SHORT COMMUNICATION

VIRUS AND VIRUS-LIKE DISEASES OF CITRUS IN EPIRUS

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SUMMARY

In 2005 a survey was conducted in the main citrus-growing areas of Epirus. Commercial groves and nurseries were inspected for symptoms of virus and virus-like diseases and a total of 123 samples were collected. Molecular hybridisation was used to test for *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), *Citrus infectious variegation virus* (CVV), *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd). Although the results are from a low number of samples, they give a significant insight into the sanitary conditions of the Greek citrus industry, disclosing the presence of CTV mild strains in the North-western area.

Key words: citrus, virus diseases, CTV, molecular hybridisation, sequencing.

Citrus is an ancient crop in Greece, where it is grown in 29 of the 54 prefectures of the country. Sweet orange is the dominant species, followed by lemon, mandarin and grapefruit, all grafted primarily onto sour orange (Kyriakopoulou, 2002). With about 3.5 millions trees, the Arta valley is the main citrus-growing area of Epirus.

Most of the virus and virus-like diseases of citrus occur in Greece, e.g. psorosis A and B, concave gum, impietratura, cristacortis, crinkly leaf, ringspot, exocortis, cachexia, gummy bark and sour orange woody gall (Kyriakopoulou, 2002). Surveys for *Citrus tristeza virus* (CTV) were started in Greece in 1995 and large-scale testing was carried out by DAS-ELISA and direct tissue blot immunoassay (DTBIA). In June 2000, CTV was detected for the first time in Argolis (North East Peloponnese) and in Chania (Crete), due to the accidental introduction of CTV-infected budwood from Spain (Dimou *et al*., 2002). We now report the results of a survey for virus and virus-like diseases affecting citrus in some areas of Epirus.

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The survey was conducted in May, July and October 2005 in the main citrus-growing areas of the Arta valley and in neighbouring areas. Trees were inspected randomly for symptoms of virus and virus-like diseases. Concavities and bark scaling were widespread and were found in most of the trees in several groves. Symptoms of oak-leaf pattern, mottling, ringspot, curling and chlorosis of the leaves were also observed, sometimes associated with bark disorders. No decline or clear-cut tristeza symptoms were noticed in field trees. A total of 123 samples (leaf and bark) were collected from symptom-bearing and symptomless trees, labelled and stored at 4°C: 12 samples (sweet orange and lemon) from groves in the Louros area; 42 samples (sweet orange and lemon) from the northern coast of Preveza; 69 samples (sweet and sour orange, clementine) in the Arta valley and a nursery in Gramenitsa (Table 1).

All samples were tested by dot-blot hybridisation for the presence of CTV, *Citrus psorosis virus* (CPsV), *Citrus infectious variegation virus* (CVV), *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), following standardized protocols. Briefly, for CTV detection (Barbarossa and Savino, 2006), total RNA was extracted from 200 mg of leaf petioles using "TRIzol Reagent" (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions, and, for each sample, an aliquot equivalent to 50 mg of tissue was spotted onto nylon membranes Hybond-N+ (Amersham Biosciences, Little Chalfont, UK), previously soaked in 2 SSC. Prehybridisation and hybridisation of dot-blots were performed following protocols for nucleic acid blots with DIG-labeled probes (Roche, Mannheim, Germany). The hybridisation solution contained a DIG-labeled minus-sense riboprobe (50 ng of probe per ml), complementary to the coat protein (CP) gene sequence (length 672 nt) of the Apulian CTV isolate CTV-0032 (Acc. n. AJ518842). After hybridisation overnight at 60°C, membranes were washed twice (5 min each) in 0.5X SSC, 0.1X SDS at room temperature and twice (30 min each) in 0.1X SSC, 0.1X SDS at 68°C. Blots were detected with the chemiluminescent substrate CSPD as recommended by the manufacturer (Roche, Mannheim, Germany).

For CPsV assay, total nucleic acids were extracted

Table 1. Trees tested by dot-blot hybridisation.

Location	Species (cultivars)	Trees infected by (No.)					
		Tested	CTV	CPsV	CVV	CEVd	HSVd
Louros	Lemon	7		2		4	7
	Sweet orange	5					
	Total	12		2		4	7
Preveza	Lemon	24		2		9	4
	Mandarin	1				1	
	Sweet Orange (Wash. Navel)	17		3		4	5
	Total	42		5		14	9
Gramenitsa (nursery)	Clementine	9		1	4	4	6
	Lemon	2		2		2	2
(groves)	Sour Orange	17				3	14
	Total	28		3	4	9	22
Arta valley	Clementine	9				7	9
	Sweet Orange (Wash. Navel, Naveline)	32	11	4		9	26
	Total	41	11	4		16	35

from 100 mg of leaf tissue, according to De Paulo and Powell (1995). For CVV and viroid assays, the extraction method was according to Foissac *et al.* (2000), by adsorption of total RNA on to silica particles after guanidine buffer-treatment of plant tissues. Before spotting, samples (equivalent to 25 mg of tissue for CPsV and CVV, and 15 mg for viroids) were denaturated in 100 mM NaOH, 5 mM EDTA. The riboprobe to CPsV was complementary to the partial CP gene sequence (position 654-1253, length 600 nt) of the Apulian CPsV isolate ps101 (Acc. n. AM409317); the riboprobe to CVV was complementary to the partial CP gene sequence (position 1521-1706, length 186 nt) of the Sicilian isolate CVV-300 (Acc. n. AJ508381); the riboprobes to CEVd and HSVd were transcribed using the plasmids (Acc. n. M30869 and X00524) previously described by Visvader and Symons, 1985; Sano *et al.,* 1984.

These hybridisations were carried out overnight at 56°C (Minafra *et al.,* 2001), but different amounts of probes (CPsV: 100 ng ml^{-1}; CVV: 50 ng ml^{-1}; CEVd and HSVd: 25 ng ml^{-1}) were used. Virus-free citrus plants were included as healthy controls: field-grown sweet (*C. sinensis*) and sour (*C. aurantium*) orange trees and greenhouse-grown sweet and sour orange, grapefruit, Mexican lime and Troyer citrange plants were used. For each hybridisation test, total nucleic acids were extracted from healthy plants using the specific protocol applied to the samples and spotted (an aliquot equivalent to 50 mg of tissue for each spot) onto a separate membrane (not shown).

cDNA synthesis and cloning of the CP gene of the Greek CTV isolates were obtained as described by Barbarossa *et al.* (2005). Clones were automatically sequenced (MWG Biotech, Ebersberg, Germany) and the complete CP sequences of isolates CTV-G17 and CTV-G9 were deposited in the EMBL database under Accession numbers AM406802 and AM406803, respectively. They were compared to existing sequences using the BLAST program (Altschul *et al.*, 1990).

Eleven out of 32 Washington Navel and Naveline orange trees collected in commercial groves of the Arta area proved to be infected by CTV (Fig. 1a), whereas 4 clementine trees from the nursery in Gramenitsa gave a positive reaction to CVV (Fig. 1b). All these virus infected plants were co-infected by HSVd and 50% of them by CEVd. The incidence of CPsV infection was about 10%, regardless of citrus species and location, although a slightly higher number of infected trees was located to the north of Preveza (Fig. 1e). CEVd was detected in 32-39% of the samples, while HSVd was detected up to 85% in samples collected from Arta and Gramenitsa (Fig. 1c and 1d). Although widely spread, viroids did not produce conspicuous symptoms in the field, because of the use of sour orange rootstock which is tolerant to viroids.

The results confirm a realistic picture of the sanitary status of Greek orchards that seems rather compromised because of the high incidence of viral diseases (Protopapadakis, 2006). In addition, it is possible that most, if not all, CPsV and CTV isolates were detected, as the variability of CPsV is very low (Martín *et al.*, 2006) and the CTV-0032 riboprobe is characterized by a broad-spectrum reaction (Barbarossa and Savino, 2006), but the same might not be true for CVV and viroids, as in many species and cultivars, citrus viroids do not accumulate at high enough titres to be detected by molecular hybridisation (Bernard and Duran-Vila, 2006). Therefore the actual incidence of some virus and virus-like diseases could be even higher. It should be also pointed out that the use of laboratory tests may have

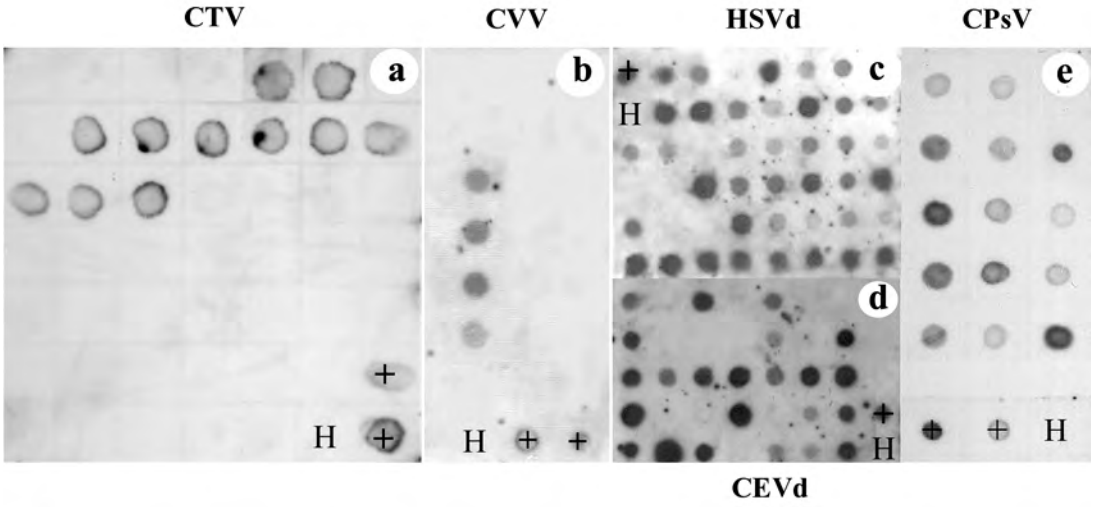

Fig. 1. Dot-blots of total RNAs from samples collected during the surveys, hybridized with riboprobes specific for CTV (**a**), CVV (**b**), HSVd (**c**), CEVd (**d**) and CPsV (**e**). H = healthy control; + = positive control.

missed potential diseases which could be detected by biological indexing, specifically concave gum, cristacortis and impietratura, which are known to occur in Greece (Kyriakopoulou, 2002).

This survey disclosed the presence of CTV in Epirus. The virus was detected in 20 year-old orange trees. The CP nucleotide sequence of these CTV isolates, compared to existing CTV CP sequences, showed a 99% identity to the T30 isolate from Florida (Acc. n. AF260651) and the Spanish isolate T385 (Acc. n. Y18420). The restriction analysis of the CP gene, based on *HinfI* and *RsaI* enzyme sites, revealed considerable resemblance to mild CTV isolates. In the classification of the *HinfI* RFLP pattern (Gillings *et al*., 1993), the isolates CTV-G17 and CTV-G9 were placed in group 2 (four restriction sites at nt positions 73, 111, 410, 501), which also contained mild isolates T30 and T385. In the classification of the *RsaI* products, the group 2 with three restriction sites (at nt positions 145, 312, 576) contained both the above Greek isolates and also mild isolates T30 and T385.

Despite the large presence of other citrus diseases, CTV is the most dangerous threat for the Greek citrus industry even though the trees were not apparently affected by the virus. Since the first report of CTV in Greece, in view of the danger of an epidemic spread of the virus and the prevalent use of the CTV-sensitive sour orange rootstock, a full certification program was initiated (Dimou *et al*., 2002). Mild CTV strains have been recently reported in several countries of the Mediterranean Basin (Barbarossa *et al*., 2003; Barbarossa and Savino, 2004; Anfoka *et al*., 2005; Barzegar *et al*., 2005; Davino *et al*., 2005), but more virulent strains may well be present in latent form in propagating material or in specific graft combinations and become destructive with more favourable environmental conditions, making the prospect of CTV outbreaks more probable.

The implementation and harmonization of citrus certification programmes should be the overall goal of the Mediterranean countries; its achievement would reduce damage caused by virus and virus-like diseases and improve the productivity of the citrus industry.

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