

## SHORT COMMUNICATION

## TRACKING THREE ILARVIRUSES IN STONE FRUIT TREES THROUGHOUT THE YEAR BY ELISA AND TISSUE-PRINTING HYBRIDIZATION

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## SUMMARY

The presence of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV) was monitored throughout 2005 in stone fruit trees by ELISA and tissue printing hybridization. The highest detection rate for all three viruses was obtained in winter with both techniques. Detection was reliable also in spring and early summer, except for ApMV. However, neither technique was able to identify successfully all infected samples in one season. Overall, ELISA was more successful for PNRSV and PDV detection and tissue-printing hybridization for ApMV.

*Key words:* PNRSV, PDV, ApMV, diagnosis, ELISA, tissue-printing hybridization.

Among the different viruses affecting stone fruit trees, *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV), all members of the genus *Illarvirus*, family *Bromoviridae* (Roosinck *et al.*, 2005) have a worldwide distribution and are the most significant economically.

PNRSV, which induces overt symptoms in many different *Prunus* species in the first year after infection (acute or shock stage), becomes latent in the years that follow, although some strains cause recurrent symptoms annually (Desvignes *et al.*, 1999).

Crop losses vary from none in Italian prune, to 15% in sweet cherry, up to 100% in peach in certain years (Barbara, 1988). The virus is also a serious threat to the nursery industry, causing poor graft take and reduced growth of grafted trees.

PDV elicits a range of different diseases of sweet and sour cherry, peach, apricot and plum (Kunze, 1988; Jones and Sutton, 1996; Desvignes *et al.*, 1999) and, when present in mixed infection with PNRSV, it may

lead to a progressive decline and death of the trees (Cropley, 1968).

ApMV has a cosmopolitan distribution (Diekmann and Putter, 1996) and a very broad spectrum of natural hosts, infecting hops, rose, hazelnut, apple and many *Prunus* species (Fulton, 1983; Németh, 1986). The extent of the damage is related to the virus strain and the type of host, with impact on vegetative growth and yield (Diekmann and Putter, 1996).

All three viruses spread through infected propagating material and nursery productions (Mink, 1992), but PNRSV and PDV are also seed- and pollen-borne and can be spread by pollinating insects (Cole *et al.*, 1982; Hamilton *et al.*, 1984; Kelley and Cameron, 1986; Aparicio *et al.*, 1999; Amari *et al.*, 2007). Seed transmission of ApMV is known only in hazelnuts (Cameron and Thompson, 1985).

The widespread presence of PNRSV, PDV and ApMV in the Mediterranean basin (Myrta *et al.*, 2003) affects the regional stone fruit industry. To prevent the spread of these viruses, the use of certified virus-tested material is required, for the production of which the use of effective detection techniques has a crucial importance.

The development of molecular detection methods based on non-radioactive hybridization tests or on RT-PCR, has increased the range of available techniques, allowing the detection of the three ilarviruses either separately (Sánchez-Navarro *et al.*, 1998; Herranz *et al.*, 2005) or simultaneously (Saade *et al.*, 2000; Herranz *et al.*, 2005; Sánchez-Navarro *et al.*, 2005). Comparison of serological and molecular detection methods showed molecular hybridization to be 25 fold and RT-PCR 15625 fold more effective than ELISA (Sánchez-Navarro *et al.*, 1998). In addition, both molecular methods offer the possibility of a simultaneous detection of the three viruses in a single hybridization reaction.

Molecular hybridization can also be applied to tissue spotted directly on a membrane (tissue printing), thus avoiding sample extraction and making this method an attractive alternative to the standard serological techniques.

In the present study, tissue-printing molecular hy-

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bridization (TP) and ELISA were compared for the detection of PNRSV, ApMV and PDV in different hosts known to be infected with one or mixture of viruses. These include 7 trees of almond, 2 of apricot, 6 of cherry, 3 of peach, and 1 of plum (Table 1). Included also were healthy trees of each stone fruit. Testing was done every month in 2005 to determine the influence of cropping seasons on the detection limits of both techniques.

All samples were comparatively tested by both DAS-ELISA (Clark and Adams, 1977) with a commercial kit (Loewe, Sauerlach, Germany) and TP (Pallás *et al.*, 1998; Herranz *et al.*, 2005), first printing the membrane then extracting tissues immediately for ELISA.

About 0.5 g of tissue were homogenized with 5 vol of extraction buffer (1x PBS supplemented with 2% PVP and 0.05% Tween 20) and 0.1 ml of the extract was directly loaded on antibody-coated ELISA plate wells in duplicate. Samples were considered positive when the absorbance value was at least three times higher than that of the healthy control after 1h incubation.

For TP, prints of two different leaves and their petioles were produced for each sample. Petioles were cut transversely and pressed gently onto the membrane, whereas leaves were first tightly rolled around the main vein and the obtained cylinder was cut in the middle

and pressed onto the membrane. During winter dormant buds were used for ELISA and TP.

Viruses were detected with both methods but optimal detection occurred in different months (Table 2). All PNRSV-infected trees gave positive reactions in January and March-April using TP and during November or January using ELISA. For the rest of the year, the highest number of positives was obtained between February and May with the detection of nine, eight or nine out of eleven PNRSV-infected trees, by both methods.

In the case of PDV, all trees tested gave positive results in March-April with both techniques and in May with ELISA. For the rest of the year a high number of positives was observed during January and February, ranging from 89% (eight positives out of nine) to 78% (seven positives out of nine), with both techniques.

ApMV was more difficult to detect since all trees gave positive results with both techniques only in November. In the rest of the year, positives ranged from 0 to 60%, the highest detection rates being in January-March-April.

There is no available information on the detection of PNRSV, PDV and ApMV in different seasons using TP. As to ELISA, Torrance and Dolby (1984) reported that the three ilarviruses were more readily detected in young rapidly growing leaves than in mature leaves, and Torrance (1981) found PNRSV and PDV in forced dormant buds collected in mid-winter. In our survey the highest detection rate for the three viruses was in winter from buds of dormant cuttings regardless of the technique used (Table 3). High detection levels were obtained also in spring to early summer from young leaves, with the exception of ApMV for which the detection rate was low. The lowest detection, regardless of the technique used, was in late summer-autumn.

As to single viruses, PNRSV was more efficiently detected in winter from dormant cuttings by ELISA (96.9%) than TP (81.8%) (Table 3). However, TP was more effective in spring and early summer (78.7%) than ELISA (75.7%). Both techniques, gave substantially lower results in late summer and autumn (August-September) (50% for ELISA and 40.9% for TP).

In the case of PDV, both techniques gave equal results (85.1% detection) in winter, whereas in spring and early summer ELISA was more effective (86.2%) than TP (62.9%). However, the virus was detected at a very low rate in August-September with both techniques, when 38.8% and 5.5% of the samples were positive in ELISA and TP, respectively.

TP was more successful in detecting ApMV in winter and spring-early summer (73.3% and 46.6% respectively), than ELISA (66.6% and 33.3% respectively) but in the hot season, neither technique performed efficiently (10% detection with both).

When figures of all seasons are taken into consideration, ELISA proved to be more efficient for PNRSV de-

**Table 1.** Virus infection pattern of surveyed trees.

N°	Host	VIRUS INFECTION		
		PNRSV	PDV	ApMV
1	Almond			
2	Peach			
3	Almond			
4	Almond			
5	Almond			
6	Almond			
7	Apricot			
8	Apricot			
9	Peach			
10	Plum			
11	Peach			
12	Almond			
13	Cherry			
14	Cherry			
15	Cherry			
16	Cherry			
17	Cherry			
18	Cherry			
19	Almond			
<b>Total</b>		<b>11</b>	<b>9</b>	<b>5</b>

**Table 2.** Virus detection by ELISA (E) and tissue printing (TP) in 2005.

Sampling time	Infected trees (No.)			Detection rate (%)		
	PNRSV	PDV	ApMV	PNRSV	PDV	ApMV
	E/TP	E/TP	E/TP	E/TP	E/TP	E/TP
January	11/11	8/8	2/4	100/100	89/89	40/80
February	10/9	7/8	3/2	91/82	78/89	60/40
March-April	8/11	9/9	4/3	73/100	100/100	80/60
May	9/9	9/7	0/3	82/82	100/78	0/60
June	8/6	7/1	1/1	73/54	78/11	20/20
August	4/2	5/0	0/0	36/18	56/0	0/0
September	7/7	2/1	1/1	64/64	22/11	20/20
November	11/7	8/7	5/5	100/64	89/78	100/100
Total	68/62	55/41	16/19	77/70	76/57	40/47

**Table 3.** Detection rate obtained by ELISA (E) and tissue printing (TP) for each season.

Sampling time	PNRSV			PDV			ApMV		
	Tested samples	Positive samples E/TP	Detection rate (%) E/TP	Tested samples	Positive samples E/TP	Detection rate (%) E/TP	Tested samples	Positive samples E/TP	Detection rate (%) E/TP
Winter (Nov, Jan, Feb)	33	32/27	96.9/81.8	27	23/23	85.1/85.1	15	10/11	66.6/73.3
Spring-Early Summer (March-June)	33	25/26	75.7/78.7	27	25/17	86.2/62.9	15	5/7	33.3/46.6
Late Summer- Autumn (August-Sep)	22	11/9	50/40.9	18	7/1	38.8/5.5	10	1/1	10/10
TOTAL	88	68/62	77.2/70.5	72	55/41	76.3/56.9	40	16/19	40/47.5

**Table 4.** Detection rate obtained by ELISA and tissue printing in different species.

MONTH	Almond			Cherry		Peach		Apricot		Plum	
	PNRSV E/TP <sup>1</sup>	PDV E/TP	ApMV E/TP	PNRSV E/TP	PDV E/TP	PNRSV E/TP	ApMV E/TP	PNRSV E/TP	ApMV E/TP	PNRSV E/TP	PDV E/TP
January	3/3	3/3	2/3	3/3	4/4	3/3	0/0	1/1	0/1	1/1	1/1
February	2/2	3/3	2/2	3/2	4/4	3/3	1/0	1/1	0/0	1/1	0/1
March-April	3/3	4/4	3/3	3/3	4/4	0/3	0/0	1/1	1/0	1/1	1/1
May	2/2	4/2	0/2	2/2	4/4	3/3	0/1	1/1	0/0	1/1	1/1
June	2/1	3/0	0/0	2/1	3/1	3/3	1/1	0/1	0/0	1/0	1/0
August	0/0	3/0	0/0	1/0	1/0	2/2	0/0	0/0	0/0	1/0	1/0
September	2/0	2/0	1/0	2/3	0/1	3/3	0/1	0/0	0/0	0/1	0/0
November	3/1	4/3	3/3	3/1	3/3	3/3	1/1	1/1	1/1	1/1	1/1
TOTAL	17/12	26/15	11/13	19/15	23/21	20/23	3/4	5/6	2/2	7/6	6/5
Detection rate (%)	71/50	81/47	46/54	79/63	72/66	83/96	38/50	63/75	25/25	88/75	75/63

<sup>1</sup> ELISA (E) and Tissue-printing (TP)

**Table 5.** Detection rate in single and mixed infections by ELISA and tissue printing.

MONTH	PNRSV		PDV		ApMV	
	Single	Mixed	Single	Mixed	Single	Mixed
	E/TP <sup>1</sup>	E/TP	E/TP	E/TP	E/TP	E/TP
January	7/7	4/4	4/4	4/4	0/2	2/2
February	6/5	4/4	4/4	3/4	0/0	3/2
March-April	5/7	3/4	4/4	5/5	2/1	2/2
May	6/6	3/3	4/3	5/4	0/0	0/3
June	4/5	4/1	3/0	4/1	0/0	1/1
August	2/1	2/1	2/0	3/0	0/0	0/0
September	5/4	2/3	1/0	1/1	0/0	1/1
November	7/4	4/3	3/3	5/4	2/2	3/3
TOTAL	42/39	26/23	25/18	30/23	4/5	12/14
Detection rate (%)	75/70	81/72	78/56	75/58	25/31	50/58

<sup>1</sup> ELISA (E) and Tissue-printing (TP)

tection (68 of 88 samples, 77.2%) than TP (62 of 88 samples, 70.5%) (Table 3). Likewise, ELISA gave 55 positives (76.3%) and TP 41 positive (56.9%) out of 72 samples for PDV, whereas TP was more successful (19 of 40 samples, 47.5%) than ELISA (16 of 40 samples, 40%) for ApMV.

With reference to the host species (Table 4), PNRSV and PDV were more efficiently detected by ELISA in almond, cherry and plum and by TP in peach and apricot. As to ApMV, almond and peach gave more positive responses by TP and apricot gave similar responses with both techniques.

As to the effect of single and mixed infections on detection level, it was found that PNRSV and PDV were detected equally well in both types of infections by both techniques. On the contrary, ApMV was detected more successfully by ELISA in mixed (50% of the samples) than in single infections (25% of the samples). ApMV was detected by TP in 58% of the samples with mixed infections and in 31% of samples with single infection (Table 5).

Although PNRSV, PDV and ApMV are closely related phylogenetically (Sánchez-Navarro and Pallás, 1997) and have similar coat protein sequence identity, they did not respond in the same way to ELISA and TP throughout the year. In some cases, more positives were obtained by ELISA than TP (e.g., November/PNRSV: 11 positives by ELISA vs 7 by TP) whereas in others, the opposite situation was recorded (e.g., March-April/PNRSV: 8 positives by ELISA vs 11 by TP) (Table 2).

An explanation to this differential behaviour can be found in the fact that TP analyzes a small portion of the host tissue (only the printed area) whereas ELISA ex-

plores a larger tissue area (*ca.* 0.5 g), which may have a bearing for viruses, like those in question, that are irregularly distributed in the host (Uyemoto *et al.*, 1989).

Furthermore, TP targets the viral RNA and ELISA the coat protein, two components whose stability and concentration are affected by the season. Thus, TP positives for PDV dropped significantly in summer (up to no detection in August) while ELISA was still effective (55.5% positives in August).

A similar situation has been reported for *American plum line pattern virus* with dot-blot hybridization (Al Rwahnih *et al.*, 2004) and for *Plum pox virus* with ELISA (Glasa *et al.*, 2003). Since both tests have a comparable detection limit (Sánchez-Navarro *et al.*, 1996; Sánchez-Navarro *et al.*, 1998) this discrepancy could reflect a lower titer of viral RNA compared with that of CP. Also, it was reported that the plant defence mechanisms that specifically target viral RNA (silencing) are more active at high temperatures (Szittyá *et al.*, 2003; Chellappan *et al.*, 2005) which, together with the alleged lower viral replication in hot months of the year may explain the differential behaviour of TP and ELISA. At lower temperatures, between November and March-April, both techniques were able to detect a comparable number of infected samples.

Since neither TP nor ELISA was able to detect all infected samples in one season it ensues that different detection approaches should be followed at different times. In any case, TP has the advantage of simplicity with no manipulation of the samples, it is adapted to large scale surveys, and it offers the possibility of a simultaneous detection of the three ilarviruses (Saade *et al.*, 2000; Herranz *et al.*, 2005).

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