SEROLOGICAL AND MOLECULAR TYPING OF *PLUM POX VIRUS* ISOLATES IN THE NORTH OF ROMANIA

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SUMMARY

Plum pox virus (PPV) is considered as the most dangerous viral pathogen of stone fruits. Although PPV is widespread in Romania and causes serious yield losses, little is known about the variability of its isolates. To secure this information we investigated 43 PPV isolates collected from five different plum orchards in the North of Romania in the Bistrita plum-growing area. PPV strains were serologically tested 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 the two major strains, D and M based on a *Rsa*I polymorphism located in (Cter)CP. All PCR products targeting (Cter)CP and 8 PCR products spanning the (Cter)NIb/(Nter)CP cistrons 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 were confirmed by nucleotide sequencing to be homologous to PPV recombinant (PPV-Rec) previously reported. All these recombinant isolates share the same recombination breakpoint and conserve the DAG motif, which is considered essential for aphid transmission. This genetic similarity confirms that PPV-Rec may represent an ancestral group with a common evolutionary origin. Overall results provided evidence for endemic distribution of PPV-Rec in plum trees grown in the North of Romania.

Key words: Sharka, PPV strains, diagnosis, ELISA, RT-PCR, 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). Therefore, this disease is among the significant limiting factors for plum production (Stoev *et al.*, 2004). Sharka has a Balkan origin and 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 and Middle East. It has also been found in America (Chile, Argentina, USA and Canada), as well as in Asia (India, China, Pakistan, Kazakhstan and Iran) (Capote *et al.*, 2006; García and Cambra 2007). In Romania, Sharka occurs in all plum-growing areas causing serious yield losses especially to sensitive cultivars (Minoiu, 1997; Zagrai *et al.*, 2001).

PPV strain identification is useful for controlling virus spreading. Breeding programmes are associated with epidemiological studies of PPV. For this reason, it is important to know the distribution of the virus and the different strains occurring in any given country (Pasquini and Barba, 1994).

Two major serologically distinguishable strains, PPV-D and PPM-M, are known (Kerlan and Dunez, 1976) which can be distinguished by strain-specific monoclonal antibodies (Cambra *et al.*, 1994; Boscia *et al.*, 1997). In addition both strains can be discriminated by *Rsa*I polymorphism in the 243 bp DNA fragment amplified by P1 and P2 primers located at the C-terminus of PPV CP gene (Wetzel *et al.*, 1991a) or by direct IC-RT-PCR typing using PD and PM specific oligonucleotides (Olmos *et al.*, 1997).

A third major group of isolates detected in Albania, Bulgaria, Czech Republic, Germany, Hungary and Slovakia was recently denoted PPV-Rec (Glasa *et al.*, 2002; 2004). Two additional minor PPV groups are represented by strains with a limited geographical distibution, i.e. El Amar (PPV-EA) originally isolated from Egypt (Wetzel *et al.*, 1991b), and Cherry (PPV-C) isolated from sour cherry in Moldavia (Kalashyan *et al.*, 1994) and from sweet cherry in southern Italy (Crescenzi *et al.*, 1996) and Romania (Maxim *et al.*, 2002).

The last PPV strain to be described is Winona (PPV-W) from Canada (James and Varga, 2004), which is genetically distinct from all other viral strains known to date (James and Varga, 2005).

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The objective of our study was to provide new data about PPV strains occurring in the North of Romania.

MATERIALS AND METHODS

PPV Isolates. Forty-three PPV isolates were collected from five different plum orchards in the Bistrita area. Sampling was initially based on typical PPV symptoms and virus infection was confirmed by serological and molecular testing.

Serological and molecular detection. Serological diagnosis was by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum (Bioreba, Switzerland) according to the manufacturer's instructions. Molecular detection was by IC-RT-PCR using the pair of primers P1/P2 and trapping with the above polyclonal antiserum. Qiagen one-step kit (Qiagen Gmbh, Germany) was used for RT-PCR.

Strain differentiation. To identify the studied PPV isolates at the serotype level serological tests were made by TAS-ELISA using the PPV-D and PPV-M specific monoclonal antibodies (Durviz, Spain). Serological differentiation was made 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 *Rsa*I polymorphism located in this genomic area. To confirm the molecular variability of the sampled PPV isolates, amplified DNAs 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 eight amplified fragments spanning (Cter) NIb-(Nter)CP region was done using the BioEdit package version 5.0.9 (Hall, 1999). Obtained sequences were then compared with those available in NCBI Data Base and GenBank. A phylogenetic tree was constructed with the Mega 3.1 programme using Minimum Evolution method Jukes-Cantor model (Bootstrap value 10 000).

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. Using TAS-ELISA, 21 of 43 isolates tested (48.8%) were identified as PPV-D, 17 (39.6%) as PPV-M and 5 (11.6%) were a mixture of D and M strains. The IC-RT-PCR analyses confirmed that 20 isolates were PPV-D type and 15 isolates PPV-M. IC-RT-PCR detected three more mixed infection. RFLP analysis confirmed these results based on the presence of the *Rsa*I polymorphism in PPV-D strain.

The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to PPV (C-ter)CP confirmed the clear-cut splitting of the two major groups D and M in this genomic area (Figure 1). Se-

Fig. 1. Phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to the C-terminus of the viral coat protein. Isolates Bistrita 4, Bistrita 9, Dumitra 6, Dumitra 10, Mihaiesti 3, Mititei 1, Mititei 2 and Mititei 3 represent mixed infection and they were both sequenced for PPV-D and PPV-M.

Table 1. Serological and molecular detection and differentiation of 43 PPV isolates from five orchards of the Bistrita area, Romania.

Orchard No.	DAS / TAS-ELISA				IC-RT-PCR (P1/P2 and P1/PD or PM)				RFLP *Rsa*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	6	2	2	10	6	2	2	6	2	2
2	10	3	7	0	10	3	7	0	3	7	0
3	10	4	5	1	10	4	4	2	4	4	2
4	10	7	3	0	10	7	2	1	7	2	1
5	3	1	0	2	3	0	0	3	0	0	3
TOTAL	43	21	17	5	43	20	15	8	20	15	8
%	100	48.8	39.6	11.6	100	46.5	34.9	18.6	46.5	34.9	18.6

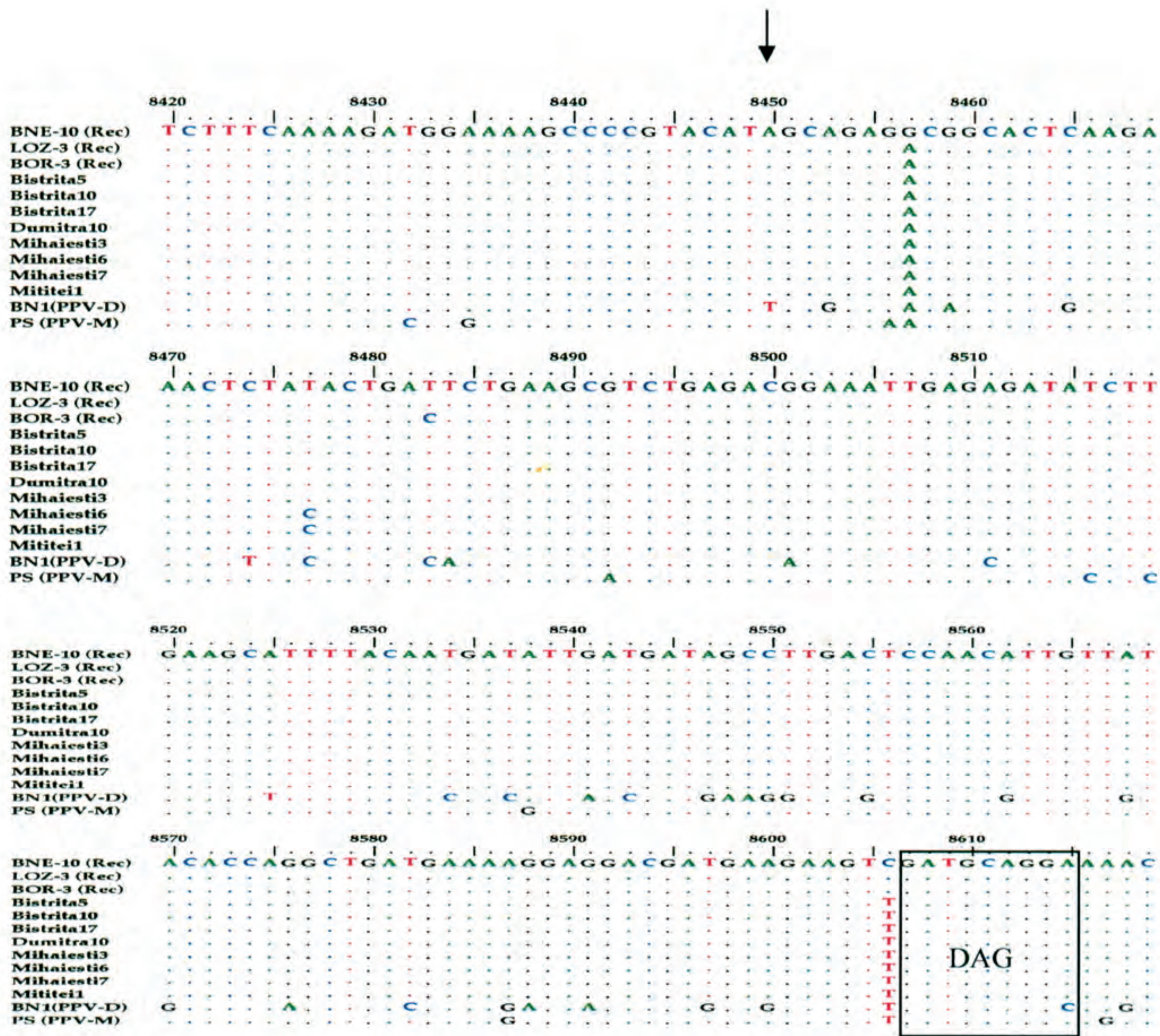

Fig. 2. Multiple alignment of sequences (NIb/CP) of eight Romanian PPV recombinant isolates (Bistrita 5, Bistrita 10, Bistrita 17, Dumitra 10, Mihaiesti 3, Mihaiesti 6, Mihaiesti 7, Mititei 1) and three PPV recombinant isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR–3 (accession number AY028309)] previously reported.

quences from Romanian PPV isolates were 98-100 % identical to sequences from the NCBI Data Base.

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

To check if the recombination breakpoint position suspected to occur in the (Cter)NIb-(Nter)CP region corresponds with those PPV-Rec previously reported by Glasa *et al.* (2002, 2004), eight PCR products spanning this genomic section were sequenced (Figure 2). Multiple sequence alignment showed that the recombination

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

Orchard No.	Isolate	PPV strain identified by			
		TAS-ELISA	IC-RT-PCR		
			(C-ter) CP	(C-ter) NIb /(N-ter) CP[a]	CI
			P1-PD/PM	mD5/mM3	CIf –CID/CIM
1	Bistrita 1	D	D	-	D
	Bistrita 2	D	D	-	D
	Bistrita 3	D	D	-	D
	Bistrita 4	D+M	D+M	Rec	D
	Bistrita 5	M	M	Rec	D
	Bistrita 6	D	D	-	D
	Bistrita 7	D	D	-	D
	Bistrita 8	D	D	-	D
	Bistrita 9	D+M	D+M	Rec	D
	Bistrita 10	M	M	Rec	D
2	Bistrita 11	M	M	Rec	D
	Bistrita 12	D	D	-	D
	Bistrita 13	M	M	Rec	D
	Bistrita 14	M	M	Rec	D
	Bistrita 15	M	M	Rec	D
	Bistrita 16	D	D	-	D
	Bistrita 17	M	M	Rec	D
	Bistrita 18	M	M	Rec	D
	Bistrita 19	M	M	Rec	D
	Bistrita 20	D	D	-	D
3	Dumitra 1	D	D	-	D
	Dumitra 2	M	M	Rec	D
	Dumitra 3	M	M	Rec	D
	Dumitra 4	D	D	-	D
	Dumitra 5	M	M	Rec	D
	Dumitra 6	D+M	D+M	Rec	D
	Dumitra 7	D	D	-	D
	Dumitra 8	M	M	Rec	D
	Dumitra 9	D	D	-	D
	Dumitra 10	M	D+M	Rec	D
4	Mihaiesti 1	D	D	-	D
	Mihaiesti 2	D	D	-	D
	Mihaiesti 3	M	D+M	Rec	D
	Mihaiesti 4	D	D	-	D
	Mihaiesti 5	D	D	-	D
	Mihaiesti 6	M	M	Rec	D
	Mihaiesti 7	M	M	Rec	D
	Mihaiesti 8	D	D	-	D
	Mihaiesti 9	D	D	-	D
	Mihaiesti 10	D	D	-	D
5	Mititei 1	D+M	D+M	Rec	D
	Mititei 2	D	D+M	Rec	D
	Mititei 3	D+M	D+M	Rec	D

[a]Only the isolates identified as PPV-M by (C-ter)CP were tested.

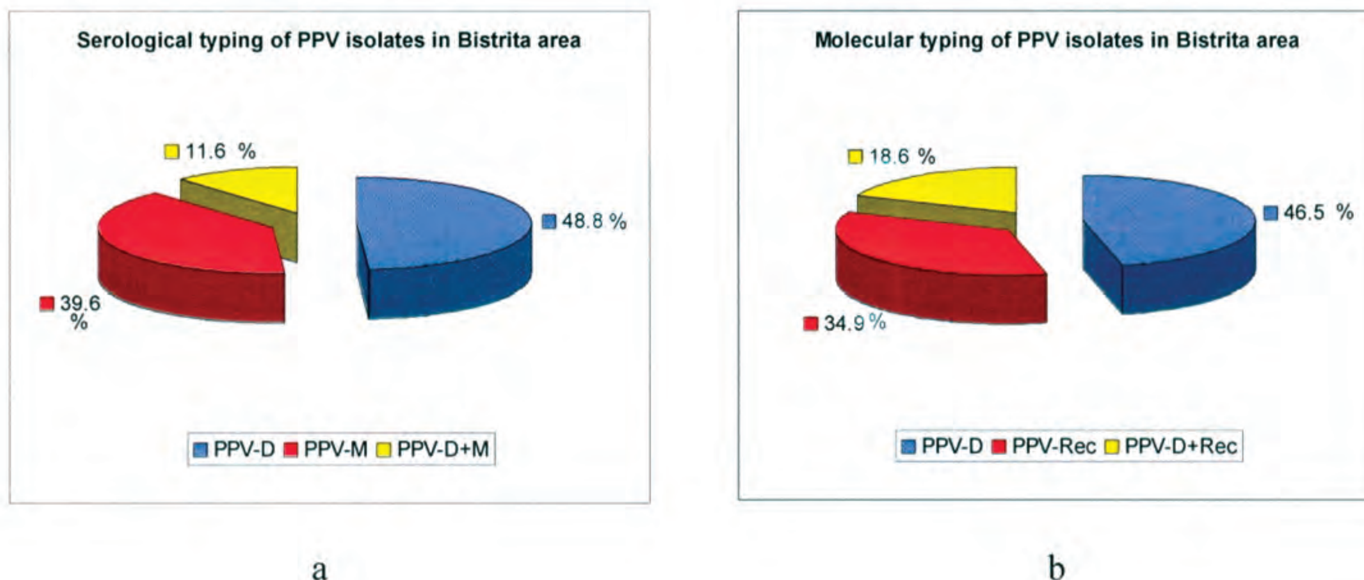

Fig. 3. Relative frequency of PPV strains in Bistrita area. (a) serological typing; (b) molecular typing.

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 present in all PPV-Rec isolates analyzed. As expected, this site was located downstream the recombination breakpoint. Based on comparative alignment, the sequencing results revealed a high similarity (98-99%) with different sequences of PPV-Rec available in GeneBank. All these recombinant isolates shared the same recombination breakpoint.

The serological and molecular typing of PPV isolates from North of Romania showed that PPV-D is the predominant strain, followed by PPV-Rec which shares the CP gene with M strain and, therefore, it is serologically detected as PPV-M with M-specific monoclonal antibodies. In this plum growing area, mixed infections (D+Rec) are also frequent, which might generate additional genetic variations by recombination (Figure 3).

Finally, evidence was provided by the present study for the endemic distribution of PPV-Rec in plum trees grown in the North of Romania.

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