Tracking *Plum pox virus* in Chile throughout the year by three different methods and molecular characterization of Chilean isolates

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Abstract

During 2007, a survey was performed to detect and identify *Plum pox virus* (PPV) in Chilean stone fruit commercial orchards. A total of 1396 trees were analyzed and 45 (3.22 %) of them resulted positive. A fragment of 467 bp, corresponding to the replicase-coat protein (Nib-CP) region from the virus genome, was amplified and the the sequences obtained permitted the characterization of all isolates as PPV-D type, confirming that, so far, this is the only serotype present in Chile. To optimize virus detection, 27 PPV-positive trees were selected and sampled monthly from December 2006 until December 2007, collecting plant tissues available at the time of sampling (leaves, cuttings, buds and flowers). Each sample was analyzed by three different techniques: DASI-ELISA, RT-PCR and non-isotopic molecular hybridization (MH). The results showed that RT-PCR was more sensitive for detection in all months excepting January 2007, when the three techniques showed the same sensitivity. In general, MH showed a better sensitivity compared with DASI-ELISA. The best plant materials for analysis were: leaves, in February, March, September and October; phloem from cuttings in June; buds in July, and flowers in August.

Keywords: PPV, detection, phylogeny, sampling.

Introduction

Plum pox virus (PPV) has been previously reported in Chile (Acuña, 1993), and its presence is related exclusively with *Dideron* strain (Reyes et al., 2003). Since 1994, the Chilean phytosanitary service (Servicio Agrícola y Ganadero - SAG) has been forcing nurseries to analyze propagating material in order to avoid virus dissemination, which mainly affect apricots, plums and peaches, by decreasing marketable production. PPV detection techniques are based on the biological indexing, serological tests and PCR-based methods (EPPO, 2004). The selected period for PPV analysis has been concentrated in spring since good results have been obtained with the different detection techniques (Olmos et al., 2007).

However, the possibility to perform several surveys throughout the year could significantly improve the certification and clean-stock programs. Limits to detection are posed by the erratic distribution of viruses within the plants, the type and age of tissue used for analysis, the season, and the environmental factors, which influence virus concentration. A study was conducted to evaluate how some of these factors influence the virus detection. Ability to determine virus presence by DASI-ELISA, non-isotopic molecular hybridization (MH) and RT-PCR was investigated on a monthly basis for different kind of tissues throughout 13 months, to establish which tissue and method is the best to use at a certain time of the year. In addition, a short survey was performed in the most important stone fruit growing zone in the country, in order to characterize PPV isolates found.

Materials and methods

During the southern hemisphere spring (September and October) of 2007, several Chilean stone fruit commercial orchards of different sizes were visited to assess their sanitary status concerning PPV in the most important fruit growing regions: Valparaiso (V), Metropolitana de Santiago (RM) and Libertador General Bernardo O'Higgins (VI). A total of 1396 trees (180, 606 and 610 from V, RM and VI regions, respectively) were sampled during the whole survey period, their geographical position was established by the GPS system and the precise coordinates of sampled plants within the orchards were recorded to facilitate identification if further sampling was required. Three samples were randomly collected in each orchard. Leaf samples (20 per tree) were collected and transferred by a tissue printing procedure (Más and Pallás, 1995; Amari et al., 2001) onto nylon membranes. PPV detection was carried out by non-isotopic molecular hybridization (MH) using a riboprobe labeled with digoxigenin and designed to hybridize the region coding for coat protein (Herranz et al., 2005). Approximately 15 % of negative and all positive samples obtained by tissue printing were tested again by RT-PCR. Total nucleic acids (TNA) extraction was carried out using a silica capture method (MacKenzie et al., 1997; Malinovski, 1997). Fifteen µl aliquots of TNA were primed with using DNA

random hexanucleotides (Roche, Switzerland) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV-RT, Promega, USA). DNA amplification was done using Invitrogen Taq DNA polymerase (Brazil) and the PPV universal primers P1/P2 (Wetzel et al., 1991).

All positive samples were analyzed to determine the strain, using specific primers for PPV-M, PPV-D, PPV-Rec, PPV-C and PPV-EA (Candresse et al., 1998; Szemes et al., 2001; Šubr et al., 2004). A fragment of 467 bp corresponding to the replicase-coat protein (Nib-CP) region from the virus genome was amplified from positive samples by using the primers P4b and P3D (Candresse et al., 1998). Fragments were directly sequenced in both directions by the dideoxynucleotide chain-termination method in an automated sequencer (ABI 3100 Genetic Analyzer; Perkin Elmer Applied Biosystem) using the primers employed for the amplification. The sequences were then aligned with the BLAST-N tool for local alignment of nucleotide sequences (version Blast N 2.2.12). The publicly available sequences of PPV strains were downloaded from TreeBase (University of Buffalo, NY, USA) and compared with the Chilean ones. A total of 30 sequences were aligned (Table 1) using the BioEdit and CLUSTAL X programs (Thompson et al., 1997; Hall, 1999); then a phylogenetic tree was constructed using the maximal parsimony algorithm of MEGA version 2.1 (Kumar et al., 2001). The statistical significance of the nodes was conferred by bootstrap analysis of 10000 pseudoreplicates.

Tab. 1. List of the isolates obtained in this work and others used as reference. [a]Serbia and Montenegro. [b]Bosnia and Herzegovina. [c] Sweet cherry.

Sequence code	Source	Origin	Strain	Accession number
CL 812 Pch	Peach cv. Loadell	Chile	D	This work
CL 803 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 811 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 816 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 790 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 795 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 814 Plm	Plum cv. D'Agen	Chile	D	This work
CL 885 Plm	Plum cv. Black Beaut	Chile	D	This work
CL 65 Ne	Nectarine cv. unknown	Chile	D	This work
CL 64 Pch	Peach cv.unknown	Chile	D	This work
CL 70 Pch	Peach cv.unknown	Chile	D	This work
CL 68 Pch	Peach cv.unknown	Chile	D	This work
CL 63 Pch	Peach cv. Nemaguard	Chile	D	This work
Cdn1 Plm	Plum cv. Italian plum	Canada	D	AY953261
Chile116 Plm	Plum cv.unknown	Chile	D	AF440743
NAT	Prunus sp.	Germany	D	NC_001445
Chile20 Pch	Peach cv. GF677	Chile	D	AF440745
Chile114 Apr	Apricot cv. unknown	Chile	D	AF440742
Chile31 Pch	Peach cv. unknown	Chile	D	AF440746
Chile12 Plm	Plum cv.Marianna 2624	Chile	D	AF440744
Chile112 Pch	Peach cv. unknown	Chile	D	AF440741
Abricotier Turquie-M	Apricot cv. unknown	Turquie	M	AY677115
PS-M	Prunus sp.	S. and M.[a]	M	AJ243957
Bos64Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749998
Bos257Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749997
Bos148Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749996
Pakistan-Rec Apr	Apricot cv unknown	Pakistan	Rec	DQ422148
Pd31-M Plm	Plum cv. unknown	Hungary	M	AJ566345
PPV-El Amar Apr	Apricot cv.unknown	Egypt	El Amar	DQ431465
PPV-C SwC	Cherry[c] cv. unknown	Italy	C	Y09851

To optimize virus detection, 27 PPV-positive trees were selected in the Metropolitana region: 16 apricots cv. Castelbrite, 1 peach cv. Loadell, 1 peach cv. Jungerman and 2 plums cv. D'Agen from the locality of Calera de Tango; 7 plums cv. Marianna 2624 from the locality of La Pintana. All the plants were positive to PPV by RT-PCR but only one (apricot cv. Castelbrite) presented symptoms at time of the study. The sampling period was from December 2006 until December 2007, collecting plant tissues available at each sampling time: leaves were sampled from December 2006 to April 2007 and from September 2007 to December 2007; cuttings from April to August 2007; buds from May to August 2007 and flowers only in August. Each sample was analyzed by RT-PCR (with P1/P2 primers) and MH as described, and DASI-ELISA (EPPO, 2004) using the kit "Realisa Reforzado" (REAL, Spain).

Results

A total of 1396 trees were analyzed by tissue printing of which 24 were positive to PPV (1.72 %). All positive plants were found in the VI (17 out of 610) and Metropolitana (7 out of 606) regions, which have the highest number of hectares planted to stone fruits. To evaluate the reliability of these results all positive samples plus 15 % of MH-negative ones were analyzed by RT-PCR. A total of 45 positive samples were obtained, including the 24 MH-positive samples, representing 3.22 % of the plants analyzed by both techniques. Regarding the three production areas the percentage of infected plants varied between 4.4 (27 out of 610), 2.6 (16 out of 606) and 1.1 (2 out of 180) for the VI, RM and V regions, respectively. The analysis of positive samples using specific primers revealed that all PPV isolates were classified as PPV-D variants. To further characterize the PPV variant present in the infected samples, we performed a phylogenetic analysis using representative sequences of the different PPV isolates present in the database and the nucleotide sequence of the PPV-D specific 467 bp amplified fragments (Figure 1).

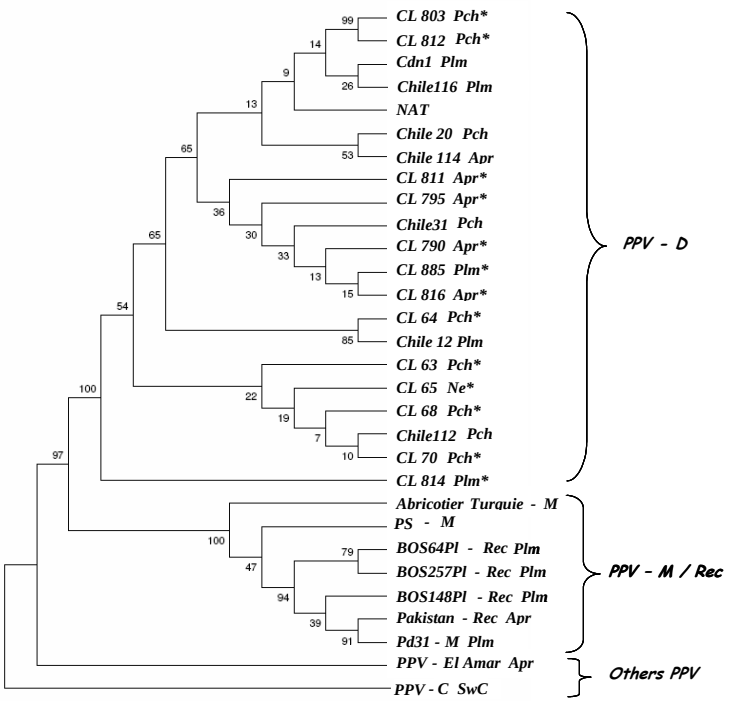

Fig. 1 Phylogenetic tree obtained with nucleotide sequences of the genomic region Nib-CP (467 bp). *Some of Chilean PPV isolates found during the survey. All the Chilean isolates clustered together with the PPV-D variant, confirming the results obtained with RT-PCR using strain specific primers.

Next, we decided to analyze different aspects affecting the detection of PPV such as the period of the year, which plant tissue to select and the detection method used. For this purpose we selected 27 PPV-infected trees (obtained from the previous surveys) that were subjected to a monthly periodical analysis during one year and using three different detection techniques: DASI-ELISA, MH and RT-PCR (Table 2).

Tab. 2 Comparison of techniques for *Plum pox virus* detection. [a]Best materials and results; [b]Second best materials and results; (+/t): Number of positives versus number of tested samples.

Month	Type of analyzed samples				Results (+/t) ELISA	MH	RT-PCR
Dec '06	Leaves[a]				1/27	3/27	16/27
Jan '07	Leaves				1/27	1/27	1/27
Feb '07	Leaves[a]				1/27	1/27	23/27
Mar '07	Leaves[a]				1/27	1/27	22/27
Apr '07	Leaves[a]	Phloem			1/27	1/27	9/27
May '07		Phloem[b]	Buds[a]		1/27	1/27	4/27
Jun '07		Phloem[a]	Buds[b]		2/27	2/27	19/27
Jul '07		Phloem	Buds[a]		1/27	6/27	25/27
Aug '07		Phloem[b]	Buds	Flowers[a]	1/27	6/27	26/27
Sep '07	Leaves[a]				1/27	16/27	24/27
Oct '07	Leaves[a]				2/27	5/27	20/27
Nov '07	Leaves[a]				2/27	2/27	16/27
Dec '07	Leaves[a]				2/27	2/27	14/27

The most sensitive method was the RT-PCR, as expected, allowing the detection of 96 % of the infected trees (26 positives out 27 samples in August), followed with the MH and ELISA with the 59.2 % (16 positive out 27 samples in September) and 7.4 % (2 positives out 27 in October), respectively. The best period to analyze PPV varied between the three techniques. For DASI-ELISA we obtained the best results between October and November, meanwhile for MH we observed good results between July and October, September being the best month. In the case of RT-PCR, we observed two different periods: one between July and September with an average detection of 92.6 % and another one between February and March with an average detection of 83.3 %. Only in January, was the sensitivity the same for all techniques, representing 3.7 % of the infected samples. The best plant material for sampling was: leaves in February, March, September and October; phloem scrapings from cuttings in June; buds in July and flowers in August (Table 2).

Discussion

In the present study we have analyzed the prevalence of PPV in the three main stone fruit production areas of Chile. The analysis of 1396 trees by MH revealed a prevalence of 1.72 %, a percentage that increased to 3.22 % when we analyzed the 15 % of MH-negative samples by RT-PCR technique. However, if we only consider the results obtained from the analysis of the 15 % of MH-negative samples by the RT-PCR, the PPV prevalence could reach 10.24 % (21 positive samples out 205). Regarding the three production areas, the prevalence of PPV ranged between 4.4 % in the VI region, followed by the RM and V areas with a prevalence of the 2.6 % and 1.1 %, respectively. In addition, we observed that all characterized PPV isolates were assigned to PPV-D by using specific strain primers or by phylogenetic analysis using the nucleotide sequence obtained from the specific amplified PCR product. These results indicate that, so far, the only PPV variant present in Chile is PPV-D. A similar observation has been reported for other North and South America PPV isolates (Damsteegt et al., 2001; Reyes et al., 2003).

In a second step, we analyzed the critical aspects that could influence routine PPV detection such as the plant tissue selected, the period of the year or the detection technique. For this purpose, we analyzed a collection of 27 PPV infected trees during one year using three different techniques. The direct comparison between the three detection methods revealed that RT-PCR was the most sensitive technique followed by MH and DASI-ELISA, detecting 96, 59.2 and 7.4 % of infected trees. Similar results have been described previously for the detection of other plant viruses, although the differences between MH and ELISA were considerably smaller (Sánchez-Navarro et al., 1998; Myrta et al., 2003; Alfaro et al., 2009). In addition, the different detection percentages obtained with RT-PCR and DASI-ELISA (96 % vs 7.4 %) are not in agreement with the results presented by Olmos et al. (2007) using the same validated ELISA kit and a collection of 205 infected trees (100 % vs 98.5 %). Since both analyses utilized the same antisera, the discrepancy observed could be attributed to the sensitivity obtained for the RT-PCR. Thus, both analyses differed in the RNA extraction procedure in which the silica chemical method (herein) gave a better detection limit for the analysis of stone fruits tissue than the Qiagen procedure (Sánchez-Navarro et al., 2005). However, we can not discard that the discrepancy could be attributed as well to the DASI-ELISA method. Thus, while we observed one period during the year that showed the high percentage of infected plants for both MH and RT-PCR methods corresponding probably to the high titre of the virus, the amount of positive samples detected by DASI-ELISA was always the same in one or two trees. This could be explained by considering that the DASI-ELISA was not sensitive to the accumulation of PPV and thus, some PPV strains would not have been detected. In this sense, we are now analyzing the CP gene of some PPV-infected trees that were negative by DASI-ELISA to look for putative amino acids changes. The results obtained with

RT-PCR, showed we were not able to detect the virus 100 % in infected trees, a fact which could be attributed to the erratic distribution of the virus and the lack of symptoms.

During December 2006 and 2007 the highest temperatures of the year were recorded. It is very probable that this has influenced the bad results of the RT-PCR in January, in which only one sample out of 27 was positive. These results suggested that the use of RT-PCR should be considered for stone fruit nursery routine control of PPV in Chile, taking into account two major sampling windows during the year: the first, from July to September (from the end of winter to early spring); the second with slightly lower sensitivity, during the months of February and March (end of summer). MH is shown as a clear alternative to the serological test for large-scale surveys, but only during September. This study allowed the optimization of PPV detection in Chile.

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