

Distribution of citrus exocortis viroid and hop stunt viroid in citrus orchards of central Italy as revealed by one-tube one-step RT-PCR

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Summary. A survey was carried out in citrus fields of Lazio and Tuscany in central Italy to determine the presence of *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), both of which cause economically important diseases in citrus. In order to make pathogen diagnosis easier and more efficient, a one-tube one-step RT-PCR assay was developed and used for the detection of CEVd and HSVd in citrus samples collected from field trees. CEVd and HSVd occurred in 100 and 30% of samples from Lazio respectively, and in 45 and 9% of samples from Tuscany. Specifically, of 21 citrus cultivars belonging to nine citrus species and one hybrid, 12 cultivars and the hybrid were infected with CEVd, one cultivar with HSVd, 3 cultivars with both viroids, and 4 cultivars were free from both viroids. The study revealed a wide distribution of the two viroids in the citrus field samples and showed that one-tube one-step RT-PCR assay is a rapid, sensitive and reliable technique for the detection of these viroids. This diagnostic technique is therefore suitable for the preliminary screening of CEVd and HSVd in routine sanitary and certification programmes because it drastically reduces the number of indicator plants needed for biological indexing of the two viroids.

Key words: citrus viroids, Lazio, Tuscany, detection.

Introduction

Citrus is an economically important crop in Italy with an estimated cultivated area of more than 132,500 hectares. The most important regions for citrus cultivation are southern Italy, particularly Sicily, Apulia and Calabria. The distribution of citrus viruses and viroids in the south of the country has been subject to a number of studies (D'Onghia, 1998; Albanese *et al.*, 2000; Malfitano *et al.*, 2005).

Currently however no information is available regarding the sanitary status of citrus plants in central Italy. For this reason an investigation was

carried out in Lazio and Tuscany in order to ascertain some epidemiological aspects of viroid diffusion. We decided to investigate the citrus viroid presence in Lazio and Tuscany because Lazio, especially southern Lazio, is renowned for its orange orchards, while Tuscany, and particularly central Tuscany, has since the 1970s been characterised by many nurseries growing ornamental citrus varieties (for instance, ×*Citro Fortunella microcarpa*, *C. latipes*). Today more than 200 ornamental citrus varieties exist worldwide (data from the Botanical garden Hesperidarium, Pescia, Pistoia, Italy).

Citrus exocortis viroid (CEVd) and *Hop stunt viroid* (HSVd) are the most widespread and economically important viroids of citrus plants (Duran-Vila and Semancik, 2003). They cause severe and serious diseases and they must not occur in

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any virus-controlled, virus-free and C.A.C propagative material.

In the light of this high-risk situation and in the framework of the national Project "Agrumicoltura" supported by the Italian Ministry of Agriculture, a survey was carried out to determine the distribution of CEVd and HSVd in central Italy.

In order to make the diagnosis of citrus viroids easier and more efficient, a one-tube one-step RT-PCR protocol (Ragozzino *et al.*, 2004) was developed to test citrus plants of different species and cultivars (Table 1) collected from several citrus orchards and nurseries located in the main citrus growing areas in Lazio and Tuscany.

Materials and methods

Plant material

Samples of green bark and leaves were collected during the summer from orchards and nurseries located in Lazio and Tuscany. Collection sites were at Aprilia (Roma) in Lazio, and at Pescia (Pistoia) in Tuscany. At Aprilia samples were collected from 2 different citrus fields, and at Pescia from nursery plants. Twenty-one varieties were analyzed belonging to 9 citrus species and one hybrid. Five samples per variety or hybrid, were collected giving a total of 105 samples. No specific symptoms were observed in the field on the collected green bark and leaves from which samples were taken, and all samples were symptomless.

Molecular tests

Viroid RNA isolation from leaf and bark tissues was performed using the RNeasy Plant Mini Kit, following manufacturer's instructions (Qiagen GmbH, Hilden, Germany). At the end of the process RNA was eluted with 50 μ l of RNase-free water. The one-tube one-step RT-PCR protocol was carried out as reported in an earlier study (Ragozzino *et al.*, 2004). Amplified products were analysed by electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. We used specific primers that amplified the full length of each viroid genome (Levy *et al.*, 1992; Astruc *et al.*, 1996). CEVd- and HSVd- infected samples, CEVd- and HSVd-free plants, and water were used as controls in all the RT-PCR tests.

To check the reliability of the RT-PCR assay, nucleic acids from the same samples were hybrid-

ized to CEVd and HSVd cRNA probes and also analyzed by a two-step RT-PCR protocol.

Biological tests

Biological indexing was performed by inoculating four plants of 'Etrog' citron with each sample that tested negative with one-step RT-PCR. The inoculated indicators were maintained at 27–32°C for 3–6 months before symptom observation. Four 'Etrog' plants were also inoculated with a CEVd- or HSVd-positive control. Samples from the indicator plants were visually inspected and tested by one-step RT-PCR, two-step RT-PCR, and spot blot hybridization 6 months after inoculation.

Results and discussion

Results are summarized in Tables 2 and 3. CEVd occurred in 100% of collected samples from Lazio and in 45% of samples from Tuscany. In Tuscany CEVd was found in two lemon varieties, 'Femminello' and 'Limone variegato', in the lemon \times citron hybrid 'Limone cedrato' (Table 1), in one lime cultivar, 'Pinocchio' (Table 1) and in one sweet orange, 'Sanguinello' (Table 1). It is significant that CEVd was detected in all the five samples of the same variety since this may indicate that the original viroid source of infection was in the citrus plants from the nursery.

Unlike the results reported from southern Italy in a number of studies (D'Onghia, 1998; Albanese *et al.*, 2000; Malfitano *et al.*, 2005), the percentage of samples infected with HSVd was rather lower in the regions of Tuscany and Lazio investigated here: it was 9% in Tuscany and 30% in Lazio. In Tuscany HSVd was found only in Kumquat (Table 1), whereas in Lazio it occurred in 2 mandarin varieties, 'San Francesco' and 'Primo Sole' and in the clementine cultivar Hernandina; these last 3 cultivars were also CEVd-positive (Table 1).

When all 105 samples from central Italy were viewed as a whole, CEVd and HSVd had average distributions across samples of 71 and 19% respectively. Both viroids were more widespread in citrus germplasm from Lazio than from Tuscany. Of the 21 cultivars belonging to 9 citrus species and one hybrid, 12 cultivars and the hybrid cultivar were infected with CEVd, one cultivar was infected with HSVd, and 3 cultivars were infected with both viroids. No particular symptoms were com-

mon to all collected samples; in the case of CEVd-positive samples, we collected commercial species normally known as symptomless hosts and grafted them onto tolerant rootstocks. HSVd-positive samples were also asymptomatic, probably on ac-

count of the HSVd isolate. Only four varieties (clementine 'Rufina', mandarin 'Tardiva di Ciaculli', citron 'Etrog' and limetta 'Pursha') were both CEVd- and HSVd-free (Table 1).

The study showed that the one-tube one-step

Table 1. List of analyzed citrus species and varieties and of infections recorded.

Species	Variety	CEVd	HSVd	Not infected
Sweet orange	Sanguinello	+		
	Navelina	+		
	Tarocco	+		
Mandarin	Tarocco nucellare	+		
	Beatrix	+		
	San Francesco	+	+	
	Tardivo di Ciaculli			+
Satsuma	Primo Sole	+	+	
	Miho	+		
Clementine	Miyagawa	+		
	Clementina	+		
	Hernandina	+	+	
Lemon	Rufina			+
	Femminello carrubaro	+		
	Femminello comune	+		
Citron	Variegato	+		
	Etrog			+
	Limetta			+
Lime	Pursha			
Lime	Pinocchio	+		
Kumquat	Fortunella		+	
Lemon × citron	Cedrato	+		

Table 2. Distribution of CEVd in Tuscany and Lazio.

Species	Tuscany		Lazio	
	IV/AV ^a	IS/AS ^b	IV/AV	IS/AS
Sweet orange	1/2	5/10	2/2	10/10
Mandarin	0/1	0/5	3/3	15/15
Clementine	0/1	0/5	2/2	10/10
Lemon	2/2	10/10	1/1	5/5
Citron	0/1	0/5		
Limetta	0/1	0/5		
Lime	1/1	5/5		
Kumquat	0/1	0/5		
Lemon × citron	1/1	5/5		
Satsuma			2/2	10/10
Total	5/11	25/55	10/10	50/50

^a Infected varieties/Analyzed varieties.

^b Infected samples/Analyzed samples.

Table 3. Distribution of HSVd in Tuscany and Lazio.

Species	Tuscany		Lazio	
	IV/AV ^a	IS/AS ^b	IV/AV	IS/AS
Sweet orange	0/2	0/10	0/2	0/10
Mandarin	0/1	0/5	2/3	10/15
Clementine	0/1	0/5	1/2	5/10
Lemon	0/2	0/10	0/1	0/5
Citron	0/1	0/5		
Limetta	0/1	0/5		
Lime	0/1	0/5		
Kumquat	1/1	5/5		
Lemon × citron	0/1	0/5		
Satsuma			0/2	0/10
Total	1/11	5/55	3/10	15/50

^a See Table 2.

^b See Table 2.

RT-PCR protocol in a single PCR reaction detected both CEVd and HSVd, widely distributed and economically important viroids, in citrus germplasm. The one-step technique made their identification much easier and more rapid as compared with other protocols currently in use for citrus viroid diagnosis. The results obtained by hybridization and two-step RT-PCR confirmed those obtained with the one-tube one-step RT-PCR protocol (data not shown). Moreover, the diagnosis was simplified by using a total RNA extraction commercial kit, thus avoiding the use of phenol. The reliability of this protocol was optimized with the use of a hot-start Taq DNA polymerase.

Spot-blot hybridization analysis, two-step and one-tube one-step RT-PCR were also performed on the leaves and bark of 'Etrog' citron inoculated with samples that were negative to one-tube one-step RT-PCR. Biological analysis confirmed the results obtained with one-tube one-step RT-PCR. After 6 months neither the indicator plants showed any specific CEVd or HSVd symptoms (Duran-Vila *et al.*, 1993) nor did molecular tests on these citrons give positive results. The negative biological tests thus confirmed the negative molecular tests and vice versa. The positive CEVd and HSVd controls showed typical viroid symptoms on the 'Etrog' indicator.

In conclusion, the distribution of CEVd and HSVd in the main citrus growing areas of central

Italy was studied for the first time. The distribution percentage of the two viroids varied between areas: CEVd was very common and widely distributed in central Italy, whereas HSVd seemed to be less common in citrus germplasm from central Italy than in that from southern Italy.

The reliability of the one-tube one-step RT-PCR technique was confirmed by the biological tests. This technique was also fast and easy to perform, making it suitable for preliminary viroid screening in sanitary and certification programmes. It will drastically reduce the number of biological tests required since these tests need to be carried out only on negative viroid samples.

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