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Assessment of *Citrus tristeza virus* (CTV) incidence in Calabria, southern Italy: results of a three-year survey

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Summary. Since 2006 a survey on *Citrus tristeza virus* (CTV) has been carried out in Calabria, southern Italy, to determine the occurrence of the virus, to evaluate its incidence, to identify and characterize the virus strains, and to monitor the aphid vector populations. Citrus samples were collected from nurseries and orchards located in the five provinces of the region. The virus was not detected in the citrus-growing areas of Catanzaro (CZ) or Crotone (KR), whereas it was found in three orchards in Cosenza (CS), three in Vibo Valentia (VV) and twelve citrus plantings in Reggio Calabria (RC). The highest infection percentages occurred in citrus orchards close to fields already infected with CTV. Infections were detected not only in foreign cultivars, but also in local cultivars such as 'Comune' clementine, 'Moro', 'Ovale' and 'Tarocco' sweet orange, suggesting that CTV was transmitted by aphids. CTV occurred in only a few plantlets sampled in two citrus nurseries located near the main areas already infected. Serological differentiation of several CTV field isolates revealed that the mild strains were prevalent. *Aphis gossypii* (Glover) and *A. spiraecola* Patch (= *A. citricola* Van der Goot) were the most frequent aphids in the orchards, whereas *Toxoptera aurantii* (Boyer de Foscoulombe) and *Myzus persicae* (Sulzer) occurred with low incidence. The absence of *T. citricidus* (Kirkaldy) was confirmed.

Key words: citrus-orchards, nurseries, aphid vectors.

Introduction

Citrus is one of the most economically important crops in Calabria; 40% of citrus production is concentrated in the Gioia Tauro (RC) area, 35% in Sibari and Corigliano (CS), about 15% in Lamezia Terme (CZ) (Figure 2), and the remaining 10% in small areas along the coasts, such as the Tyrrhenian and the south Ionian, where citron and bergamot cultivation is particularly important. Citrus nurseries are mainly located in the areas around Lamezia Terme and Sibari, and with all other citrus activities comprise 25% of the total agricultural output of the region.

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Diseases caused by viruses and virus-like agents can represent a limiting factor for citriculture; *tristeza* and greening are the greatest threats to the citrus industry worldwide (Roistacher and da Graça, 2006). In Italy greening has not been reported, but *tristeza* is a very serious disease because almost all citrus trees are grafted on sour orange, which is highly susceptible to the virus. *Citrus tristeza virus* (CTV) epidemics have recently been reported in Sicily (Davino *et al.,* 2003), adjacent to Calabria, so that measures to control and prevent the spread of this virus should be implemented in Calabria. Following initial reports of CTV in Calabria by Catara (1968) and Davino *et al.* (1983), new outbreaks have recently occurred in this region (Caruso *et al.,* 2006) which led to the initiation of a disease containment program by the Calabrian Agricultural Bureau during 2006–2009. This program, coordinated by SIAL SpA Services, was carried out under the supervision of the Calab-

rian Phytosanitary Service in collaboration with the Dipartimento di Gestione dei Sistemi Agrari e Forestali, Università degli Studi *Mediterranea* of Reggio Calabria and the CRA-Centro di Ricerca per la Patologia Vegetale of Rome. The objectives of the program were to ascertain whether CTV occurred in commercial citrus orchards and nurseries, to evaluate the incidence of CTV infection in areas where outbreaks occurred, to characterize the CTV isolates, and to monitor the CTV vector aphid population.

Materials and methods

CTV monitoring

As required by the Italian law on CTV control (D.M. 22 November 1996), commercial citrus orchards and nurseries were monitored for CTV from spring 2006 to autumn 2008, in five Calabrian provinces: Catanzaro (CZ), Cosenza (CS), Crotone (KR), Reggio Calabria (RC) and Vibo Valentia (VV). Surveys were carried out annually in June and during September–November. In the orchards, 20 composite samples were collected per 10 hectares, a 'composite sample' consisted of 5 plants. In the nurseries composite samples were collected from seedling and plantlet stocks (1% of homogeneous stock) and from incremental sections (10% of homogeneous stock), while mother plants were individually tested. During the three years of monitoring, 248 citrus farms in the five provinces were surveyed and 8,023 composite samples for a total of 40,115 plants were tested. Twelve nurseries, located in CZ, CS, RC and VV, were also surveyed and 2,258 samples (1,419 from seedlings and plantlets, 360 from incremental sections, and 479 from mother plants) for a total of 9,374 plants were tested. CTV was detected with the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using a polyclonal antiserum (Agritest Srl, Valenzano [BA], Italy) following manufacturer's instructions. Samples which gave doubtful readings (low OD405) were tested further by reverse transcription-polymerase chain reaction (RT-PCR) with specific CTV primers as described by Olmos *et al*. (1999).

CTV field incidence and symptoms

In 2007–2008 infection incidence was evaluated in some citrus orchards of RC where CTV outbreaks had previously occurred. The percentage of infected plants per homogeneous plot (= citrus orchards with trees of the same species/variety, identical origin and age, and grafted on the same rootstock) was determined. A total of 2,190 trees was individually sampled, 12.5% of the trees on 32 plots distributed in 45 hectares, following the scheme of Gottwald and Hughes (2000) modified as shown in Figure 1. From each plot, some CTV-positive trees were selected and their CTV symptoms evaluated. Fifty-five infected plants (37 'Navelina', 7 'Washington Navel', 3 'Navelate', 2 'Ovale', 3 'Tarocco' and 3 'Moro'), grafted on sour orange, were scored for infection according to the following scale: 0, no symptoms; 1, mild decline; 2, severe decline. The presence/absence of wood inverse pitting below the grafting line was also determined.

Serological characterization

CTV isolates of the fifty-five trees evaluated for symptoms were serologically characterized with the triple antibody sandwich-ELISA (TAS-ELISA) using the selective monoclonal antibody MCA-13 (Permar *et al*., 1990). This antibody does not react with most of the mild isolates identified in various parts of the world, and is therefore used for a preliminary ranking of isolates according to their virulence.

Aphid population monitoring

Aphids were collected from various infected citrus orchards. In each field the composition of the aphid population was defined and the possible presence of *Toxoptera citricidus* (Kirkaldy), the most efficient CTV vector, not yet detected in Italy, was also monitored. The dichotomy keys of the Natural History Museum in London were used to identify the aphids. The insect samples collected in the orchards were stored in 75% ethanol and labelled with a unique key. Aphid specimens, after passing through 10% NaOH, were dehydrated in increasing sequential alcohol concentrations, mounted on microscopy slides and finally luted with Canadian balsam.

To evaluate the spatial distribution of the aphid populations, a CTV-infected citrus orchard was selected, local coordinates of each tree were registered with GPS apparatus (GEO Explorer XT - Trimble®), and the number of aphid species collected from four young shoots per tree during the spring was recorded. The data were tested

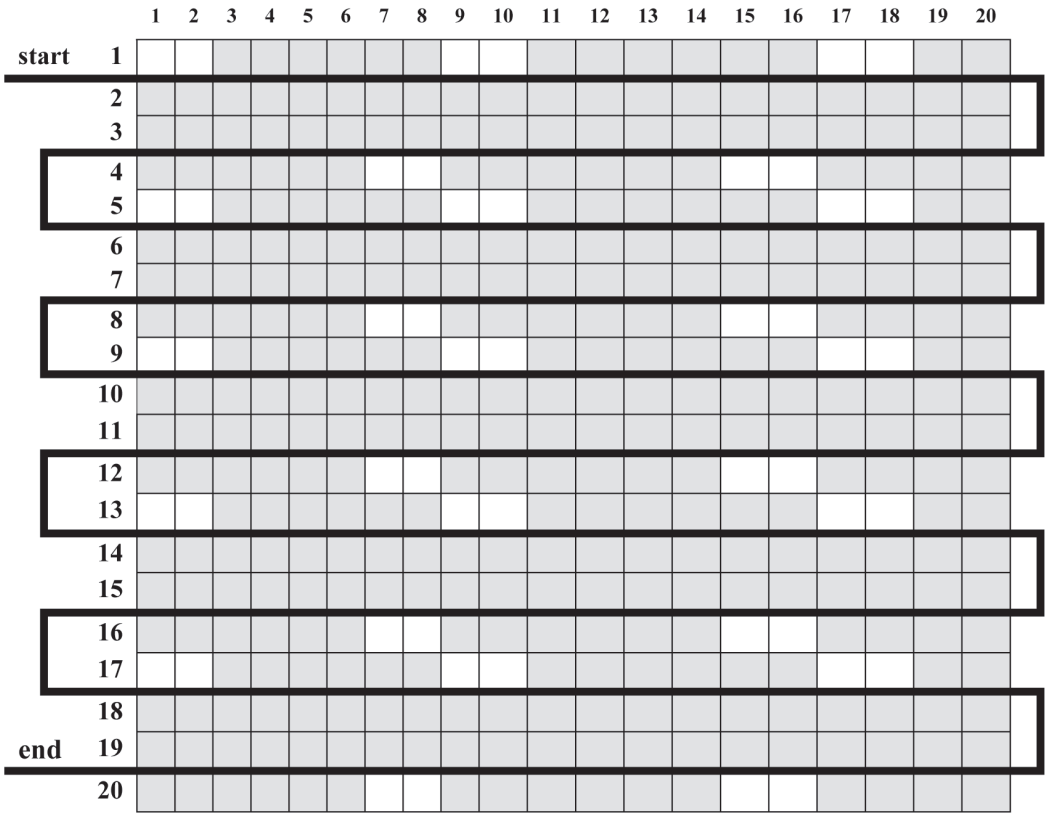

Figure 1. Scheme followed for sampling (Gottwald and Hughes [2000] with modifications): white squares indicate sampled trees, grey squares are trees not sampled.

with parametric univariate analysis of variance, applied with SPSS 16 software; the least sum of squares was used to compute all F-values. Differences between the means were determined by the Student-Neuman-Keuls (SNK) procedure. The mean number of aphid species recorded per tree was used to determine the spatial autocorrelation by Moran's index (Sokal, 1978).

Results

CTV monitoring

Citrus tristeza virus was not found in any of the citrus orchards in CZ and KR, but it was detected in a few orchards in CS. Twelve new CTV outbreaks were identified in RC, whereas three citrus orchards were found to be infected for the first time in VV (Table 1, Figure 2). In each province, outbreaks occurred in various citrus species/varieties as shown in Table 2. CTV was detected for the first time in samples from one nursery each in RC and VV. Seven positive samples were found in plantlets ready for marketing, but all samples from mother plants and incremental stocks were CTV-negative. The DAS-ELISA technique confirmed its effectiveness, detecting CTV even in samples in which only one out of five shoots was infected (data not shown), but this test was not reliable when daily average temperature exceeded 25°C for several days.

CTV field incidence and symptoms

The analysis conducted in 2007–2008 on a total of 2,190 plants detected widely variable percentages of infection in the homogeneous plots investigated. High infection rates were found with 'Navelate' (30–50%), medium rates with 'Washington Navel' (12.5–17.5%) and 'Navelina ISA 315' (12%)

Table 1. *Citrus tristeza virus* monitored in citrus orchards of Calabrian provinces.

Province	No. infected farms/ No. monitored farms	No. infected samples/ No. tested samples
Catanzaro	0/26	0/1,320
Cosenza	3/76	23/2,729
Crotone	0/37	0/1,588
Reggio Calabria	12/97	45/2,240
Vibo Valentia	3/12	10/146
Total	18/248	78/8,023

Table 2. Citrus species/varieties, located in farms of different municipalities per province, found infected with citrus tristeza virus.

Province	Municipalities (No. infected farms)	Species/varieties infected with CTV [a]
Reggio Calabria	Candidoni (6)	'Washington Navel', satsuma, **'Moro'**, 'Navelina', 'Navelate', **'Ovale'**, **'Tarocco'**, **'Comune' clementine**
	San Ferdinando (4)	'Washington Navel', 'Navelina', tangelo
	Gioia Tauro (1)	**'Comune' clementine**
	Rosarno (1)	'Valencia', 'Nova', 'Navelina'
Vibo Valencia	Nicotera (1)	**'Comune' clementine**
	San Calogero (2)	**'Moro'**
Cosenza	Corigliano Calabro (2)	'Washington Navel'
	Terranova da Sibari (1)	**'Comune' clementine**

[a] In boldface: typical Italian cultivars.

and low or zero rates with 'Moro', 'Ovale' and 'Tarocco' sweet orange. However, the same cultivars on the same farm exhibited widely variable infection rates depending on where the monitored plots were located. Variations for 'Washington Navel' were: 88, 57.5, 48, 34, 28, 25, 17.6 and 0%; for clementine: 24, 16, 10, 4.16 and 0%. When these data were compared with topographic map of the corresponding farms, it became clear that the plots with the highest percentages of infection were adjacent to fields that had been infected in previous years. The average infection percentage of the surveyed area was 20% (435 infected/2,190 tested plants). Of the fifty-five CTV infected trees whose symptoms were scored in 2007, only the 'Washington Navel' trees had severe symptoms (Figure 3), whereas 'Navelina', 'Navelate', 'Ovale', 'Tarocco' and satsuma had mild symptoms, which became more severe in 2008. Wood inverse pitting under the grafting line was seen in 'Moro', 'Ovale', 'Tarocco' and 'Washington Navel' plants grafted on sour orange.

Serological characterization

Serological characterization using MCA-13 monoclonal antibody identified three positive CTV

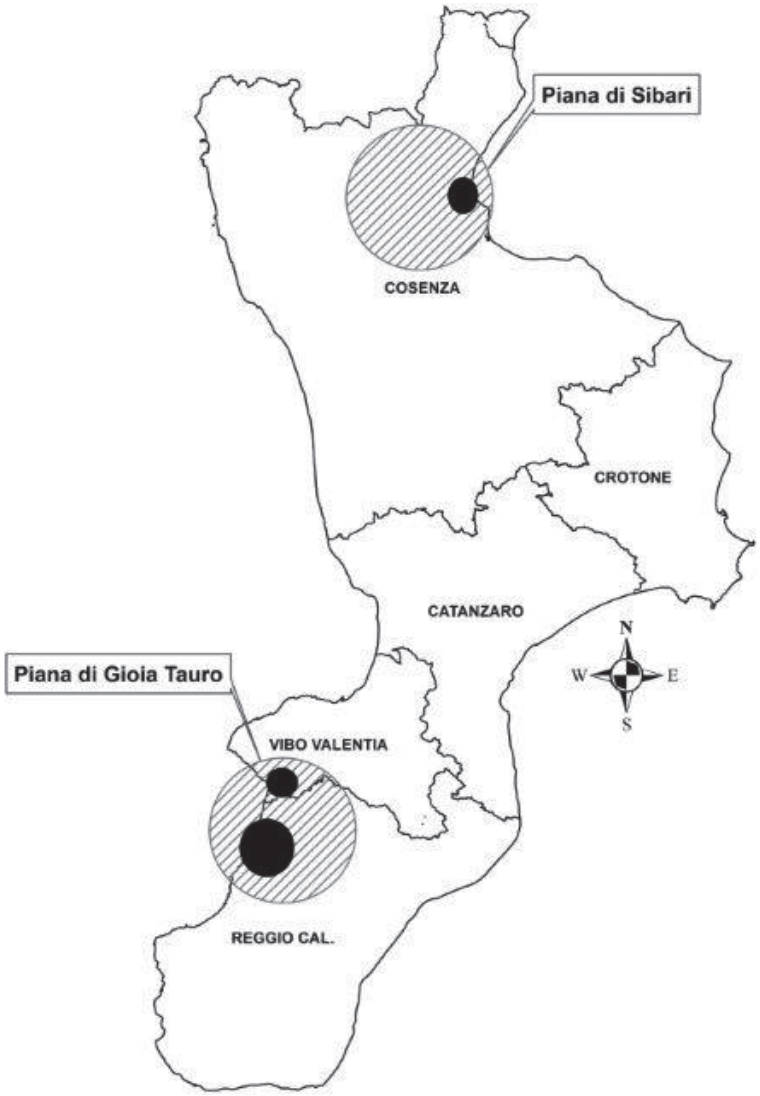

Figure 2. Citrus areas (black circles) where new outbreaks of *Citrus tristeza virus* (black spots) were found.

samples (two 'Navelate' and one 'Navelina') out of fifty-five tested plants, indicating that some of the CTV strains may be severe.

Aphid population monitoring

The following aphid species were found: *Aphis gossypii* (Glover), *Aphis spiraecola* Patch (= *A. citricola* Van der Goot), *Toxoptera aurantii* (Boyer de Foscoulombe) and *Myzus persicae* (Sulzer). In 2007, 709 samples were collected from different citrus areas. The most frequent aphid species, in terms of both numbers and frequency was *A. gossypii,* found in 52.61% of samples (373 samples). *A. spiraecola* represented 40.2% of the aphid population (285 samples), *T. aurantii* 5.50% (39) and *Myzus persicae* 1.69% (12). In 2008, 263 aphid samples were collected. *A. spiraecola* was the most abundant (41.06%); *A. gossypii* and *T. aurantii* were found in 34.98% and 15.97% of samples respectively. *T. citricidus* was not found in either year of monitoring. In their spatial distribution, the aphid species differed significantly in

Figure 3. Severe decline of sweet orange 'Washington Navel' grafted on sour orange (in front) in comparison with healthy plants (at the back).

their abundance ($F_{83.869,}$ $DF_{2,}$ $P<0.01$) (Figure 4). *A. gossypii* was the most abundant (39.44±22.57); *A. spiraecola* and *T. aurantii* ranked second and third (18.23±15.81; 3.83±4.12). Moran's index showed a random distribution of all aphid species (*A. gossipii*, I: -0.02, Z score: -0.012; *A. spiraecola*: I: -0.02, Z score: -0.82; *T. aurantii*: I: -0.03 Z score: -1.20).

Discussion

The monitoring carried out in this study gives an overview of the current CTV situation in Calabria. The absence of the virus on all 63 monitored farms in CZ and KR suggests that in these areas the disease is under control. The finding that only three farms out of 76 tested in CS were infected suggests that eradication can still be effective to control CTV spread here. But the extensive CTV outbreaks in the area between RC and VV is alarming and suggests that CTV eradication in these orchards should be reinforced by other containment measures. Since CTV incidence was highest in fields close to previous CTV outbreaks, and since CTV was detected in local cultivars such as 'Comune' clementine, 'Moro', 'Ovale' and 'Tarocco' sweet orange, it is probable that in this area the virus is transmitted by aphid vectors. The likelihood that CTV is transmitted by aphids is supported by the finding that CTV was also found in two nurseries adjacent to the main affected orchards, but only in a few plantlets ready for marketing, and never in mother plants or in incremental sections.

Monitoring of aphid populations confirmed that the citrus aphids already described in Italy are widespread (Barbagallo *et al.,* 1996) and that *T. citricidus* does not occur. The role and the effectiveness of these aphids in the transmission of CTV must be further investigated. In the meantime,

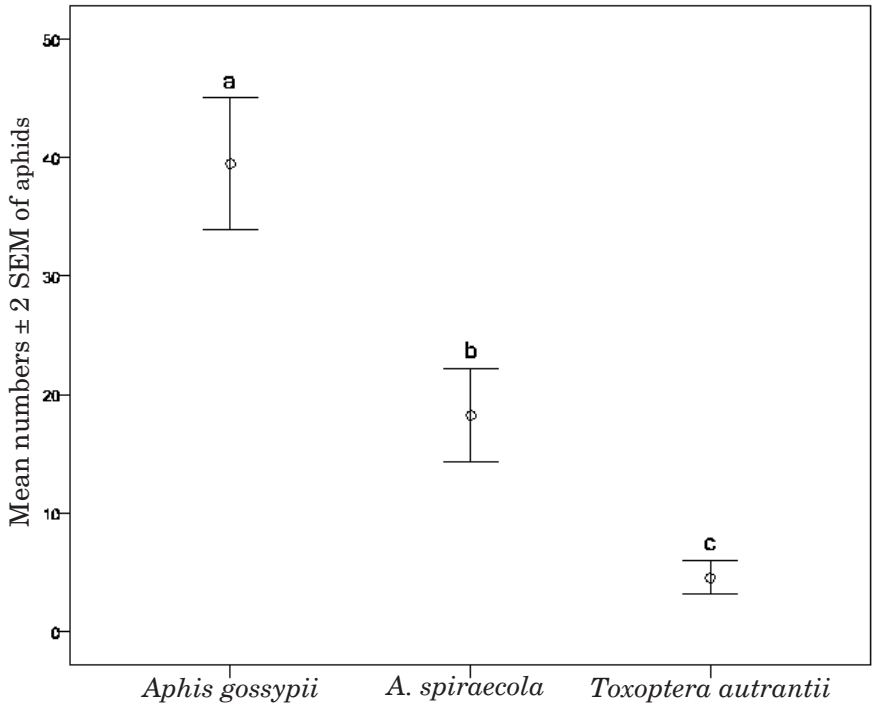

Figure 4. Mean number ± SE of different aphid species collected during the investigation.

aphids should be controlled especially in nurseries, and monitored to prevent the selection of these species for transmission effectiveness and/or the introduction of new and more efficient species.

Serological characterization of the Calabrian CTV isolates confirmed previous results obtained by the molecular characterization of 26 CTV isolates with single-strand conformation polymorphism (SSCP) (Albanese *et al*., 2007). As in that study, no correlation was found between the symptoms and the serological characterization since severe decline and inverse pitting were observed in trees infected by either severe or mild strains. The predominance of mild strains reduces the risk of economic damage that citrus *tristeza* could induce in the short term; however, a few virulent isolates were found: any plants infected with them should be immediately uprooted to prevent these isolates from spreading in the field.

Experiences in other countries have shown that when CTV incidence is high and when it is transmitted by aphids, the disease can no longer be controlled only by eradicating infected plants. Tolerant rootstocks, cross-protection and the selective removal of severe viral strains should then be considered as combined strategies to contain the disease. Moreover, when mild strains are prevalent, the high cost of replacing all infected trees could be buffered over time by removing plants only when it is no longer economic to keep them. Since CTV incidence differs considerably in the 5 Calabrian provinces, containment strategies should take the local epidemiological situation into account. A CTV management program should be adapted to specific needs: where incidence is low and aphid transmission does not occur, infected trees should be removed, but where CTV is endemic (contaminated areas) the impact of the disease should be contained by the selective removal of severe strains, the use of tolerant rootstocks, cross-protection and/or other means.

While CTV was first reported in Calabria on foreign cultivars imported from other countries (Catara, 1968; Davino *et al*., 1983), more recent studies (Caruso *et al*., 2006; Schimio *et al*., 2007; the present report) have examined trees introduced from other regions in Italy. This has shown that virus spread occurs also with the local movement of infected propagation material and that

mother plants and nursery stocks must therefore be carefully inspected.

In conclusion, the problem of *Citrus tristeza virus* was addressed in a timely manner by the Calabrian Institutions initiating the program through which this work has been carried out. A new and more appropriate law of the Italian Ministry of Agriculture is now essential without delay, so that all Italian regions where citriculture plays an important economic role are not penalized.

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