Characterization of Additional *Citrus tristeza virus* Isolates in a Highly Infected Citrus Area of Sicily

A. Catara[1,2], A. Lombardo[2], G. Nobile[2], and S. Rizza[1]

[1]*Department of Phytosanitary Sciences e Technologies, University of Catania, Via S. Sofia, 102, 95123, Catania, Italy*
[2]*Laboratory of Phytosanitary Diagnosis and Biotechnologies, Science and Technology Park of Sicily, Stradale V. Lancia, 95030, Catania, Italy. srizza@unict.it*

ABSTRACT. In recent years, *Citrus tristeza virus* (CTV) has often been reported in Italy. Interestingly enough, despite the wide use of sour orange rootstock the typical inverse pitting commonly associated with CTV infection has only been reported on trees derived from introduced budsticks of citrus varieties, and recently on some 30 yr-old Sanguinello sweet orange trees. Recently, thousands trees of different varieties at distant locations were examined for inverse pitting without success. Preliminary studies showed that the isolate of CTV infecting two representative Sanguinello orange trees was different from other CTV isolates found close to this area which do not induce inverse pitting. All the sources indexed on Mexican lime seedlings showed typical vein clearing after only 10 days, whereas only sour orange, sweet orange and grapefruit inoculated with the Sanguinello sources showed stunting, yellowing, small and/or cupped leaves and vein corking to different degrees. The gene coding for p23 was amplified and the nucleotide sequences were determined in both directions. BLAST analysis showed a nucleotide identity of 99% with seedling yellow strains like BaraoB, Val-CB and C271-2. Results confirm that more than a single introduction of CTV could have occurred in Italy over the years.

Citrus tristeza virus (CTV) has been reported in Italy since 1953, mostly on imported citrus varieties (3). The first outbreak on local sweet orange varieties, as Tarocco and Navelina, in a relevant citrus area of Sicily occurred many years later (4). Since then, there have been reports based on DAS-ELISA assays, immunoprinting tests and molecular analysis, describing the spread of the virus and its destructive effects in some areas. Surveys during the last 5 yr show that CTV is unevenly distributed in nearly all the citrus areas of Sicily, with disastrous results when trees are grafted onto sour orange (2). Affected trees show dwarfing and dieback, size reduction and interveinal chlorosis, small and malformed fruit, and root death. Sour orange inverse pitting has been reported only on trees obtained from introduced CTV-infected buds. SSCP analysis has demonstrated that the present population in Apulia is comparable to the mild T-30 strain from Florida, whereas Sicilian isolates are close to the Californian isolates SY568 and SY107 (5, 6), suggesting that at least two independent introductions into Italy have occurred. A further genotype of CTV, dissimilar to any reference strain, has been recently characterized in Apulia (1).

Unfortunately, all these the results are not supported by conventional indexing on standard reference indicator host plants (7) and the evaluation of symptom severity was based on field symptoms shown by different citrus varieties, in different soil and climatic conditions. Therefore, the pathogenic characters of the Italian isolates are still lacking.

During a survey in 2005 we found some Sanguinello sweet orange trees showing a slight, but clear inverse pitting on sour orange rootstock (Fig. 1), which has never been observed before on trees of local varieties. Therefore, we undertook a study to characterize the causal agent of the disease through ELISA tests, indexing on indicator plants, and nucleotide sequence analysis. In order to provide a better understanding on the knowledge of the pathogenic aspects, they were evaluated in comparison with CTV isolates present in two orchards within a radius of only 1.5 km (9).

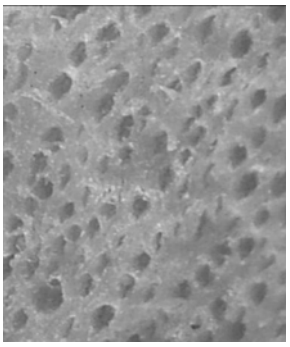

Fig. 1. Inner bark inverse pitting on sour orange rootstock of a Sanguinello tree infected with *Citrus tristeza virus* (CTV) (S25 isolate).

MATERIALS AND METHODS

Virus Isolates. CTV isolates used in this study were collected in the citrus-growing area of Belpasso (Catania province GPS N 37°-28'-18,1" / E 014°-54'-56,7"), very close to the orchard investigated by Davino et al. (5; 6). Two Sanguinello sweet orange old clone trees grafted on sour orange rootstock (named S24 and S25), a Tarocco Tapi and a Tarocco TDV both on Troyer citrange, were tested by ELISA and diagnosed as positive for CTV. The three field isolates were inoculated into 18 mo-old sour orange and Troyer citrange seedlings, and maintained in a growth chamber (26°C – 16 hrs day / 22°C – 8 hrs night).

Biological indexing. Biological characterization was undertaken on standard citrus indicator hosts under controlled greenhouse conditions. Three 8-mo old seedlings of sour orange, Hamlin and Pineapple sweet orange, Duncan grapefruit and Mexican lime were graft inoculated by T-cut with three bark chips per seedling. Moreover, 2-mo old Mexican lime seedlings were inoculated by leaf punch inserting five inoculum leaf discs per seedling. A healthy seedling for each experiment was used as control.

ELISA test. Plants were tested for the presence of CTV by indirect-double antibody sandwich ELISA (DAS-ELISA). Assays were performed with a mixture of two monoclonal antibodies (Ingenasa, Spain), following the EPPO protocol (8).

cDNA synthesis and PCR amplification of the p23 gene. Total RNA was extracted from infected leaves, with Trizol® Reagent (Invitrogen, Italy) following manufacturer's instructions. The primers P23F(5'GGACAAACTTTYRTT TCTGTGAACCTTTC-3') and P23R (5'-GATGAAGTGGTGTTCACGGAGAAC TC) were used to amplify the CTV p23 gene by a two step reverse transcription - polymerase chain reaction (RT-PCR). cDNA synthesis was performed with the Superscript^{TM} III First-Strand Synthesis System for RT-PCR (Invitrogen) according to manufacture's instructions. An aliquot (2 µl) of cDNA product was PCR amplified in a 50 µl reaction mixture containing 1X PCR buffer, 1mM $MgSO_4$, 0,2 mM each dNTPs, 0,2 µM each primer and 1U di PLATINUM *Pfx* DNA Polymerase (Invitrogen). The following PCR conditions were used: 94°C for 2 min; 35 cycles each of 94°C for 20 s, 60°C for 20 s, 68°C for 40 s; 68°C for 5 min.

SSCP analysis. Following amplification, 1 µl of the PCR product was mixed with 9 µl of denaturing solution (95% v/v deionized formamide, 20 mM EDTA, 0,25% w/v bromophenol blue and 0.25% xylene-cyanol), heated at 94°C for 10 min and chilled on ice. Samples were submitted to electrophoresis in a non-denaturing 8% polyacrylamide gel using 1X TBE buffer. Conditions used were 200 V for 3 h at 4°C. SSCP analysis (10) was carried out to screen PCR products in comparison with a mild strain previously isolated in the same area.

Cloning and nucleotide sequence analysis. The sequence variants detected with SSCP were cloned into a pETBlue-1 vector according to Perfectly Blunt Cloning kit (Novagen, San Diego, USA). The nucleotide sequence of the inserts, in both directions, was determined at the MWG biotech (Ebersberg; Germany).

Sequences obtained were submitted on BLAST for database searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Budsticks taken from inverse pitted plants and grafted on sour orange gave apparently normal shoots and grew almost regularly for 2 yr, then declined slowly and died. Whereas those obtained from budsticks infected with the isolates Tarocco Tapi and Tarocco TDV are still alive after 3 yr. All of them were CTV-infected according to ELISA tests.

Regardless of the source, within 1 mo, Mexican lime seedlings leaf punch inoculated showed vein clearing of young leaves and water spots of the main veins on the lower side. Seedlings bark-inoculated showed also stem pitting within 6-8 mo. Sour orange seedlings bark-inoculated with the Sanguinello sources showed seedling yellows (chlorosis, yellowing, small leaf size and vein corking). After 6 mo a marked stunting was observed (Fig. 2). In contrast, Tarocco sources were symptomless. None of the five sources of either Sanguinello nor Tarocco induced stem pitting on sweet orange Hamlin and Pineapple, nor on Duncan grapefruit.

Fig. 2. Chlorosis, vein corking, leaf size reduction and stunting on sour orange seedlings (right) in response to inoculation of *Citrus tristeza virus* (S25 source). Healthy control (left).

SSCP profiles showed two bands in the case of TDV and Tapi whereas three bands were obtained from inverse pitted sources (Fig. 3).

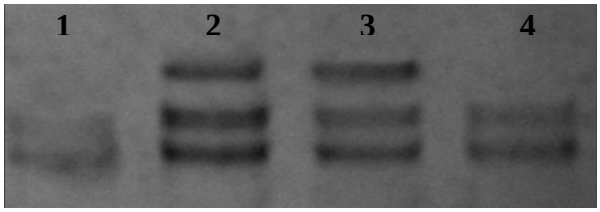

Fig. 3. SSCP profiles of the CTV p23 gene from isolates S24 and S25 (lane 2 and 3) compared to Tapi and TDV isolates (lanes 1 and 4, respectively).

BLAST analysis showed that isolates S24 (EU487601) and S25 (EU483657) have a nucleotide identity of 99% with seedling yellow strains from South America such as BaraoB (AJ579775), Val-CB (AJ579779) and C271-2 (AY750752), whereas isolates TAPI (EU487602) and TDV (EU487603) have a nucleotide identity of 99% with the strain T385. Therefore, the CTV isolate infecting those plants is different from those reported before (5, 6) and suggests it belongs to a Brazilian strain of the seedling yellows type.

DISCUSSION

According to the field inverse pitting symptoms on sour orange and the indexing results, the source plants of Sanguinello orange carry a CTV strain different from those reported up to now in Italy. The molecular analyses strongly support such conclusion showing a 99% of nucleotide identity with some South American strains (BaraoB, Val-CB and C271-2). This was unexpected since these strains usually induce stem pitting on sweet oranges and grapefruit (11) whereas in our tests they did not show any symptom. To our knowledge this is the first report in Italy of CTV isolates close to South America strains.

In spite of the proximity of the orchard to the one investigated by others, the two isolates are different to those previously reported in Sicily and in Apulia (1, 5, 6).

The evidence of this research shows that although the two examined citrus orchards are planted in quite a confined area, different isolates of CTV seedling yellows are present. Some of them belong to a South America-like group and induce inverse pitting on sour orange rootstocks, leaf yellowing, vein corking and stunting, whereas those of the California-like group are rather symptomless. This confirms that more than a single introduction of CTV has occurred in Italy in general (1, 5, 6), as well as in Sicily, over the years. Most likely, they were introduced from different areas and the infections have been masked for many years until the right combinations of isolates, vectors and host plants resulted in modified phenotypes.

ACKNOWLEDGEMENTS

This work was funded by MUR PRIN 2006 prot. 2006071323.

LITERATURE CITED

1. Barbarossa, L. and V. Savino
2006. Comparative sequence analysis of coat protein gene of Apulian citrus tristeza virus isolates. J. Plant Pathol. 88: 32.
2. Catara, A. and M. Davino
2006. Il virus della tristezza degli agrumi in Sicilia. Riv. Frutticolt. Ortofloricolt. 68 (1):18-23.
3. Davino M., R. Areddia and G. Reforgiato Recupero
1988. Effect of inoculation of a strain of tristeza on Tarocco budlings on nine rootstocks. Proc. 6th Int. Citrus Congr. 2: 863-866.
4. Davino, S., M. Davino, A. Sambade, M. Guardo and A. Caruso
2003. The first *Citrus tristeza virus* outbreak found in a relevant citrus producing area of Sicily, Italy. Plant Dis. 87: 314.
5. Davino, S., L. Rubio and M. Davino
2005. Molecular analysis suggests that recent *Citrus tristeza virus* in Italy were originated by at least two independent introductions. Eur. J. Plant Pathol. 111: 289-293.
6. Davino S., M. Guardo, G. Sorrentino, A. Caruso and M. Davino
2005. Variability among Italian *Citrus tristeza virus* isolates revealed by SSCP analysis, cloning and sequencing. In: *Proc. 16th Conf. IOCV*: 137-142, IOCV, Riverside, CA.
7. Garnsey S. M., E. L. Civerolo, D. J. Gumpf, C. Paul, M. E. Hilf, R.F. Lee, R. H. Brlansky, R. K. Yokomi and J. S. Hartung
2005. Biological characterization of an international collection of *Citrus tristeza virus* (CTV) isolates. In: *Proc. 16th Conf. IOCV*: 75-93, IOCV, Riverside, CA.
8. OEPP/EPPO
2004. Diagnostic protocols for regulated pests/Protocoles de diagnostic pour les organismes réglementés - *Citrus tristeza closterovirus.* OEPP/EPPO Bull. 34: 239 –246.
9. Rizza, S., A. Lombardo, G. Nobile and A. Catara
2007. Biological and molecular characterization of two additional *Citrus tristeza virus* isolates associated with sour orange inverse pitting rootstock. J. Plant Pathol. 89: 57.
10. Rubio L., M.A. Ayllon, J. Guerri , H. Pappu , C. Niblett, and P. Moreno
1996. Differentiation of citrus tristeza closterovirus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. Ann. Appl. Biol. 129: 479-489.
11. Sambade A., L. Rubio, S. M. Garnsey, N. Costa, G. W. Müller, M. Peyrou, J. Guerri, and P. Moreno.
2002. Comparison of viral RNA populations of pathogenically distinct isolates of *Citrus tristeza virus*: application to monitoring cross-protection. Plant Pathol. 51: 257-265.