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RESEARCH PAPERS

Sour and duke cherry viruses in South-West Europe

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Summary. This study investigated the phytosanitary status of sour and duke cherry genetic resources in the Iberian Peninsula, and the incidence and leaf symptoms induced by the *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple chlorotic leaf spot virus* (ACLSV). Young leaf samples were taken from 204 sour and duke cherry trees belonging to ten cultivars, and were assayed by DAS-ELISA. Samples positive for any of the three viruses were also tested by RT-PCR. To associate the leaf symptoms with virus presence, 50 mature leaves from each infected tree were visually inspected during the summer. The ELISA and RT-PCR results indicated that 63% of the cherry trees were infected by at least one of these viruses. PNRSV occurred in all cultivars sampled and presented the highest infection rate (46%), followed by PDV (31%) and ACLSV (6%). Many trees, (60 to 100%), were asymptomatic while harbouring single and mixed virus infections. The leaf symptoms associated with the viruses included chlorotic and dark brown necrotic ringspots on secondary veins and interveinal regions, for PNRSV, generalized chlorosis around the midveins, for PDV, chlorotic and reddish necrotic ringspots, for ACLSV, and generalized interveinal chlorosis, for mixed PNRSV and PDVinfections.

Key words: *Prunus cerasus*, *Prunus* x *gondouinii*, ELISA, RT-PCR, symptoms.

Introduction

Sour cherry (*Prunus cerasus* L., Rosaceae) is a deciduous tree that originated from around the Black and Caspian seas, and then spread to other regions through human and animal migrations. It is a tetraploid species (2n = 4× = 32), allogamous, generally self-incompatible, and is mainly cultivated for its sour and succulent fruit. Sour cherry fruits are mostly used for industrial preserves (frozen and dried cherries, jam, juice, liquor, and other uses). Duke cherry (*Prunus* x *gondouinii* (Poit. & Turpin) Rehd., Rosaceae, 2n = 4× = 32) is considered to be a taxon stemming from the fertilisation of sour cherry by the unreduced gametes of sweet cherry (Iezzoni *et al.*, 1990). Owing to disturbances during meiosis, these hybrids are often sterile, but can be clonally propagated. The fruit characteristics of duke cherry trees are intermediate between those of their progenitors (Tavaud *et al.*, 2004; Pérez-Sánchez *et al.*, 2008). Duke and sour cherries are harvested at the same time and are used for the same purposes. In 2012, the Iberian Peninsula (the tenth European producer) dedicated 1,032 ha to sour and duke cherry production, and produced 3,521 metric tons of fruit (FAO, 2012). The main sour and duke cherry-producing areas are the Jerte River Valley and neighbouring regions, northern Portugal and Galicia, and the D'Óbidos area.

Sour and duke cherry trees are susceptible to a range of pests and diseases, and these jeopardize profitable production. Virus diseases are of special relevance because they cause significant economic losses through lower yields and reduced quality of plant products. Some of the most widespread viruses affecting sour and duke cherry trees are *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple chlorotic leafspot virus* (ACLSV) (Pérez-Sánchez *et al.*, 2015).

PNRSV and PDV are members of the genus *Ilarvirus* (Bromoviridae), and are composed of tripartite

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genomes in isometric to bacilliform particles (Fauquet *et al.*, 2005). Cherry trees infected by PNRSV exhibit an array of symptoms ranging from none to severe rugose mosaic disease, depending on the virus strain (Hammond and Crosslin, 1998). PDV-infected trees often remain symptomless, but chlorotic and necrotic spots on cherry leaves have also been reported as associated with this virus (Massart *et al.*, 2008). These ilarviruses are mechanically transmitted through grafting, and through pollen and seeds (Matić *et al.*, 2008). ACLSV is the type species of the genus *Trichovirus* (Betaflexiviridae), with flexuous filamentous particles containing positive-sense single-stranded RNA (Martelli *et al.*, 2007; Al Rwahnih *et al.*, 2004). Infected trees are normally symptomless, but deformation and discoloration (russet rings, pox and necrotic spots) in cherry fruit and severe graft incompatibilities in nurseries have also been reported for ACLSV infections (Rana *et al.*, 2011). ACLSV is transmitted by mechanical inoculation and grafting (Martelli *et al.*, 2007).

There have been several studies addressing PNRSV, PDV and ACLSV in sour cherry trees (Ulubaş and Ertunç, 2004; Milusheva and Borisova, 2005; Sipahioglu and Baloglu, 2006; Mandic *et al.*, 2007; Myrta and Savino, 2008; Suchá and Svobodobá, 2010; Çevik *et al.*, 2011; Radičević *et al.*, 2012; Soltani *et al.*, 2013). However, there have been no studies about the presence, distribution or symptoms of these viruses in sour and duke cherry trees within the Iberian Peninsula. The study reported here has analysed the incidence and leaf symptoms of PNRSV, PDV and ACLSV in *Prunus cerasus* and *Prunus* x *gondouinii* trees in the main sour and duke cherry-producing areas of the Iberian Peninsula.

Materials and methods

Sources of plant material

Field inspections and sample collections were carried out during 2009 in the Jerte River Valley and neighbouring regions, D'Óbidos, and northern Portugal and Galicia. A total of 204 sour and duke cherry trees, from ten different cultivars considered as local or native genotypes [Cordeiro *et al.* (2008); Pérez-Sánchez *et al.* (2008)], were selected by square root biased sampling (n = 41,600 trees). The sour cherry cultivars sampled were Martinho D'Óbidos, Pedro Miguel D'Óbidos, Sobral D'Óbidos, Guindo Común, Guindo Silvestre and Galega, and the duke cherry cultivars were Guindo Garrafal Negro, Guindo Tomatillo, Guindo Garrafal Rosa and Seixas. Five samples of young leaves were collected from the yearly flush at the tip of lower branches of each tree from April to June. They were kept in thermal bags during transportation to the laboratory. All analyses were carried out in the Plant Production Laboratory at the University of Salamanca, Spain.

DAS-ELISA

The samples were tested using the Double Antibody Sandwich (DAS) ELISA method (Clark and Adams, 1977). Leaves (0.2 g) were homogenized in 4 mL of extraction buffer [2.40 g Tris-hydroxymethyl, 8 g NaCl, 20 g polyvinylpyrrolidone (PVP, MW = 2,400), 0.20 g KCl, 0.50 g tween-20 and 0.20 g NaN_3 in 1 L of distilled H_2O (pH 7.4)]. DAS-ELISA was carried out by coating the plates with polyclonal IgG diluted in coating buffer [1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.20 g NaN_3 in 1 L of distilled H_2O (pH. 9.6)]. The prepared samples were then incubated with specific polyclonal IgG overnight at 4ºC. A positive reaction was revealed by alkaline phosphatase-linked goat antimouse (IgG-AP) conjugate and substrate, which was p-nitrophenyl phosphate (pNPP) in diethanolamine buffer (Cambra *et al.*, 1994). Two wells were included in each plate for positive, negative and buffer controls. Commercial antisera for PDV, PNRSV and ACLSV (Bioreba, Switzerland) were used as described by the manufacturer. The wells were read at 405 nm (Multiscan Plus, Labsystem) and the immunological responses were assessed. All samples exceeding two and a half times the absorbance value of the negative controls were considered as positive.

RT-PCR assays

The leaf samples from cherry trees that were positive for any of the three viruses (PNRSV, PDV or ACLSV) in DAS-ELISA were also tested by reverse transcription - polymerase chain reaction (RT-PCR). Plant RNA was extracted using the protocol of Noorani *et al.* (2013), and RT-PCR was carried out as described by Zong *et al.* (2015). The oligonucleotide primers used for the detection of the three viruses were as described by Zong *et al.* (2014; 2015).

Visual evaluation of leaf symptoms

Fifty adult leaves from each infected tree were inspected in the field and laboratory during the summer of 2009 to detect the main symptoms indicative of the presence of these three viruses, either as single or mixed infections. This large number of leaves per tree was inspected to form a general representation of the entire treetop, even for the larger trees, since the distribution of the viruses can sometimes be irregular.

Results and discussion

Serological detection

The results of the ELISA virus-positive cherry tree samples from the main producing areas in the Iberian Peninsula are shown in Table 1. Sixty-three percent of the samples were infected by at least one of the viruses assayed. Similar mean infection rates were also recorded for these three viruses by Mandic *et al.* (2007) in a sour cherry collection in Serbia (64.3%). The duke cherry cultivars Guindo Tomatillo, Guindo Garrafal Rosa and Guindo Garrafal Negro had the greatest infection levels (between 68 and 74%). In contrast, less than 57% of the trees from the sour cherry cultivars Guindo Común, Guindo Silvestre and Sobral D'Óbidos were infected. These results indicate that in general the prevalence of infection was less in sour cherry (overall mean infection 58%) than in duke cherry trees (68%).

The most commonly detected virus was PNRSV (46%), which was present in 73% of the infected sour and duke cherry tree samples. Sour cherry cultivars were generally more infected by PNRSV (mean infection rates 55%) than duke cherry cultivars (35%). Other researchers who have also recorded high infection rates for this virus in sour cherry trees include: Soltani *et al.* (2013) in Iran (100% trees infected), Milusheva and Borisova (2005) in Bulgaria (95%), Suchá and Svobodová (2010) in the Czech Republic (89%) and Mandic *et al.* (2007) (85%) and Radičević *et al.* (2012) in Serbia (75% of trees infected trees). Paprštein *et al.* (1995), Ulubaş and Ertunç (2004), Çevik *et al.* (2011) and Olivier *et al.* (2009) also found higher PNRSV infection levels in sour cherry trees than in sweet cherries. There are no published data

Table 1. Relative incidence of single infections by PDV, PNRSV and ACLSV in sour (so) and duke (du) cherry cultivars from the Iberian Peninsula. ELISA results.

Cultivar	Tested trees	Infected trees		PNRSV		PDV		ACLSV	
		No.	%	No.	%	No.	%	No.	%
Guindo Garrafal Negro (du)	25	17	68.00	8	32.00	11	44.00	2	8.00
Guindo Garrafal Rosa (du)	19	13	68.42	7	36.84	9	47.36	3	15.78
Guindo Tomatillo (du)	23	17	73.91	9	39.13	12	52.17	3	13.04
Seixas (du)	24	15	62.50	8	33.33	10	41.66	2	8.33
Subtotal (duke cherry)	91	62	68.13	32	35.16	42	46.15	10	10.98
Galega (so)	21	13	61.90	11	52.38	4	19.04	1	4.76
Guindo Común (so)	16	9	56.25	9	56.25	3	18.75	0	0.00
Guindo Silvestre (so)	15	8	53.33	8	53.33	3	20.00	0	0.00
Martinho D'Óbidos (so)	22	13	59.09	12	54.54	5	22.72	0	0.00
Pedro Miguel D'Óbidos (so)	20	13	65.00	12	60.00	3	15.00	1	5.00
Sobral D'Óbidos (so)	19	10	52.63	10	52.63	4	21.05	0	0.00
Subtotal (sour cherry)	113	66	58.40	62	54.86	22	19.46	2	1.76
TOTAL (duke and sour cherry)	204	128	62.74	94	46.07	64	31.37	12	5.88

regarding the PNRSV infection of duke cherry trees.

PDV had lower infection levels than PNRSV (31%) and was detected in 50% of the infected sour and duke cherry trees. Mandic *et al.* (2007) also reported similar rates of PDV infection in sour cherry trees (37% of trees infected). Duke cherry cultivars were generally more infected by PDV (mean infection rates of 46%) than sour cherry cultivars (19%). Milusheva and Borisova (2005), Suchá and Svobodová (2010), Çevik *et al.* (2011) and Radičević *et al.* (2012) also found lower PDV infection levels in sour cherry trees than in sweet cherries. There are no published data regarding the PDV infection of duke cherry trees.

ACLSV was not widespread in the main sour and duke cherry-producing regions of the Iberian Peninsula. This virus exhibited the lowest degree of infection among the three viruses tested (6%). Myrta and Savino (2008) also reported a low incidence of ACLSV in the cherry-growing regions of Mediterranean areas (14%). Duke cherry cultivars were generally more infected by ACLSV (mean infection rate 11%) than sour cherry cultivars (2%). 'Galega' and 'Pedro Miguel D´Óbidos' were the only sour cherry cultivars that presented one ACLSV-positive tree. We conclude that ACLSV has very limited distribution in the sour and duke cherry-growing areas studied. In other countries, such as Bulgaria, Turkey and Serbia, no infection by ACLSV in sour cherry trees has been reported (Borisova, 2005; Sipahioglu and Baloglu, 2006; Mandic *et al.*, 2007). There are no published data regarding the ACLSV infection of duke cherry trees.

The results for PNRSV, PDV and ACLSV mixed infections in sour and duke cherry cultivars in the Iberian Peninsula are shown in Table 2. No relationships occurred for infections by the three viruses studied (correlation coefficients less than 0.80). PNRSV and PDV occurred most frequently in combination (24% of infected sour and duke cherry trees), followed by PDV with ACLSV (3%), PNRSV with ACLSV (2%) and PNRSV with PDV and ACLSV (2%). The PDV-ACLSV, PNRSV-ACLSV, PNRSV-ACLSV-PDV co-infections were not found in the sour cherry cultivars. PDV-ACLSV and PNRSV-PDV-ACLSV co-infections

Table 2. PDV, PNRSV and ACLSV mixed infections in sour (so) and duke (du) cherry cultivars from the Iberian Peninsula. ELISA results.

Cultivar	Mixed infections PNRSV-PDV		Mixed infections PDV-ACLSV		Mixed infections PNRSV-ACLSV		Mixed infection PNRSV-PDV-ACLSV	
	No.	%	No.	%	No.	%	No.	%
Guindo Garrafal Negro (du)	3	12.00	1	4.00	0	0.00	0	0.00
Guindo Garrafal Rosa (du)	2	10.52	1	5.26	1	5.26	1	5.26
Guindo Tomatillo (du)	3	13.04	1	4.34	1	4.34	1	4.34
Seixas (du)	3	12.50	1	4.16	1	4.16	0	0.00
Subtotal (duke cherry)	11	12.08	4	4.39	3	3.29	2	2.19
Galega (so)	3	14.28	0	0.00	0	0.00	0	0.00
Guindo Común (so)	3	18.75	0	0.00	0	0.00	0	0.00
Guindo Silvestre (so)	3	20.00	0	0.00	0	0.00	0	0.00
Martinho D'Óbidos (so)	4	18.18	0	0.00	0	0.00	0	0.00
Pedro Miguel D'Óbidos (so)	3	15.00	0	0.00	0	0.00	0	0.00
Sobral D'Óbidos (so)	4	21.05	0	0.00	0	0.00	0	0.00
Subtotal (sour cherry)	20	17.69	0	0.00	0	0.00	0	0.00
TOTAL (duke and sour cherry)	31	15.19	4	1.96	3	1.47	2	0.98

were also not found in sour cherry trees in Serbia by Mandic *et al.* (2007). The cultivars "Seixas", "Guindo Garrafal Rosa" and "Guindo Tomatillo" had the greatest number of trees with mixed virus infections (between 4 and 5%). Moreover, "Guindo Garrafal Rosa" and "Guindo Tomatillo"were the only host genotypes that presented PNRSV-PDV-ACLSV co-infections. In contrast, "Galega", "Guindo Común", "Guindo Silvestre" and "Pedro Miguel D´Óbidos" were the cultivars with the fewest trees with mixed virus infections (2%).

RT-PCR

The ELISA results for the three viruses were confirmed by RT-PCR assay. Electrophoretic analysis of the RT-PCR products with the expected sizes confirmed the presence of PNRSV, PDV and ACLSV.

Visual evaluation of leaf symptoms

Table 3 shows the results of the leaf symptom study carried out for PNRSV, PDV and ACLSV, and co-infections, in sour and duke cherry cultivars. Many of the trees were asymptomatic (60 to 100%).

PNRSV was infecting the greatest number of symptomatic trees (40%). Their adult leaves showed annular chlorotic spots, which evolved to dark brown necrotic areas on secondary veins and the interveinal regions of the leaf blades (Figure 1). Smith *et al*. (1988), Myrta and Savino (2008) and Oliver *et al*. (2009) also observed chlorotic or necrotic spots on the leaf blades of sour cherry trees infected by PNRSV. They reported that the centres of these necrotic spots often disappeared, resulting in a "shot-hole" effect. However, Paunovic *et al.* (2011) indicated that this type of symptom is generally not of diagnostic significance, because similar symptoms may also be produced in *Prunus* spp. by other *Ilarviruses* such as the *Apple mosaic virus* (ApMV). All of the duke and sour cherry genotypes infected with this virus exhibited leaf symptoms.

Overall, the mean percentage of symptomatic trees with PDV infection was 30%. The leaves of these trees showed generalized chlorosis around the main veins, which increased towards the bases

Table 3. Symptomatic trees for the PDV, PNRSV and ACLSV viruses and their mixed infections in sour (so) and duke (du) cherry cultivars from the Iberian Peninsula.

Cultivar	PNRSV		PDV		ACLSV		Mixed infections PNRSV-PDV		Mixed infections PDV-ACLSV		Mixed infections PNRSV-ACLSV		Mixed infection PNRSV-PDV-ACLSV	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Guindo Garrafal Negro (du)	2	40.00	2	28.57	2	100.00	1	33.33	0	0.00	-	-	-	-
Guindo Garrafal Rosa (du)	1	33.33	2	40.00	-	-	1	50.00	0	0.00	0	0.00	0	0.00
Guindo Tomatillo (du)	1	25.00	3	42.85	-	-	0	0.00	0	0.00	0	0.00	0	0.00
Seixas (du)	1	25.00	1	16.66	-	-	0	0.00	0	0.00	0	0.00	-	-
Galega (so)	4	50.00	0	0.00	1	50.00	1	33.33	-	-	-	-	-	-
Guindo Común (so)	2	33.33	-	-	-	-	0	0.00	-	-	-	-	-	-
Guindo Silvestre (so)	2	40.00	-	-	-	-	0	0.00	-	-	-	-	-	-
Martinho D'Óbidos (so)	3	37.50	0	0.00	-	-	1	25.00	-	-	-	-	-	-
Pedro Miguel D'Óbidos (so)	5	55.55	-	-	0	0.00	1	33.33	-	-	-	-	-	-
Sobral D'Óbidos (so)	2	33.33	-	-	-	-	0	0.00	-	-	-	-	-	-
Mean		39.65		29.62		33.33		16.12		0.00		0.00		0.00

-, Unrecorded virus / virus combination

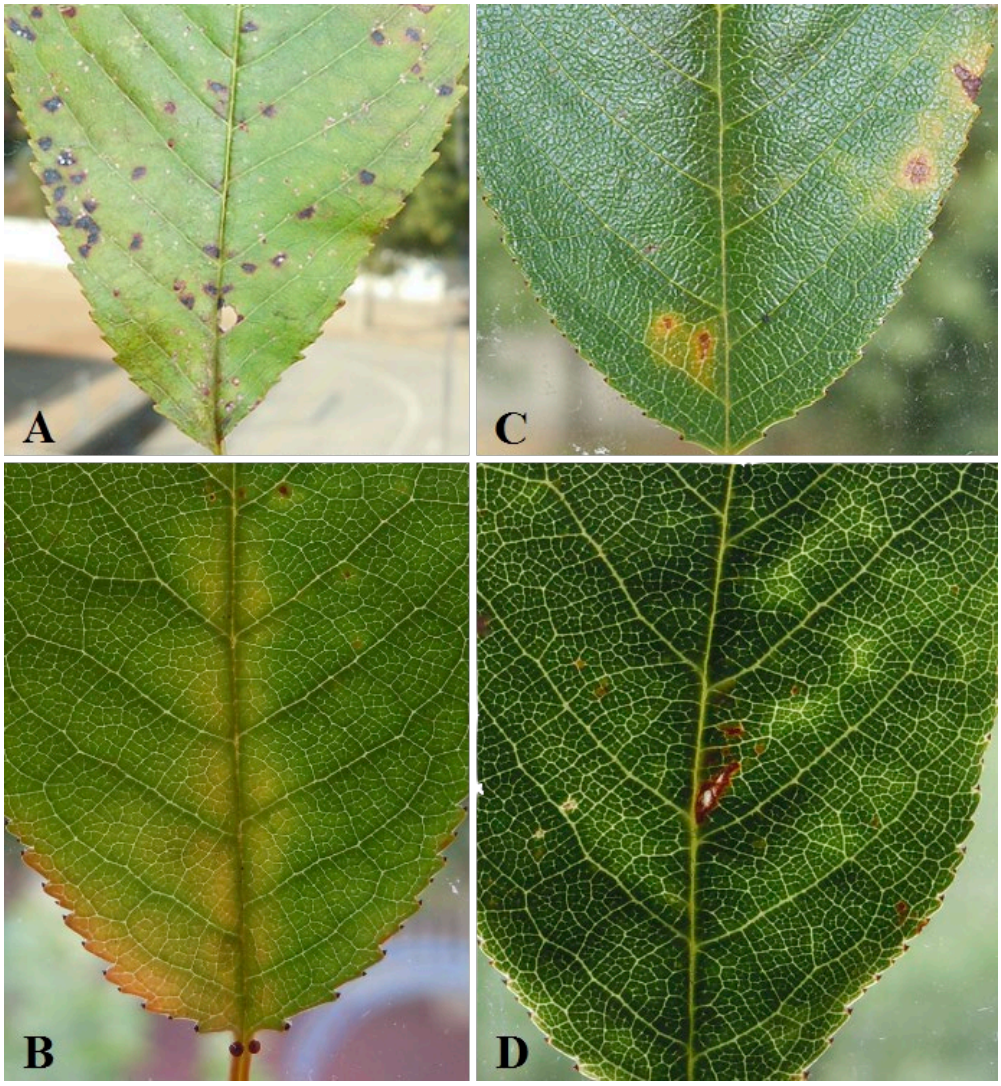

Figure 1. Leaf symptoms associated with single or mixed viral infections; (A) chlorotic and dark-brown necrotic ringspots induced by PNRSV; (B) generalized chlorosis around the midvein due to PDV; (C) chlorotic and reddish necrotic ringspots induced by ACLSV; (D) generalized interveinal chlorosis due to PNRSV-PDV mixed infection.

of the leaf blades (Figure 1). The leaves with symptoms of PDV infection were completely expanded, in contrast to those infected with PNRSV. Smith *et al.* (1988) also reported that the leaf symptoms of PDV infection, such as chlorotic spots, rings and diffuse mottling, only appeared on leaves that were almost completely expanded. They also reported that PDV-infected cherry trees often remained symptomless in subsequent years. Other researchers who have associated PDV sour cherry infection with yellow leaf disease include Ogawa and English (1991) in the United States of America, Verma and Sharma (1999) in India, Cağlayan *et al.* (2011) in Turkey, Pallás *et al.* (2012) in Spain, and Soltani *et al.* (2013) in Iran. The cultivars Guindo Tomatillo, Guindo Garrafal Rosa and Guindo Garrafal Negro had the greatest number of PDV symptomatic trees; in contrast, Galega and Martinho D´Óbidos trees infected with PDV were symptomless.

ACLSV infection was observed in 33% of symptomatic trees. Their leaves displayed annular chlorotic spots on the leaf blades, which evolved to reddish

necrotic areas with more intensely coloured edges (Figure 1). Myrta and Savino (2008) and Myrta *et al.* (2011) also reported that cherry trees with ACLSV infections showed chlorotic spotted leaves. Regarding the cultivars, only the "Guindo Garrafal Negro" and "Galega" trees showed leaf symptoms related to ACLSV infections.

Symptomatic sour and duke cherry trees (16%) were only detected for the PNRSV-PDV co-infection. Their leaves showed generalized interveinal chlorosis (Figure 1). Smith *et al*. (1988) reported that this mixed viral infection could induce increased damage to sour cherry trees. Nonetheless, there are no data in the literature about the leaf symptoms shown by sour and duke cherry trees with the PNRSV-PDV co-infections. 'Guindo Garrafal Negro', 'Guindo Garrafal Rosa', 'Galega', 'Martinho D´Óbidos' and 'Pedro Miguel D´Óbidos' were the only duke and sour cherry cultivars that had leaf symptoms due to the presence of this co-infection. On the contrary, the cultivars "Guindo Tomatillo", "Seixas", "Guindo Común", "Guindo Silvestre" and "Sobral D´Óbidos" with the PNRSV-PDV co-infections did not exhibit leaf symptoms. The other co-infections were infrequent and none of the trees with these co-infections displayed characteristic symptoms.

Conclusions

A significant number of sour and duke cherry trees (63%) in the Iberian Peninsula were infected by PNRSV, PDV or ACLSV. PNRSV was the most widespread virus followed by PDV and ACLSV, and PNRSV-PDV was the most frequent viral co-infection recorded in this study. High numbers of trees (ranging from 60 to 100%) were asymptomatic while carrying single or mixed virus infections. The leaf symptoms associated with the viruses analysed were: annular chlorotic and dark brown necrotic spots on secondary veins and interveinal regions (PNRSV); generalized chlorosis around the main veins (PDV); annular chlorotic and reddish necrotic spots (ACLSV); and generalized interveinal chlorosis (PNRSV-PDV). Sour and duke cherry trees are widely affected by single and mixed virus infections involving the three viruses studied. This indicates that host plant material certification programmes should be implemented to ensure productive and high quality sour and duke cherry orchards in South-West Europe.

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