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First report on *Hop stunt viroid* (HSVd) from some Mediterranean countries

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Summary Hop stunt viroid (HSVd) has a very wide host range including most stone fruit trees. Among them, apricot is one of the most important host crops in the Mediterranean basin. In this study non-isotopic molecular hybridisation revealed, for the first time, the presence of HSVd on apricot in four Mediterranean countries (Cyprus, Greece, Morocco and Turkey). The results obtained by this technique were confirmed by northern-blot and RT-PCR analyses. The data presented in this work indicate a wider geographical distribution of this viroid than hitherto known and emphasise the need for this kind of study as part of the control effort.

Key words: Hop stunt viroid, apricot, Mediterranean countries, HSVd.

Introduction

Hop stunt viroid (HSVd) (Ohno *et al*., 1983) has been found in a wide range of hosts including hop, cucumber, grapevine, citrus, plum, peach, pear (Shikata, 1990) and, recently, apricot and almond (Astruc *et al*., 1996; Cañizares *et al*., 1999). In some hosts, such as grapevine (Shikata, 1990; Polivka *et al*., 1996) and apricot (Astruc *et al*., 1996), the infection seems to be latent; in others it is associated with serious disorders of economic importance, i.e. hop stunt (Shikata, 1990), dapple fruit disease of plum and peach (Sano *et al*., 1989) and citrus cachexia (Diener *et al*., 1988; Semancik *et al*., 1988).

Apricot is one of the most important crops of the Mediterranean stone-fruit industry and this region produces approximately half of the world apricot supply (Anonymous, 1998). The cultivation of apricot in the area is still broadly based on a high number of local varieties (Bassi and Pirazzoli, 1998).

A recent study (Cañizares *et al*., 1998) revealed HSVd in 81% of the apricot trees tested in south-eastern Spain. Although HSVd is latent in apricot, this host could represent a natural reservoir from which the viroid can potentially be transmitted to

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other susceptible host crops, including other stone fruits. In an attempt to further evaluate the presence of the HSVd in the region, we tested apricot and plum samples from different Mediterranean countries.

Materials and methods

Plant material

The first survey was made in 1997 and comprised five countries: Albania, Cyprus, Greece, Morocco and Turkey. Leaf samples were collected from apricot and plum of different cultivars, including, where possible, the native ones. The second was carried out in 1998 in the same countries as before (except Morocco) plus Malta. Collected samples were assayed by applying the non-radioactive molecular detection technique for viroid detection. To unequivocally confirm the presence of the viroid, positive samples were re-analysed by northern-blot and RT-PCR.

Preparation of tissue extracts

The tissue processing procedure, which does not use organic solvent is adapted to handle a large number of small-volume samples (Cañizares *et al*., 1998) and has been previously described (Pallás *et al*., 1987; Astruc *et al*., 1996). Aliquots (0.5 g) of leaf tissue were homogenised inside sealed plastic bags in the presence of 5 ml of extraction buffer (0.1 M Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl, 10 mM MCE) using a hand-homogeniser. A 1 ml aliquot of the homogenate was transferred to an Eppendorf tube, 50 ml of 20% SDS were added and the sample was incubated at 65ºC for 20 min, followed by the addition of 250 µl of 5 M potassium acetate and incubation on ice for another 20 min. Samples were centrifuged at 12,000 rpm for 15 min and the nucleic acids present in the supernatant were recovered by ethanol precipitation and resuspended in 40 µl autoclaved water.

Dot-blot and northern-blot hybridisation

The non-radioactive digoxigenin-labelled RNA HSVd probe was obtained by T7 RNA polymerase transcription of the linearised plasmid pHSVd.EB, which contains an HSVd insert of 272 nucleotide residues (Astruc *et al*., 1996).

For dot-blot analysis, samples were denatured at 60ºC with 7.4% formaldehyde in the presence of 6xSSC, and 4 µl spots were applied to nylon membranes (Boehringer, Mannheim, Germany). Prehybridisation and hybridisation were carried out in both cases at 68ºC essentially as described by Más *et al*. (1993). Chemiluminescent detection using CSPD (Boehringer) as a substrate were carried out as described in Pallás *et al*., 1998a.

For northern-blot, preparations of total RNAs enriched for viroids were analysed by sequential polyacrylamide gel (sPAGE) (Semancik and Harper, 1984; Flores *et al*., 1985) and electrotransferred to nylon membranes by standard procedures. Prehybridisation, hybridisation and chemiluminescent detection were carried out as described above.

RT-PCR

RT-PCR was performed as described by Astruc *et al*. (1996). Samples were heated to 90ºC for 3 min in the presence of the HSVd-specific oligonucleotide VP-19 (5'-dGCCCCGGGGCTCCTTTCTCAGGTAAG-3', complementary to HSVd residues 60-85), and slowly cooled to room temperature. The mixture was then subjected to reverse transcription (RT) with avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA) at 42ºC for 45 min in 20 µl volume. One-fifth of the RT product was directly subjected to PCR amplification in a 50 µl volume reaction in the presence of the oligonucleotide VP-19 and the 27-mer VP-20 (5'-dCGCCCGGGGCAACTCTTCTCAGAATCC-3' which contains HSVd residues 78-102). PCR cycling parameters were: denaturation at 94ºC for 1 min, followed by 30 cycles of 94ºC for 40 sec, 60ºC for 40 sec and 72ºC for 1 min, to finish with extension at 72ºC for 5 min. PCR products were analysed in TAE 1% agarose gel and visualised using ethidium bromide.

Results and discussion

A total of 245 apricot and 82 plum samples from 65 and 28 identified varieties respectively, were collected from the six Mediterranean countries listed in Table 1. By applying non-isotopic molecular hybridisation, which has proved to be a very suitable procedure for viroid diagnosis (Hull, 1993; Badenes and Llacer, 1998; Cañizares *et al*., 1998; Pallás *et al*., 1998b), HSVd was detected in 4 out of the 6 countries surveyed: Cyprus, Greece, Morocco and Turkey (Table 1 and Fig. 1).

Table 1. HSVd incidence in some Mediterranean countries.

Country	No. of samples (infected/tested)		Infection rate[a] (%)
	Apricot	Plum	
Albania	0/20	0/15	0.0
Cyprus	9/86	0/0	10.4
Greece	3/59	0/10	5.0
Malta	0/2	0/17[b]	0.0
Morocco	3/29	0/21	10.3
Turkey	1/49	0/19	2.0
Total	16/245	0/82	

[a] Only for apricot samples.
[b] Considered together with 10 peaches.

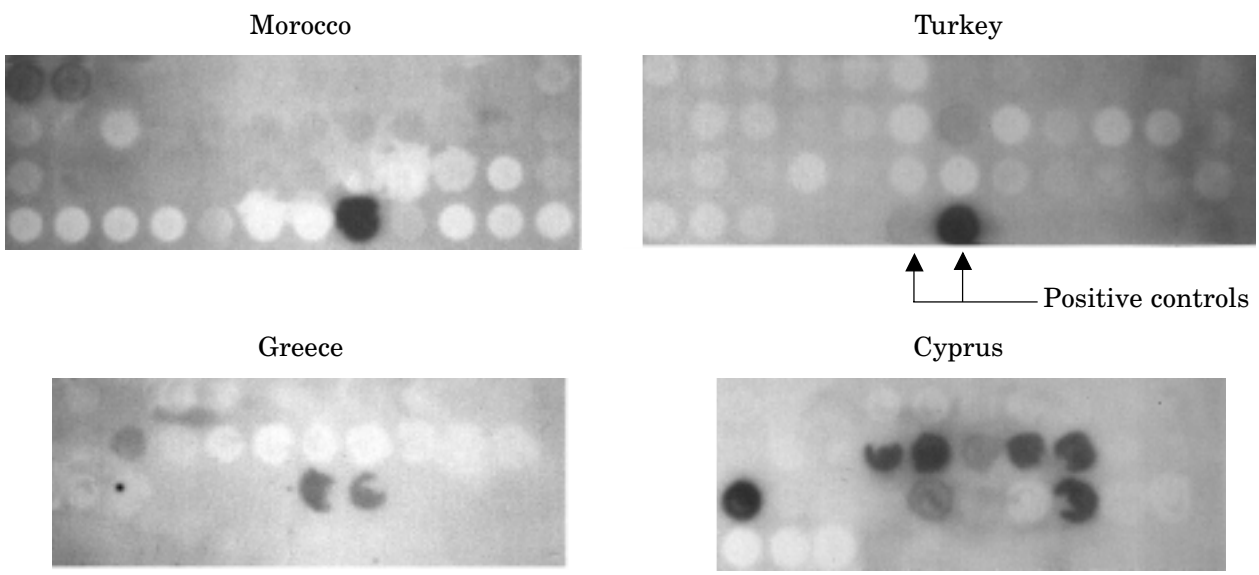

Fig. 1. HSVd detection by non-isotopic molecular hybridisation of those samples from four countries in which at least one positive reaction was observed (3 for Morocco, 1 for Turkey, 9 for Cyprus and 3 for Greece). Positive controls for the Turkey membrane are marked. Leaf samples were processed by a non-organic extraction procedure, denatured and applied to nylon membranes. The films were exposed for 30 min.

In this study, HSVd was detected only in apricot samples. The highest infection levels were recorded in Cyprus and Morocco where respectively 9 out of 86 samples (10.4%) and 3 out of 29 samples (10.3%) were positive (Table 1 and Fig. 1). In Turkey, by contrast, only one sample out of 49 (2%) was positive. The viroid incidence observed in all these countries was much lower than that on apricot in south-eastern Spain (81%) reported by Cañizares *et al*. (1998). It is worth noting that out of 65 apricot varieties tested, 12 were infected, including 5 local varieties (Table 2).

Non-radioactive molecular hybridisation can, in a very few cases, give false positives due to the pres-

Table 2. HSVd-infected varieties and analysis methods used.

Origin	Variety	Molecular hybridisation	Northern transfer	RT-PCR
Cyprus	Monaco Bello	+	+/-[a]	+
	Cafona[b]	+	+	+
	Boccuccia spinosa	+	+/-	+
	Don Gaetano	+	n.t.	n.t.
	Bulida	+	n.t.	n.t.
	Palumella[b]	+	+	+
	Canino	+	+	+
Greece	Koliopoulou	+	+/-	+
	Pr. Porou	+	+	+
	Bebecou-Paros	+	+	+
Morocco	Canino[b]	+	+	+
	Maoui	+	+	+
Turkey	Septik	+	+	+

[a] +/-, doubtful result.
[b] Two infected samples.
n.t., not tested.

ence of host RNAs with partial high similarity to the viroid sequence (see Cañizares *et al*., 1999 and references therein). In order to unequivocally demonstrate the presence of HSVd in the samples that tested positive by this technique, these samples were further analysed by northern-blot hybridisation and by RT-PCR. As can be seen from Fig. 2, after northern-blot analysis, an electrophoretic band migrating to the expected position of HSVd was clearly observed for the three positive Moroccan samples (lanes 2, 3 and 6) and for the Turkish sample (lane 5). No hybridisation was observed when an uninfected control extract was tested (lane 4). Fig. 3 shows that all the samples that tested positive by non-isotopic molecular hybridisation, produced a clear electrophoretic band of the expected size after RT-PCR analysis. Table 2 summarises the results obtained with the three techniques and it can be seen there was good correlation between the data obtained by non-isotopic molecular hybridisation and those from northern-blot and RT-PCR analysis.

To conclude, in this study, the presence of HSVd in four Mediterranean countries (Greece, Cyprus, Morocco and Turkey) was demonstrated for the first time. This new information complements the existing data about HSVd in other countries of the

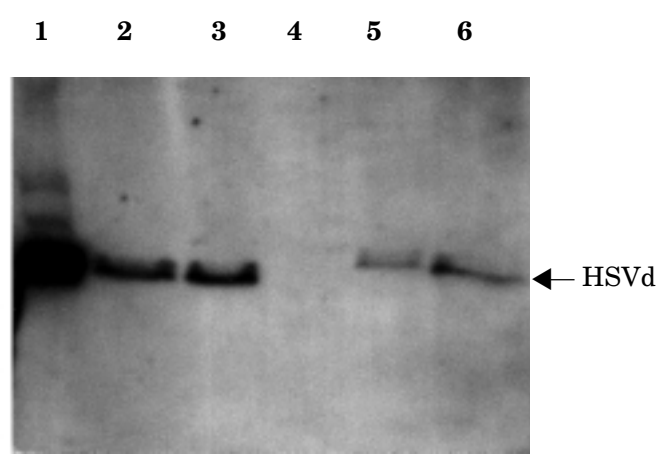

Fig. 2. Northern-blot analysis of some of the samples that gave a hybridisation signal in Fig. 1. After sequential polyacrylamide gel electrophoresis (sPAGE), the gel was electrotransferred to a nylon membrane and hybridised against a HSVd-specific riboprobe (see Materials and methods section for details). Lane 1, positive control consisting of HSVd-infected Japanese plum cv. Taiyo; lanes 2, 3 and 6, apricot samples from Morocco; lane 5, apricot sample from Turkey; lane 4, uninfected negative control.

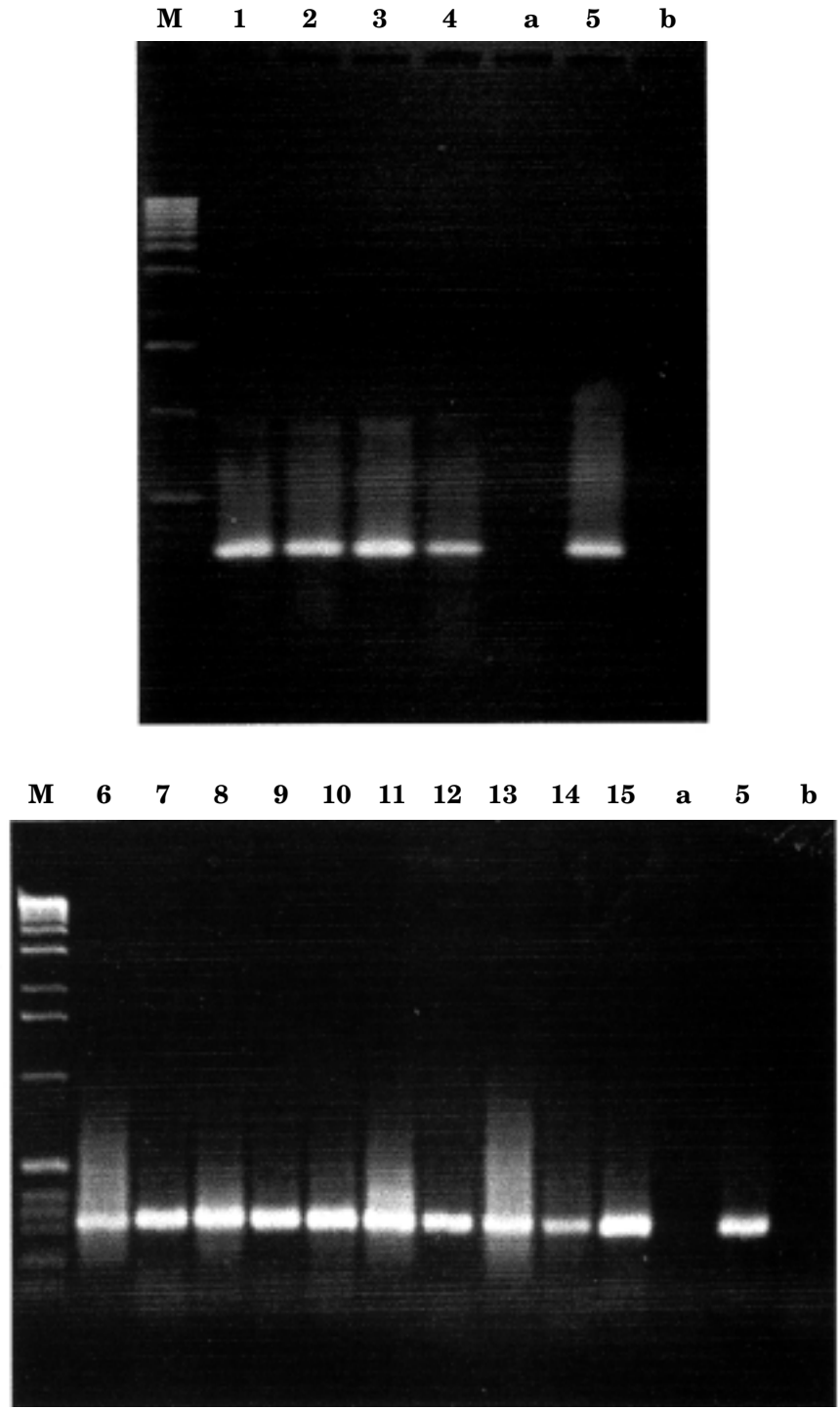

Fig. 3. Detection of HSVd by RT-PCR. Samples that gave a positive hybridisation signal by non-isotopic molecular hybridisation were amplified by RT-PCR and the products analysed by 1% agarose gel electrophoresis. Apricot samples from Morocco (lanes 1, 2 and 3); from Turkey (lane 4); positive control consisting of HSVd-infected Japanese plum cv. Taiyo (lane 5) ; samples from Cyprus (lanes 6-12); from Greece (lanes 13-15). Lanes a and b, uninfected apricot extract and water controls respectively. DNA molecular weight markers were loaded in lane M.

region, Spain, France and Italy (Astruc *et al*., 1996; Kofalvi *et al*., 1997; Cañizares *et al*., 1998; Loreti *et al*., 1998). Although the viroid has been detected only in apricot trees, in which it is reported to be latent (Astruc *et al*., 1996), the demonstration of its presence here should enable control measures to be taken so that its transmission to other, susceptible crops, can be prevented.

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