SHORT COMMUNICATION

DETECTION OF VIROIDS IN FORCED CITRUS CUTTINGS

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SUMMARY

A procedure was developed for detecting viroids in citrus cuttings collected in the field and forced to sprout in a growth chamber. Leaves collected 15 days or one month after sprouting were tested in comparison with leaf and bark samples from field-grown source plants. Tests were made by using RT-PCR with primers specific for *Citrus exocortis viroid* (CEVd), the citrus strain of *Hop stunt viroid* (HpSVd-cit), *Citrus bent leaf viroid* (CBLVd), *Citrus viroid III* (CVd-III), or *Citrus viroid IV* (CVd-IV). Whereas no evidence was obtained for the presence of CBLVd and CVd-IV were not found whereas HpSVd-cit, CEVd, and CVd-III were detected consistently in forced cuttings even when no positive amplification was obtained from field samples. This method may be a useful in the laboratory detection of citrus viroids.

Key words: citrus, viroid, diagnosis, RT-PCR.

A number of viroids are known to infect citrus, some of which, such as the agents of exocortis and cachexia, cause severe diseases (Duran Vila and Semancik, 2003).

In Italy, a voluntary programme of citrus certification is being implemented that requires certified propagation material to be free from viroids, as ascertained by biological indexing (Roistacher *et al.*, 1973, 1977) and sequential polyacrylamide gel electrophoresis (sPAGE) (Boccardo *et al.*, 1984). The first procedure is time-consuming and expensive. The second allows the processing of a small number of samples at a time and its success depends on viroid concentration, which can vary with the environmental conditions (Tessitori *et al.*, 1996).

Quicker and more reliable laboratory assays based on RT-PCR are now available (Levy *et al.*, 1992; Yang *et al.*, 1992; Tessitori *et al.*, 1996; Ito *et al.*, 2002a) and protocols have recently been developed for the simultaneous detection of multiple citrus viroids (Ito *et al.*, 2002b; Ragozzino *et al.*, 2004).

Our experience with diverse viroid-infected citrus species and varieties from different southern Italian areas has shown that sPAGE can detect only *Citrus exocortis viroid* (CEVd) from field samples throughout the year, whereas other viroids can be detected consistently only in warmer months, i.e. from May onwards (A. Caruso, unpublished information). Similar problems were encountered with RT-PCR, which, however, proved more sensitive than sPAGE.

In a previous survey, *ca.* 25-year-old citrus trees grafted on sour orange from Sicily (lemon cvs Monachello and Zagara bianca, grapefruit cv Star ruby) and Calabria (bergamot cv Castagnaro) were tested by indexing and sPAGE and found to be infected by CEVd, *Hop stunt viroid* (HpSVd-cit), and *Citrus viroid III* (CVd-III). In the present investigation, the same sources were analysed by one-step RT-PCR as described by Caruso *et al.* (2000), with the primers listed in Table 1, for the presence of CEVd, HpSVd-cit CVd-III, *Citrus bent leaf viroid* (CBLVd), and *Citrus viroid IV* (CVd-IV).

Two types of starting material were used in these experiments, bark and leaves from field-grown trees collected monthly from December 2001 to May 2002, and leaves from citrus cuttings that had been collected in February 2002. These were forced to sprout in a growth chamber in sterilized agriperlite and grown under artificial illumination (1000 lux and a phoroperiod of 16 h) at a temperature between 27 and 32°C (Fig. 1). Cuttings were collected in February because HpSVd-cit and CVd-III could not be detected by one-step RT-PCR in that month.

Leaves from forced cuttings were collected and tested 15 days or one month after sprouting. Cuttings from healthy lemon, grapefruit and bergamot trees were used as negative controls. Field temperatures were recorded throughout the collection period (Table 3).

As shown in Table 2, regardless of the citrus species examined, CEVd was readily detected in bark and leaves of all samples from December 2001 to May 2002. By contrast, no HpSVd-cit amplification was obtained from 'Castagnaro' bergamot from January onwards and from the other citrus species from February until May. CVd-III

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Table 1. Primer list.

Viroid	Primers[a]	Sequence	Position	References
CEVd	CEVd (c)	5¹-CCGGGGATCCCTGAAGGA-3¹	81-98	(Gross *et al.*, 1982)
	CEVd (h)	5¹-GGAAACCTGGAGGAAGTCG-3¹	99-117	
HpSVd-cit	HSVd (c)	5¹-GGCTCCTTTCTCAGGTAAG-3¹	61-79	(Levy *et al.*, 1992)
	HSVd (h)	5¹CGGGGCAACTCTTCTCAGAATCCA-3¹	80-104	
CBLVd	CVd-I (c)	5¹-TTCGTCGACGACGACAAGTC-3¹	84-103	(Semancik *et al.*, 1997)
	CVd-I (h)	5¹-GGCTCGTCAGCTGCGGAGT-3¹	104-123	
CVd-III	CVd-III (c)	5¹-TTCGTCGACGACGACAGGTA-3¹	76-95	(Rakowski *et al.*, 1994).
	CVd-III (h)	5¹-GGCAGCTAAGTTGGTGACGC-3¹	96-115	
CVd-IV	CVd-IV (c)	5¹-GGGTAGTTTCTATCTCAG-3¹	199-216	(Puchta *et al.*, 1991)
	CVd-IV (h)	5¹-GGTGGATACAACTCTTGG-3¹	217-235	

[a] (c) complementary primer; (h) homologous primer.

Fig. 1. Citrus cuttings after one month forcing in growth chamber.

was consistently undetectable from January until May and no amplification was obtained from any of the samples with primers specific for CBLVd and CVd-IV.

In forced cuttings, HpSVd-cit and CVd-III, but not CEVd, were readily detected in the leaves collected 15 days after sprouting (Table 3). However, leaves collected after 30 days yielded amplicons of the size expected for each of the three viroids, i.e. CEVd (371 bp) for HpSVd-cit (300 bp) and CVd-III (296 bp) (Fig. 2). RT-PCR assays using primers specific for CBLVd and CVd-IV were consistently negative, thus confirming the results obtained by sPAGE.

No amplification was ever obtained from healthy samples with any set of primers.

Viroid detection from field-grown trees can be influenced by climatic conditions and host species (Tessitori *et al.*, 1996; Palacio Bielsa *et al.*, 1999). Like Tessitori *et*

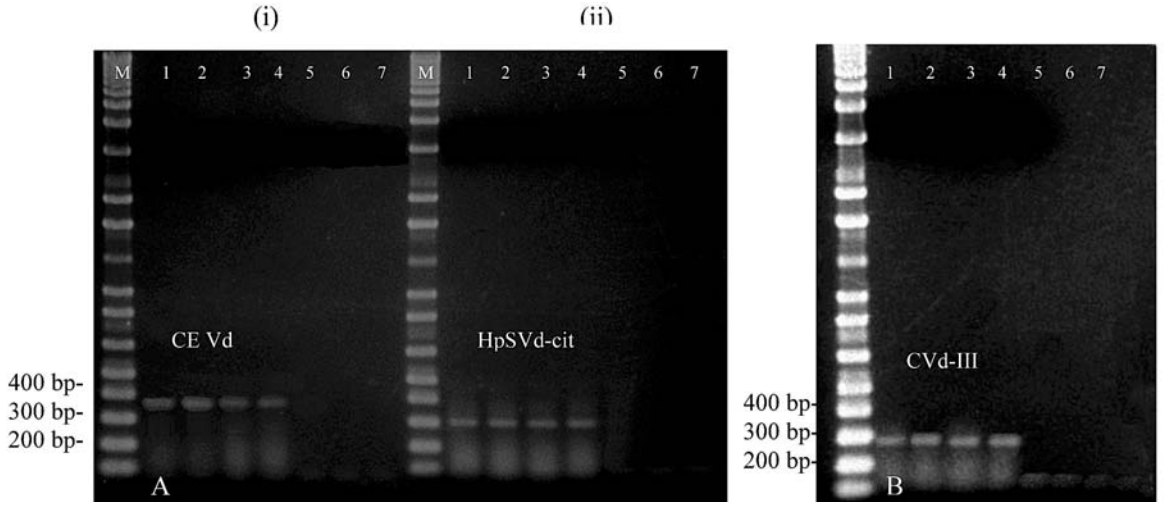

Fig. 2. A) agarose gel electrophoresis of fragments amplified by RT-PCR with CEVd (Panel i) and HpSVd-cit primers (Panel ii). Lane M: marker 1 KB plus DNA Ladder (Invitrogen); lane 1: 'Monachello' lemon; lane 2: 'Star Ruby' grapefruit; lane 3: 'Zagara Bianca' lemon; lane 4: 'Castagnaro' bergamot; lane 5: healthy 'Monachello' lemon; lane 6: healthy 'Star Ruby' grapefruit; lane 7: healthy 'Castagnaro' bergamot. **B)** agarose gel electrophoresis of fragments amplified by RT-PCR with CVd-III primers. Lane M: marker 1 KB plus DNA Ladder (Invitrogen); lane 1: 'Monachello' lemon; lane 2: 'Star Ruby' grapefruit; lane 3: 'Zagara Bianca' lemon; lane 4: 'Castagnaro' bergamot; lane 5: healthy 'Monachello' lemon; lane 6: healthy 'Star Ruby' grapefruit; lane 7: healthy 'Castagnaro' bergamot.

Table 2. Detection of viroids by one-step RT-PCR in samples collected in the field.

Date of collection	Average temperature	Viroids	'Monachello' lemon[a]		'Star Ruby' grapefruit		'Zagara Bianca' lemon		'Castagnaro' bergamot	
			Leaves	Bark	Leaves	Bark	Leaves	Bark	Leaves	Bark
12-12-01	15.8°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	+	+	+	+	+	+	+	+
		CVd-III	+	+	+	+	+	+	+	+
14-01-02	10.8°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	+	+	+	+	+	+	–	–
		CVd-III	–	–	–	–	–	–	–	–
14-02-02	8.5°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	–	–	–	–	–	–	–	–
		CVd-III	–	–	–	–	–	–	–	–
10-03-02	10.6°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	–	–	–	–	–	–	–	–
		CVd-III	–	–	–	–	–	–	–	–
10-04-02	14.0°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	–	–	–	–	–	–	–	–
		CVd-III	–	–	–	–	–	–	–	–
10-05-02	17.0°C	CEVd	+	+	+	+	+	+	+	+
		HpSVd-cit	+	+	+	+	+	+	+	+
		CVd-III	+	+	+	+	+	+	+	+

[a] + positive amplification; **–** no amplification

al. (1996), we succeeded in identifying CEVd by RT-PCR in every infected field sample analyzed in the present study, regardless of the host species, place of collection, and temperature. However, this was not the case with HpSVd-cit and CVd-III, both of which became undetectable when the ambient temperature dropped below an average of 15°C, and remained around 10°C for about one month.

We have shown that the forced cutting method allows citrus viroid detection when field conditions are unfavourable for their identification in samples taken directly from the grove. Forcing cuttings to sprout in a growth chamber at temperatures around 30°C seems to promote viroid replication without the need of using 'Etrog 861-S1' citron for their amplification.

However, it seems to be necessary to collect leaf tissues a month after sprouting in order to obtain consistent responses with all of the three viroids present in the trees examined. Whether this is because of a different replication rates is unknown.

For the last three years, we have been using this technique routinely together with sPAGE and indexing on citron with consistently comparable results. This makes it plausible to conclude that RT-PCR using tissues from

Table 3. Detection of viroids by one-step RT-PCR in leaves from cuttings collected in February 2002 and forced for15 or 30 days.

	Viroid	'Monachello' lemon[a]	'Star Ruby' grapefruit	'Zagara Bianca' lemon	'Castagnaro' bergamot
15 days	CEVd	–	–	–	–
	HpSVd-cit	+	+	+	+
	CVd-III	+	+	+	+
30 days	CEVd	+	+	+	+
	HpSVd-cit	+	+	+	+
	CVd-III	+	+	+	+

[a] + positive amplification; – no amplification.

forced cuttings is a useful and simple protocol for diagnosis of certain viroids (e.g. HpSVd-cit and CVd-III) in periods when unfavourable environmental conditions impair their detection in field samples.

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