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## Survey of *Citrus tristeza virus* in Southern Italy

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**ABSTRACT.** After a recent detection of *Citrus tristeza virus* (CTV) in Calabria (Southern Italy), an investigation was initiated in the spring 2006 to survey for the virus in this Region. The survey showed that CTV was not present in the nurseries which indicated that citrus mother plants and citrus plantlets ready for marketing have fulfilled completely the phytosanitary requirements. CTV was not detected in citrus-growing areas of Catanzaro, Cosenza and Crotona Provinces, but was found, at different levels in infected plants, in some orchards located in the Reggio Calabria and Vibo Valentia Provinces. Since the virus was detected not only in material illegally imported from abroad but also in local varieties, aphid transmission of the virus to local cultivars is suspected. Molecular characterization of some of the detected CTV isolates showed that at least three different CTV accessions have been introduced in this Region.

*Citrus tristeza virus* (CTV) is the causal agent of one of the most damaging and economically important citrus diseases. CTV, a Closterovirus with a filamentous particle of 2000x11 nm, is transmitted long distances by infected propagating material and locally by aphids, in a semi-persistent mode (1). The virus was detected 25 yr ago in Calabria (Southern Italy). While it was found rather sporadically from 1982 to 1986 (4), its detection has become more frequent in recent years (3). Because of that report as well as for its alarmingly rapid spread in the surrounding Regions (5, 6, 8), the local administration of Calabria has supported recently a special grant for 3-yr (2006-2009) aimed at improving screening, prevention and eradication of CTV discovered in Calabria Region. In order to unravel the degree of CTV spread in this Region, several actions were undertaken, including: i) monitoring and determining CTV presence and prevalence in various citrus areas; ii) molecular characterization of CTV isolates discovered, if any; iii) studying the citrus aphid population, in order to identify possible CTV vectors. Results of the first 18 mo of this investigation are reported here.

**Collection of samples and observation of field symptoms.** Leaf samples of different citrus species and cultivars were collected in nurseries and orchards located in Catanzaro, Cosenza, Crotona, Reggio Calabria and Vibo Valentia Provinces. Sampling in the nurseries was accomplished by collecting one plant/sample for mother plants and five plants/sample (1% of homogeneous stock) for seedlings and plantlets. In commercial orchards, the procedure of sampling was: five plants/sample (20 samples/10 ha) according to the Italian law on mandatory control of CTV (D.M. 22/11/1996). In foci where CTV was detected, a second sampling was done by with samples from 25% of plants in the orchards. In addition, 26 CTV-affected trees (four Comune Clementine, 10 Washington Navel, six Navelina and six Ovale sweet oranges) were selected in order to detect symptoms and to collect biological material for the molecular characterization of CTV isolates. Symptoms of decline and inverse pitting below the bud union line were observed in Washington Navel sweet orange trees grafted on sour orange. No symptoms were found in any of the other plants (Table 1).

TABLE 1  
RATIO OF AFFECTED OVER TESTED PLANTS AND SYMPTOMS IN *CITRUS TRISTEZA VIRUS* FOCUS AREAS

Citrus species and varieties	Plants affected/ plants tested (%)	Symptoms *
Comune Clementine	16/109 (15%)	None
Washington navel sweet orange	76/143 (53%)	Decline and inverse pitting on sour orange
Navelina & Ovale sweet oranges	40/539 (7%)	None

\* All plants were grafted on sour orange

**Diagnosis of CTV.** In order to detect CTV, DAS-ELISA was performed using a commercial kit with polyclonal antibodies (Agritest S.r.l., Valenzano, Bari – Italy). CTV was not detected in the nurseries which indicated that both citrus mother plants and citrus plantlets, which are ready for marketing, have fulfilled completely the phytosanitary requirements. CTV was not detected in citrus-growing areas of Catanzaro, Cosenza and Crotona, but was found in some orchards located in Reggio Calabria and Vibo Valentia Provinces. Since the virus was detected not only in non-Italian imported species and cultivars such as Satsuma, Navelina, Washington Navel and Valencia but also in local varieties such as Comune Clementine, Moro and Ovale sweet orange (Table 2), aphid transmission of CTV to local cultivars was strongly

suspected. The percentage of CTV infection was different across orchards (Table 3); this finding is likely to be attributable to the introduction of infected material for Washington Navel sweet orange (with a high % of infection), and to the initial aphid spread to other sweet orange cultivars and species (with a low % of infection). In addition, it is worth noting that in Southern Italy, the presence of potential CTV vectors *Toxoptera aurantii* (Boyer-de-Foscouombe.), *Aphis spiraecola* Patch (= *A. citricola* Van der Goot), *A. gossypii* (Glover) and *Myzus persicae* (Sulzer) has been reported, despite the absence of *T. citricidus* (Kirkaldy) (11). Taken together, these data strongly suggest that, in Calabria, the natural spread by vector transmission has to be further investigated.

TABLE 2  
*CITRUS TRISTEZA VIRUS*-AFFECTED SPECIES AND VARIETIES IN LOCALITIES  
 OF REGGIO CALABRIA AND VIBO VALENTIA PROVINCES

Province	Localities	Citrus species and varieties *
Reggio Calabria	Candidoni	Washington navel, <b>Moro</b> , Navelina, & <b>Ovale</b> sweet oranges, Satsuma
	San Ferdinando	Washington navel & Navelina sweet oranges
	Rosarno	Valencia sweet orange
	Laureana di Borrello	Navelina sweet orange
Vibo Valentia	Nicotera	<b>Comune Clementine</b>

\* Cultivars in bold are local varieties

TABLE 3  
 RATIO OF *CITRUS TRISTEZA VIRUS*-AFFECTED OVER TESTED PLANTS IN VARIOUS  
 PROVINCES OF CALABRIA

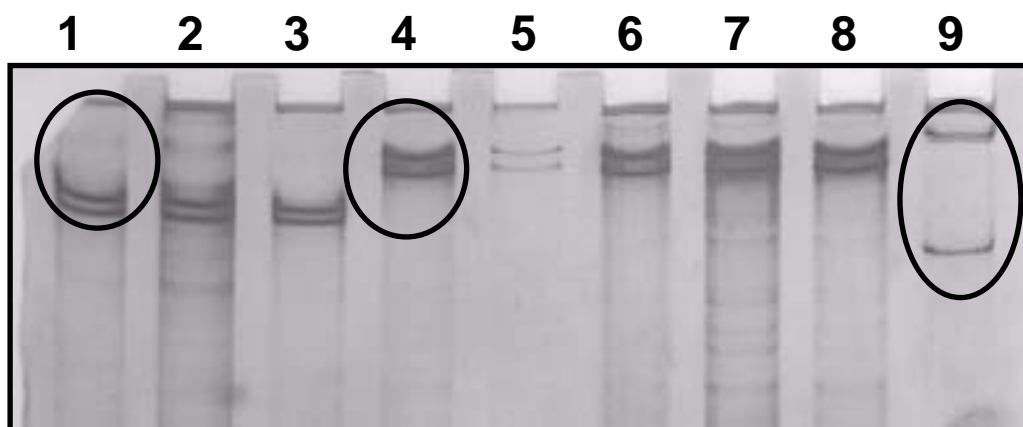
Province	Seedlings and plantlets in nursery	Mother plants	Citrus orchards
Catanzaro	0/2,195	971	-
Cosenza	0/395	-	0/3,220
Crotone	-	-	0/3,460
Reggio Calabria	0/915	-	39/3,570
Vibo Valentia			6/410
<b>Total</b>	<b>0/3,505</b>	<b>0/971</b>	<b>45/10,660</b>

**Molecular characterization.**  
 Twenty-six CTV isolates collected in five different citrus CTV-infected orchards were analyzed by the single-strand conformation polymorphism (SSCP) technique. Total RNA was extracted by the

RNeasy Plant Mini Kit, according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany) and finally eluted with 50 µl of RNase-free water. cDNA was synthesized from total RNA extracts by reverse transcription and amplification

(RT-PCR), using primers P20A 5'-ACAATATGCGAGCTTACTTTA-3' and P20B 5'-AACCTAACAGCAAGATGGA-3' of the p20 CTV gene (9). In detail, 2 µl of target RNA solution were heat-denatured at 94 °C for 2 min, chilled on ice and then added to 23 µl of the reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, one nM of each primer, 4 units RNase Out, 10 units of Superscript II reverse transcriptase RNaseH and 2 units of Taq DNA polymerase (Invitrogen Corporation, Paisley, Scotland, UK). Thermo cycling conditions were: 1 cycle of 45 min at 42°C; 1 cycle of 15 min at 75°C; 40 cycles of 20 s at 94°C, 20 s at 50°C, 20 s at 72°C and a final elongation of 4 min at 72°C. RT-PCR products were examined on a 1.5% agarose gel stained with ethidium bromide. For SSCP analysis, 2 µl of RT-PCR product were mixed with 18 µl of denaturing solution (95% formamide, 20mM EDTA pH 8.0,

and 0.5 mg/ml of phenol blue) heated at 99°C for 10 min and immediately chilled on ice. cDNA strands were separated by electrophoresis in a non-denaturing gel of 8% acrylamide using TBE electrophoretic buffer and 200 V for 4 h at 4°C (10). Four different isolates [DS1SR and T385 Spain isolate (mild isolates), DS2CT and SY568 (severe isolates) (7)] were used as markers. Gels were stained with silver nitrate (2). After RT-PCR, all CTV isolates gave a 545-nt band, typical of the p20 gene; SSCP analysis showed that 24 of the 26 patterns of tested isolates were similar to DS1SR and T385. Of the 10 Washington Navel isolates analyzed, one sample gave an electrophoretic profile analogous to DS2CT and SY568 and another, a distinct atypical profile (Fig. 1). Results obtained suggest that at least three different CTV accessions have been introduced in this Region. Further isolates characterization is needed to evaluate this hypothesis.



**Fig. 1.** Single-strand conformation polymorphism (SSCP) analysis of RT-PCR products for *Citrus tristeza virus* (CTV) p20 gene showing profiles of marker isolates [DS1SR and T385 Spain isolate (mild isolates), DS2CT and SY568 (severe isolates)] and CTV isolates detected in Calabria. CTV isolates are: 1, 8 and 9 Washington Navel sweet orange; 2, DS2CT; 3, SY 568; 4, Ovale sweet orange, Navelina; 5, T385; 6, DS1SR; 7, Navelina sweet orange.

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