

Genetic diversity of *Plum pox virus* Isolates in Muntenia, Romania

Received for publication, September 22, 2009

Accepted, June 20, 2010

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Abstract

Plum pox virus (PPV) is considered the most devastating viral pathogen of stone fruits causing serious yield losses. Although PPV is endemic in Romania, limited data about the occurrence of its strains in some plum regions like Muntenia are available. Considering this fact, fifty PPV isolates collected from five different commercial plum orchards from Muntenia were investigated. PPV detection was made by DAS-ELISA and by IC-RT-PCR. 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 at the C-ter of CP cistron was used to distinguish the two major strains, D and M based on a RsaI polymorphism located in this genomic section. All PCR products targeting (Cter)CP and two PCR product spanning the (Cter)Nib/(Nter)CP were sequenced. All PPV isolates typed as PPV-M by serological analysis and by molecular differentiation in the genomic region corresponding to (C-ter)CP proved to be PPV recombinant (PPV-Rec) when the molecular analysis were performed in the region corresponding to Nib/CP. Sequencing results confirmed a high similarity with different sequences of PPV-Rec previously reported. Overall results provided that in Muntenia the prevalent strain is PPV-D. PPV-Rec is also present with a much lower frequency both in singular and mixed infections (PPV-D+PPV-Rec).

Keywords: Plum, Sharka, PPV strain, DAS/TAS-ELISA, IC/RT-PCR, sequencing.

Introduction

Plum pox virus (PPV), the causal agent of the Sharka disease, is the most dangerous quarantine viral pathogen of stone fruits. This disease is highly detrimental because reduce the fruit quality and causes premature dropping [1, 2]. Sharka disease was described for the first time around 1917 in Bulgaria [3]. 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) [4, 5]. In Romania, Sharka occurs in all plum growing areas causing serious yield losses especially on sensitive cultivars [6, 7].

To control the virus spreading it is important to know the distribution of the virus and the strains occurring [8]. Seven strains of PPV have been reported so far. Two major groups, PPV-D and PPV-M [9] can be distinguished by strain-specific monoclonal antibodies [10, 11], and also by *RsaI* polymorphism in the 243 bp DNA fragment amplified by P1/P2 primer pairs located at the C-terminus of PPV CP gene [12] or by direct RT-PCR typing using PD and PM specific oligonucleotides [13].

The third major group was identified and denoted PPV-Rec [14]. This natural recombinant between PPV-D and PPV-M was reported in Albania, Bulgaria, Czech Republic, Germany, Hungary, Slovakia [14, 15], Bosnia and Herzegovina [16], Pakistan [17], Romania [18, 19], Turkey [20] and Canada [21]. Three additional minor PPV groups are represented by geographically limited strains El Amar (PPV-EA) originally isolated from Egypt [22], Cherry

(PPV-C) isolated from sour cherry in Moldavia [23] and from sweet cherry in southern Italy [24] and Romania [25, 26], and Winona (PPV-W) from Canada [27]. A new PPV strain was recently isolated from apricot in Turkey and called PPV-T [28].

A recent study performed in Transylvania, Romania, revealed that PPV-D is the prevalent strain followed by PPV-Rec, and the mixed infections (PPV-D + PPV-Rec) are also frequent [19, 29]. The prevalence of PPV-D strain and the occurrence of PPV-Rec in singular and mixed infections were also showed in Moldavia region [30].

The objective of the present study was to provide new data about PPV strains occurring in the Muntenia plum growing area.

Materials and Methods

Fifty PPV isolates were collected from five different plum orchards in the Muntenia area. 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 [31] using a commercial polyclonal antiserum (Bioreba, Switzerland) according to the manufacturer's instructions. Molecular detection was made 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 differentiation was made by TAS-ELISA using the PPV-D and PPV-M specific monoclonal antibodies (Durviz, Spain) [32]. Aliquots of PCR products, corresponding to (Cter)CP, were subjected to RFLP analysis to distinguish D strains from M strains based on *RsaI* polymorphism located in this genomic area.

Further 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) N1b/(Nter)CP, using mD5/mM3 pair of primers [33] that detect natural recombinants between D and M (PPV-Rec); (iii) CI, using C1f/C1D or C1f/C1M primer sets [14] to confirm the presence of PPV-Rec.

To confirm the molecular variability of the sampled PPV isolates, amplified DNAs were first purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA), 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 from all PCR products corresponding to (Cter)CP and two amplified fragment spanning (Cter) N1b/(Nter)CP region was done using the BioEdit package version 5.0.9 [34]. Obtained sequences were then compared with those available in NCBI Data Base and GeneBank. A phylogenetic tree was constructed with the Mega 3.1 programme using Minimum Evolution method Jukes-Cantor model (Bootstrap value 10 000) for sequences corresponding to (Cter)CP.

Results and Discussion

Some differences were obtained in the discrimination of PPV isolates by TAS-ELISA using D and M monoclonal antibodies and by IC/RT-PCR using PD and PM specific primers or RFLP using the *RsaI* on P1/P2 products (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. Using TAS-ELISA, 45 (90%) of 50 isolates tested were identified as PPV-D and 5 (10%) as PPV-M. IC/RT-PCR analyses confirmed that 34 isolates were PPV-D type and 4 isolates PPV-M, and also revealed the presence of 12 mixed infection involving D and M strains. RFLP analysis confirmed these results based on the presence of the *RsaI* polymorphism in PPV-D strain.

Table 1. Serological and molecular detection and differentiation of 50 PPV isolates from five orchards in Muntenia, Romania

Orchard No.	DAS/TAS-ELISA				IC/RT-PCR (P1/P2 and P1/PD or PM)				RFLP <i>RsaI</i>		
	PPV poly	PPV-D	PPV-M	PPV D+M	PPV poly	PPV -D	PPV-M	PPV D+M	PPV-D	PPV-M	PPV D+M
1	10	10	0	0	10	10	0	0	10	0	0
2	10	9	1	0	10	7	1	2	7	1	2
3	10	8	2	0	10	8	1	1	8	1	1
4	10	10	0	0	10	4	0	6	4	0	6
5	10	8	2	0	10	5	2	3	5	2	3
TOTAL (%)	50 (100)	45 (90)	5 (10)	0 (0)	50 (100)	34 (68)	4 (8)	12 (24)	34 (68)	4 (8)	12 (24)

The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to PPV (C-ter)CP confirmed the differentiation of the two major groups D and M in this genomic section (Figure 1). Sequences from Muntenia PPV isolates were 98-100 % identical to different sequences from the NCBI Data Base (accessions AF354268.1, AF354269.1, AF360579.1, AF401295.1, AF401296.1, AF440741.1, AF440743.1, AJ566345.1, AM260934.1, AM260937.1, AY591253.1, AY591254.1, AY750961.1, AY795603.1, AY912056.1, AY953261.1, AY953263.1, AY953264.1, AY953265.1, AY953266.1, DQ299538.1, DQ465242.1, EF569214.1, EF611241.1, EF611242.1, EF611243.1, EF611244.1, EF640933.1, EF640934.1, EF640935.1, EF640936.1, EF640937.1, EF640938.1, EF640939.1, EU117116.1, EU818840.1, FN179152.1, GQ411056.1, S57404.1, X16415.1, X57975.1, X57976.1, X81077.1, X81079.1, X81080.1, X81081.1, X81082.1)

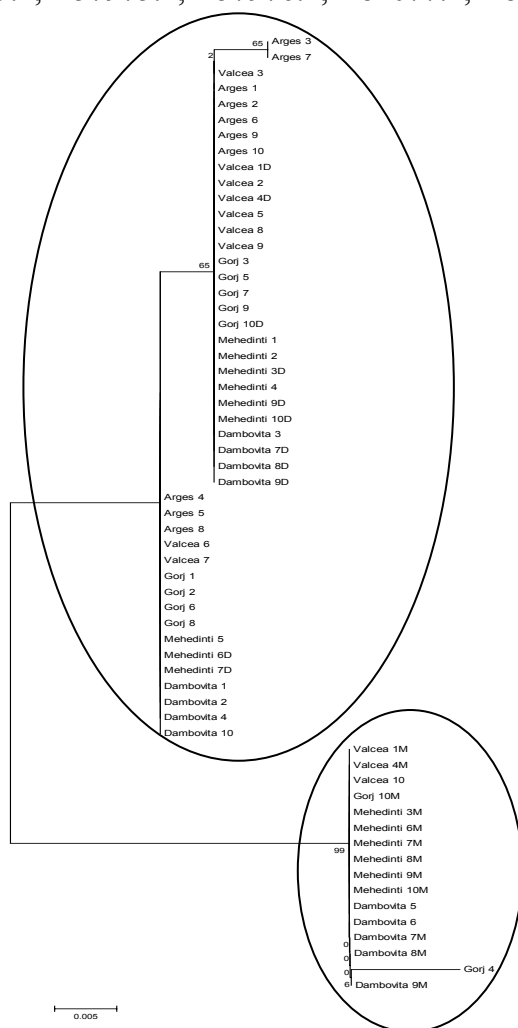


Figure 1. The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to C-terminus of PPV coat protein.

Using the primer pair (mD5/mM3) targeting (Cter)NIb/(Nter)CP region, was observed that all PPV isolates typed as PPV-M were in fact PPV-Rec. Using specific primers to distinguish the two strains D and M in CI region were detected only fragments belonging to PPV-D. That confirmed the presence of PPV-Rec (Table 2).

Table 2. Results of serological and molecular typing based on different targeted regions of the genome of PPV isolates selected from 5 orchards from Muntenia, Romania

Plots no.	Isolate	PPV strain identified by				
		TAS-ELISA	IC/RT-PCR			RFLP (C-ter) CP
			(C-ter) CP P1-PD/PM	(C-ter) NIb /(N-ter) CP ^(a) mD5/mM3	CI Cif – CID/CIM	
1	Arges 1	D	D	-	D	D
	Arges 2	D	D	-	D	D
	Arges 3	D	D	-	D	D
	Arges 4	D	D	-	D	D
	Arges 5	D	D	-	D	D
	Arges 6	D	D	-	D	D
	Arges 7	D	D	-	D	D
	Arges 8	D	D	-	D	D
	Arges 9	D	D	-	D	D
	Arges 10	D	D	-	D	D
2	Valcea 1	D	D+M	Rec	D	D+M
	Valcea 2	D	D	-	D	D
	Valcea 3	D	D	-	D	D
	Valcea 4	D	D+M	Rec	D	D+M
	Valcea 5	D	D	-	D	D
	Valcea 6	D	D	-	D	D
	Valcea 7	D	D	-	D	D
	Valcea 8	D	D	-	D	D
	Valcea 9	D	D	-	D	D
	Valcea 10	M	M	Rec	D	M
3	Gorj 1	D	D	-	D	D
	Gorj 2	D	D	-	D	D
	Gorj 3	D	D	-	D	D
	Gorj 4	M	M	Rec	D	M
	Gorj 5	D	D	-	D	D
	Gorj 6	D	D	-	D	D
	Gorj 7	D	D	-	D	D
	Gorj 8	D	D	-	D	D
	Gorj 9	D	D	-	D	D
	Gorj 10	M	D+M	Rec	D	D+M
4	Mehedinti 1	D	D	-	D	D
	Mehedinti 2	D	D	-	D	D
	Mehedinti 3	D	D+M	Rec	D	D+M
	Mehedinti 4	D	D	-	D	D
	Mehedinti 5	D	D	-	D	D
	Mehedinti 6	D	D+M	Rec	D	D+M
	Mehedinti 7	D	D+M	Rec	D	D+M
	Mehedinti 8	D	D+M	Rec	D	D+M
	Mehedinti 9	D	D+M	Rec	D	D+M
	Mehedinti 10	D	D+M	Rec	D	D+M
5	Dambovita 1	D	D	-	D	D
	Dambovita 2	D	D	-	D	D
	Dambovita 3	D	D	-	D	D
	Dambovita 4	D	D	-	D	D
	Dambovita 5	M	M	Rec	D	M
	Dambovita 6	M	M	Rec	D	M
	Dambovita 7	D	D+M	Rec	D	D+M
	Dambovita 8	D	D+M	Rec	D	D+M
	Dambovita 9	D	D+M	Rec	D	D+M
	Dambovita 10	D	D	-	D	D

^(a)Only the isolates identified as PPV-M in the region corresponding to (C-ter)CP were tested.

To check if the recombination breakpoint position suspected to occur in the (Cter)Nib/ (Nter)CP region corresponds with those PPV-Rec previously reported [14, 15], two PCR product spanning this genomic section were sequenced (Figure 2). Multiple sequence alignment showed that the recombination breakpoint is located in the region corresponding to (Cter)Nib at the nucleotide position 8450. The DAG motif that is considered as essential for aphid transmission was also present. As expected, this site was located downstream the recombination breakpoint. Based on 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.

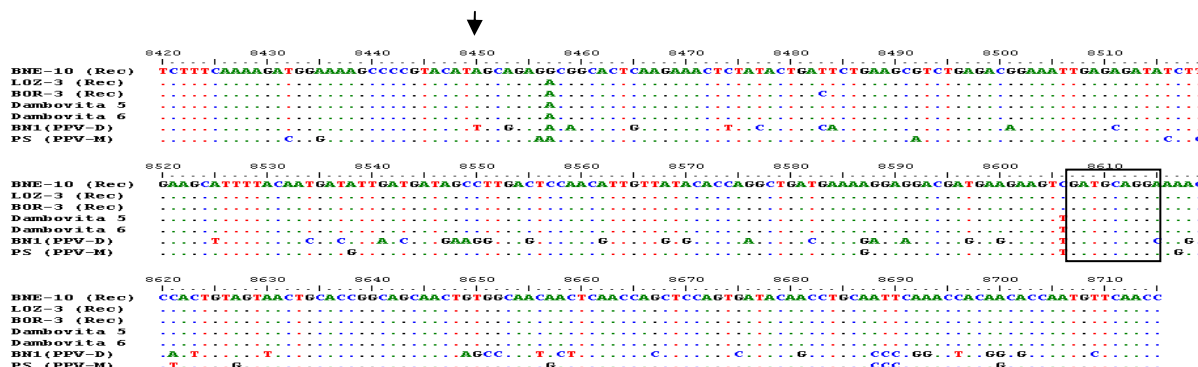


Figure 2. Multiple alignment of recombinant sequences (Nib/CP) of two Romanian PPV isolate (Dambovita 3 and Dambovita 6) and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR-3 (accession number AY028309)] previously reported.

The synthesis of serological and molecular typing of the 50 PPV isolates from Muntenia showed a relative frequency of 68% in the case of PPV-D and of 8% in the case of PPV-Rec (Figure 3). The mixed infections (PPV-D+PPV-Rec) had a rate of 24%.

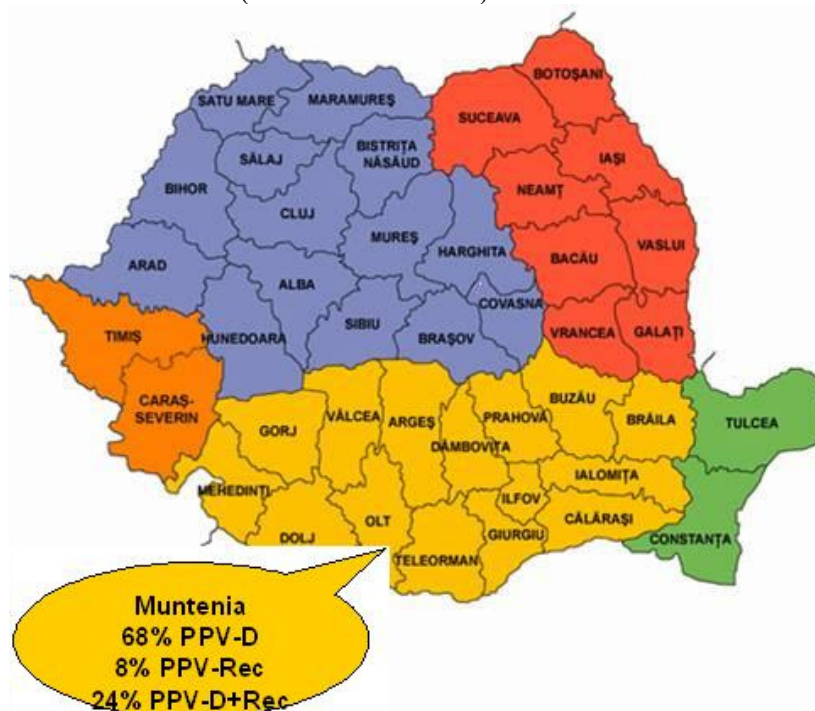


Figure 3. Relative frequency of PPV strains in Muntenia.

Conclusions

Overall results provided that in Muntenia the prevalent strain is PPV-D. PPV-Rec is also present with a much lower frequency both in singular and mixed infections (PPV-D+PPV-Rec).

Acknowledgments

This work was supported mainly by Romanian Research Ministry under the CEE- BIOTECH program, contract no. 102/2006, and also by European Community's Seven Framework Program under Grant Agreement n° 204429.

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