Phytopathol. Mediterr. (2005) 44, 285–290

Occurrence of stone fruit viroids in Bosnia and Herzegovina

Slavica Matić, Maher Al-Rwahnih and Arben Myrta

Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano, Bari, Italy

Summary. Tissue-imprint hybridization (TIH) assays were used to determine the occurrence and incidence of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) in stone fruit trees in Bosnia and Herzegovina. Our collections included trees of plum, peach, cherries, apricot, myrobalan and blackthorn from 33 commercial orchards and 2 nurseries, in the areas of Banja Luka, Gradacac, Sarajevo and Mostar. Of the 410 trees assayed, 44 (11%) tested positive by TIH assays. PLMVd was detected in 39 peach trees, including two old (seed grown) vineyard peach trees (*Prunus persica* subsp. *vulgaris*). Tests for HSVd were positive in 3 apricot and 2 plum trees. PLMVd was widely distributed throughout the country. In contrast, HSVd was found only in the northern part of the country. Both native and imported cultivars of *Prunus* were infected. This is the first record of PLMVd and HSVd in Bosnia and Herzegovina. In a separate experiment, peach trees with PLMVd were monitored in the autumn, winter and spring seasons, with tissue imprints of leaf petioles, dormant cuttings and forced sprouts from dormant cuttings. Irrespective of the tissues assayed, nearly all samples tested positive for PLMVd.

Key words: *Prunus*, PLMVd, HSVd, tissue-imprint hybridization, dot-blot hybridization

Introduction

The stone fruit industry is important for the agriculture sector of Bosnia and Herzegovina, in particular European plum (*Prunus domestica*), which has a long cultivation tradition and numbers in the millions of trees planted. The northern part of the country (Bosnia) is primarily devoted to plum production, while the southern part (Herzegovina) grows cherry, peach and apricot.

Although in the former Yugoslavia *Peach latent mosaic viroid* (PLMVd) was reported to occur in peach and plum (Shamloul *et al.,* 1995; Hadidi *et al.,* 1997), the search for viroids was limited. Recently a large-scale survey for PLMVd and *Hop stunt viroid* (HSVd) was completed in orchards located in Bosnia and Herzegovina, and the findings of this survey are presented here.

Corresponding author: A. Myrta
Fax: +39 080 4606275
E-mail: myrta@iamb.it

Materials and methods

Validation of tissue-imprint hybridization (TIH) assays as compared with dot-blot hybridization (DBH)

Prior to our large-scale surveys we tested 81 peach trees comparing the two assays, with PLMVd as the target viroid.

With DBH, total RNA extraction was made by a non-organic method as described by Astruc *et al*. (1996). SP6 and T7 RNA polymerase-generated full-length cRNA was used for Digoxigenine labeled probes (Shamloul *et al*., 1995; Astruc *et al*., 1996). Samples were blotted onto membranes, and prehybridization and hybridization steps performed as described in Más and Pallás (1995) and Pallás

et al. (1998). Chemiluminiscent detection was carried out according to manufacturer's instructions, using Fab-fragments conjugated to alkaline phosphatase.

For TIH, fresh cut ends of leaf petioles were pressed onto a nylon membrane (in duplicates) and processed according to Pallás *et al*. (2003).

Large-scale survey for viroids

The main stone fruit growing areas of Bosnia (Banja Luka, Gradacac and Sarajevo) and Herzegovina (Mostar) were surveyed. A total of 230 trees of European plum (*P. domestica*), 66 of peach (*P. persica*), 11 of wild peach (*P. persica* ssp. *vulgaris*), 11 of apricot (*P. armeniaca*), 65 of cherry (*P. avium* and *P. cerasus*), 16 of myrobalan (*P. cerasifera*) and 11 of blackthorn (*P. spinosa*). Commercial orchards varied in age and cultivation technology. Both native and imported cultivars were sampled.

Leaves were randomly sampled for tissue imprinting in autumn and were imprinted in the field. The membranes were stored at 4°C and developed two weeks later at the Mediterranean Agronomic Institute of Bari (Italy). The collections, also stored at 4°C, were held as backup for RT-PCR assays on those samples that had given a doubtful reaction (Astruc *et al*., 1996; Ambrós *et al*., 1998).

Tracking of PLMVd in peach trees

PLMVd was monitored by TIH in 10-year-old peach trees in autumn (leaf petioles), winter (dormant cuttings and forced sprouts from dormant cuttings), and spring (leaf petioles).

Results

Validation of TIH assay

Of the 81 peach trees tested, DBH detected 30 PLMVd-positive trees (Fig. 1b) and TIH 29 (Fig. 1a). Twenty-eight PLMVd trees were detected by both assays but three PLMVd trees were not in common, i.e. two were positive by DBH only (C_2 and H_9) and one by TIH only (B_2). Based on the relative ease of tissue imprinting, the TIH assay was selected in our survey.

Large-scale survey

In Bosnia and Herzegovina, collections were made in 33 commercial orchards and 2 nurseries. PLMVd-like symptoms (fruits with small discolored spots and suture cracks) were observed in some peach samples. No particular symptoms associated to HSVd were observed. A total of 230 plums, 77 peaches, 65 cherries, 11 apricots and 27 other *Prunus* spp., were collected during October 2003 and assayed for PLMVd and HSVd. Forty-four (11%) samples tested positive for viroids (Table 1), with PLMVd in 39 peach samples and HSVd in 3 apricots and 2 plums. PLMVd incidence in peach was 51% and that of HSVd in apricot was 27%.

RT-PCR test, carried out on doubtful positive samples, confirmed the hybridization results (data not shown).

Both native and imported cultivars of *Prunus* were infected by viroids and incidences were relatively high irrespective of cultivars (Table 2). PLMVd infection rate in peach was highest in the imported cvs.: i.e. Loedel (80%), Red Haven (74%), Sun Crest (67%) etc. Interestingly, two mature seedling trees of vineyard peach (*Prunus persica* ssp. *vulgaris*) assayed positive for PLMVd (Table 2). Among six plum cultivars, HSVd was detected only in the native cv. Bjelica (1 out of 8) and the imported cv. Stanley (1 out of 55). In a limited number of apricots samples (11) 3 trees tested positive for HSVd.

Table 1. Results of tissue-imprint hybridization (TIH) assays for PLMVd and HSVd.

Species	No. of trees		Infection rate (%)	Viroids detected	
	Tested	Infected		PLMVd	HSVd
Plum	230	2	1	–	2
Peach	77	39	51	39	–
Cherry	65	–	–	–	–
Apricot	11	3	27	–	3
Other *Prunus* spp.	27	–	–	–	–
Total	410	44	11	39	5

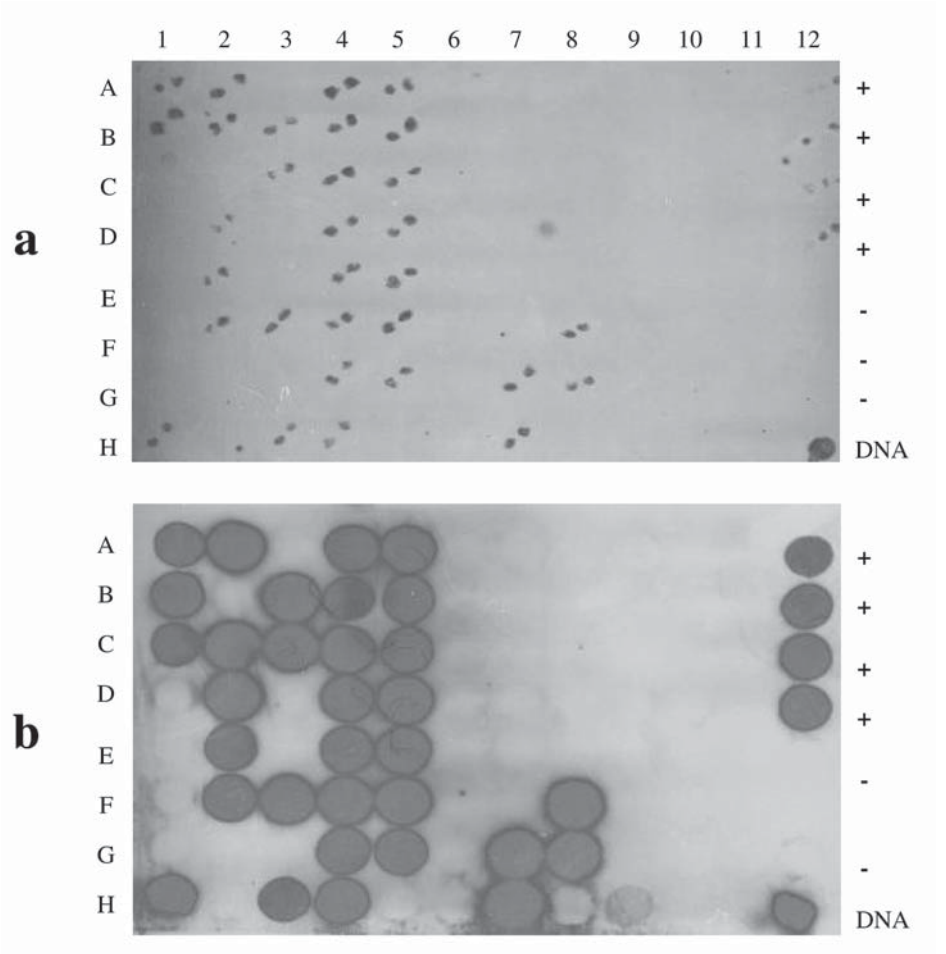

Fig. 1. Comparison of TIH (a) and DBH (b) for detection of PLMVd. Columns 1-H, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 are tested samples. A, B and C in column 1, and A, B, C and D in column 12 are positive controls. D, E, F and G in lane 1, and E, F and G in column 12 are negative controls. DNA plasmid control is spotted in column 12-H on both membranes.

PLMVd was widely distributed throughout the country, while HSVd appeared limited in two areas in the Northern regions (Fig. 2). Taking in consideration the small number of tested apricots, a host reported as highly susceptible to HSVd, a larger survey may prove worthwhile to more accurately reflect its incidence in orchards.

Tracking of PLMVd in peach

Twenty-four PLMVd infected peach trees were monitored for the presence of the viroid, in different seasons during the year: October, January and May. PLMVd was detected nearly in all samples, even in dormant wood (Table 3). The highest rate of detection (100%) was in autumn using leaf petioles, but a very good result (96% of positive detection) was obtained by testing also dormant cuttings and young sprouts forced from them in greenhouse (not shown). Although the highest detection rate was in autumn, reliable levels of detection were obtained during winter and spring also. Comparing the results of dormant cuttings, collected in January, with those obtained from young sprouts forced from them in a greenhouse at 18–22°C, both tissues gave identical results (23 positives out of 24 tested), but more clear-cut results were obtained from young sprouts than directly from dormant cuttings. The same positives were detected from leaf petioles collected in May.

Table 2. Viroid infection related to *Prunus* cultivars.

Species	Varieties	Origin	No. of trees		Viroid infection		Infection rate (%)
			Tested	Infected	PLMVd	HSVd	
Peach	Red Haven	Imported	19	14	14	–	74
	Vesna	Native	10	5	5	–	50
	Early Red Haven	Imported	9	5	5	–	56
	Loadel	Imported	5	4	4	–	80
	Dixired	Imported	5	3	3	–	60
	Spring Crest	Imported	5	2	2	–	40
	Sun Crest	Imported	3	2	2	–	67
	Conus	Imported	2	1	1	–	50
	Cardinal	Imported	2	0	–	–	0
	Vineyard peach	Native	11	2	2	–	18
	Unknown	–	6	1	1	–	17
Plum	Stanley	Imported	55	1	–	1	2
	Pozegaca	Native	49	0	–	–	0
	Cacanska Rodna	Native	48	0	–	–	0
	Cacanska Lepotica	Native	43	0	–	–	0
	Cacanska Najbolja	Native	27	0	–	–	0
	Bjelica	Native	8	1	–	1	13
Apricot	Novosadska Rana	Native	7	1	–	1	14
	Luizet	Imported	1	1	–	1	100
	Magiar Kajszi	Imported	1	1	–	1	100
	Unknown	–	2	0	–	–	0

Table 3. PLMVd tracking in peach.

Season	Tested tissue	Collection location	Average temperature (°C)	No. of trees	
				Infected/Tested	Detection rate (%)
Autumn	Leaf petioles	Field	25–28	24/24	100
Winter	Dormant cuttings	Field	0–3	23/24	96
Winter	Sprouts from cuttings	Green house	18–22	23/24	96
Spring	Leaf petioles	Field	16–18	21/22	96

Discussion

PLMVd was recorded previously from the former Yugoslavia, although the number of tested samples was limited (Shamloul *et al*., 1995; Hadidi *et al*., 1997). Results of our surveys report for the first time the occurrence of HSVd in Bosnia and Herzegovina. Use of the TIH assay, as proposed by Pallás *et al*. (2003), allowed us to process a large number of samples at each collection site in a rapid, efficient manner.

In our study, TIH was successful in detecting 97% of the positives obtained by dot-blot hybridization. Considering those results, tissue-imprinting, thanks to its easy and rapid approach, was chosen to be used in the large-scale survey for the viroids of stone fruit trees.

The high incidence of PLMVd in peach and occurrence of HSVd in apricot fit well with data reported in other Mediterranean countries, i.e. with PLMVd in Spain (Badenes and Llácer, 1998), Italy (Barba and Faggioli, 1999), Syria (Ismaeil *et al*., 2001), Albania and Western Turkey (Torres *et al*., 2004), and for HSVd in South-Eastern Spain (Cañi-

Fig. 2. Distribution (in circles) of PLMVd (solid) and HSVd (open) in Bosnia and Herzegovina.

zares *et al*., 1998), Syria (Ismaeil *et al*., 2001), Western Turkey and Egypt (Torres *et al*., 2004).

The PLMVd-infected "vineyard peaches", originated by seed, raise the problem of viroid epidemiology. Actually, there is no clear information on PLMVd epidemiology, although the natural spread of the viroid in peach orchards has been reported (Desvignes, 1980). Aphid- and cutting tool-transmissions are possibilities (Desvignes, 1986; Flores *et al*., 1992; Hadidi *et al*., 1997), but more studies are needed to clarify this epidemiological aspect.

PLMVd was readily detected in leaf petioles and dormant shoots in all examined seasons and from all types of tissues used, similarly with what reported by Amari *et al*. (2001) for HSVd. The detection of PLMVd from field cuttings in winter (at about 0°C) was consistent with the data obtained by Torres *et al*. (2004). The wide temperature range, from about 0°C in winter to 25–28°C in autumn, within which PLMVd is detected, indicates that the viroid concentration in peach trees is high under a wide range of natural conditions. This offers a good opportunity to carry out reliable sanitary tests for propagating material even during dormancy.

Acknowledgements

Grateful thanks are expressed to Dr. Vicente Pallás for supplying the viroid-specific probes and Dr. Jerry K. Uyemoto for the critical reading and improvement of the manuscript

Literature cited

Amari K., M.C. Cañizares, A. Myrta, S. Sabanadzovic, B. Di Terlizzi and V. Pallás, 2001. Tracking Hop stunt viroid infection in apricot trees during a whole year by non-isotopic tissue printing hybridisation. *Acta Horticulturae* 550, 315–320.

Ambrós S., C. Hernández, J.C. Desvignes and R. Flores, 1998. Genomic structure of three phenotypically different isolates of peach latent mosaic viroid: implications of the existence of constraints limiting the heterogeneity of viroid quasi–species. *Journal of Virology* 72, 7397–7406.

Astruc N., J.F. Marcos, G. Macquaire, T. Candresse and V. Pallás, 1996. Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. *European Journal of Plant Pathology* 102, 837–846.

Badenes M.L. and G. Llácer, 1998. Occurrence of peach latent mosaic viroid in American peach and nectarine cultivars in Valencia, Spain. *Acta Horticulturae* 472(2), 565–570.

Barba M. and F. Faggioli, 1999. I viroidi agenti causali di malattie degenerative dei fruttiferi. *Rivista di Frutticoltura* 2, 69–73.

Cañizares M.C., J.F. Marcos and V. Pallás, 1998. Studies on the incidence of hop stunt viroid in apricot trees (*Prunus armeniaca)* by using an easy and short extraction method to analyze a large number of samples. *Acta Horticulturae* 472(2), 581–585.

Desvignes J.C., 1980. Different symptoms of the peach latent mosaic. *Acta Phytopathologica Academiae Scientiarum Hungaricae* 15, 183–190.

Desvignes J.C., 1986. Peach latent mosaic and its relation to peach mosaic and peach yellow mosaic virus diseases. *Acta Horticulturae* 193, 51–57.

Flores R., C. Hernández, L. Avinent, A. Hermoso, G. Llácer, J. Juárez, J.M. Arregui, L. Navarro and J.C. Desvignes, 1992. Studies on the detection, transmission and distribution of peach latent mosaic viroid in peach trees. *Acta Horticulturae* 309, 325–330.

Hadidi A., L. Giunchedi, A.M. Shamloul, C. Poggi Pollini and A.M. Amer, 1997. Occurrence of peach latent mosaic viroid in stone fruits and its transmission with contaminated blades. *Plant Disease* 81, 154–158.

Ismaeil F., N. Abou Ghanem-Sabanadzovic, A. Myrta, B. Di Terlizzi and V. Savino, 2001. First record of Peach latent mosaic viroid and Hop stunt viroid in Syria. *Journal of Plant Pathology* 82(2), 227.

Más P. and V. Pallás, 1995. Non-isotopic tissue printing hybridization: a new technique to study long-distance plant virus movement. *Journal of Virological Methods* 52, 317–326.

Pallás V., P. Más and J.A. Sánches-Navarro, 1998. Detection of plant RNA viruses by non isotopic dot-blot hybridisation. In: *Plant Virus Protocols: From Virus Isolation to Transgenic Resistance* (G. Foster, S. Taylor, ed.), Humana Press, Totowa, NJ, USA, 461–468.

Pallás V., H. Torres, A. Myrta and G. Gomez, 2003. Validation of the 'tissue-printing' technique for detecting stone fruit viroids. In: *Virus and Virus-like Diseases of Stone fruits, with Particular Reference to the Mediterranean Region*. Option Méditerranéennes, Sér. B/ N° 45, (A. Myrta, B. Di Terlizzi, V. Savino, ed.), Bari, Italy, 135–137.

Shamloul A.M., A. Minafra, A. Hadidi, H.E. Waterworth, L. Giunchedi and E.K. Allam, 1995. Peach latent mosaic viroid: nucleotide sequence of an Italian isolate, sensitive detection using RT-PCR and geographic distribution. *Acta Horticulturae* 386, 522–530.

Torres H., G. Gómez, B. Stamo, A. Shalaby, B. Aouane, I. Gavriel, P. Kominek, K. Caglayan, M. Sipahioglu, R. Michelutti, A. Myrta, N. Fiore and V. Pallás, 2004. Detection by Tissue Printing of Stone Fruit Viroids, from Europe, the Mediterranean and North America. *Acta Horticulturae* 657, 379–383.

Accepted for publication: November 11, 2005