INDEXING OF VIROIDS IN CITRUS ORCHARDS OF CAMPANIA, SOUTHERN ITALY

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SUMMARY

During the last three years a survey was made to evaluate the presence and distribution of citrus viroids in the Campania region (Southern Italy). A total of 112 citrus trees of different species and cultivars were inspected visually and sampled. One hundred and four out of the 112 samples graft-inoculated into Arizona 861-S1 "Etrog" citron gave typical viroid symptoms. Viroid infection was confirmed by sequential polyacrylamide gel electrophoresis (sPAGE) analysis and molecular hybridization of imprinted membranes. *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus exocortis viroid* (CEVd) were the most widely detected, accounting respectively for 87, 85 and 68% of the tested sources. *Citrus viroid IV* (CVd-IV) and *Citrus bent leaf viroid* (CBLVd), never reported in Italy before, were found in 24 and 13% of the sources. Most of the trees (82%) were infected with more than a viroid. The most frequent viroid combinations were CEVd + HSVd + CVd-III (43%), HSVd + CVd-III (17%) and CEVd + CBLVd + HSVd + CVd-III + CVd-IV (13%).

Key words: citrus, viroids, indexing.

INTRODUCTION

Commercial citrus harbours several viroids and viroid variants that have been classified in five different species: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd; formerly called Citrus viroid (CVd)-I), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV) (Flores *et al.*, 2000; Duran-Vila and Semancik, 2003).

CEVd and particular variants of HSVd are causal agents of exocortis (Semancik and Weathers, 1972; Gross *et al.*, 1982; Visvader and Symons, 1985) and cachexia (Semancik *et al.*, 1988; Reanwarakorn and Semancik, 1998), respectively. Exocortis and cachexia are two economically important diseases present in almost all citrus-growing areas of the world. It has been reported that certain combinations of citrus viroids may also induce the exocortis-like symptoms in the absence of CEVd (Ito *et al.*, 2002a).

Exocortis is characterized by bark scaling and splitting in sensitive species, such as trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and most of its hybrids, Rangpur lime (*Citrus limonia* Osb.) and Palestine sweet lime (*Citrus limettioides* Tan.). Most citrus species grown commercially, such as sweet orange, grapefruit, and mandarin are tolerant to CEVd. Therefore, these species can act as symptomless carriers and, when infected and propagated on sensitive rootstocks, develop the stunting and bark scaling symptoms characteristic of the exocortis disease.

Cachexia induces wood pitting and gumming on the trunk, above or below the bud-union according to the position of the sensitive species [mandarin, mandarin hybrids, kumquats, alemow (*Citrus macrophylla* Wester), rough lemon (*Citrus jambhiri* Lush) and Rangpur lime], as well as stunting, chlorosis and tree decline.

CBLVd, CVd-III, CVd-IV and the non-pathogenic variants of HSVd have not been linked to specific symptoms in commercial citrus species and varieties. However, infections by CBLVd and CVd-III have been reported to reduce the canopy size and fruit harvest of trees grafted on trifoliate orange rootstock (Roistacher *et al.*, 1993; Semancik *et al.*, 1997; Hutton *et al.*, 2000; Vernière *et al.*, 2004). It has been recently demonstrated that CVd-IV and all the variants of HSVd cause severe bark cracking in trifoliate orange (Vernière *et al.*, 2004).

Citrus viroids are currently diagnosed by biological indexing on sensitive indicator plants, usually Arizona 861-S1 Etrog citron (*Citrus medica* L.) grafted onto rough or Volkamer lemon (*Citrus volkameriana* Ten. and Pasq.) and grown in a temperature-controlled (27-32°C) greenhouse (Roistacher *et al.*, 1977). Characteristic symptoms of viroid infection include stunting, leaf epinasty and necrosis of midvein, petiole and leaf tip. The time taken for symptoms to develop, and for their range to be displayed in inoculated citrons, are viroid-specific if the tested citrus plants contain single viroid

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species. Since field sources usually contain multiple viroid infections, symptom expression may be influenced by synergistic or inhibitory interactions. In such cases, a negative or positive reaction of the indicators allows an accurate assessment if the tree is viroid-free or not, but the test does not allow the identification of the viroid species. In such cases, the species involved can only be determined by additional molecular analysis.

Nucleic acid analyses of viroids by gel sequential electrophoresis (sPAGE), Dot and Northern-blot hybridisation and reverse transcription polymerase chain reaction (RT-PCR) have been proposed as alternatives to biological indexing procedures (Owens and Diener, 1981; Flores, 1988; La Rosa *et al*., 1988; Sano *et al*., 1988; Yang *et al*., 1992) and these methods are used for viroid detection and identification. However, the sensitivity and reliability of molecular methods depend on the titre and distribution of viroids in the host plants (Albanese *et al*., 1991; Tessitori *et al*., 1996; Palacio *et al*., 2000). For this reason, the use of citron as a bioamplification host combined with molecular analysis has been suggested as the most sensitive and specific procedure for viroid indexing (Duran-Vila *et al*., 1993; Palacio *et al*., 2000).

Previous studies, carried out in commercial orchards of Southern Italy (Sicily, Apulia, Calabria), proved the presence of CEVd, HSVd and, rarely, CVd-III (Albanese *et al*., 1995; Turturo *et al*., 1998). More recently, data obtained from a preliminary survey conducted in Campania indicated the presence of HSVd, CVd-III, CEVd, CBLVd and CVd-IV in this region. HSVd and CVd-III proved to be the most widespread viroid species (Malfitano *et al*., 2005).

In this study, we report the results of a comprehensive survey conducted in the main citrus-growing areas of Campania. The survey and indexing tests were carried out over a three-year period and viroid incidence was determined by biological and molecular tests.

MATERIALS AND METHODS

Plant material. Surveys were made in 14 citrus fields randomly selected in 7 citrus-growing areas of the Campania region during summer 2001-2004. Citrus trees were inspected visually. Sampling was carried out randomly regardless of whether or not the plants showed viroid-like symptoms. About 5-10 g of green bark were collected from 2-3-month-old flushes of 112 field citrus plants of different sweet orange, lemon, clementine and grapefruit cultivars.

Biological indexing. Each sample was graft-inoculated into three Arizona 861-S1 'Etrog' citrons grafted on rough lemon rootstock and maintained in a greenhouse under controlled temperatures (28-32°C). Symptom development was recorded at monthly intervals.

Nucleic acid extraction and viroid analysis. Bark (5 g) collected from field citrus trees was triturated in 5 ml of extraction buffer (0.4 M Tris-HCl, pH 8.9; 1% SDS; 5 mM EDTA, pH 7.0; 4% 2-mercaptoethanol) and 15 ml of water-saturated phenol neutralized at pH 7.0. The nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl TKM buffer (10 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4) (Semancik *et al*., 1975).

Polyacrylamide gel electrophoresis and Northern and Dot-blot analyses. Aliquots (20 µl) of partially purified nucleic acids were analyzed by 5% sPAGE. The first gel was polymerized in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2), and subjected to a constant current of 60 mA at 4°C for 2.5 h. A segment of the gel defined by the xylene-cyanol dye and 1 cm below was excised and placed on the top of a second gel containing 8 M urea and polymerized with TBE buffer (22.25 mM Tris, 22.25 mM boric acid, 0.625 mM EDTA, pH 7.5). The second gel was subjected to a constant current of 16 mA at room temperature for 4 h (Rivera-Bustamante *et al*., 1986). Avocado sunblotch viroid (ASBVd) and citrus exocortis viroid (CEVd) were co-electrophoresed as markers. In each test, the nucleic acids were run in duplicate. In order to perform Northern-blot analysis, one of the gels was electroblotted to a positively charged Nylon membrane (Boeringer Mannheim, Mannheim, Germany) with an LKB 2500 transphor apparatus and immobilized by UV cross-linking; the other was silver stained (Igloi, 1983).

To carry out the Dot-blot analysis, aliquots (10 µl) of the partially purified nucleic acids, including negative and positive controls containing CEVd, CBLVd, HSVd, CVd-III and CVd-IV were denatured with 7.4% formaldehyde in 6x SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA) at 60°C for 15 min (Palacio *et al*., 2000) and loaded under vacuum on positively charged nylon membranes with a Bio-Dot-SF apparatus (Biorad Laboratories, Hercules, CA, USA) and immobilized by 5 min of UV treatment.

Northern and Dot-blot analyses were performed using digoxigenin (Dig)-labeled cDNA probes specific for CEVd, CBLVd, HSVd, CVd-III and CVd-IV (Palacio *et al*., 2000). Pre-hybridization and hybridization were performed in 50% formamide and 6x SSPE. The membranes were pre-hybridized at 42°C for 2 h and hybridised overnight at 50°C. After hybridisation, they were washed twice in 2x SSC, 0.1% SDS at room temperature for 15 min, followed by another wash in 0.1x SSC, 0.1% SDS at 68°C for 1 h. The Dig-labelled hybrids were detected with an anti-Dig-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminescent substrate CSPD (Roche Diagnostic, Indianapolis, USA).

RESULTS

Field surveys. Field inspections carried out in Campania over a three-year period showed the presence of cachexia-like symptoms in 37 out of 38 sampled mandarin and clementine trees (Fig. 1). Severe bark scaling characteristic of exocortis was observed on a single 'Ovale di Sorrento' lemon, the only sampled tree found to be grafted onto trifoliate orange (Fig. 2).

Fig. 1. Wood pitting on a clementine tree affected by cachexia.

Fig. 2. Bark scaling symptoms on Ovale di Sorrento grafted onto trifoliate orange rootstock.

Biological indexing. Out of the 112 citrus field sources graft-inoculated into Etrog citron plants, 104 (93%) induced viroid-like symptoms. The times required for symptom development differed. The average for initial symptom appearance was about 4 weeks. Symptoms included epinasty (Fig. 3A) and/or stunting and/or necrosis of the midrib and tip of the leaf (Fig. 3B).

Electrophoretic and hybridisation tests. Analysis by sPAGE of nucleic acid extracts of the 112 citrus field trees revealed that 8 sources did not contain viroid-like RNAs. Twelve trees contained a single RNA band with the molecular size of HSVd (7 samples) or CEVd (2 samples) or CVd-III (3 samples). In the remaining citrus field sources, two or more viroid-like RNAs with the electrophoretic mobility of CEVd and/or CBLVd and/or HSVd and/or CVd-III and/or CVd-IV were detected demonstrating the presence of more than one viroid in the same tree (data not shown). An example of the results obtained by sPAGE from field citrus trees is shown in Fig. 4.

The results of sPAGE were consistent with those obtained by Northen and Dot-blot analyses using CEVd-, CBLVd-, HSVd-, CVd-III- and CVd-IV-specific Dig-labeled probes.

Results of sPAGE and molecular hybridisation analyses of the 112 field trees were confirmed over a three-year period, with the exception of thirteen samples where one or more viroids failed to be detected from year to year using all the techniques. These erratic results might be due to the uneven distribution of viroids in the field hosts and to the seasonal variations of viroid titre. On the basis of this, samples that tested positive at least once were considered as positive. The results are summarized in Table 1.

CEVd, HSVd and CVd-III were the most widely detected viroids, accounting respectively for 68, 87 and 85% of the tested sources. CVd-IV and CBLVd were only found in 24 and 13% of the sources. CEVd, HSVd and CVd-III were found in practically all the citrus species and cultivars analyzed. CVd-IV was detected in 'Tarocco' (4 of 10), 'Biondo commune' (2 of 13) and 'Washington Navel' (1 of 8) sweet oranges, in 'Com-

A

B

Fig. 3. Symptoms of severe epinasty (**A**) and midvein and tip browning of the leaf (**B**) on Arizona 861-S1 Etrog citron indicator plant.

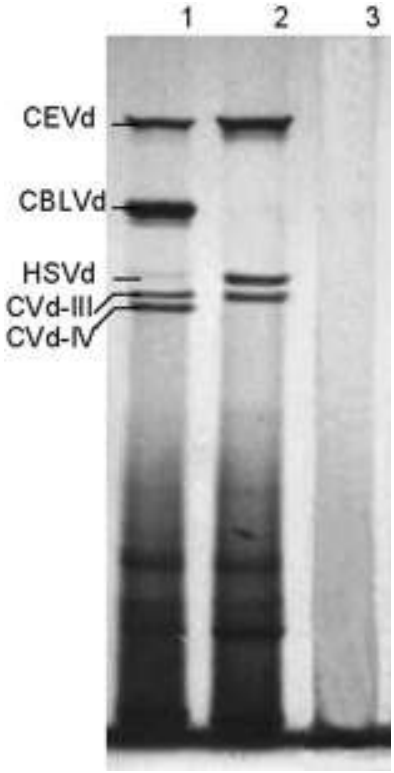

Fig. 4. Samples from lemon (lane 1) and from 'W. Navel' sweet orange (lane 2) containing several viroids as revealed by sPAGE and silver staining. Healthy control (lane 3).

mune clementine' (5 of 21), in 'Ovale di Sorrento' (7 of 8) and 'Zagara' (6 of 6) lemons and in sour orange (2 of 4). CBLVd was detected in 'Tarocco' (1 of 10) and 'Biondo commune' (1 of 13) sweet oranges, in 'Ovale di Sorrento' (7 of 8) and 'Zagara' (6 of 6) lemons (Table 1).

The five viroids were frequently found in mixed infections. The overall results show that 82% of the sampled trees were infected with more than one viroid (data not shown).

The most frequent viroid combinations were CEVd + HSVd + CVd-III (43%), HSVd + CVd-III (17%) and CEVd + CBLVd + HSVd + CVd-III + CVd-IV (13%). The last combination was frequently found in 'Ovale di Sorrento' and 'Zagara' lemons. Other combinations, CEVd + HSVd + CVd-III + CVd-IV (12%), CEVd + CBLVd + HSVd + CVd-III (1%) and CEVd + CVd-III (1%), were infrequent (Table 2).

DISCUSSION

The results of this study indicate that viroids are widespread in all citrus orchards surveyed in the Campania region and show for the first time the presence of CBLVd and CVd-IV in Italy.

HSVd, CVd-III, and CEVd were found to be the most widespread viroids in Campania, infecting 87%, 85% and 68% of the tested samples respectively, and were detected in almost all citrus species and cultivar (Table 1). They were frequently found in mixed infections, as in 43% of tested trees (Table 2). These results are in line with previous studies on viroid indexing conducted in many parts of the world in which it was observed that HSVd and CVd-III followed by CEVd were the viroids more frequently present in citrus orchards (Ito *et al*., 2002b; Duran-Vila and Semancik, 2003).

Despite the relatively high incidence of CEVd, exocortis symptoms were hardly ever observed in citrus orchards of the Campania region because most cultivars were grafted onto sour orange rootstock, which is tolerant to exocortis (Duran-Vila and Semancik, 2003). The only tree displaying bark scaling and stunting symptoms was an 'Ovale di Sorrento' lemon grafted onto trifoliate orange (Fig. 2), which was found to be affected by CEVd, CBLVd, HSVd, CVd-III and CVd-IV.

Cachexia symptoms were observed only on mandarin and clementine trees (Fig. 1) affected by HSVd since these were the only tested species that were sensitive to the disease (Duran-Vila and Semancik, 2003).

CVd-IV and CBLVd were found only in 24 and 13% of the analyzed trees. CVd-IV was detected in sweet orange ('Tarocco', 'Biondo Commune' and 'Washington navel'), lemon ('Ovale di Sorrento' and 'Zagara'), clementine ('Commune') and sour orange whereas CBLVd was detected in sweet orange ('Tarocco' and 'Biondo Commune') and lemon ('Ovale di Sorrento' and 'Zagara') (Table 1).

The percentages of CVd-IV and CBLVd found in Campania may be considered significantly high since in other countries these viroids, particularly CVd-IV, have been reported as the least widespread (Duran-Vila and Semancik, 2003; Najar and Duran-Vila, 2004).

Nevertheless, the results of this study show that, in Campania, the presence of citrus viroids in mixed infections is not occasional but rather frequent considering that the 82% of the sampled trees were infected with more than a viroid. This is in agreement with the results of indexing tests conducted in several countries where different combinations of citrus viroids were frequently detected. The high incidence of citrus viroids, particularly HSVd, CVd-III and CEVd, and the high percentage of these pathogens in single or mixed infections in Campania suggest that they may have been introduced a long time ago in infected propagation material and later spread and accumulated in a single tree by the use of infected budwoods, contaminated tools and top-grafting, a common horticultural practice in Italy. The less frequent distribution of CVd-IV and CBLVd, not only in Campania but all over the world, could be due to a their more recent origin or to a their accidental introduction from an unknown host into citrus plants.

To avoid a further viroid dissemination, especially in

Table 1. Detection of CEVd, CBLVd, HSVd, CVd-III and CVd-IV in different citrus species and cultivars of Campania by sPAGE and molecular hybridization (Dot and Northen-blot). All positive plants were found positive with the three methodologies applied.

Species	Varieties	Total No. trees	CEVd	CBLVd	HSVd	CVd-III	CVd-IV
Sweet orange	Biondo Commune	13	9	1	12	12	2
	Tarocco	10	8	1	9	8	4
	Navelina	14	9	0	12	12	0
	W. Navel	8	6	0	7	8	1
	Sanguinello	1	1	0	1	1	0
	Valencia	6	3	0	1	1	0
	Vanilla	1	0	0	1	1	0
Mandarin	Commune	8	3	0	8	8	0
	Avana	2	1	0	1	2	0
Clementine	Commune	21	14	0	21	17	5
	Monreale	7	5	0	7	6	0
Lemon	Ovale di Sorrento	8	8	7	7	8	7
	Zagara	6	6	6	6	6	6
Grapefruit		1	0	0	0	1	0
Sour orange		4	3	0	4	4	2
Tangelo		2	0	0	0	0	0
Total		112	76	15	97	95	27
% infection			68	13	87	85	24

Table 2. Incidence of citrus viroid combinations in citrus species and cultivars of Campania.

Species	Varieties	Total No. of infected trees	CEVd+CBLVd +HSVd+CVd-III+CVd-IV	CEVd+CBLVd+HSVd+CVd-III	CEVd+HSVd+CVd-III+CVd-IV	CEVd+HSVd+CVd-III	HSVd +CVd-III	CEVd+CVd-III
Sweet orange	Biondo Commune	12	1	-	1	7	3	-
	Tarocco	9	1	-	3	4	-	-
	Navelina	12	-	-	-	9	3	-
	W. Navel	8	-	-	1	5	1	-
	Sanguinello	1	-	-	-	1	-	-
	Valencia	4	-	-	-	-	-	1
	Vanilla	1	-	-	-	-	1	-
Mandarin	Commune	8	-	-	-	3	5	-
	Avana	2	-	-	-	1	-	-
Clementine	Commune	21	-	-	5	9	3	-
	Monreale	7	-	-	-	5	1	-
Lemon	Ovale di Sorrento	8	6	1	1	-	-	-
	Zagara	6	6	-	-	-	-	-
Sour orange		4	-	-	2	1	1	-
Tangelo		1	-	-	-	-	-	-
Total		104	14	1	13	45	18	1
% infection			13	1	12	43	17	1

new citrus plantations, it is advisable to develop certification programmes for citrus in Campania in order to produce healthy planting material.

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