Present Status of Citrus Tristeza in Italy

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ABSTRACT. Results of a large survey for citrus tristeza virus (CTV) in Italy are reported. Over 20,000 trees from the main citrus area of Italy have been indexed by enzyme-linked immunosorbent assay (ELISA) and inoculation on Mexican lime seedlings. About 100 trees have been found infected by CTV and removed. Among the local varieties tested, three Clementine mandarin trees have shown to host symptomless infections of CTV. Some isolates of CTV tested on citrus hosts have shown a mild virulence. No evidence for seedling yellows was found. Results suggest natural spread of CTV infection under field conditions, but experimental tests showed low transmission efficiency with the aphids tested.

Index words. ELISA, virus survey, dsRNA, seedling yellows, aphid transmission.

Citrus tristeza virus (CTV) is the most destructive disease for many species of citrus. The virus is widespread in almost all citrus areas of the world (8).

The recent discovery of a substantial focus of the disease in Calabria (6) has prompted a survey of the trees introduced into Italy from abroad because about 90% of the citrus grown in our country is grafted on sour orange, known for its high susceptibility to all different strains of CTV. The present paper reports the results of an indexing carried out in Italy during 3 yrs (1984-1986) and reviews the results of other research (7, 9) on this topic in order to give information on the present status of CTV in Italy.

MATERIALS AND METHODS

Field survey. Different localities of Basilicata, Calabria, Sardinia and Sicily, which are the most important citrus regions of Italy, were surveyed. The survey was carried out in May-June and September-October of each year, mostly in orchards where trees propagated from budsticks introduced from abroad were noticed. Over 100,000 trees were inspected for symptoms and those considered suspicious for any reason (such as introduction from abroad, poor growth, scion overgrowth, inverse pitting below the budunion, closeness to known infected trees) were tested.

ELISA test. For these assays an antiserum prepared to the T4 isolate of CTV (11) was used. The citrus extracts were prepared from leaf samples and/or from bark taken the day before or stored for a few days at –20 C. The standard ELISA procedure was used (2). A CTV isolate from Japan (TJ) (4) and a mild isolate (T4) from Florida, free from other viruses, were used as positive controls. Recently (July 1986), a CTV monoclonal antibody (MAb), purchased from Immunologia y Genetica Applicada, S.A., Spain, was also used. Over 20,000 trees belonging to different citrus species and varieties were tested.

Lime Indexing. All the trees found infected by ELISA tests were also indexed on Mexican lime seedlings grown in 3-liter pots on a composite substrate of 2/3 peat and 1/3 volcanic soil sterilized by moist heat and fertilized adequately. Plants were kept in a glasshouse at 20-30 C. Four seedlings were inoculated for every sample, using three pieces of bark from each donor tree. Five seedlings were inoculated with T4 and TJ isolates as controls. The inoculated seedlings were inspected weekly for about 18 months. All seedlings were protected by nets to avoid contact with aphids or other insects.

Biological characterization of CTV isolates. Two isolates of CTV discovered in infected trees of Golden Buckeye sweet orange (T-GB) and satsuma mandarin (T-SM) were inoculated into container-grown seedlings of sour orange, Femminello S.

Teresa lemon and Mexican lime and into budlings of Madam Vinous sweet orange and Marsh seedless grapefruit grafted on sour orange. Two known CTV isolates (T4 and TJ) were used as controls. Adequate precautions were used in order to prevent mechanical contamination (12).

Ten young plants were inoculated by bark patch (two inocula per plant) per each virus isolate and 10 uninoculated plants were used as controls. During the test period the plants were kept in a air-conditioned room (25-26 C and 60-80% RH) and protected with thin-mesh nets. The plants and the nets were periodically sprayed with an aphicide (pirimicarb). The plants were inspected weekly and after 6 months bark was peeled and checked for symptoms.

Analysis of dsRNA. The dsRNA was purified from bark peeled from 10-20 cm long twigs of greenhouse-grown citron plants individually graft-inoculated with the above mentioned isolates. Extraction was performed in buffer containing 10% SDS, STE (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 7.8) saturated with phenol and bentonite (10). The mixture was shaken for 20-30 min. After centrifugation at low speed, the aqueous phase was mixed with 95% ethanol (5:1, v:v). The samples were passed through a small column of CF11 cellulose. After three washes with STE containing 16.5% ethanol, the dsRNA was collected by elution of the column with STE. After another passage through a small column, the dsRNA eluates were precipitated by adding sodium acetate, pH 5.5 to a concentration of 5% and 3 volumes of 95% ethanol. The mix was stored at –20 C overnight, and the precipatate recovered by centrifugation. The pellet was resuspended in a small volume of 95% ethanol containing sodium acetate and recovered by centrifugation after storage overnight at –20 C. The pellet was resuspended in a small volume of electrophoresis buffer (0.04 M Tris, 0.033 M sodium acetate, 0.001 M sodium EDTA) containing 2.5% bromophenol blue tracer dye and 12% RNAase-free sucrose. Ten µl of each sample (equal to about 3-3.5 g of fresh tissue) were electrophoresed in a 6% polyacrylamide gel minislab (Mini protean II, BioRad, Richmond, California) in buffer for 1 hr at 55 V. The minislab was stained with ethidium bromide (25 ng/ml) for 15 min in electrophoresis buffer or alternatively, with silver stain (BioRad, Richmond, California).

Aphid transmissibility. Different approaches were attempted to ascertain the aphid transmissibility of CTV in our conditions. At first, all the trees (mostly local varieties) located in the same field where Golden Buckeye sweet orange and satsuma mandarin trees had been found infected were tested by ELISA to check for natural spread. Moreover, different species of aphids were tested in the greenhose using two different procedures. Aphid colonies collected on trees of satsuma mandarin and Golden Buckeye sweet orange which had tested positive for CTV infection, were assayed for the presence of CTV by ELISA and by feeding on Mexican lime seedlings. The aphid species recovered from the infected trees were: *Aphis craccivora*, *A. fabae*, *A. gossypii*, *Aulacorthum solani*, *Macrosiphum euphorbiae* and *Myzus persicae*. The colonies collected from young shoots were put in a plastic bag, refrigerated, and then transferred to the laboratory. After 24 hr, single species samples of 100-120 specimens (adult apterous and fourth instar nymphs), were transferred, with the help of a brush, to a mortar and homogenized in a phosphate-buffered saline solution, pH 7.2, with 1% polyvinyl-pyrrolidone added and tested by ELISA (3). Other groups of the same colonies were transferred to Mexican lime seedlings and allowed to feed for 24 hr under a cage. Both assays were replicated at least three times.

In other experiments, the following aphid species were tested: *A. cit-*

ricola, A. gossypii, M. persicae, Toxoptera aurantii and *A. fabae* (table 1). The first four aphid species were collected in different citrus trees near Catania, whereas *A. fabae* was collected on broad bean crops, growing in the same area. The young shoots of citrus trees, infested with a single aphid species, were placed on young leaves of donor plants of Mexican lime or Madam Vinous sweet orange inoculated with T4, TJ, T-GB and T-SM isolates. After a 24 hr acquisition period, aphids were removed. The young shoots of the acquisition host that were covered with feeding aphids were cut and attached to the young leaves of the Mexican lime acceptor host. After an inoculation feeding of 24 hr, plants were sprayed with an aphicide (pirimicarb). The Mexican lime seedlings were inspected every week and tested by ELISA every month for one year for CTV infections. Transmission tests were carried out during May-October 1984 and 1985 in an isolated greenhouse, where temperatures ranged between 22-32 C and relative humidity from 30% to 100%.

RESULTS

In the last three years 20,787 trees have been tested (table 2). In

TABLE 1
RESULTS OF TRANSMISSION ATTEMPTS WITH FOUR CITRUS TRISTEZA VIRUS (CTV) ISOLATES BY FIVE APHID SPECIES

Aphids	Average no. of aphids	No. of plants positive of total inoculated with each isolate[z]			
		T4	T-GB	T-SM	TJ
A. fabae	30-120	0/25	0/20	0/15	0/25
A. citricola	50-150	3/39	0/10	0/10	0/15
A. gossypii	50-120	0/25	0/10	0/10	0/10
M. persicae	40-110	0/21	0/10	0/10	0/10
T. aurantii	75-150	0/22	0/15	0/20	0/10

[z]T4 and TJ were known CTV isolates from Florida and Japan respectively. T-GB and T-SM were isolated in Italy from field trees of Golden Buckeye sweet orange and satsuma manderin.

TABLE 2
RESULTS OF ASSAYS FOR CITRUS TRISTEZA VIRUS (CTV) IN DIFFERENT CITRUS OF LOCAL VARIETIES AND VARIETIES INTRODUCED FROM ABROAD

Citrus species	No. of trees	
	tested	infected
Calamondin	391	0
Grapefruit	82	4
Kumquat	89	0
Lemon	29	0
Lime	12	0
Mandarin and similar	7,194	47
Pummelo	6	0
Sweet orange	12,984	27
Total	20,787	78

trees introduced from abroad CTV infections were found in 27 trees of Golden Buckeye sweet orange, 36 trees of Wase satsuma mandarin, four trees of Marsh seedless grapefruit and eight groups of budsticks of satsuma mandarin introduced clandestinely from Yugoslavia. Golden Buckeye sweet orange and satsuma mandarin trees grafted on sour orange showed dieback, loss of leaves and a severe stunting. In some cases we noticed a scion overgrowth and honeycombing below the budunion. The young roots were severely damaged and secondary roots were dead. Other CTV-infected satsuma and Marsh seedless grapefruit trees grafted on trifoliate orange did not show any symptoms.

None of about 15,000 trees of local varieties of different citrus species was positive for CTV, but three healthy looking Clementine mandarin trees of 580 trees of local varieties located near the infected Golden Buckeye sweet orange and satsuma mandarin trees were infected.

Two different sources assayed on citrus behaved in a different way in comparison with known sources. Two months after inoculation, the young leaves of Mexican lime seedlings inoculated with bark patches showed the characteristic vein clearing. Later, vein corking appeared on some

leaves and light stem pitting in the wood. None of citrus hosts inoculated with different isolates of CTV found in Italy showed yellowing on young leaves. The four isolates tested, expecially T-GB, reduced the growth of inoculated trees about 46% for sour orange, 43% for Madam Vinous sweet orange, 37% for Femminello S. Teresa lemon and 41% for Marsh seedless grapefruit.

The electrophoretic pattern of dsRNAs of citrus extracts was similar to that reported for CTV (10) without any difference among the four tested isolates. No dsRNA band referable to seedling yellows (SY) component was found.

In over 30 attempts all the aphid species collected from citrus plants carrying CTV tested negatively by ELISA and were not able to transmit the virus to Mexican lime seedlings. About 330 transmission trials were carried out with aphids collected from CTV-free citrus hosts and fed on CTV infected plants (table 1). The percentage of aphids found alive after the feeding period in our greenhouse was about 70-90%. Only three attempts of 39 carried out with *A. citricola* yielded transmission of the T4 isolate (9).

DISCUSSION

In the last 3 years of the survey for CTV only 78 trees have been found infected. In the past, 47 trees were discovered affected by CTV (8) so up to now only 125 infected trees have been found in Italy (table 3).

The main infection site appears to be in Calabria, an important citrus area. This could be a serious threat for the entire citrus industry in Italy, and although infected trees have been pulled out, that area is continuously monitored. Fortunately, up to now the tests carried out on the trees near the infected ones show very poor transmission. In fact of 580 trees of different citrus species of local varieties close to the infected ones, only three have been found infected. This can be due to a very low spread or either to a regraft of previously infected trees. However, the transmissibility of the isolates may change in a short time (13).

The indexing on indicator plants points out that among the different

TABLE 3
HISTORY OF CITRUS TRISTEZA VIRUS (CTV) INFECTIONS DETECTED IN ITALY ON DIFFERENT CITRUS SPECIES OR BUDSTICKS UNTIL 1986

Year Detected	Cultivar	Locality[y]	No. Infected trees	Reference no.
1955	Meyer lemon	Acireale (CT)	2	15
1955	Meyer lemon	Palermo (PA)	2	15
1955	Satsuma mandarin (Wase)	Acireale (CT)	3	15
1967	Satsuma mandarin (Wase)	Muravera (CA)	8	16
1967	Meyer lemon	Monasterace (RC)	1	5
1974	Satsuma mandarin[z]	Catania (CT)	8	4
1982	Marsh seedless grapefruit	Monasterace (RC)	5	6-7
1983	Golden Buckeye sweet orange	Monasterace (RC)	42	6-7
1983	Ceylon lemon	Copanello (CZ)	1	6-7
1983	Satsuma mandarin (Wase)	Monasterace (RC)	42	6-7
1984	Comune Clementine	Monasterace (RC)	3	7
1984	Satsuma mandarin[z]	Basilicata	8	8
	Total		125	

[z]Budsticks introduced from abroad.
[y]CA—Cagliari (Sardinia); CT—Catania; PA—Palermo (Sicily); CZ—Catanzaro; RC—Reggio Calabria (Calabria).

CTV isolates found in Italy during recent years, the most virulent one is the TJ isolate introduced by chance from Japan. Since no yellowing has been observed on leaves of indicator plants we assume that SY component was not present in the CTV isolates found in Italy. These results are supported also by the electrophoretic pattern for dsRNA, which is a physical method of discriminating CTV isolates (10).

The absence of the SY component of CTV is highly important, because this is usually the most destructive component of the CTV complex. Its transmissibility by aphids may be high and the destructiveness is impressive (14). Changes in transmissibility of CTV have occurred in other countries and must not be underestimated (1).

Moreover, even if in a low rate and limited to a single CTV isolate, results of aphid transmission experiments indicate that a very small population of *A. citricola* could be a significant threat to the citrus groves in Italy. Further investigations are in progress.

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