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MOLECULAR AND SEROLOGICAL DIFFERENTIATION OF PLUM POX STRAINS IN THREE PLUM-GROWING AREAS SITUATED IN TRANSYLVANIAN FRUIT CENTRAL AREA, ROMANIA

A. BRICIU*, D. PAMFIL and D. BRICIU
University of Agricultural Sciences and Veterinary Medicine, 3-5 Manastur Street, Cluj-Napoca 400372, Romania

Abstract

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Plum Pox virus (PPV) is the most destructive viral pathogen of stone species and is responsible for a serious loss of production particularly for susceptible varieties. This virus is very dangerous because it reduces the quality and causes the premature fall of fruits. Therefore, PPV is considered one of the main factors that make the plum crop to be considered no longer profitable. The virus has gradually spread to most of Europe, around the Mediterranean basin and Middle East. Also, the virus was found in India and America (Chile, USA and Canada). In Romania, Plum pox virus is prevalent in virtually all areas of the plum crop, causing a significant loss of production particularly for some susceptible varieties.

Ninety PPV isolates were collected from three fruit-growing centers from Transylvania. Molecular strain differentiation was done by RT-PCR analyzed three genomic regions of the virus (Cter)CP, (Cter)Nib-(Nter)CP and CI. With RFLP analysis we could distinguish the two major strains, D and M based on Rsa I polymorphism located in (Cter)CP. Serological analysis were performed using DASI-ELISA technique with PPV-D and PPV-M specific monoclonal antibodies. All PPV isolates typed as PPV-M by molecular and serological analysis in the (C- ter)CP genomic region proved to be recombinants (PPV-REC) between D and M when we make the analysis in (Cter) Nib – (Nter)CP region.

Differentiation and distribution of isolates shows that in Reghin and Cluj regions PPV-D are the predominant strain, with the highest percentage in Reghin (80%) and in Bistrita mixed infections prevail (60%). PPV-Rec strain has the highest percentage in Bistrita and Reghin regions with the same percentage of 13%.

Key words: PPV, virus strains, diagnosis, differentiation, RT-PCR, RFLP

**E-mail: ciprianbriciu@yahoo.com*

Introduction

The European and Mediterranean Plant Protection Organization (EPPO) - 1975 proposed two lists of quarantine viruses of fruit trees. The most important virus, which is the subject to quarantine fruit trees, is Plum Pox. He also is considered a highly dangerous virus by the Inter-African Plants and by the North American Plant Protection and is the subject of some regulations in Australia and USA (Cree, 1999).

So far, seven strains were identified and characterized: D strain (Dideron) isolated for the first time on apricot in Southeastern of France, M strain (Marcus) identified on peach in northern of Greece (Kerlan et al., 1979; Myrta et al., 1998), EA (El Amar) strain described on apricot in Egypt (Wetzel, 1991), SOC (sour cerry) strain detected in Moldova (Kalashyan, 1993), SwC (sweet cerry) strain identified in Italy (Crescenzi, 1996) and PPV-Rec (name proposed and accepted) resulting in recombinant strains of the two major (D and M), the highlight being reported in Albania, Bulgaria, Czech Republic, Germany, Hungary, Slovakia (Glasa et al., 2004) and Romania (Zagrai et. al., 2006, 2008, 2009). The last PPV strain 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). First report on natural infection of plum recombined strain PPV-Rec in Romania was presented in 2006. All isolates initially identified as PPV-M strains were found belonging to PPV-Rec strain following molecular analysis.

Also, following sequencing of the PCR (Polymerase Chain Reaction) corresponding regions (Cter) CP and (Cter) NIB - (Nter) CP proved that nucleotide alignments correspond to those of Gene Bank, reported by Glas et al., in 2002 and 2004 (Zagrai et al., 2006). Genetic similarity with other PPV-Rec strains suggests a common evolutionary origin. (Zagrai et al., 2006; 2008).

Thanks to modern methods of characterization of PPV viral isolates, it is known that two strains are most spread, (PPV-D) and (PPV-M) (Bousalem et al., 1994). PPV-D has a large spread in western of Europe, being non-seed transmitted, difficult to be transmitted to experimental hosts and effectively reduced to be spread with vectors (Nemeth and Koelber, 1983). Unlike PPV-D, transmitted through the seed of necrotic strains (PPV_M) was reported by Nemeth and Koelber in 1983 (Levy et al., 2000). Also can be transmitted easily through afids (Nemeth and Koelber, 1983).

Materials and Methods

PPV isolates

Ninety PPV isolates were collected from three fruit-growing areas from Transylvanian fruit central area. Identification of infections and selection of infected trees has been done on the basis of typical symptoms of PPV and then samples of leaves with obvious symptoms were taken from different parts of the crowns of trees with generalized infection and analyzed with molecular and serological testing.

ARN extraction

For ARN extraction was used the usual procedure, dehydration and tissue crushing them under liquid nitrogen, to a fine powder and tissue resuspendation in a reaction buffer that protects the RNA which is released from cells.

Extraction of total RNA from leaf plum tree was done using the kit extraction Rneasy Plant Mini Kit (Qiagen). Protocol was used so that recommended by the manufacturer and the alleged crossing of ten steps.

Molecular and serological diagnosis

For viral detection were used primers pair P1/P2, which are designed to amplify a fragment of 243 bp region corresponding to C-terminus of the

protein capsid. With Qiagen One-Step RT-PCR kit we could perform both, reverse transcription (RT) and amplification (PCR) in a single reaction. The sequence of primers used in reaction is as follows: P1: 5`-3` ACC GAG ACC ACT ACA CTC CC; P2: 5`-3` CAG ACT ACA GCC TCG CCA GA.

Thermal cycles required for RNA reverstranscription and amplification of DNA fragments were performed in the Eppendorf Mastercycler gradient, optimized as follows: RT- 40 min at 50^0C, activating polymerases- 15 min at 95^0C followed by 35 cycles: denaturation- 1 min at 94^0C, primer annealing – 45 s at 61^0C and DNA elongation – 1 min at 72^0C. DNA amplified was elongated for 10 min at 72^0C.

Amplified products (10 µl + 2 µl loading dye) were fractionated onto 1.4% agarose gel electrophoresis in 1 x TAE buffer. Bands were visualized by ethidium-bromide staining under UV light. Serological diagnosis was made by DASI-ELISA technique using the specific monoclonal antibodies 5B-IVIA.

Molecular and serological differentiation

Molecular strain typing was done by RT-PCR targeting three genomic regions: (Cter) CP- using the pairs of primers P1/PD and P1/PM that distinguish PPV-D and PPV-M; (Cter) Nib/(Nter) CP, using the pair primer mD5/mM3 that detect a natural recombinants between PPV-D and PPV-M strains (PPV-Rec) (Glasa et al., 2004); CI, using CIF/CID and CIF/CIM pair primers to confirm the presence of PPV-Rec (Glasa et al., 2002).

PCR products corresponding to (Cter) CP were analyzed with RFLP in order to distinguish the PPV-D and PPV-M strains based on R*sa* I polymorphism located in this genomic section. Digested products were fractionated onto 8% polyacrylamide gel electrophoresis in 1 x TBE buffer and photographed under UV light.

To identify the serotype level of PPV isolates we performed the serological tests with DASI-ELISA technique using the specific monoclonal antibody 4DG5 for PPV-D (Cambra et al., 1994) and AL for PPV-M (Boscia et al., 1997).

Results and Discussion

Specific primers P1/P2, have confirmed the presence of the virus in the leaves that present clear symptoms of PPV. These primers amplify a 243 bp fragment corresponding to the C-terminus region of the capsid protein (CP).

This pair of primers proved to be very effective in molecular diagnosis in our country (Zagrai et al., 2006) and abroad (Wetzel et al., 1991). The fact that the virus is present in all isolates indicates a massive infection with PPV in the orchard where the samples were collected. Similar results were obtained for the differentiation of PPV isolates by RT-PCR using PD and PM specific primers and by RFLP using Rsa I restriction enzyme which is also useful to distinguish PPV-D and PPV-M strains for Plum pox virus.

From 30 isolates collected from SCDP Cluj, a total of 20 (67%) belong to PPV-D strain, 9 (30%) isolates are mixed infections belonging to D and M strain (PPV-D + PPV-M) and one isolate belongs to PPV-M strain (3%).

Concerning the molecular differentiation of isolates from Reghin, 2 (7%) isolates were identified as mixed infection (PPV-D + PPV-M), 4 (13%) isolates belonging to PPV-M strain and the rest of 24 (80%) are related to PPV-D strain.

The results obtained using samples from SCDP Bistrita are the following: from 30 isolates, a total of eight (27%) belong to PPV-D strain, 18 (60%) are mixed infections, belonging to the both strain, PPV-D and PPV-M, and four isolates belong only to PPV-M strain (13%).

All isolates typed as PPV-M in (Cter) CP region were in fact PPv-Rec in (Cter) Nib- (Nter) CP region. Using specific primers CIF/CID and CIF/CIM, corresponding to helicaze gene, has been observed that in case of recombinant strain PPV-M,

we obtain PCR product with the pair primers CIF / CID (band size is 962pb). This confirms that PPV-Rec is a recombinant strain (Table 1).

The differentiation and distribution of isolates indicates that the predominant strain is PPV-D for Reghin area and Cluj, with the highest percentage being found in Reghin, respectively 80%. Also, in Bistrita mixed infections are predominant with a procentage of 60 %. PPV-Rec strain has a higher percentage in Reghin and Bistrita (13%).

Table 1

Results of molecular and serological detection, differentiation and typing based on different targeted regions of the genome of 90 PPV isolates collected from three fruit-growing centers from Transylvania, Romania

No	Isolated	RT-PCR (P1/P2 and P1/PD or PM)				Target region			PCR-RFLP Rsa I				DASI-ELISA (4DG5 and AL)		
		PPV-poly	PPV-D	PPV-M	PPV-D+M	CP	Nib	CI	PPV-D	PPV-M	PPV-D+M	Alu I	PPV-D	PPV-M	PPV-D+M
1	Cluj 1	+	+	-	-	D	-	D	+	-	-	+	+	-	-
2	Cluj 2	+	+	-	-	D	-	-	+	-	-	+	+	-	-
3	Cluj 3	+	+	-	-	D	-	-	+	-	-	+	+	-	-
4	Cluj 4	+	+	-	-	D	-	D	+	-	-	+	+	-	-
5	Cluj 5	+	+	-	-	D	-	D	+	-	-	+	+	-	-
6	Cluj 6	+	+	-	-	D	-	D	+	-	-	+	+	-	-
7	Cluj 7	+	+	-	-	D	-	D	+	-	-	+	+	-	-
8	Cluj 8	+	+	-	-	D	-	D	+	-	-	+	+	-	-
9	Cluj 9	+	+	-	-	D	-	D	+	-	-	+	+	-	-
10	Cluj 10	+	+	-	-	D	-	D	+	-	-	+	+	-	-
11	Cluj 11	+	+	-	-	D	-	D	+	-	-	+	+	-	-
12	Cluj 12	+	+	-	-	D	-	D	+	-	-	+	+	-	-
13	Cluj 13	+	+	-	-	D	-	D	+	-	-	+	+	-	-
14	Cluj 14	+	+	-	-	D	-	D	+	-	-	+	+	-	-
15	Cluj 15	+	+	-	-	D	-	D	+	-	-	+	+	-	-
16	Cluj 16	+	+	-	-	D	-	D	+	-	-	+	+	-	-
17	Cluj 17	+	+	-	-	D	-	D	+	-	-	+	+	-	-
18	Cluj 18	+	+	-	-	D	-	D	+	-	-	+	+	-	-
19	Cluj 19	+	+	-	-	D	-	-	+	-	-	+	+	-	-
20	Cluj 20	+	+	-	-	D	-	D	+	-	-	+	+	-	-
21	Cluj 21	+	+	+	+	D+M	Rec	-	+	+	+	+	+	-	-
22	Cluj 22	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
23	Cluj 23	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
24	Cluj 24	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
25	Cluj 25	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
26	Cluj 26	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-

continued

Table 1 (continued)

27	Cluj 27	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
28	Cluj 28	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
29	Cluj 28	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
30	Cluj 30	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
31	Uila 1	+	+	-	-	D	-	D	+	-	-	+	+	-	-
32	Uila 2	+	+	-	-	D	-	D	+	-	-	+	+	-	-
33	Uila 3	+	+	-	-	D	-	D	+	-	-	+	+	-	-
34	Uila 4	+	+	-	-	D	-	D	+	-	-	+	+	-	-
35	Uila 5	+	+	-	-	D	-	D	+	-	-	+	+	-	-
36	Reghin 1	+	+	-	-	D	-	D	+	-	-	+	+	-	-
37	Reghin 2	+	+	-	-	D	-	D	+	-	-	+	+	-	-
38	Reghin 3	+	+	-	-	D	-	D	+	-	-	+	+	-	-
39	Reghin 4	+	+	-	-	D	-	D	+	-	-	+	+	-	-
40	Reghin 5	+	+	-	-	D	-	D	+	-	-	+	+	-	-
41	Reghin 6	+	+	+	+	D+M	Rec	D	+	+	+	+	+	+	+
42	Reghin 7	+	+	-	-	D	-	D	+	-	-	+	+	-	-
43	Reghin 8	+	+	-	-	D	-	D	+	-	-	+	+	-	-
44	Reghin 9	+	+	-	-	D	-	D	+	-	-	+	+	-	-
45	Reghin 10	+	+	-	-	D	-	D	+	-	-	+	+	-	-
46	Reghin 21	+	+	-	-	D	-	D	+	-	-	+	+	-	-
47	Reghin 22	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
48	Reghin 23	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
49	Reghin 24	+	+	-	-	D	-	D	+	-	-	+	+	-	-
50	Reghin 25	+	+	-	-	D	-	D	+	-	-	+	+	-	-
51	Reghin 26	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
52	Reghin 27	+	+	-	-	D	-	D	+	-	-	+	+	-	-
53	Reghin 28	+	+	-	-	D	-	D	+	-	-	+	+	-	-
54	Reghin 29	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
55	Reghin 30	+	+	+	+	D+M	Rec	D	+	+	+	+	+	+	+
56	Reghin 31	+	+	-	-	D	-	D	+	-	-	+	+	-	-
57	Reghin 32	+	+	-	-	D	-	D	+	-	-	+	+	-	-
58	Reghin 33	+	+	-	-	D	-	D	+	-	-	+	+	-	-
59	Reghin 34	+	+	-	-	D	-	D	+	-	-	+	+	-	-
60	Reghin 35	+	+	-	-	D	-	D	+	-	-	+	+	-	-
61	Bistrița 1	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
62	Bistrița 2	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
63	Bistrița 3	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
64	Bistrița 4	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
65	Bistrița 5	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
66	Bistrița 6	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-

continued

Table 1 (continued)

67	Bistrița 7	+	+	-	-	D	-	D	+	-	-	+	+	-	-
68	Bistrița 8	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
69	Bistrița 9	+	+	-	-	D	-	D	+	-	-	+	+	-	-
70	Bistrița 10	+	+	-	-	D	-	D	+	-	-	+	+	-	-
71	Bistrița 11	+	+	-	-	D	-	D	+	-	-	+	+	-	-
72	Bistrița 12	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
73	Bistrița 13	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
74	Bistrița 14	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
75	Bistrița 15	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
76	Bistrița 16	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
77	Bistrița 17	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
78	Bistrița 18	+	+	-	-	D	-	D	+	-	-	+	+	-	-
79	Bistrița 19	+	+	+	+	D+M	Rec	D	+	+	+	+	+	-	-
80	Bistrița 20	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
81	Bistrița 21	+	+	-	-	D	-	D	+	-	-	+	+	-	-
82	Bistrița 22	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
83	Bistrița 23	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
84	Bistrița 24	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
85	Bistrița 25	+	+	-	-	D	-	D	+	-	-	+	+	-	-
86	Bistrița 26	+	+	-	-	D	-	D	+	-	-	+	+	-	-
87	Bistrița 27	+	-	+	-	M	Rec	D	-	+	-	+	-	+	-
88	Bistrița 28	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
89	Bistrița 29	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-
90	Bistrița 30	+	+	+	+	D+M	Rec	D	+	+	+	+	-	+	-

Conclusions

Our research allowed obtaining data concerning the serological and molecular variability of Plum pox virus isolates in the three studied regions, namely Cluj, Bistrita and Reghin.

The results provide a very high PPV infection rate and a critical situation for the plum orchards located in the studied regions. The molecular and serological typing of PPV isolates from Transylvanian fruit central area relived that PPV-D is the predominant strain, followed by PPV-REC which share the CP gene with M strain. Mixed infections are also frequent.

Knowing the distribution of virus strains is essential for developing strategies to eradicate or limit PPV impact, given the huge plum crops losses that exist in our country.

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