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## First Report of *Citrus tristeza virus* (CTV) in Greece

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**ABSTRACT.** Surveys for the detection of *Citrus tristeza virus* (CTV) in Greece started in 1995 using DAS-ELISA. In June 2000 the virus was recorded for the first time near Argos (North East Peloponnese) on one Lane Late navel orange grafted on the CTV tolerant rootstock Carrizo citrange. The tree was illegally imported from Spain in 1994, but was labelled *Conformitas agraria communitatis* (CAC) quality. Twenty trees of the same lot were then traced and nine were found infected. All of these, as well as all their progeny (60 trees in all), were destroyed. Further surveys, however, revealed 15 more CTV-infected trees of various varieties, all close to a tree of the initial source, strongly indicating aphid transmission. Three of these trees showed some slight stem-pitting. The other 12 were symptomless. At the same time another 18 trees of the initial illegal consignment of Lane Late navel were found in the area of Hania, Crete, and two, both CTV-positive, had been used to establish an orchard of 50 trees which had in turn been used as a source of budwood and thus the number of CTV-positive trees that had to be eradicated in the Hania area went up to 3,500. Recently, CTV was detected in another consignment of nursery plants, certified Clemenpons mandarin on Carrizo citrange rootstock, from Spain. Large-scale testing for CTV by ELISA continues and since 1995 more than 22,000 trees have been tested all over the country. In 2001 testing by immunoprinting was initiated on 3,800 trees.

Citrus has been grown in Greece for many centuries and is an important component of Greek agriculture (1). Since 1959, importation of citrus propagating material from other countries had been prohibited and *Citrus tristeza virus* (CTV), the major viral threat of citrus (2), was not reported up to now (8). This situation changed in 1995, when free entry of citrus propagating material from European Community (EC) countries only was allowed (EEC Directive 77/93, PD 332/1995, G.G.F. 178/29.8.1995). As a result, during the past few years citrus propagating material has been introduced from the EC in unprecedented quantities, even by growers, who have found themselves under great pressure to change over to other varieties than the predominant Washington navel, in order to obtain a longer and better balanced production season.

To comply with the new situation and EC demands, the Greek Ministry of Agriculture launched a DAS-ELISA survey for CTV in 1995, focusing at first mainly on mother trees, as no mother trees had ever been re-tested after their initial indexing. All varieties are main-

tained at the Arbicultural Station, Poros (ASP), while mother blocks with a limited number of varieties of local interest have also been established in other areas using ASP budwood. Furthermore, many nurserymen, producing *Conformitas agraria communitatis* (CAC) material, have their own mother trees. Thus, the survey was carried out in cooperation between the Ministry and local authorities. Since 1999, grove trees have been included in the survey and immunoprinting to detect the virus was also introduced.

### MATERIALS AND METHODS

**Sampling and testing.** The survey was carried out as described by Cambra et al. (3). Mother trees and grove trees were registered with the name of the owner, the site, and the tree's precise location. Four 10 cm long tender shoots of the last flush were taken from each quadrant of each tree. For nursery plants one shoot per plant was sampled. Samples were kept in a refrigerator until shipment to the Control Station for Vegetative Propagating Material (CSVPM) at Aspropyrgos, Attiki.

Prior to 1998, samples were individually tested by DAS-ELISA (5), but later four tree samples were pooled (16 shoots per extract). All samples were kept separately for re-testing, in case of a positive or doubtful result from a composite sample. Except for the first year, when bark from young shoots was hand scraped into extraction buffer held on ice, shoots were sliced, using surgical blades, into 1-2 mm pieces, and transferred to firm plastic bags, containing cold extraction buffer (ca 1:2 w/v). Extraction bags were then tightly squeezed, and the extracted juice refrigerated overnight. These extracts were then loaded onto a pre-coated microplate and incubated in the refrigerator overnight. Positive and doubtful samples were retested individually in two wells per sample. Over the years various commercially available plates, extraction buffers and antibodies have been used according to the manufacturer's protocols. For reasons of convenience, over the last 2 yr antisera and extraction buffer from LOEWE Biochemica GmbH, Germany have been used on Costar high-binding EIA plates.

Since the spring of 2001, grove trees have also been tested locally by immunoprinting (DTBIA) (6) from Plant Print Diagnostics S.L., Spain. At least four, but often more, shoots were taken per tree and printed in the laboratory. Before printing, each shoot was freshly cut transversely using a razor blade. Each shoot was printed twice. When possible, samples positive by DTBIA were also tested by DAS-ELISA and vice versa.

**Eradication of infected trees.** Trees found to be CTV positive were pulled mechanically or were cut, and the stumps killed with glyphosate. Growers were compensated with 20.6 per tree.

## RESULTS AND DISCUSSION

**Detection of CTV.** It was verified in the laboratory that in composite samples of 16 non-lignified shoots

a single CTV-infected shoot can be reliably detected by DAS-ELISA. Results from DAS-ELISA and immunoprinting matched fully. However, immunoprinting appeared to be more sensitive, as it visualized an infection even at a level too low to ensure an unmistakable signal in DAS-ELISA. Storing samples in the refrigerator for several weeks had no impact on the detectability of CTV by either test. However, extracts of CTV-positive samples, stored refrigerated for more than a week, were usually no longer ELISA-positive, whereas printed membranes could be kept for several months before testing, without problems.

**Propagating Material.** All tests conducted to date on propagating material were found negative. Since the beginning of the survey, 3,545 mother trees have been tested by DAS-ELISA, covering the varieties currently marketed and representing about 70% of all listed varieties. All mother trees, that were tested for the first time in 1995 have been re-tested. Satsuma mandarin mother trees have been tested twice, as Satsuma mandarins have been reported elsewhere to carry orange stem pitting strains of CTV latently (10). So far, 2,003 nursery trees have been tested, as well as a number of potential mother trees, still under horticultural and phytosanitary testing.

**Grove trees.** Up to June 2000, more than 3,100 grove trees were tested from various parts of Greece and found CTV-negative. Information was obtained at that time that bare-rooted nursery material had been illegally introduced from Spain to Argolis in 1994. At first, two trees were located and one was found to carry CTV (4). The original consignment was found to have been composed of 50 trees forwarded to Argolis in the Peloponnese and another 18 to Hania in Crete (Fig. 1). As the plants had been soil-free, only 18 survived in Argolis and two in Hania. However, 16 of the trees



**Fig. 1.** Map of Greece showing location of two prefectures where *Citrus tristeza virus* was introduced; 1 = Argolis, 2 = Hania.

were distributed over the whole plain of Argos, while two were taken to Lakonia. Six more trees in Argolis were found positive, bringing the total number in that area to seven. The two trees in Hania were found

positive for CTV, while the two in Lakonia tested negative.

One of the trees in Argolis carried a complete label, showing it to be Lane Late orange on Carrizo citrange rootstock and of CAC status. Budwood from this material had been used for further propagation, and thus eight more trees were found to be infected, all but one Washington Navel on sour orange re-grafted in 1997 and 1998 with infected scions (Fig. 2). In the Hania area, Lane Late proved to be a very promising variety and a grove of 50 trees was established with budwood from the two CTV-infected trees.

The illegal introduction of CTV-infected citrus prompted the adoption of the following strategy: 1) inspection services in all citrus area were alerted; 2) a call was made on local broadcast stations all over Greece inviting growers and users to report any Lane Late trees obtained privately, as well as any citrus material brought from abroad,



**Fig. 2.** Locations of apparent natural spread of *Citrus tristeza virus* in Argolis Prefecture.

and urge them to have it tested; 3) a systematic survey of all citrus groves into which infected material had been introduced would be undertaken; and 4) trees found CTV-infected would be eradicated (with compensation), as would all trees of the suspicious consignment of Lane Late Navel, imported in 1994 and all trees re-grafted with scions of this lot, whether the graft was successful or not and with no regard to test outcome. As a result, in Hania 28 growers came forward who had established 3,531 Lane Late navel grove trees, all of which were eradicated.

Since September, 2000 more than 13,000 grove trees have been tested for CTV by DAS-ELISA and another 3,800 by immunoprinting, in the CTV eradication program. Sampling has mainly been focused on Lane Late navel orange, material of foreign origin and groves in which CTV-infected trees had been established. However, grapefruit, lemon other sweet orange varieties, bergamot, calamondin, citron, kumquat, pummelo and various rootstocks were also sampled. Samples were

collected in 18 prefectures around the country.

In the spring of 2001, a consignment of 1,100 bare-rooted nursery plants of certified Clemenpons mandarin on Carrizo citrange rootstock was brought from Spain and planted by a grower in Argolis. Seven of these trees were found positive for CTV.

**Natural spread of the virus.**

The Lane Late navel trees on Carrizo citrange rootstock showed no symptoms except for slight “cupping” of the leaves despite regular irrigation. The groves in Argolis, into which infected Lane Late navel orange trees had been introduced, were surveyed by testing all trees by immunoprinting, DAS-ELISA or both. At four localities CTV was detected in trees of various citrus varieties near the “source”. Thus far 15 trees have indexed as CTV-positive and most of these were fully productive, 20-50 yr old, of various varieties. Infected trees were usually near the “source tree” (Fig. 3). None of the trees showed symptoms in the field, but after eradication, stem-pitting symptoms were noted under the peeled bark (9) in one

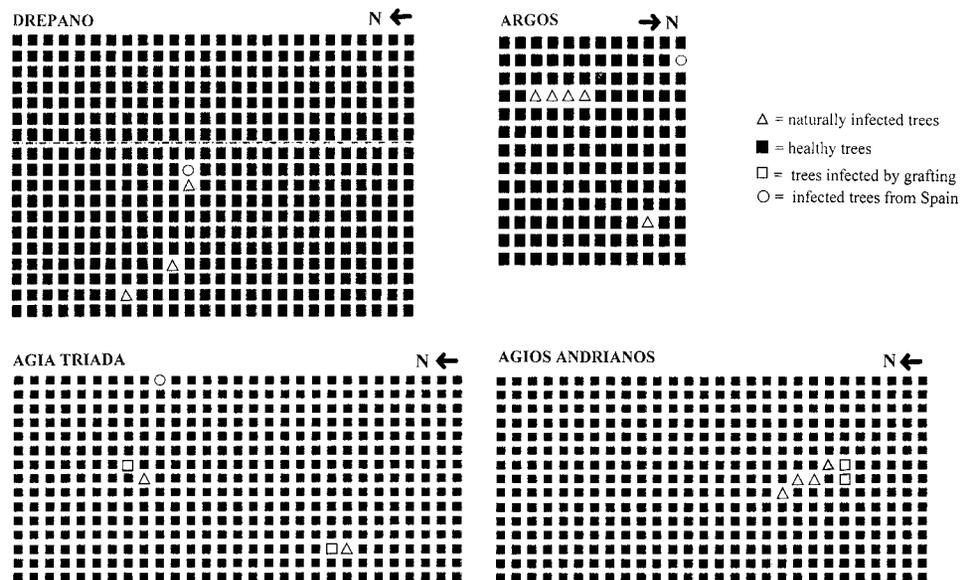


Fig. 3. Distribution of *Citrus tristeza virus*-infected trees in groves in Argos, Agia Triada, Agios Adrianos and Drepano.

Washington navel and two Clementine trees.

These infections can be either attributed to virus spread by aphids from the known sources, or to an "old", unrelated, infection. CTV incidence in the Argolis area is still very low, and it is highly unlikely that inoculum would have been present before 1994, without resulting in a much higher incidence (7). Furthermore, all "naturally infected" trees were situated downwind of the "source" tree. In immunoprints of the "naturally infected" trees, only two or three of the five shoots were positive, in most cases, suggesting a recent infection. *Aphis gossypii*, occurring in high numbers in Argolis, was probably responsible for virus spread.

To control the situation, the following action is planned:

1. The survey will be continued, including regular re-testing of mother trees.
2. All infected trees will be promptly eradicated.
3. The groves into which CTV was introduced will be re-tested

annually until no new infections are detected. At localities where transmission was observed, adjoining groves will be screened.

4. Intensive screening for CTV will be carried out in the Hania area.
5. As no data are available for Greece on aphids in citrus, an effort will be made to monitor and characterize aphid species in the citrus areas of Argolis and Hania.

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