

SHORT NOTE

Relationship of viruses and viroids with apricot “viruela” disease

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Summary. In this study, 34 apricot orchards in south-eastern Spain planted with the Spanish cultivar ‘Búlida’ and showing “viruela” symptoms were studied for 2 years. Leaf and fruit samples from four trees in each orchard, either with or without “viruela” symptoms, were collected and analysed by multiplex RT-PCR for the detection of *American plum line pattern virus* (APLPV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apricot latent virus* (ApLV), *Plum bark necrosis and stem pitting-associated virus* (PBNSPaV), *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), and *Prunus necrotic ring spot virus* (PNRSV). In addition, molecular hybridization assays were performed for the detection of *Hop stunt viroid* (HSVd) and *Peach latent mosaic viroid* (PLMVd). All fruits showing “viruela” symptoms were infected with ACLSV and HSVd, suggesting that these pathogens may be responsible for “viruela” disease. Other viruses including PNRSV, PBNSPaV, ApLV, PDV, ApMV and PPV, were detected to a lesser degree. Detection of ACLSV and HSVd in samples without symptoms could be explained by the influence of environmental conditions and/or the physiological stage of fruits on the expression of symptoms.

Key words: *Prunus*, ACLSV, HSVd, stone fruits.

Introduction

Since the 1960s, apricot culture in Spain has been significantly affected by a disease called “viruela” (pox) (Peña-Iglesias and Ayuso, 1975). Apricot trees with “viruela” produce fruits with dark irregular fissures and spots, corky flesh and notable malformations that make them unmarketable, while apricot leaves usually do not show symptoms (Peña-Iglesias, 1988; Cañizares *et al.*, 2001; Pallás *et al.*, 2003). The apricot cultivar ‘Búlida’, representing more than 50% of the Spanish apricot production, is the most affected with “viruela”. The disease has spread rapidly and is now present in the main areas of apricot production in Spain, becoming one of the main disorders affecting this crop (Cañizares *et al.*, 2001).

Despite the considerable impact of the disease, its causal agent remains unknown. Most previous studies

indicate that *Apple chlorotic leaf spot virus* is responsible for this disease (Peña-Iglesias and Ayuso, 1975; Cañizares *et al.*, 2001), although without absolute certainty. Other authors have suggested the relationship between “viruela” and *Hop stunt viroid* (Flores *et al.*, 1990).

Domínguez *et al.* (1998) found that 30% of apricots in the Region of Murcia were infected with viruses, and 23% of the total were infected with ACLSV. In the case of ‘Búlida’, these authors detected ACLSV by ELISA-DAS in 50% of their samples. In addition, other studies showed a very high incidence of HSVd in apricot (81%) in most production areas in Murcia (Cañizares *et al.*, 1998), and this virus was associated with “degeneration of the fruit” in apricot (Amari *et al.*, 2007).

The objective of the present study was to assess the presence of viruses and viroids in ‘Búlida’ apricot plants with “viruela” to determine the causal agent of this disease in apricot.

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Materials and methods

Fruit and leaf samples were analysed from 'Búlida' apricots located in 34 orchards affected by "viruela" disease in four different areas (Cehegín, Mula, Pliego and Hellín) of apricot culture in south-eastern Spain. Trees between 10 and 35 years old were identified and located by GPS and SIGPAC (<http://sigpac.mapa.es/feqa/visor/>). In 2009 and 2010, four trees were selected in each orchard, two with and two without "viruela" symptoms the previous year. Five fruits and 20 leaves were collected from each tree at harvest time. During this study, 136 leaves and 680 fruit samples from 136 different trees were analysed each year.

In 2009 and 2010, leaf and fruit samples were analysed by multiplex RT-PCR to detect eight viruses, including *American plum line pattern virus* (APLPV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apricot latent virus* (ApLV), *Plum bark necrosis and stem pitting-associated virus* (PBNSPaV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Plum pox virus* (PPV) (Sánchez-Navarro *et al.*, 2005) (Figure 1A). Furthermore, the viroids *Hop stunt viroid* (HSVd) and *Peach latent mosaic viroid* (PLMVd) were tested by molecular hybridization (Dot-Blot) (Cañizares *et al.*, 1998; Amari *et al.*, 2007) (Figure 1B). Taking into account the number of years, plots, trees, fruits, viruses and viroids studied, more than 13,000 tests were performed.

A tree was considered infected when at least one leaf or fruit sample tested positive by RT-PCR or molecular hybridization for one of the viruses or viroids.

Results

Sanitary state

We observed that most of the trees were infected with ACLSV (94 and 89%, respectively, in 2009 and 2010) and HSVd (99% and 98%) (Table 1). PNRSV (35 and 44%) and PBNSPaV (23 and 29%) were also frequently detected but to a lesser degree. The incidence of other viruses was low, and APLPV and PLMVd were not detected. Only in three trees (2%) were none of the analysed viruses or viroids detected. In general, no differences between seasons were observed for each virus and growing area. However, the PNRSV infection rate increased in Mula from 30% to 64% for the 2 years, the highest rate observed.

Symptoms and annual variability

Table 1 shows the percentage of infection for each virus or viroid in leaves and fruits with or without "viruela" symptoms by area and year. Despite the fact that the same trees were analysed in both years (except for nine trees removed by farmers), the number of fruits with "viruela" symptoms by area varied significantly from 2009 to 2010. For example, in Cehegín, the number decreased from 55 to 3, and in Hellín, the number increased from 5 to 49. No symptoms of "viruela" were observed on leaves.

The relationship between virus and symptoms

Among the 680 fruits studied in 2009 and 635 fruits studied in 2010, only 90 and 67, respectively, showed "viruela" symptoms (Table 1). In both years, all fruits showing "viruela" symptoms were positive for ACLSV and HSVd. Despite the high number of fruits without symptoms, ACLSV was detected in 85% of fruits in 2009 and in 73% in 2010. In leaves, the rate of ACLSV infection was 46% in 2009 and 60% in 2010.

HSVd was detected in most fruit samples, both with symptoms (100%) and without symptoms (97%), in 2009 and 2010. Only eight trees (three in Pliego, two in Mula and three in Hellín) were infected exclusively with HSVd. In these trees, "viruela" symptoms were never observed. However HSVd was detected in all tested fruits but not in leaves even in HSVd infected trees. Other viruses were detected but at lower incidence (0 to 43%) than ACLSV and HSVd, in fruits with or without "viruela" symptoms.

Discussion

Sanitary state

This research shows the high rate of infection, mainly with ACLSV and HSVd, in the traditional 'Búlida' apricot orchards, particularly those affected by "viruela". ACLSV and HSVd were detected in nearly all samples and usually together.

The presence of other viruses was highly variable and they were less widespread, which indicates that they are not likely to be related to "viruela" disease.

In a study of apricot in Murcia, Domínguez *et al.* (1998) found up to 50% of trees were infected with ACLSV. Our data indicate an exponential progression of virus infection in south-eastern Spain, even

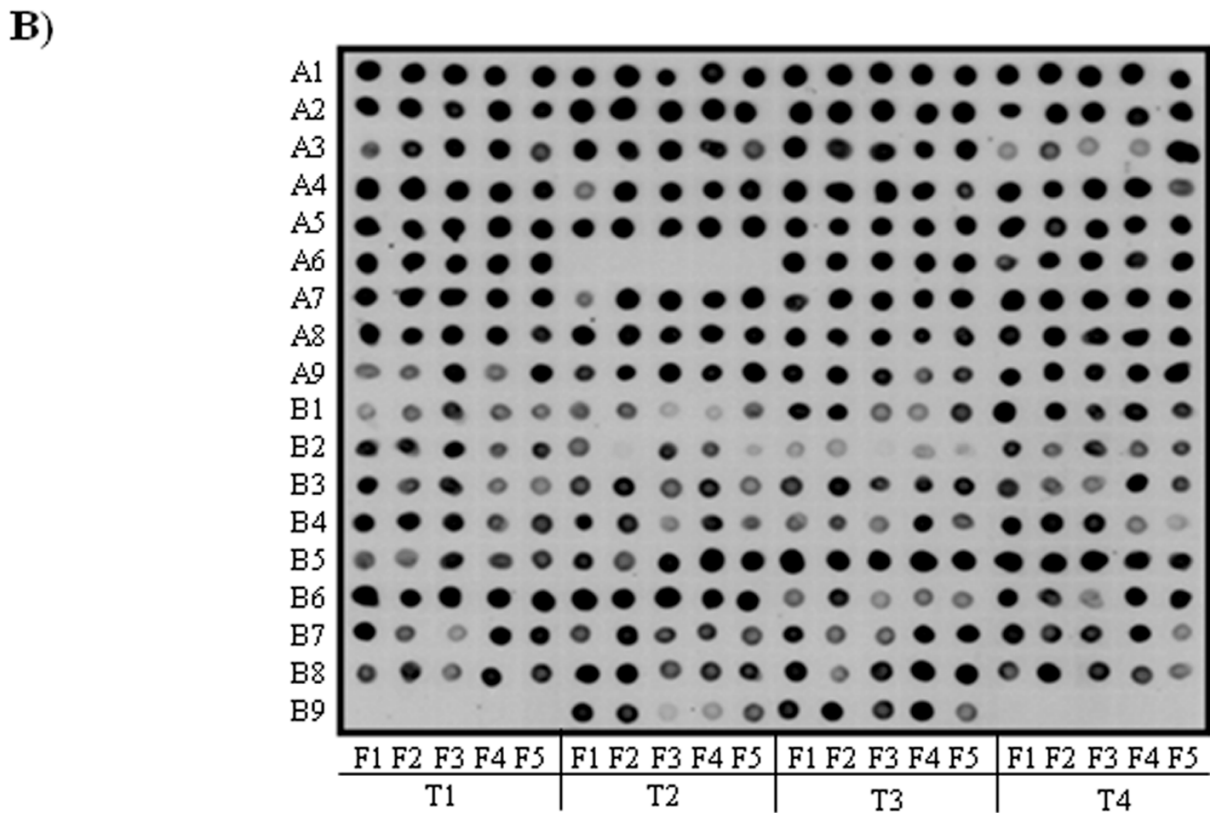
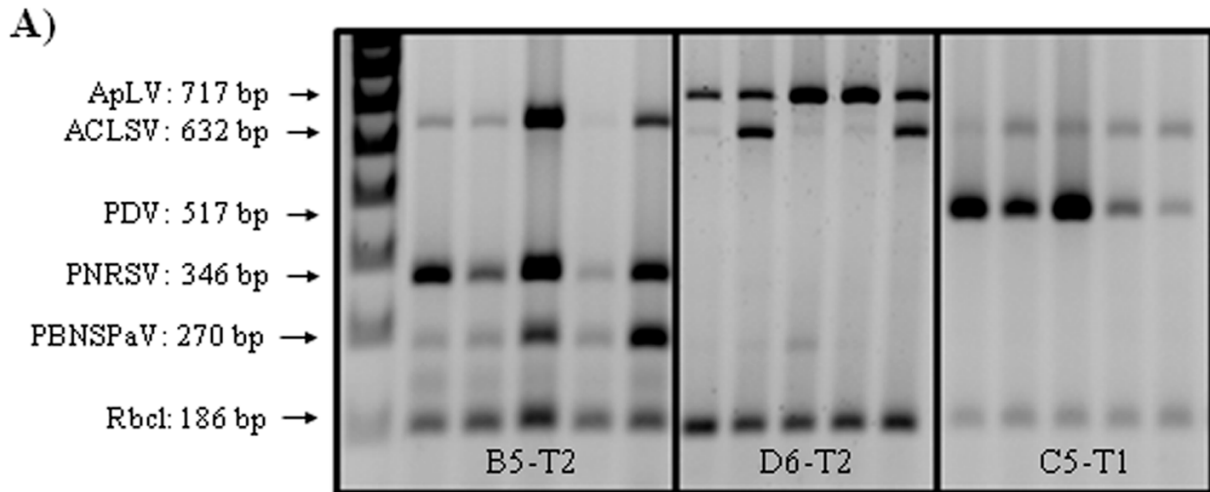


Figure 1. A) Multiplex RT-PCR for eight viruses (ACLSV, ApLV, ApMV, APLPV, PDV, PNRSV, PBNSPaV and PPV). In the agarose gel with five different fruits of three trees (B5-T2, D6-T2 and C5-T1) from three plots (B5, D6 and C5), the presence of bands with specific size corresponds to different viruses. B) Hybridization Dot-Blot of HSVd at nine plots of Pliego (A1-A9) and nine of Mula (B1-B9) in 2010. Four trees (T1 to T4) by orchard and five fruits (F1 to F5) by tree are represented. In the chemiluminiscent film we observe the presence of the viroid in almost all analyzed fruits. Only three trees (A6-T2, B9-T1 and B9-T4) were free of HSVd in their five analyzed fruits, showing no spots in the film.

Table 1. Percentage of fruits, leaf samples and trees infected with different viruses (ACLSV, APLPV, ApLV, ApMV, PBNSPaV, PDV, PNRSV and PPV) studied by RT-PCR and viroids (HSVd and PLMVd) by molecular hybridization (2009 and 2010), in 34 apricot cv. 'Búlida' orchards showing symptoms of "viruela" disease in four areas: A, Pliego; B, Mula; C, Cehegín and D, Hellín. Since APLPV and PLMVd were not detected, neither is included in the table.

Virus	Leaves (%)				Fruits without symptoms (%)				Fruits with symptoms (%)				Studied trees (%)				
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
	36	40	32	28	173	177	105	135	590	7	23	55	5	90	36	40	32
2009																	
ACLSV	56	42	22	66	46	90	78	98	78	85	100	100	100	100	92	90	100
ApLV	8	3	3	13	7	2	2	13	6	0	0	0	100	6	8	3	3
ApMV	0	0	6	3	2	0	0	1	3	1	0	0	15	0	0	0	6
PBNSPaV	11	10	9	15	12	20	24	17	19	21	0	22	5	0	19	28	19
PDV	0	0	3	7	2	0	0	4	2	1	0	0	2	0	0	0	6
PNRSV	14	25	31	25	24	14	25	31	35	25	14	35	55	0	14	30	53
PPV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HSVd	0	0	0	0	0	97	94	100	100	97	100	100	100	100	97	98	100
2010																	
ACLSV	62	56	53	67	60	71	65	79	75	73	100	100	100	100	83	86	93
ApLV	3	0	0	15	4	4	0	0	14	3	21	0	10	12	6	0	0
ApMV	0	0	11	3	3	0	0	9	2	3	0	0	33	0	0	0	14
PBNSPaV	20	25	8	12	17	21	38	13	19	23	36	0	14	18	28	44	15
PDV	0	4	7	7	4	0	6	6	1	3	0	0	0	14	0	5	11
PNRSV	26	45	30	25	31	16	51	47	26	35	14	100	100	16	25	64	53
PPV	3	0	0	0	1	2	0	0	0	1	7	0	0	1	3	0	0
HSVd	0	0	0	0	0	97	94	100	97	97	100	100	100	100	97	94	100

taking into account the higher sensitivity of RT-PCR for virus detection (Sánchez-Navarro *et al.*, 2005) compared with the ELISA method used previously, and the focussed nature of our study.

Similarly high infection rates were also observed for HSVd. Cañizares *et al.* (1998) reported HSVd incidence of 81%, less than that observed in our survey using the same viroid detection technique.

Virus and viroid infections are likely to be related to repeated grafting, vegetative multiplication, pollen transmission and virus vector activity over the previous 10 to 35 years for the apricot trees surveyed.

Symptoms and annual variability

Despite the general infection of trees with ACLSV and HSVd in 2009 and 2010 and the fact that the same trees were studied in both years, the influence of season on “viruela” symptoms expression in fruits was different in each area. This result emphasizes the influence of environment on symptom expression (Peña-Iglesias and Ayuso, 1975; Martínez-Cutillas and Llácer, 1987). For example, cool and wet periods during ripening, appear to stimulate the appearance of “viruela” symptoms in fruits. In our study, leaves were largely asymptomatic, except for some samples infected with ApMV, PNRSV and PPV. No symptoms were ever observed on leaves infected with ACLSV or HSVd. García-Ibarra *et al.* (2012) also observed this absence of ACLSV symptoms in leaves of infected plants grown under controlled greenhouse conditions. This seems to be typical of Spanish isolates from the same subgroup (A) which have a high degree of homology (94–98%) (Al Rwahnih *et al.* 2004).

The relationship between viruses and symptoms

Only ACLSV and HSVd were present in most fruits, both with or without symptoms. Alioto *et al.* (1995), Cañizares *et al.* (2001) and Pallas *et al.* (2003) suggested a synergistic effect by ACLSV with other viruses, especially with PNRSV and PDV, in the expression of “viruela” symptoms. Our results, using more samples and more sensitive detection techniques than in previous studies, did not show these relationships.

With the high number of mixed HSVd and ACLSV infections it is still not clear whether HSVd or ACLSV (or the interaction of the two) is responsi-

ble for “viruela” disease. However, the presence of eight trees in which only HSVd was detected with no fruits showing “viruela” symptoms suggests that HSVd is not alone responsible for the disease. Similarly data in previous studies dismiss HSVd as the causal agent of “viruela”. Amari *et al.* (2007) only detected HSVd and observed fruit “degeneration”, but no “viruela” symptoms in apricot fruits. Flores *et al.* (1990) hypothesised a possible relationship between “viruela” disease and HSVd, but could find no clear evidence. In Italy, Di Terlizzi *et al.* (1991) described a similar disease called “butteratura” with identical symptoms to those of “viruela”, which was previously associated with ACLSV (Ragozzino and Pugliano, 1974). Thereafter, Liberti *et al.* (2005) identified a new Trichovirus (*Apricot pseudo-chlorotic leaf spot virus*; APCLSV,) closely related to ACLSV in apricot trees with “butteratura” (“viruela”) symptoms. However, they could not determine whether either APCLSV or ACLSV alone was responsible for the symptoms, or whether these symptoms were the result of an ACLSV / APCLSV co-infection.

In Spain this relationship between “viruela” disease and ACLSV was previously suggested (Peña-Iglesias and Ayuso, 1975; Cañizares *et al.*, 2001; Pallás *et al.*, 2003) but not proven. Similarly the lack of evidence of association between “viruela” and ACLSV, observed by Llácer *et al.* (1985), Peña-Iglesias (1988) and Martínez-Cutillas and Llácer (1987), could be due to the fact these authors detected the viruses by ELISA, which is less sensitive than RT-PCR for detecting low concentrations of ACLSV in leaves.

The lack of symptoms in ACLSV positive fruits could have a number of explanations. Viral concentration over the season could affect the appearance and intensity of symptoms. Cañizares *et al.* (2001), using molecular hybridization, which is less sensitive than RT-PCR, only detected ACLSV in symptomatic fruits, and not in those without symptoms. In the present study, the high sensitivity of RT-PCR allowed detection of ACLSV in symptomatic plants (100%), and also in plants without symptoms (85–73%), but actually infected. Environmental influence on “viruela” expression has been observed. Cool and wet periods before harvest favour the appearance of “viruela” (García-Ibarra, 2011). Fruit maturity could also affect symptom expression. For example unripened ACLSV-infected fruits usually do not show symptoms. It is also known that “viruela” may develop over time in storage chambers in asymptomat-

ic but infected fruits (A. Soler unpublished). These three factors (virus concentration, environmental conditions and fruit stage) are obviously related, but we do not yet know the way they interact to result in presence or absence of symptoms in infected trees.

Our results suggest that ACLSV is responsible for "viruela" disease, either alone or with HSVd. However, the presence of ACLSV is not always enough to promote symptom appearance, since environmental conditions and fruit stage probably modulate the concentration of viruses and therefore symptom intensity.

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