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Molecular and serological differentiation of Plum pox strains in Transylvanian fruit central area - Mures plum-growing area

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Abstract Plum pox virus (PPV) is the causal agent of sharka disease, which causes severe damage and important economic losses in the stone fruit industry. The disease mainly affects apricot, plum and peach. Sharka is originated from Eastern Europe and was described for the first time around 1915 in Bulgaria [1]. In this study we collected thirty PPV isolates from two plum comercial orchards from Mures plum-growing area. 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.

Key words

PPV strains, RT-PCR, DASI-ELISA, Sharka

Plum Pox virus (PPV) is the most destructive viral pathogen of stone fruits species causing serious loss of production particularly susceptible varieties, that can be 100% disaster. This virus is very dangerous because it reduces the quality and causes premature fall of fruit. Therefore, PPV is considered one of the main factors that make the plum crop is no longer profitable. The virus have gradually spread to most of Europe, around the Mediterranean basin and Middle East. Also, the virus was found in India and America (Chile, USA, Canada) [11].

In Romania, Plum pox virus is prevalent in virtually all areas of the plum crop, causing loss of production particularly in susceptible varieties. [12]. 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[5]. 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 [10,13], EA (El Amar) strain described on apricot in Egypt [15], SOC (sour cerry) strain detected in Moldova [9], SwC (sweet cerry) strain identified in Italy [6] 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[7] and PPV-W detected in Canada[8]. Thanks to modern methods of characterization of PPV viral isolates, it is known that two strains are most spread, (PPV-D) and (PPV-M) [2]. 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 [14]. Unlike PPV-D, transmitted through the seed of necrotic strains (PPV_M) was reported by Nemeth and Koelber in 1983[11]. Also can be transmitted easily through afids. [14].

Materials and Methods

PPV isolates. Thirty PPV Isolates were collected from two plum comercial orchards from Mures plum-growing 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 (infection with PPV was over 20%) and analyzed with molecular testing.

ARN extraction. For ARN extraction was used the usual procedure, deshydration 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 detection.** For viral diagnosis 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 94^0 C, primer annealing – 45 s at 61^0 C and DNA elongation – 1 min at 72^0 C. DNA amplified was elongated for 10 min at 72^0 C. Amplified products (10 µl + 2 µl loading dye) was 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 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 D and M (PPV-Rec) [7]; CI, using Cif/Cid and Cif/Cim pair primers to confirm the presence of PPV-Rec. PCR products corresponding to (Cter) CP were analized with RFLP in order to distinguish the D and 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 [4] and AL for PPV-M[3].

Results and Discussions

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[16] and abroad [15]. 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 in the PPV isolates typing using RT-PCR and DASI-ELISA. RFLP analysis confirmed these results based on the presence of the Rsa polymorphism in PPV-D strain. Thus, from 30 isolates tested, 24 were identified as PPV-D (80%), 4 (13%) as PPV-M and 2 (7%) were mixed infection (D and M strains). 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).

Table 1

Results of molecular and serological typing based on different targeted regions of the PPV genome isolates colected from two plum comercial orchards from Mures plum-growing area

NO.	ISOLATED	DASI-ELISA	RT-PCR			PCR-RFLP
		4DG5/AL	CP P1-PD/PM	Nib mD5/mM3	CI Cif/Cid	Rsa I
0	1	2	3	4	5	6
1	Uila 1	D	D	-	D	D
2	Uila2	D	D	-	D	D
3	Uila 3	D	D	-	D	D
4	Uila 4	D	D	-	D	D
5	Uila 5	D	D	-	D	D
6	Reghin 1	D	D	-	D	D
7	Reghin 2	D	D	-	D	D
8	Reghin 3	D	D	-	D	D
9	Reghin 4	D	D	-	D	D
10	Reghin 5	D	D	-	D	D
11	Reghin 6	D+M	D+M	Rec	D	D+M
12	Reghin 7	D	D	-	D	D
13	Reghin 8	D	D	-	D	D
14	Reghin 9	D	D	-	D	D
15	Reghin 10	D	D	-	D	D

0	1	2	3	4	5	6
16	Reghin 21	D	D	-	D	D
17	Reghin 22	M	M	Rec	D	M
18	Reghin 23	M	M	Rec	D	M
19	Reghin 24	D	D	-	D	D
20	Reghin 25	D	D	-	D	D
21	Reghin 26	M	M	Rec	D	M
22	Reghin 27	D	D	-	D	D
23	Reghin 28	D	D	-	D	D
24	Reghin 29	M	M	Rec	D	M
25	Reghin 30	D+M	D+M	Rec	D	D+M
26	Reghin 31	D	D	-	D	D
27	Reghin 32	D	D	-	D	D
28	Reghin 33	D	D	-	D	D
29	Reghin 34	D	D	-	D	D
30	Reghin 35	D	D	-	D	D

Conclusions

These results highlighted a very high PPV infection rate and a critical situation in plum orchards infected with Plum pox virus in the region we conducted the study.

The molecular and serological typing of PPV isolates from Mures plum-growing area releaved 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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