Typing and distribution of *Plum pox* virus isolates in Romania

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Abstract

Plum pox or Sharka, caused by *Plum pox virus* (PPV) is considered the most destructive disease of plum. Although PPV is widespread in all plum growing areas of Romania and causes serious yield losses, little is known about the variability of its isolates at a country level. For this reason, a large-scale study was performed with the aim of obtaining a picture of the prevalence and distribution of PPV strains in plum. During a three year survey, 200 PPV isolates collected from 23 different plum orchards from Transylvania, Moldavia and Muntenia areas were investigated. DAS-ELISA and IC-RT-PCR were used for PPV detection. PPV strains were serologically determined by TAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies. Molecular strain typing was done by IC/RT-PCR targeting three genomic regions corresponding to (Cter)CP, (Cter)NIb/(Nter)CP and CI. RFLP analysis was used to distinguish D and M strains, based on the *Rsa*I polymorphism located in (Cter)CP. To confirm the presence of PPV-Rec strain, 13 PCR products spanning the (Cter)NIb/(Nter)CP were sequenced. Overall results showed that in Romania the predominant strain is PPV-D (73%), followed, with a much lower frequency, by PPV-Rec (14%). Mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent (13%).

Keywords: Romania, PPV strains, DAS/TAS-ELISA, IC/RT-PCR, RFLP, sequencing

Introduction

Plum pox or Sharka is the most devastating disease of stone fruits. The disease is highly detrimental because it reduces the quality of the fruits and causes their premature dropping. (Dunez and Sutic, 1988; Nemeth, 1994). Sharka disease was described for the first time around 1917 in Bulgaria (Atanasoff, 1932). Since then, the disease has progressively spread to a large part of the European continent, around the Mediterranean basin, in Asia (India, China, Pakistan, Kazakhstan and Iran) as well as in America (Chile, Argentina, USA and Canada) (Capote et al., 2006; García and Cambra 2007). Therefore, this disease is among the most significant limiting factors for plum production (Stoev et al., 2004). In Romania, Sharka occurs in all plum growing areas causing serious yield losses especially to sensitive cultivars (Minoiu, 1997; Zagrai et al., 2001).

To control the virus spreading it is important to know the distribution of the virus and the different strains occurring (Pasquini and Barba, 1994). Seven strains of PPV have been reported so far. Two major groups, PPV-D and PPV-M (Kerlan and Dunez, 1976) can be distinguished by strain-specific monoclonal antibodies (Boscia et al., 1997; Cambra et al., 1994), and also by the *Rsa*I polymorphism in the DNA fragment amplified by P1/P2 primer pairs located at the C-terminus of the PPV CP gene (Wetzel et al., 1991a) or by direct IC/RT-PCR typing using PD and PM specific oligonucleotides (Olmos et al., 1997). The third major group was identified and denoted PPV-Rec (Glasa et al, 2002). This natural recombinant between PPV-D and PPV-M was reported in Albania, Bulgaria, Czech Republic, Germany, Hungary, Slovakia (Glasa et al., 2002, 2004), Bosnia and Herzegovina (Matic et al., 2006), Pakistan (Kollerova et al., 2006), Romania (Zagrai et. al., 2006, 2008), Turkey (Candresse et al., 2007) and Canada (Thompson et. al., 2009). Three additional minor PPV groups are represented by geographically limited strains El Amar (PPV-EA) originally isolated from Egypt (Wetzel et al., 1991b), Cherry (PPV-C) isolated from sour cherry in Moldavia (Kalashyan et al., 1994) and from sweet cherry in southern Italy (Crescenzi et al., 1997) and Romania (Maxim et al., 2002a, 2002b), and Winnona (PPV-W) from Canada (James and Varga, 2004). A new PPV strain was recently isolated from apricot in Turkey and called PPV-T (Serce et al., 2009).

The objective of the present study was to provide a picture of the prevalence and distribution of PPV strains occurring in Romania plum orchards.

Materials and methods

Two hundred PPV isolates were collected from 23 different plum orchards from Transylvania, Moldavia and Muntenia areas of Romania. Sampling was initially based on typical PPV symptoms and virus infection was confirmed by serological and molecular testing. Serological diagnosis was made by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum (Bioreba, Switzerland) according to the manufacturer's instructions. Molecular detection was done by IC-RT-PCR using the pair of primers P1/P2 and trapping with the above polyclonal antiserum. Qiagen one-step kit (Qiagen, Germany) was used for RT-PCR.

Serological discrimination was made by TAS-ELISA using the PPV-D and PPV-M specific monoclonal antibodies (Durviz, Spain) according to Cambra et al. (2004). Molecular strain typing was done by IC/RT-PCR targeting three genomic regions corresponding to:

(i) (Cter) CP, using P1/PD and P1/PM pair of primers that distinguish PPV-D and PPV-M, respectively;
(ii) (Cter) NIb/(Nter)CP, using mD5/mM3 pair of primers (Subr et al., 2004) that detect natural recombinants between D and M (PPV-Rec);
(iii) CI, using CIf/CID or CIf/CIM primer sets (Glasa et al., 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter)CP were subjected to RFLP analysis to distinguish D strains from M strains based on the *Rsa*I polymorphism located in this genomic region. To check if the recombination breakpoint position suspected to occur in the (Cter)NIb/ (Nter)CP region corresponds with those previously reported for PPV-Rec, 13 PCR products spanning (Cter) NIb/(Nter)CP region were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA), and then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The samples were run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The alignment of nucleotides was done using the BioEdit package version 5.0.9 (Hall, 1999). Obtained sequences were then compared with those available in GeneBank.

Results and discussion

Similar results were obtained in the differentiation of PPV isolates by TAS-ELISA using D and M monoclonal antibodies and by IC/RT-PCR using PD and PM specific primers (Table 1). All isolates reacted positively to at least one of the two monoclonal antibodies as well as PPV-D or/and PPV-M specific primers. In a few cases, the mixed infection could not be detected by TAS-ELISA. This could be explained by the lower sensitivity of serological techniques in comparison with molecular techniques. RFLP analysis confirmed the IC/RT-PCR results based on the presence of the *Rsa*I polymorphism in the PPV-D strain.

Tab. 1 Serological and molecular differentiation of 200 PPV isolates from Transylvania, Muntenia and Moldavia

			DAS-ELISA			IC/RT-PCR				RFPL				Rate of
Area	No of isolates		PPV poly	PPV-D	PPV-M	P1/P2	P1/PD	P1/PM	mD5/mM3	CIf/CID	CIf/CIM	*Rsa*I	Strain status	infection %
		70	+	+	-	+	+	-		+	-	+	PPV-D	70
Transylvania	100	18	+	-	+	+	-	+	+	+	-	-	PPV-Rec	18
		12	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	12
		34	+	+	-	+	+	-		+	-	+	PPV-D	84
Muntenia	50	4	+	-	+	+	-	+	+	+	-	-	PPV-Rec	12
		12	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	4
		42	+	+	-	+	+	-		+	-	+	PPV-D	68
Moldavia	50	6	+	-	+	+	-	+	+	+	-	-	PPV-Rec	8
		2	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	24

Using the primer pair (mD5/mM3) targeting the (Cter)NIb/(Nter)CP region, it was observed that all PPV isolates typed as PPV-M in (Cter) CP were in fact PPV-Rec. Using specific primers to distinguish the two strains D and M in the CI region only fragments belonging to PPV-D were detected, thus confirming the presence of PPV-Rec. The typing of PPV isolates from Transylvania, Moldavia and Muntenia areas revealed that PPV-D and PPV-Rec occurred in the plum orchards from Romania. In all three areas PPV-D is the prevalent strain. The higher incidence of PPV-D was noticed in Moldavia (84%) and the higher rate of PPV-Rec was recorded in Transylvania (18%). Mixed infections (D+Rec) were more frequent in Muntenia (24 %). Multiple sequence alignment of the 13 PCR products spanning (Cter) NIb/(Nter)CP region showed that the recombination breakpoint is located in the region corresponding to (Cter)NIb at nucleotide position 8450 (Figure 1).

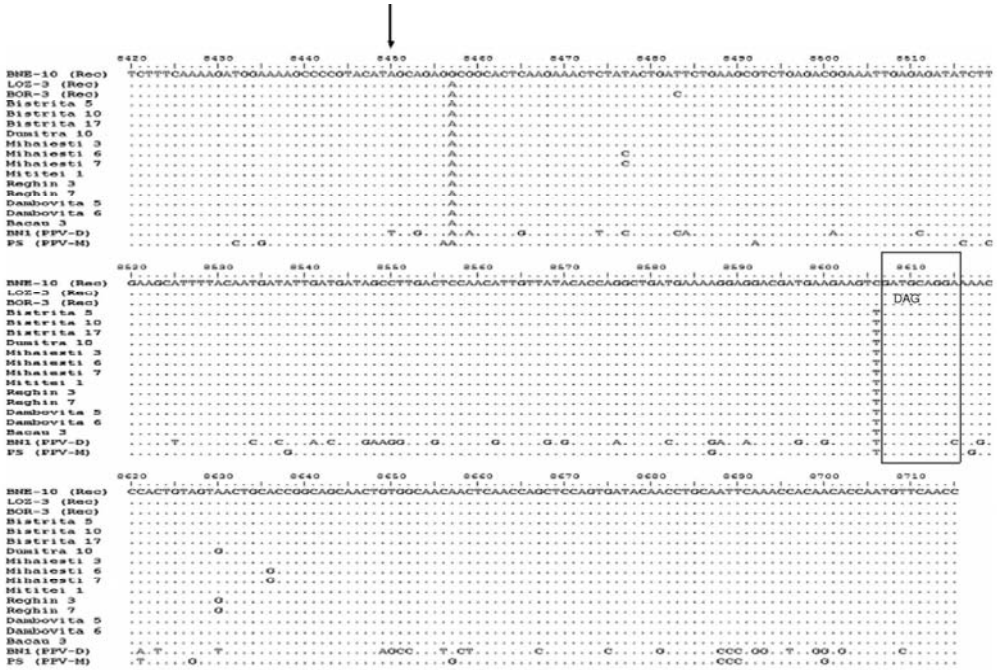

Figure 1 Multiple alignment of recombinant sequences (NIb/CP) of 13 Romanian isolates (Bistrita 5, Bistrita 10, Bistrita 17, Dumitra 10, Mihaiesti 3, Mihaiesti 6, Mihaiesti 7, Mititei 1, Reghin 3, Reghin 7, Dambovita 5, Dambovita 7, Bacau 3) and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR–3 (accession number AY028309)] previously reported.

The DAG motif that is considered as essential for aphid transmission was also present. As expected, this site was located downstream of the recombination breakpoint. Based on a comparative alignment, the sequencing results revealed a high similarity (98%) with different sequences of PPV-Rec available in GeneBank. All these recombinant isolates shared the same recombination breakpoint. Overall results presented in the table 2 showed that in Romania the predominant strain is PPV-D (73%), follow with a much lower frequency by PPV-Rec (14%). Although a big difference between the incidence of PPV-D and PPV-Rec was recorded, our results confirmed that the recombinant strain represents a major PPV group. Mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent (13%).

Tab. 2 Synthesis of serological and molecular differentiation of 200 PPV isolates from Romania

	DAS/TAS-ELISA					IC/RT-PCR				RFLP		
No. of isolates	PPV poly	PPV-D	PPV-M	P1/P2	P1/PD	P1/PM	mD5/ mM3	CIf/ CID	CIf/ CIM	*RsaI*	Strain status	Rate of infection %
146	+	+	-	+	+	-	-	+	-	+	PPV-D	73
28	+	-	+	+	-	+	+	+	-	-	PPV-Rec	14
26	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+PPV-Rec	13

Conclusions

Both PPV-D and PPV-Rec occurred in plum orchards of Romania. PPV-D is the prevalent strain in all the three plum growing areas and at country level, too. There was a higher incidence of PPV-D in Moldavia and a higher rate of PPV-Rec in Transylvania. The mixed infections (D+Rec) were more frequent in Muntenia.

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