

SURVEY RESULTS OF CITRUS TRISTEZA VIRUS (CTV) IN CRETE AND DETECTION BY DIRECT IMMUNOPRINTING-ELISA METHOD

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Abstract

Citrus tristeza virus (CTV), an aphid-borne closterovirus, is the causal agent of one of the most devastating citrus diseases, causing serious economic losses for the citrus industry all over the world. Currently, CTV is considered a quarantine virus in Greece and systemic surveys are conducted annually in the major citrus fruit growing districts of Crete to assess the incidence of CTV. Over a period of two years, more than 5,000 citrus trees in Crete were screened for CTV infection by immunoprinting-ELISA method. A total of 38 citrus samples (ca 1.9% of the total tested samples) were positive. This result represents almost a ten-fold increase in a number of detected CTV-positive samples compared to the previous study (2010). This observation calls for further in-depth studies in order to understand to a full extent CTV *loci* in Crete, and moreover design appropriate measures of CTV containment and control. Interestingly, CTV incidence at the newly identified locus (Koufos) in Western Crete accounted for 87% (33 out of 38) positive samples identified during the survey.

Key words: CTV, Crete, survey, detection, immunoprinting-ELISA

Introduction

Citrus tristeza virus (*Closterovirus, Closteroviridae*) is the most destructive and economically important virus of citrus worldwide. Losses from *Citrus tristeza virus* attacks and its economic impact on the citrus industry in particular regions depend on four main factors: presence of sour orange as susceptible rootstock, severity of CTV strains, ability of local aphid species to disseminate the virus and presence of *Toxoptera citricida*, the most efficient virus vector (Bar-Joseph *et al.* 1989; Cambra *et al.* 2000; Moreno *et al.*, 2008). Generally speaking, CTV can cause three main syndromes: tristeza or decline, stem pitting (SP) and seedling yellows (SY) (Moreno *et al.*, 2008). Some isolates are mild and essentially symptomless, and it is not infrequent to find infected trees that do not manifest any noticeable symptoms (Papayiannis *et al.*, 2007). Tristeza is a disease manifested by the progressive decline and death of most citrus species grafted on the susceptible sour orange rootstock (*Citrus aurantium*) or lemon (*Citrus limon* (L.) Burn. F.) (Moreno *et al.*, 2008; Papayiannis *et al.*, 2007). The general decay is a result of malfunction of the phloematic tissue induced by CTV infections (Schneider, 1959) which leads to continuous reduction of the root system with a deficient supply of water and minerals, which results in wilting, chlorosis and dieback symptoms (Fig.1, a). Stem pitting syndrome usually does not have a lethal effect on the host, but induces economic losses due to the prolonged reduction in yield quality and quantity. SP results in reduced vigor that causes stunting, thin foliage with small yellow leaves, low yields and small, poor quality fruits that are unmarketable (Fig. 1, b & c).



Figure 1. Progressive dieback of citrus tree in Chania (a); Leaf chlorosis of the citrus plant affected with CTV (b); Small-sized fruits from an orange tree severely affected by stem pitting (c)(Chania, November, 2010)

Although tristeza poses a tremendous threat to the Mediterranean citrus industry and it is widely distributed, CTV infections appear mostly as isolated foci and/or without showing clear-cut tristeza symptoms (Bové, 1995). However, tristeza has caused significant damages to the citrus industry in this region, in particular in Spain in 1957 (Moreno *et al.*, 2008) and Israel in 1970 (Bar-Joseph *et al.*, 1983) due to the adaptation of local CTV strains to *A. gossypii*. Tristeza outbreaks have also been reported from Cyprus (Kyriakou *et al.*, 1996) and Italy (Davino *et al.*, 2003). Moreover, CTV is currently considered a quarantine virus in Greece and was recently detected in relatively high rates in Crete (Afifi, 2010) illustrating the need for implementation of certification programs in Mediterranean countries, along with eradication efforts in order to prevent its spread. Bearing in mind that the citrus industry is very important for the Greek agriculture and economy in general, surveys for *Citrus tristeza virus* (CTV) in Greece were started in 1995 and large-scale testing was carried out by DAS-ELISA and direct tissue blot immunoassay (DTBIA). In June 2000, due to the accidental introduction of CTV-infected budwood from Spain, CTV was detected for the first time in Argolis (North East Peloponnese) and in Chania (Crete) (Dimou *et al.*, 2002). The scope of this research was to conduct a survey in 2009 and 2010 to estimate the incidence of CTV infections in Crete.

Materials and methods

Plant material. Newly pushed stems and leaves from four branches of 2,565 and 2,665 citrus trees were obtained in two periods (May-June and September-October) in 2009 and 2010 from four Cretan prefectures (Chania, Rethymno, Heraklion and Lasithi).

Immunological detection. For the detection of CTV in citrus plant tissues, the Direct immunoprinting-ELISA method kit (PlantPrint, Spain) was used, which is comprised of the following: nitrocellulose membranes, pre-spotted positive and negative control tissue prints, alkaline phosphatase conjugated CTV-specific monoclonal antibodies, mineral salts and substrate tablets [5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP), Nitro-Blue Tetrazolium Chloride (NBT)]. The tender shoots, leaves pedicels or fruit peduncles of the citrus plants were recommended to be used for the detection procedure. The printing was made by pressing the fresh crosswise cuts on the nitrocellulose membrane surface. A pack of ten membranes were firstly blocked by using 1% of blocking solution for one hour incubation at room temperature. Subsequently, for the antibody conjugation step, the antibody solution was added over the membranes for three hours incubation. Later, three washes were made to remove the excess of the antibody. Each wash was done for 10 min at room temperature. As a final step, the membranes were shacked in the substrate buffer which was poured on the membranes until the appearance of purple-violet in the positive control. After the reaction was stopped by adding the tap water, the membranes were air-dried and the results were observed by using a power magnification (X5-X10). All the solution and buffers were prepared with the recommended dilutions according to the manufacture's instructions.

Mapping of CTV presence in Crete. Geographic position system (GPS) data for each sampled plot were registered and then compiled in an ArcObject-based database and topographic maps geo-referenced in a Greek Grid (GCS-GGRS-1987) coordinate system.

Results and Discussion

CTV survey in Crete. An extensive survey was conducted in 2009 and 2010, in order to determine the incidence and distribution of *Citrus tristeza virus* (CTV) in Crete. Sampling was carried out by the personnel of different Cretan prefectures, and samples (stems and leaves) were delivered for lab analysis. Citrus trees and each sample were visually inspected for the presence/absence of visible virus-like symptoms ranging from mild leaf vein clearing to general plant stunting (Fig. 2.) Overall, samples from over 5,000 citrus trees were collected and analyzed for the presence of CTV. Young leaves/shoots collected from different points of the canopy were subjected to a standard diagnostic immunoprinting-ELISA test using a commercial kit (PlantPrint, Valencia-Spain). The nitrocellulose membrane of the kit included positive and negative control tissue prints and accommodated two sets (duplicates) of section prints from each of the four sides of the sampled trees. After completion of these tests, prints from 38 in total citrus samples exhibited purple-violet reactions in the vascular area of the printed sections indicating CTV infections (Fig. 3, and not shown), representing 1.9% of the total number of samples tested. Interestingly, the intensity of the color reactions was not uniform as expected in all four prints from the same tree-sample (Fig. 3) indicating a non-homogenous distribution of the virus among different branches of the same tree.

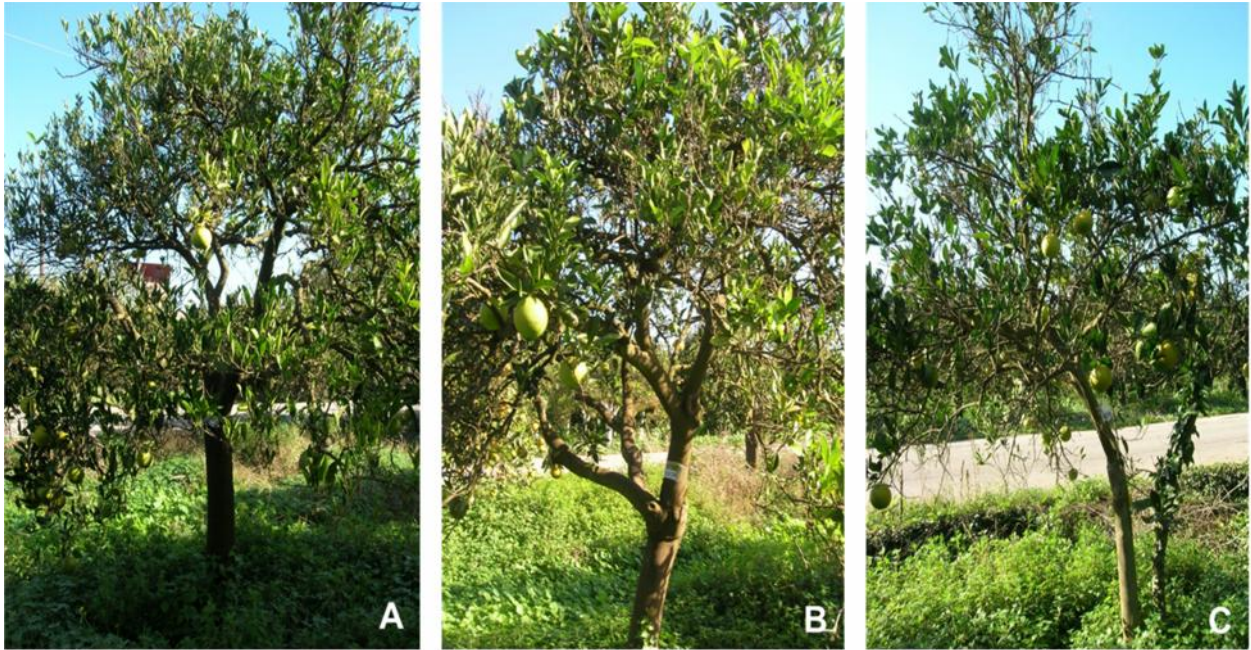


Figure 2. CTV-infected trees visited during the survey carried out during November 2010. Panels A-C show CTV-infected trees #1823, 1829 and 1851, respectively.

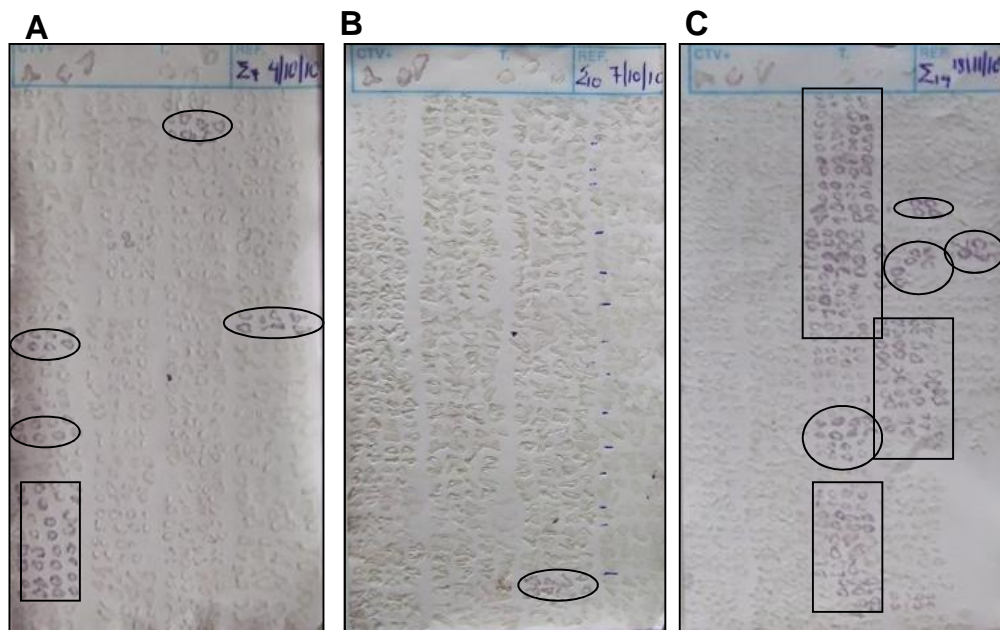


Figure 3. Immunoprinting-ELISA test. General view of three membranes (A, B and C) containing stem prints from surveyed plants. Boxed and/or encircled are CTV-infected samples.

CTV-infected trees were all identified at different locations of Chania prefecture (Fig. 4) with the highest incidence at the Koufos location.

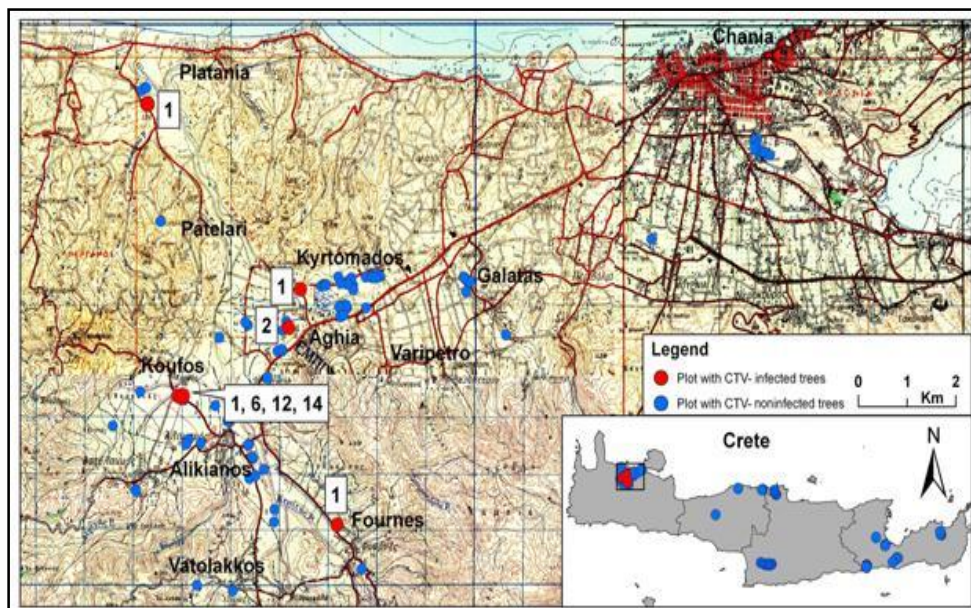


Figure 4. Map showing citrus orchards scouted during the survey in 2009-2010. Surveyed areas are indicated by dots; blue dots represent CTV-free orchards; red dots indicate CTV *loci*. The number of infected trees per orchard is indicated in the white boxes.

Conclusion

Bearing in mind a quarantine status of CTV in Greece, surveys should be executed routinely on a yearly basis on Crete and throughout Greece and combined with systematic monitoring for the aphid vector *T. Citricida* and epidemiological studies. Over a period of two years, more than 5,000 citrus trees in Crete were screened for CTV infection by immunoprinting-ELISA method, resulting in 38 infected trees, what represents almost a ten-fold increase in a number of detected CTV-positive samples compared to the previous study (Affifi, 2010). Our observations may be of use to extension and phytosanitary services and assist epidemiological studies to promote sustainable strategic solutions for the local and national citrus industry.

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