Short Communication

SURVEY OF STONE FRUIT VIRUSES AND VIROIDS IN CHILE

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SUMMARY

Stone fruits orchards from three Chilean regions were visited to collect leaf samples which were tested for the presence of the most important viruses and viroids. Molecular nonradioactive hybridization (MH) testing of 2,456 samples and confirmatory RT-PCR of some MH-negative samples gave the following infection rates: 31.5% for *Prunus necrotic ringspot virus* (PNRSV); 31.3% for *Prune dwarf virus* (PDV); 25.7% for *Peach latent mosaic viroid* (PLMVd); 3.1% for *Tomato ringspot virus* (ToRSV) only in rootstocks; 1.8% for *Plum pox virus* (PPV); 1.2% for *Hop stunt viroid* (HSVd); 1.1% for *Apple mosaic virus* (ApMV); and 0.9% for *Apple chlorotic leaf spot virus* (ACLSV). *American plum line pattern virus* (APLPV) was not detected. The overall infection rate in the surveyed orchards was 29.6%, specifically 28.2% (Región Metropolitana), 23.4% (Valparaiso) and 33% (Bernardo O'Higgins) in the three regions surveyed. ApMV and HSVd, detected only in peach and nectarine, are new records in stone fruits in Chile.

Key words: Detection, molecular nonradioactive hybridization, RT-PCR.

The stone fruits industry is very important in Chile, occupying 62,110 hectares in 2014, and comprising plantings of almond, cherry, plum, apricot, peach and nectarine (ODEPA, 2014). From 2006 and 2011, a number of orchards of different size were visited to assess their sanitary status in austral hemisphere spring (September to November) in the most important fruit growing regions (48,300 hectares): Valparaiso, Libertador General Bernardo O'Higgins and Metropolitana of Santiago.

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From one to three stone fruit trees were randomly sampled in the central area of each orchard for a total of 2,456 trees during the whole survey period. One plant every 20 hectares was sampled. The samples were then recorded, and their locations was determined with GPS, to enable future inspection/sample collection from infected plants. Leaves used for testing were collected from several points of the canopy of an individual tree for a total of twenty per plant. Samples were tested for the presence of viruses and a viroid previously reported in Chile, i.e. *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leaf spot virus* (ACLSV), *Tomato ringspot virus* (ToRSV) and *Peach latent mosaic viroid* (PLMVd) (Auger, 1989; Herrera, 2001; Herrera and Madariaga, 2002; Fiore *et al*., 2003; Torres *et al*., 2004) and for viruses and a viroid hitherto unrecorded in the country, but detected in stone fruit in other areas of the world, i.e. *Apple mosaic virus* (ApMV), *American plum line pattern virus* (APLPV) and *Hop stunt viroid* (HSVd). Not all samples were analyzed for all the above listed agents. Additionally, the detection of ToRSV was carried out on leaves collected from rootstocks (189 plants out of 2,456).

Detection was carried out by molecular nonradioactive hybridization (MH) using a specific single probe for each viral/viroidal sequence (one membrane for each virus/viroid detection) (Herranz *et al*., 2005; Peiró *et al*., 2012). The specific probe for detection of ToRSV was designed during this study. Reverse transcription-polymerase chain reaction (RT-PCR) was used on selected positive and negative samples, and in all the samples which showed questionable and weak reactions using MH.

Total nucleic acids (TNA) were extracted by the silica capture method (Malinovski, 1997; Rott and Jelkmann, 2001) and used for both MH and RT-PCR assays. For RT-PCR TNA aliquots were primed with DNA random hexanucleotides (Roche, Basel, Switzerland) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Madison, Wisconsin, USA). Target-specific primers were P1/P2 for PPV (Wetzel *et al*., 1991); PDV-R/F and ApMV-F/R for PDV and ApMV, respectively (Sanchez-Navarro *et al*., 2005); C537/H83 for PNRSV (McKenzie *et al*., 1997); ACLSV-C/H for ACLSV (Nemchinov *et al*., 1995); APLPV1/2 for APLPV

Table 1. Viruses and viroids detected in Chilean stone fruit trees by molecular nonradioactive hybridization.

Viruses and viroids[a]	Samples tested (number)	Positive samples (number)	Positive samples (%)
ACLSV	980	9	0.9
APLPV	1139	0	0
ApMV	1070	0	0
HSVd	1070	0	0
PDV	1139	324	28.4
PLMVd	1139	272	23.9
PNRSV	1139	347	30.5
PPV	2456	24	1.0
ToRSV	1139	35[b]	3.1
Overall infections	2456	681	27.7

[a]ACLSV = *Apple chlorotic leaf spot virus*; APLPV = *American plum line pattern*; ApMV = *Apple mosaic virus*; HSVd = *Hop stunt viroid*; PDV = *Prune dwarf virus*; PLMVd = *Peach latent mosaic viroid*; PNRSV = *Prunus necrotic ringspot virus*; PPV = *Plum pox virus*; ToRSV = *Tomato ring spot virus*.

[b]ToRSV detected only in rootstocks.

Table 2. RT-PCR detection of viruses and viroids in Chilean stone fruit samples that were negative in molecular nonradioactive hybridization.

Viruses and viroids	Samples tested (number)	Positive samples (number)	Positive samples (%)
ACLSV	4	0	0
APLPV	15	0	0
ApMV	25	12	48.0
HSVd	35	13	37.1
PDV	33	32	97.0
PLMVd	21	21	100
PNRSV	13	12	92.3
PPV	222	21	9.5
ToRSV	n.t.	n.t.	n.t.

n.t. = not tested

(Li *et al*., 2008); U1/D1 for ToRSV (Griesbach, 1995); VP19/20 for HSVd (Astruc *et al*., 1996); c/hPLMVd for PLMVd (Loreti *et al*., 1999). To confirm RT-PCR analyses, selected amplicons of all the viruses and viroids detected were purified using the Concert Rapid PCR Purification System (Invitrogen, Carlasbad, CA, USA). To identify PPV strain, for each of the isolates found during the survey RT-PCR was carried out with the primer pair P4b/P3D to amplify an approximately 467 bp fragment (Candresse *et al*., 1998). DNA fragments were directly sequenced in both directions using the BIG DYE sequencing terminator Kit (PE Biosystems, Warrington, UK). The sequences obtained in this study were aligned and compared with those present in GenBank using CLUSTAL-W program inside BioEdit (Thompson *et al*., 1997; Hall, 1999).

Positive controls for MH and RT-PCR were either fresh or lyophilized leaf tissues from infected plants.

During this survey, typical symptoms caused by PPV, PNRSV, PDV and PLMVd were frequently seen in most of the orchards. Some peach plants, infected contemporarily with PNRSV and PDV, showed stunting, and those infected with PLMVd were asymptomatic or expressed mild mosaic, necrosis of buds or fruit cracked sutures. Plum D'Agen grafted on cv. Myrobalan infected by ToRSV showed "prune brown line". Foliar mild yellow chlorotic symptoms caused by ACLSV, were rarely observed and only in peach plants. No symptoms were observed in plants in which ApMV and HSVd were detected.

Viruses and viroids were detected by MH in the three regions surveyed (Table 1). Of 2,456 MH-tested plants, 681 were positive for at least one virus or viroid (27.7%). Several samples that tested negative in MH, found to be positive by RT-PCR (Table 2). All RT-PCR tests carried out in selected MH-positive samples confirmed the presence of ACLSV, PDV, PNRSV, PPV, ToRSV, and PLMVd (data not shown). Combining the results of RT-PCR to those obtained by MH, 728 trees were positive for at least one virus or viroid (29.6%) (Table 3). No evidence was obtained for presence of APLPV in any of the 1139 plant assayed by MH and RT-PCR (Tables 1 and 2).

PNRSV, PDV and PLMVd are the main virus and virus-like disease agents infecting stone fruits in Chile with 31.5, 31.3, and 25.7% infected plants, respectively. These viruses and viroid occurred in all regions surveyed with prevalence in the Libertador General Bernardo O'Higgins and Metropolitana regions for PNRSV (38.8%) and PLMVd (27.1%), and PDV (47.8%) and PLMVd (26.5%), respectively. Besides using infected plant material for propagation, the wide distribution of these ilarviruses and PLMVd is largely favored by their mode of spread in the field, which occurs mainly by infected pollen, in the case of PNRSV and PDV, and by contaminated pruning tools for the viroid. Infections by ToRSV, PPV, HSVd, ApMV, and ACLSV reached for 3.1, 1.8, 1.2, 1.1, and 0.9%, respectively (Table 3). Cherry had 41.9% positive samples because of PNRSV and PDV infections. In nectarine and peach, the levels of infection were slightly lower (38.8 and 35.4%, respectively), but all viruses and viroids tested in the survey (excluding APLPV), were detected with a prevalence of PNRSV, PDV and PLMVd. European plum, apricot, Japanese plum, and almond had lower rates of infection: 15.8, 13.1, 11.1, and 8.3%, respectively. Apricot, European and Japanese plums were infected by PDV, PNRSV, PPV, and ToRSV, with prevalence of PDV infections in the three species. Only in one sample of Japanese plum was PLMVd also detected (Table 4).

Single and mixed infections (two to five viruses and/or viroids) were present in 416 and 312 samples, respectively. The most recurrent pathogens found in mixed infection were PNRSV and PLMVd with 132 samples, followed by PDV and PLMVd, and PNRSV and PDV with 121 and 120 samples, respectively (Table 5). The viral and viroidal

Table 3. Regional distribution of the six most important viruses and two viroids detected in Chile based on molecular nonradioactive hybridization and RT-PCR results.

Region	Viruses and viroids[a]								Overall infection for region[b]
	ACLSV	ApMV	HSVd	PDV	PLMVd	PNRSV	PPV	ToRSV[c]	
Metropolitana	9/401 (2.2%)	9/422 (2.1%)	9/447 (2.0%)	231/483 (47.8%)	128/483 (26.5%)	119/483 (24.6%)	16/1134 (1.4%)	15/483 (3.1%)	320/1134 (28.2%)
Valparaiso	0/143 (0%)	3/149 (2.0%)	2/143 (1.4%)	11/151 (7.3%)	28/151 (18.5%)	44/151 (29.1%)	2/295 (0.7%)	1/151 (0.7%)	69/295 (23.4%)
Libertador General Bernardo O'Higgins	0/436 (0%)	0/499 (0%)	2/480 (0.4%)	114/505 (22.6%)	137/505 (27.1%)	196/505 (38.8%)	27/1027 (2.6%)	19/505 (3.8%)	339/1027 (33.0%)
Overall infection for virus or viroid[d]	9/980 (0.9%)	12/1070 (1.1%)	13/1070 (1.2%)	356/1139 (31.3%)	293/1139 (25.7%)	359/1139 (31.5%)	45/2456 (1.8%)	35/1139 (3.1%)	**728/2456[e] (29.6%)**

[a]Number and percentage of positives against all analyzed samples for each virus and viroid.
[b]Total number and percentage of positive samples for at least one virus or viroid against all analyzed samples for each region.
[c]ToRSV detected only in rootstocks.
[d]Total number and percentage of positive samples for each virus or viroid.
[e]Overall infection.

Table 4. Distribution of the six most important viruses and two viroids detected in stone fruit species growing in three regions of Chile based on molecular nonradioactive hybridization and RT-PCR results.

Stone fruit species	Viruses and Viroids[a]								Overall infection[b]
	ACLSV	ApMV	HSVd	PDV	PLMVd	PNRSV	PPV	ToRSV[c]	
Almond	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	0/12 (0%)	0/10 (0%)	1/12 (8.3%)
Cherry	0/35 (0%)	0/37 (0%)	0/37 (0%)	12/37 (32.4%)	0/37 (0%)	11/37 (29.7%)	0/43 (0%)	0/37 (0%)	18/43 (41.9%)
European plum	0/14 (0%)	0/17 (0%)	0/17 (0%)	3/17 (17.6%)	0/17 (0%)	2/17 (11.8%)	1/38 (2.6%)	1/17[d] (5.9%)	6/38 (15.8%)
Japanese plum	0/239 (0%)	0/245 (0%)	0/245 (0%)	40/245 (16.3%)	1/245 (0.4%)	19/245 (7.8%)	7/559 (1.3%)	2/245[d] (0.8%)	62/559 (11.1%)
Apricot	0/40 (0%)	0/44 (0%)	0/42 (0%)	5/44 (11.4%)	0/44 (0%)	5/44 (1.4%)	2/84 (2.4%)	1/44[e] (2.3%)	11/84 (13.1%)
Peach	4/442 (0.9%)	6/470 (1.3%)	6/476 (1.3%)	185/518 (35.7%)	136/518 (26.3%)	206/518 (39.8%)	22/1106 (2.0%)	20/518[e] (3.9%)	392/1106 (35.4%)
Nectarine	5/200 (2.5%)	6/247 (2.4%)	7/243 (2.9%)	111/268 (41.4%)	156/268 (58.2%)	115/268 (42.9%)	13/614 (2.1%)	11/268[e] (4.1%)	238/614 (38.8%)

[a]Number and percentage of positives against all analyzed samples for each virus and viroid.
[b]Total number and percentage of positive samples for at least one virus or viroid against all analyzed samples for each stone fruit species.
[c]ToRSV detected only in rootstocks.
[d]ToRSV detected in Myrobalan rootstock.
[e]ToRSV detected in Nemaguard rootstock.

origin of the amplicons was confirmed by sequence analysis using BLAST tools.

This is the first extensive survey of stone fruit viruses and viroids in Chile. The high rate of infections, especially for PNRSV, PDV and PLMVd, allows us to conclude that these pathogens play an important role in decreasing of stone fruits production observed in the country. ApMV and HSVd were detected only in peach and nectarine but these are new records in stone fruits in Chile. Sequence comparisons of PPV amplicons obtained with the primer pair P4b/P3D confirmed that strain D of PPV is present in Chile. The low percentage of infected plants with PPV-D is the consequence of mandatory control in mother plants, which is regulated by the National Plant Protection Service (Servicio Agrícola y Ganadero – SAG) (SAG, 1994). ToRSV was detected only in rootstocks, specifically in Nemaguard and Myrobalan. Horizontal transmission of this virus is favored by the presence in Chile of its vector *Xiphinema americanum sensu lato* (Lamberti *et al*., 1988; Insunza *et al*., 2001). RT-PCR was confirmed as being a more sensitive detection technique when compared with HM. All the viruses and viroids detected during this survey should be taken into consideration if a national certification program for the production and marketing of sanitarily improved stone fruit propagative material will be updated, as it appears highly desirable.

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Table 5. Number and rates of single and mixed infections.

Number of viruses and viroids in mixed infections	Species of viruses and/or viroids in mixed infection	Positive samples (number)	Positive samples (%)
5		**1**	**0.04**
	PDV PLMVd PNRSV ApMV HSVd	1	0.04
4		**11**	**0.45**
	PDV PLMVd PNRSV ToRSV	3	0.12
	PDV PLMVd PNRSV ACLSV	2	0.08
	PDV PLMVd ApMV ACLSV	1	0.04
	PDV PLMVd ToRSV ACLSV	1	0.04
	PDV PLMVd PNRSV ApMV	1	0.04
	PDV PLMVd PNRSV HSVd	1	0.04
	PDV PLMVd ToRSV HSVd	1	0.04
	PDV PNRSV ToRSV HSVd	1	0.04
3		**57**	**2.32**
	PDV PLMVd PNRSV	39	1.59
	PDV PLMVd ToRSV	4	0.16
	PNRSV PLMVd ToRSV	4	0.16
	PDV PLMVd ACLSV	1	0.04
	PDV PNRSV ApMV	1	0.04
	PDV HSVd ApMV	1	0.04
	PDV ApMV ACLSV	1	0.04
	PDV ApMV PPV	1	0.04
	PDV PLMVd PPV	1	0.04
	PDV PNRSV PPV	1	0.04
	PNRSV PLMVd PPV	1	0.04
	PNRSV ToRSV HSVd	1	0.04
	PNRSV HSVd PPV	1	0.04
2		**243**	**9.89**
	PNRSV PLMVd	80	3.26
	PDV PNRSV	70	2.85
	PDV PLMVd	65	2.65
	PDV ToRSV	5	0.20
	PDV PPV	5	0.20
	PNRSV ToRSV	5	0.20
	PDV HSVd	2	0.08
	PDV ApMV	2	0.08
	PDV ACLSV	2	0.08
	PLMVd HSVd	1	0.04
	PLMVd ToRSV	1	0.04
	PNRSV ApMV	1	0.04
	PNRSV PPV	1	0.04
	ToRSV HSVd	1	0.04
	ToRSV ACLSV	1	0.04
	HSVd PPV	1	0.04
1		**416**	**16.94**

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