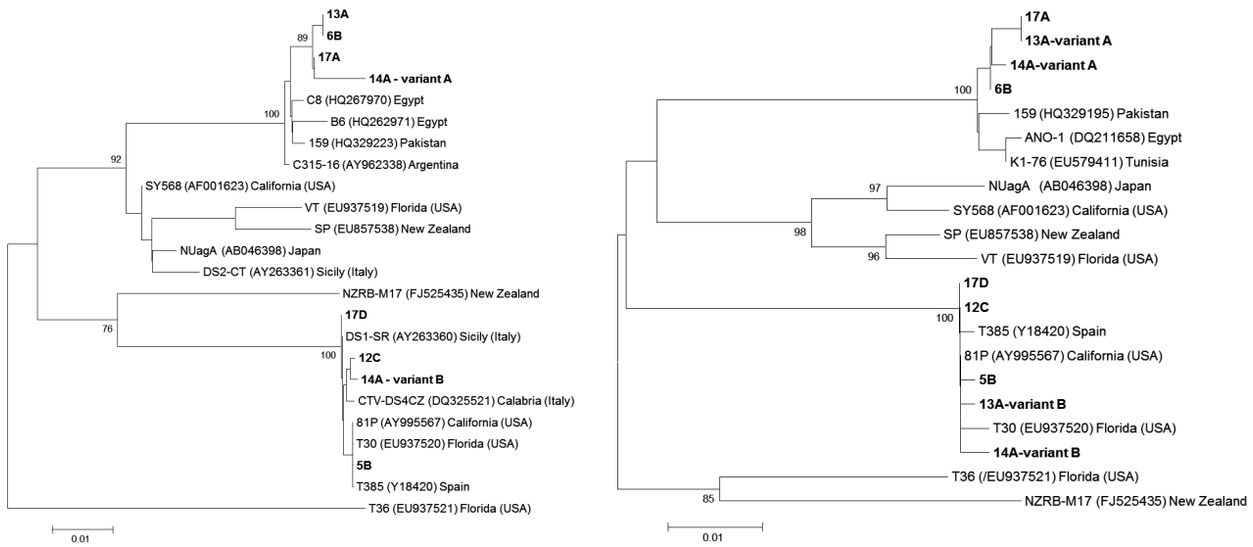

Figure 2. Phylogenetic trees generated by the neighbour-joining method from the alignment of the nucleotide sequences of the genes *p20* (left) and *p25* (right) of selected CTV isolates using MEGA (Version 5). Bootstrap values (percentage) for 1000 replicates are indicated at the main branches. Branch lengths are proportional to number of nucleotide changes. The sequences in bold were obtained in the present study. GenBank accession numbers for the Calabrian isolates are reported in the Table 3.

clones harboring the *p25* amplified gene were either associated with the SSCP pattern A or B. For both isolates the recombinant *p20*-clones all displayed the SSCP pattern I, except one clone from the isolate 14A which showed pattern II.

Phylogenetic analysis

Nucleotide sequence analysis of the *p20* gene showed 99% of sequence identity among the 6B, 13A, 14A (variant A) and 17A isolates. From the comparison with the sequences retrieved from GenBank, these Calabrian isolates shared 99% of homology with the isolates C8 and B6 (Egypt), 159 (Pakistan) and C315-16 (Argentina). Phylogenetic analysis of the nucleotide sequences of the *p20* gene grouped these isolates in the same main clade with several severe reference strains (SP, SY568, VT, NUagA and DS2-CT), although sharing nucleotide sequence

Table 3. GenBank accession numbers for the nucleotide sequences determined for the selected *Citrus tristeza virus* isolates from Calabria.

Isolate	Genomic region sequenced				
	p20	p25	T30POL	VTPOL	T3K17
5B	JX402740	JX402760	JX402761	-	-
6B	JX402741	JX090143	-	JX106445	JX106441
12C	JX402742	JX090144	JX106451	-	-
13A	JX402743	JX090145-JX106438	JX106449	JX106446	JX106442
14A	KC584008-KC584009	JX106436 -JX106440	JX106450	JX106447	JX106444
17A	JX402746	JX106437	-	JX106448	JX106443
17D	JX402747	JX106439	JX106452	-	-

identities ranging from 94% (with SP and VT) to 96% (with NUagA, SY568 and DS2-CT). On the other hand, isolates 5B, 12C, 14A (variant B) and 17D from Calabria shared 99% identity among them and with the mild reference strains T30, T385, DS1-SR, CTV-DS4CZ and the 81P isolate from California (USA), grouping all together in the same main cluster of the phylogenetic tree (Figure 2A).

Phylogenetic analysis based on the nucleotide sequences of the *p25* gene clearly differentiated the Calabrian isolates in two distinct groups. Isolates 6B, 13A (variant A), 14A (variant A) and 17A clustered on the same main branch as the severe reference strains (SP, SY568, VT and NUagA), but in a distinct clade along with isolates reported in Egypt (AN0-1), Tunisia (K1-76) and Pakistan (159). The nucleotide sequence identities shared with these isolates ranged from 94% (with SP, SY568, VT and NUagA) to 99% (with AN0-1, K1-76 and 159). The second phylogenetic group included the isolates 5B, 12C, 13A (variant B), 14A (variant B) and 17D, which clustered all together with the mild reference strains T30 and T385 (Figure 2B). Based on sequence analysis of both genes coding for *p20* and *p25*, none of the analysed CTV isolates showed phylogenetic correlation with the T36 and NZRB-M17 reference strains.

The nucleotide sequence of the amplicons obtained with the MMM T3K17, VTPOL and T30POL confirmed their specificity as well the phylogenetic correlations previously described for the *p20* and *p25* genes. Specifically, the T3K17 and VTPOL sequences recovered from isolates 6B, 13A, 14A and 17D, showed 98-99% nucleotide identities among them and with the most closely related SP reference isolate associated to a T3 genotype. Similarly, the T30POL amplicons recovered from isolates 5B, 12C, 13A (variant A), 14A (variant A) and 17D, showed 99% nucleotide identities among them and with both T30 and T385 reference isolates.

Isolates 13A and 14A, showing complex SSCP and MMM patterns, and contained genetically diverse haplotypes, with *p20* and *p25* sequences clustering in separate phylogenetic clusters. However, for the isolate 13A, none of the 30 *p20*-recombinant clones screened by SSCP contained the *p20* sequence related to the T30-like genotype.

GenBank accession numbers for the *p20*, *p25*, T30POL, VTPOL and T3K17 sequences are listed in Table 3.

Biological characterization

Severe seedling yellow (SY) reactions were recorded on SO, DGF and Eureka lemon graft-inoculated with the isolates 6B, 13A, 14A and 17A (Figure 3). All replicates of these indicator plants developed small yellow leaves and severely reduced plant growth. Stem pitting (SP) and intense vein clearing were recorded for the above mentioned isolates on ML (Figure 3). Occasionally, vein clearing was observed on the new flush leaves of MV, but no stem pitting was recorded on the MV and DGF peeled

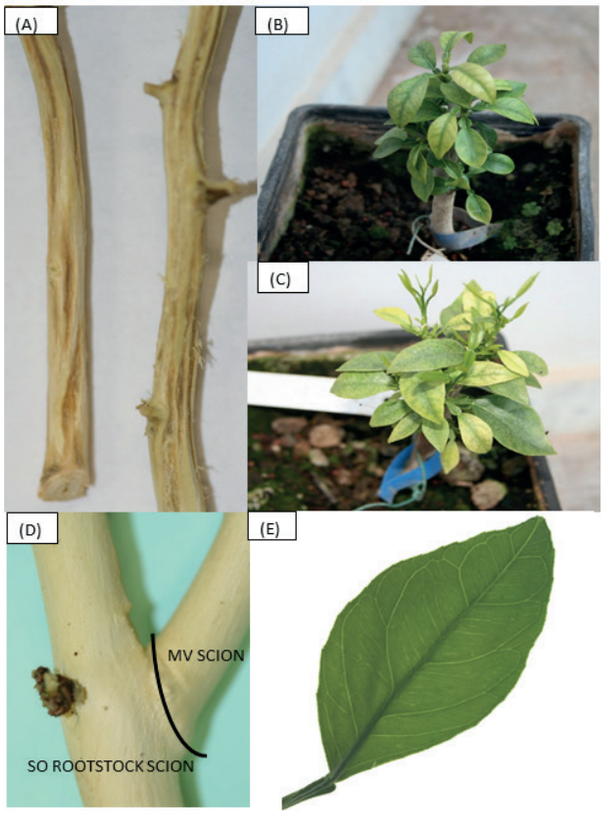

Figure 3. Symptoms recorded for isolate 6B associated with the T3-like genotype. (A) Stem pitting on Mexican lime. (B) Severe seedling yellow reaction on sour orange. (C) Severe seedling yellow reaction on Duncan grapefruit. (D) Inverse pitting on sour orange (SO) grafted with Madame vinous (MV). (E) Vein clearing on Mexican lime.

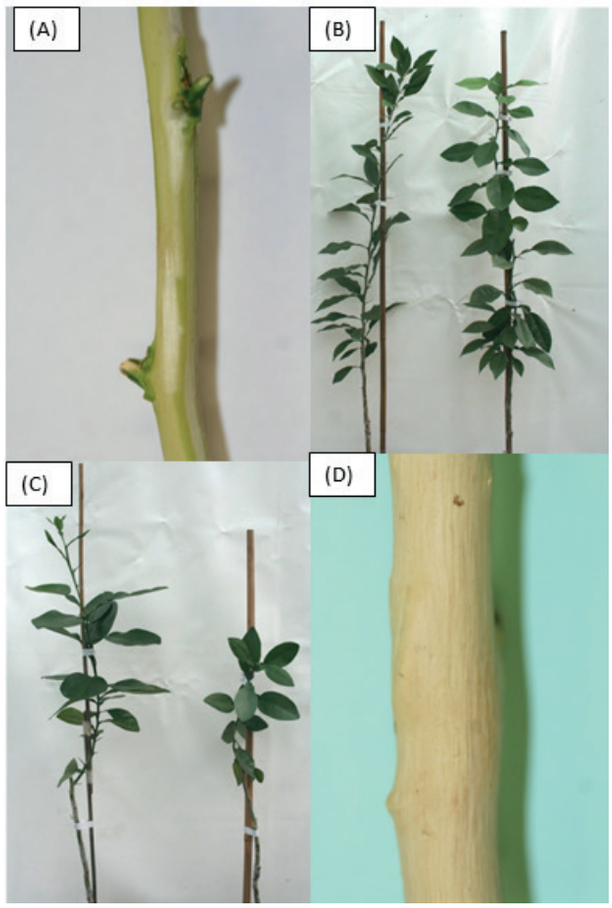

Figure 4. Results of the biological indexing for isolate 12C associated with the T30-like genotype. (A) Mexican lime stem. (B) Sour orange seedlings (healthy control on the left). (C) Duncan grapefruit seedlings (healthy control on the left). (D) No inverse pitting or woody bristle were present on peeled stem of sour orange (SO) grafted with Madam vinous (MV).

stems. Inverse pitting and woody bristle were recorded on SO grafted with MV, with this alteration being associated with reduced growth and leaf yellowing.

Other than mild vein clearing on ML, no significant alterations were recorded on any other indicator plants graft-inoculated with isolates 5B, 12C and 17D (Figure 4).

Discussion

Genetic and biological diversity are common among isolates of CTV worldwide, and disease symptoms depend on scion and rootstock combination (Bar-Joseph and Lee, 1989; Garnsey *et al.*, 2005). Mild strains of the virus either cause economic damage in citrus grafted on SO rootstock only after long periods, or are asymptomatic on tolerant or resistant rootstocks. Severe strains induce SP and SY in indicator plants and SP in commercial grapefruit, mandarin and sweet orange cultivars regardless of rootstock. The most virulent SP strains result in stunting of trees, bushy appearance, and loss of fruit quality and production of plants (Timmer *et al.*, 2000). Mandarins and pummelos show SP symptoms when infected with very severe strains of CTV (Koizumi, 1991; Tsai *et al.*, 1993). Rapid identification of strain severity is critical for disease control because CTV

Table 4. Biological characterization of the *Citrus tristeza virus* isolates from Calabria.

Isolate	Severity rating					Total	Notes
	Mexican lime[a]	Sour orange[b]	Duncan grapefruit[b]	Eureka lemon[b]	MV/ SO[c]		
5B	0.5	0.5	0.5	0.5	0	2	Mild
6B	3	3	3	3	1	13	Severe SY
12C	0.5	0.5	0.5	0.5	0	2	Mild
13A	3	3	3	3	1	13	Severe SY
14A	2	2.5	2.5	3	1	11	Severe SY
17A	2.5	2.5	2.5	N.D.	1	8	Severe SY
17D	0.5	0.5	0.5	0.5	0	2	Mild

[a] Scores refer to mild vein clearing and mild stunting for isolates 5B, 12C and 17D; to severe stunting, leaves with intense vein clearing, reduced size and stem pitting for isolates 6B, 13A, 14A and 17A.
[b] Scores refer to mild growth reduction for isolates 5B, 12C and 17D; to severe stunting, leaves small in size and with strong yellowing for isolates 6B, 13A, 14A and 17A. Stem pitting on DGF seedlings was also searched for but never detected (N.D.).
[c] Score refers to stunting and woody alterations (inverse pitting, woody bristles) in SO grafted with MV for isolates 6B, 13A, 14A and 17A. Stem pitting on MV was also searched for but never detected.

is readily spread by propagation and aphid vectors (Yokomi *et al.*, 2010). The present study was undertaken because of recent concern of increasing spread and preliminary evidence of the presence of severe CTV strains in the two major citrus growing areas of Italy (Davino *et al.*, 2005; Rizza *et al.*, 2007; Ferretti *et al.*, 2009, 2010).

ELISA using MCA13 allowed screening for potential severe CTV strains from Calabria. Different SSCP patterns obtained for genes *p20* and *p25* showed the presence of genetically diverse variants or genotypes among the tested isolates. MMM and sequence analysis validated these results by identifying two distinct groups of isolates associated with T3-like or T30-like genotypes. Furthermore, few isolates proved to contain a mixture of these two genotypes.

Bioindexing assays showed that CTV isolates from Calabria could be classified into mild and severe pathotypes. Biological results were consistently correlated with MCA13-reactivity, SSCP and MMM results.

Phylogenetic analysis showed that the Calabrian isolates with a T3-like genotype were distinct from the Sicilian isolate DS2-CT, the first known severe T3-like CTV strain found in Italy. Moreover, sequence comparison with reference genotypes associated to severe CTV strains showed that the T3-like genotype from Calabria shared only 94–96% nucleotide sequence identity with isolates VT, NUagA, SY568 and SP, whereas, they had 98-99% identity with partially characterized isolates from different Mediterranean and Middle East countries. These findings suggest that the severe isolates from Calabria are likely to have different origins from that of isolate DS2-CT. A similar conclusion was reached by Davino *et al.* (2005) for isolates found in a previous CTV outbreak in other Italian regions.

Biological indexing showed that the isolates associated with the co-presence of T30- and T3-like genotypes (13A and 14A) induced severe symptoms similar to those induced by the isolates containing the single T3-like genotype. Thus, in the mixed infections, the mild component (T30-like) did not alleviate the effect of the severe component (T3-like).

Field observations did not show the presence of SP but only symptoms of decline on the citrus/SO combination. While no strict correlation was found between the infections caused by CTV isolates containing T30- or T3-like genotypes and decline severity on sweet orange (Swt) in the field, isolates with T3-like genotype were always associated with inverse pitting below the bud unions in the Swt/SO indicator plants, stem pitting on Mexican lime and severe

seedling yellow (SY) reactions on SO, DGF and Eureka lemon seedlings. Nevertheless, since the greatest degree of decline was mostly associated with both T30- and T3-like genotypes, this can be explained by the citrus varieties and the time of CTV inoculation. Comparing the data presented here with those previously described in the same area (Giuliana Albanese, personal communication), it can be confirmed that severe CTV strains are spreading and represent a serious threat for the local citrus industry, which remains predominantly on SO rootstock (Lacirignola and D'Onghia, 2009).

CTV is a quarantine pest in the EU and is included in the "A2 list" of EPPO. Mandatory programmes are therefore required to prevent or suppress further introduction and spread of the virus (D'Onghia, 2009). Effective quarantine, eradication and budwood certification programmes are the best measures to avoid CTV introduction or dispersal in CTV-free areas. However, due to the increase of CTV incidence in multiple contaminated areas, Italian phytosanitary regulations for CTV eradication are under revision, and the selective removal of trees infected with virulent strains is being considered. Rapid elimination of infected trees is, in fact, the best strategy to avoid or delay an epidemic because this keeps CTV at low incidence and infected trees remain in a limited number of foci (Bar-Joseph and Lee, 1989; Gottwald *et al.*, 2002). However, when eradication becomes unfeasible due to inoculum pressure, budget limitations and lack of co-operation, use of tolerant rootstocks combined with selective removal of trees infected with severe strains becomes necessary CTV control measures. California has recently adopted such a CTV control strategy (Barnier *et al.*, 2010).

In conclusion, our study reveals the presence of CTV isolates containing T3-like genotype and inducing severe seedling yellows in sour orange, lemon and grapefruit for the first time, in the major Italian inland citrus-growing region. Knowledge of the strains in this area is critical for developing effective disease management and control measures to prevent or limit economic impact of this virus. To this end, the work described here supports the effectiveness of approaches already validated for CTV management in other important citrus growing areas (Yokomi *et al.*, 2011), namely the combined use of MCA13 and molecular tools to identify field trees infected by potentially virulent CTV strains.

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