LOCALIZATION OF *PLUM POX VIRUS* IN STEM AND PETIOLE TISSUES OF APRICOT CULTIVARS BY IMMUNO-TISSUE PRINTING

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Summary. – Localization of *Plum pox virus* (PPV) in stem and petiole tissues of four susceptible and four resistant apricot cultivars has been studied. Consecutive 1-mm spaced transverse sections were taken from the tissues and were printed onto nitrocellulose membrane in duplicate. For virus-specific detection, one series of prints was probed with an antibody to PPV coat and the density of stains was evaluated by light microscopy. Another series of prints was treated with a substrate but not with the antibody to reveal non-specific staining due to endogenous peroxidases. The virus was currently detected in all inoculated susceptible cultivars but only in one inoculated resistant cultivar (Harcot). In the stem tissues, the virus was localized in the pith and in the xylem. In the petiole tissues, it was localized in the epidermis and in cortical and medullae parenchyma. Non-specific staining was observed only in the stem sclerenchyma and in the petiole phloem.

Key words: Prunus armeniaca L; sharka; Plum pox virus; immuno-tissue printing

Introduction

Sharka, a disease caused by PPV, (*Potyviridae* family, *Potyvirus* genus, *Plum pox virus* species) is the most important viral disease affecting European fruit trees (Nemeth, 1994; Roy and Smith, 1994). One of the characteristics that makes difficult the detection of this virus in fruit trees is its low concentration and uneven distribution (Albrechtova, 1986; Audergon *et al.*, 1989).

Immuno-tissue printing on nitrocellulose membrane has been used successfully for the localization of viruses such as tomato yellow leaf curl virus (Navot *et al.*, 1989), tobacco mosaic virus (Wisniewski *et al.*, 1990), soybean mosaic virus (Mansky *et al.*, 1990), tomato spotted wilt virus (Hsu, 1991), potato viruses X and Y (Bravo-Almonacid *et al.*, 1992), and plum pox virus (Adamolle, 1993; Dicenta and Audergon, 1995a,b) in tissues. Reid *et al.* (1992) have reported a review of applications of this technique. It permits the observation of the marks left by the tissues on the membrane. Moreover, the contents of the cut tissues transferred to the membrane can be exposed to an immunological reaction to locate the virus.

In this work, the immuno-tissue printing was used to compare the location of PPV in the stem and petiole of eight apricot cultivars, of which four were susceptible and four were resistant to PPV.

Materials and Methods

Plant material. Eight apricot cultivars were studied. Four of them are generally considered susceptible (Screara, Tirinto, Colomer, and Bebeco) while the others resistant (Henderson, Stella, Stark Early Orange, and Harcot) to PPV (Martínez-Gómez *et al.*, 2000). Six plants of each cultivar grafted on peach GF305 rootstock were employed. Three of them were graft-inoculated, while three non-inoculated plants served as controls. A sample consisting of a piece of stem with a leaf was taken from each of them. In the case of inoculated susceptible cultivars samples showing strong sharka symptoms were chosen. In the case of inoculated resistant cultivars

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Abbreviations: PPV = *Plum pox virus*; TBS = Tris buffered saline

Table 1. Number of prints with stains and mean density of stains (in brackets) in stem tissues of eight apricot cultivars

Cultivar		Virus-specific assay						Non-specific assay					
		Pi	Xy	Ph	Sc	Co	Ep	Pi	Xy	Ph	Sc	Co	Ep
Screara (susc.)	C-1												
	C-2												
	C-3												
	I-1	5 (0.9)	8 (0.8)										
	I-2												
	I-3	5 (0.5)	3 (0.3)										
Tirinto (susc.)	C-1				1 (0.1)								
	C-2				1 (0.1)								
	C-3												
	I-1		2 (0.2)		8 (0.8)								
	I-2	1 (0.1)	5 (0.5)										
	I-3												
Colomer (susc.)	C-1												
	C-2												
	C-3												
	I-1		4 (0.4)		5 (0.5)								
	I-2	4 (0.5)	5 (0.5)										
	I-3												
Bebeco (susc.)	C-1												
	C-2												
	C-3												
	I-1				4 (0.4)								
	I-2				6 (0.6)								
	I-3												
Henderson (resist.)	C-1				2 (0.2)								
	C-2												
	C-3												
	I-1												
	I-2												
	I-3												
Stella (resist.)	C-1				3 (0.3)								
	C-2												
	C-3												
	I-1				4 (0.4)							2 (0.2)	
	I-2												
	I-3												
Stark Early Orange (resist.)	C-1				1 (0.1)								
	C-2				1 (0.1)								
	C-3				1 (0.1)								
	I-1				2 (0.2)								
	I-2												
	I-3												
Harcot (resist.)	C-1												
	C-2												
	C-3												
	I-1												
	I-2				1 (0.1)	1 (0.1)							
	I-3												

Pi = pith, Xy = xylem, Ph = phloem, Sc = sclerenchyma, Co = cortex, Ep = epidermis.
C = control, non-inoculated plants. I= inoculated plants. susc. = susceptible to PPV. resist. = resistant to PPV.

and non-inoculated cultivars of any kind the samples did not show any symptoms.

Detection of the virus by immuno-tissue printing. In the stem, twenty 1-mm spaced cuts were taken from each sample (10 above and 10 below the insertion of the petiole) and were printed on nitrocellulose membrane in duplicate. In the petiole, 10 cuts were made and printed similarly. Finally, each membrane contained 10 stem prints and 5 petiole prints.

In virus-specific assay, one series of membranes was subjected to the protocol described by Dicenta and Audergon (1995a). The membranes were fixed at 80°C for 1 hr and then blocked with 3% gelatine in a Tris buffer (TBS) for 60 mins. After washing with TBS-Tween 20 (0.5 ml of Tween-20 per 1 l of TBS) 3 times for 5 mins the membranes were incubated with a goat antibody against PPV capsid developed in goat (Sanofi; 9.5 µl of the antibody was reconstituted in 1 ml of TBS-Tween 20 with 1% gelatine) for 2 hrs. After additional washing with TBS-Tween 20 three times for 5 mins the membranes were incubated with a rabbit anti-goat peroxidase-labeled antibody (Sigma A.5420; 0.25 µl of the antibody was reconstituted in 1 ml of TBS-Tween-20 with 1% gelatine) for 2 hrs. The membranes were then washed again with TBS-Tween-20 three times for 5 mins, and once with TBS, and were immersed in a 4-chloro-1-naphtol solution. A dark blue color developed in about 15 mins. Finally the membranes were dried and were observed by light microscopy at the magnification of 40–100x.

In non-specific assay, duplicate membranes were treated similarly as above but without the addition of the antibody against PPV capsid. Altogether 1440 prints were evaluated.

Density of stains was visually scored for each tissue in each print, using a subjective scale (0 = none, 1 = low, 2 = intermediate, 3 = maximum). The mean density of each sample was calculated from 10 prints for the stem and from 5 prints from the petiole.

Results and Discussion

PPV localization in susceptible apricot cultivars

The samples from the stem of inoculated susceptible cultivars showed in the virus-specific assay stains for the pith, xylem and sclerenchyma. The control samples did not show any stains except for two prints from the sclerenchyma of cv. Tirinto (Table 1). No stains were observed in the inoculated samples or in the controls in the non-specific assay. In the samples from the petiole of inoculated susceptible cultivars some stains appeared in the virus-specific assay, mainly in the cortical parenchyma and in the epidermis but also in the medullae parenchyma. In the control plants no specific stains appeared except for one sample from the phloem of a control plant for cv.Tirinto. Non-specific stains appeared in the phloem of two samples (Table 2).

These results basically agree with previous ones for other susceptible cultivars (Adamolle, 1993; Dicenta and Audergon, 1995a,b; Knnap *et al.*, 1995). Moreover, as far as heavily infected plants are concerned, it has to be considered that only a little number of prints revealed virus by immuno-tissue printing. The distribution of PPV, both in the plant and in the tissues of *Prunus*, has been studied by different authors who, in general, have found a low signal of the inmunological reaction as a consequence either of low concentration of the virus in *Prunus* or of its uneven distribution (Morvan and Castelain, 1976; Albrechtova, 1986; Dosba *et al.*, 1986; Dicenta and Audergon, 1995a,b).

PPV localization in resistant apricot cultivars

Very few stains were observed in the prints of the stem of resistant cultivars in virus-specific assay. These were mainly localized in the sclerenchyma, both in inoculated plants and in controls. Only a stain in the cortex of a sample of cv. Harcot was observed (Table 1). This could be related to the finding that the resistance of this cultivar could be overcome by the infection (Audergon *et al.*, 1995). Non-specific stains were observed only in two prints from the sclerenchyma of cv. Stella.

The prints from the petiole of resistant cultivars showed very few virus-specific stains. Only cvs. Stark Early Orange and Harcot presented stains in the phloem, both in the inoculated and in the control samples. A very few non-specific stains were present only in the phloem, both in the control and in the inoculated samples from these cultivars (Table 2).

Presence of stains in the stem sclerenchyma and in the petiole phloem

The frequent presence of stains in the stem sclerenchyma appears to be due more to the non-specific reactions than to the presence of the virus in this tissues, since 7 control samples in the virus-specific assay (Tirinto C-1 and C-2, Henderson C-1, Stella C-1, Stark Early Orange C-1, C-2 and C-3) and 1 sample in the non-specific assay (Stella I-1) showed stains. In fact, it may be the result either of the oxidation of phenolic compounds (Quadt-Hallmann and Hamacher, 1996) or of the induction of peroxidases by a pathogen (Visedo *et al.*, 1990, 1991).

It remains unclear why stains appeared more frequently in the prints from the sclerenchyma in the virus-specific assay. It seems necessary to improve this technique by eliminating non-specific reactions, e.g. by the optimization of the enzyme linked to the specific antibody. However, the results of Adamolle (1993) suggest that the method used in our study was most efficient.

The stains observed in the petiole phloem can be easily interpreted. Although in the susceptible cultivars the stains were almost none in both the virus-specific and the non-specific assays, the stains in resistant cultivars appeared in

Table 2. Number of prints with stains and mean density of stains (in brackets) in petiole tissues of eight apricot cultivars

Cultivar		Virus-specific assay					Non-specific assay				
		MP	Xy	Ph	CP	Ep	MP	Xy	Ph	CP	Ep
Screara	C-1										
(susc.)	C-2										
	C-3										
	I-1										
	I-2	1 (0.4)			1 (0.4)	1 (0.4)					
	I-3	1 (0.2)			1 (0.2)	1 (0.2)					
Tirinto	C-1			1 (0.2)						2 (0.4)	
(susc.)	C-2										
	C-3										
	I-1	5 (2.2)			5 (2.0)	5 (3.0)				1 (0.2)	
	I-2	2 (0.4)			3 (0.6)	1 (0.2)					
	I-3	2 (0.4)			5 (2.0)	4 (1.2)					
Colomer	C-1										
(susc.)	C-2										
	C-3										
	I-1				1 (0.2)	2 (0.4)					
	I-2				3 (0.6)	1 (0.2)					
	I-3				3 (0.6)	2 (0.4)					
Bebeco	C-1										
(susc.)	C-2										
	C-3										
	I-1				4 (1.4)	5 (1.2)					
	I-2				5 (2.4)	5 (2.6)					
	I-3										
Henderson	C-1										
(resist.)	C-2										
	C-3										
	I-1									1 (0.2)	
	I-2										
	I-3										
Stella	C-1										
(resist.)	C-2										
	C-3										
	I-1									1 (0.2)	
	I-2									4 (0.8)	
	I-3										
Stark Early	C-1			1 (0.2)							
Orange	C-2										
(resist.)	C-3										
	I-1										
	I-2										
	I-3			1 (0.4)						3 (0.6)	
Harcot	C-1			1 (0.2)						1 (0.2)	
(resist.)	C-2										
	C-3			1 (0.2)							
	I-1			1 (0.2)							
	I-2			1 (0.2)							
	I-3			2 (0.4)							

MP = medullae parenchyma, Xy = xylem, Ph = phloem, CP = cortical parenchyma.
Ep = epidermis. C = control, non-inoculated plants. I = inoculated plants. susc. = susceptible to PPV. resist. = resistant to PPV.

both a ays in the inoculated as well as in the control samples, indica g non-specific reactions.

Inte - and intra-cultivar variability

As could be expected, a difference in the behavior of susceptible and resistant cultivars was observed. In susceptible cultivars, despite the fact that the inoculated samples with numerous symptoms of sharka were chosen, a great variability both between cultivars and between replicate samples of each cultivar in the detection of the virus was observed. There were even samples that did not show any stains (the stem of cvs. Screara I-2, Tirinto I-3, and Colomer I-3). It is to be pointed out that cv. Bebeco, in spite of being apparently seriously infected did not show any virus-specific stains in the stem in any sample. Cvs. Screara and Colomer presented more stains in the stem than did cvs. Tirinto and Bebeco (Table 1).

In the petiole the number of stains was higher than in the stem, mainly in cv. Tirinto (Table 2). In the susceptible cultivars the petiole seemed to be more suitable than the stem for the detection of the virus; the virus was detected in 10 of 12 petiole samples against 6 of 12 stem samples.

This variability shows again a low concentration and uneven distribution of PPV in *Prunus* as described earlier by other authors (Morvan and Castelain, 1976; Albrechtova, 1986; Dosba *et al.*, 1986). Similar reports concerning other potyviruses *in vitro* have been published by Lin *et al.* (1990), Martínez-Gómez and Dicenta (2000).

Thus our results indicate (1) that PPV in the apricot stem is mainly located in pith and xylem tissues, and in the apricot petiole in cortical parenchyma and epidermis tissues and also in the medullae parenchyma tissues, (2) the distribution of PPV in apricot is uneven, and (3) the petiole is more suitable than the stem for the detection of the virus by immuno-tissue printing.

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