SEROLOGICAL AND MOLECULAR DETECTION AND DIFFERENTIATION OF PLUM POX VIRUS ISOLATES FROM BISTRIȚA AREA

DETECȚIA ŞI DIFERENȚIEREA SEROLOGICĂ ŞI MOLECULARĂ A IZOLATELOR VIRUSULUI PLUM POX DIN ZONA BISTRIȚA

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Abstract. *Plum pox virus (PPV) is the most dangerous viral pathogen of stone fruits causing serious yield loss in Romanian plum orchard. Different PPV isolates from five experimental plots estate to Fruit Research-Development Station Bistrita were collected and investigated. First, the virus was detected by DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) using polyclonal antibodies and after that by IC-RT-PCR (Immunocapture-Reverse Transcriptase-Polymerase Chain Reaction) using P1/P2 primers that amplify a 243 bp fragment in the C-terminus of the coat protein coding region. Subsequently, PPV strain determination was serologically achieved by TAS (Triple Antibody Sandwich) -ELISA with the PPV-D and PPV-M specific monoclonal antibodies. Molecular strain typing was done by IC-RT-PCR using specific primers PD and PM. All isolates reacted positively to at least one of twomonoclonal antibody. Thus, from 200 isolates tested, 105 (52,5%) were identified as belonging to PPV-D strain, 88 (44%) belonging to PPV-M and 7 (3.5%) to mixed infection. A slight difference was registered between the results obtained by serological and molecular analysis. IC-RT-PCR analysis confirmed the presence of PPV-D in 105 isolates while the PPV-M was identified in 86 isolates. Nine cases reflected a mixed infection involving D and M strain. These results show that PPV strains D and M are naturally present in Bistrița area orchard and their association in a mixed infection can also occur.*

Rezumat. *Virusul Plum pox (PPV) este cel mai periculos patogen viral al speciilor pomicole sâmburoase, cauzând serioase pierderi de producție în livezile de prun din România. Diferite izolate de PPV din cinci loturi experimentale aparținând SCDP Bistrița au fost colectate şi investigate. Inițial, virusul a fost detectat prin DAS-ELISA (Double Antibody Sandwich-Enzime Linked Immunosorbent Assay) utilizând antiseruri policlonale, iar apoi prin IC-RT-PCR (Immunocapture-Reverse Transcriptase-Polymerase Chain Reaction) folosind amorse P1/P2 care flanchează un fragment C-terminal de 243 bp din regiunea genomică ce codifică sinteza proteinei capsidale. Ulterior, s-a efectuat diferențierea serologică prin TAS (Triple Antibody Sandwich)-ELISA utilizând antiseruri monoclonale (PPV-D şi PPV-M) şi moleculară prin IC-RT-PCR folosind primeri specifici (PD şi PM). Toate izolatele au reacționat pozitiv la cel puțin unul dintre cele două antiseruri monoclonale. Astfel, din 200 izolate testate, 105 (52,5%) au fost identificate ca aparținând suşei PPV-D, 88 (44%) aparținând PPV-M şi 7 (3.5%) infecțiilor mixte. O uşoară diferență a fost înregistrată între rezultatele obținute prin analizele serologice şi moleculare. Analizele IC-RT-PCR au confirmat prezența PPV-D la 105 izolate, în timp ce, PPV-M a fost identificat la 86 izolate. In nouă cazuri s-a înregistrat prezența infecțiilor mixte implicând tulpinile D şi M. Aceste rezultate arată că tulpinile D şi M ale virusului plum pox sunt prezente în livezile din zona Bistrița iar asocierea lor în infecții mixte poate fi întâlnită.*

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INTRODUCTION

Plum pox virus (PPV*)* or Sharka is considered one of the most devastating diseases of stone fruit from the point of view of agronomic impact and economic importance (*Nemeth, 1994*). The virus is very detrimental because it reduced significant the quality and produces premature dropping of fruits. Sharka is originated from Eastern Europe and was described for the first time around 1915 in Bulgaria (*Atanasoff, 1932*). Since then, the disease has progressively spread to a large part of the European continent, around the Mediterranean basin and near Middle East. Also, it has been found in India and America (Chile, USA, Canada)

In Romania, Sharka is spread in all plum areas causing serious yield loss especially on sensitive cultivars (*Minoiu, 1997, Zagrai et al, 2001*)

The differentiation of plum pox virus strain is a basic condition to work out the efficient control measures to minimize damages caused by this virus.

The PPV strains can be differentiated biological and epidemiological (symptomatology, aggressiveness, aphid transmissibility) but the accuracy in differentiation is achieved by serological and molecular analysis. Two main groups named PPV-D (Dideron or chlorotic strain) and PPV-M (Marcus or necrotic strain) with a different epidemiological behaviour were serologically established (*Kerlan and Dunez, 1976*). The M strain spread more rapidly than D strain and causes more severe symptoms. The serological differentiation of the two groups can be achieved by using specific monoclonal antibody for PPV-D (*Cambra et al., 1994*) and PPV-M (*Boscia et al., 1997*). The use of the specific primers in PCR (Polymerase Chain Reaction) analysis applied on fragments corresponding to the C-terminal region of the coat protein gene allow a precise differentiation to the two strains (*Olmos et al., 1997*).

MATERIALS AND METHODS

Different PPV isolates were selected initial based on typical symptoms. Two hundred isolates from five experimental plots estate to Fruit Research-Development Station Bistrita were collected from different varieties and investigated by serological and molecular analysis. Leaves from infected plants were used as biological material for study.

First, the virus was serological detected by DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) using polyclonal antibodies and protocol provided by Bioreba. Aliquots of 200 µl of plant extract were applied to the wells.

The next step was the molecular detection by IC-RT-PCR (Immunocapture-Reverse Transcriptase-Polymerase Chain Reaction) using a pair of primers (P1/P2) that amplify a 243 bp fragment located within the C-terminus of the PPV capsid-protein gene (*Wetzel et al., 1991*). Enhanced Avian kit provide by Sigma was used for RT-PCR applied after a preliminary immunocapture of virus particles by PPV polyclonal antibodies adsorbed on an eppendorf microtube. The thermal cycling scheme used was the following: RT reaction - 30 min at 50° C, denaturation / RT inactivation - 2 min at 94° C followed by 35 cycles: template denaturation - 30 s at 94° C, primer annealing - 45 s at 61° C and DNA elongation - 60 s at 72° C. After the last cycle DNA elongation was done for 10 min. at 72° C. In every case, 10 µl aliquots of the amplification products were analyzed by 1.5 % agarose gel electrophoresis in 1 x TBE buffer. Bands were visualized under UV light by ethidium-bromide staining.

Subsequently, PPV strain determination was serologically achieved by TAS (Triple Antibody Sandwich) -ELISA with the PPV-D and PPV-M specific monoclonal antibodies. TAS-ELISA was performed according to the protocol described by *Cambra et al. (2004*) Molecular strain typing was done by IC-RT-PCR using specific primers PD and PM (*Olmos et al., 1997*).

RESULTS AND DISCUSSION

The table data show a good correlation between the results obtained by serological and molecular analysis. Without exception, all isolates reacted positively at polyclonal antibody and to at least one of two monoclonal antibody. Thus, from 200 isolates tested, 105 (52,5%) were identified as belonging to PPV-D strain, 88 (44%) belonging to PPV-M and 7 (3.5%) to mixed infection.

An amplified fragment of the expected size (243 bp) was obtained from all 200 isolates tested and from the positive controls - figure 1. That confirmed the polyvalence of the primers developed by *Wetzel et al. (*1991). A slight difference was registered between the results obtained by TAS-ELISA using monoclonal antibodies (PPV-D and PPV-M) and IC-RT-PCR using specific primers PD and PM. The molecular analysis confirmed the presence of PPV-D in 105 isolates detected by TAS-ELISA while the PPV-M was identified in only 86 isolates. Nine cases reflect a mixed infection involving D and M strain.

Table 1

The detection and differentiation of PPV strain using DAS/TAS -ELISA and IC-RT-PCR

No. plot	No. cultivars and hybrids / No. isolates tested	DAS/TAS-ELISA results (OD = 405nm)				IC-RT-PCR results			
		PPV poly-clonal (%)	PPV-D (%)	PPV-M (%)	PPV-D+M (%)	PPV poly-valent (%)	PPV-D (%)	PPV-M (%)	PPV-D+M (%)
1	14 / 41	41 (100)	30 (73.2)	8 (19.5)	3 (7.3)	41 (100)	30 (73.2)	8 (19.5)	3 (7.3)
2	19 / 51	51 (100)	22 (43.1)	29 (56.9)	0 (0)	51 (100)	22 (43.1)	29 (56.9)	0 (0)
3	5 / 14	14 (100)	5 (35.7)	9 (64.3)	0 (0)	14 (100)	5 (35.7)	9 (64.3)	0 (0)
4	22 / 72	72 (100)	41 (56.9)	28 (38.9)	3 (4.2)	72 (100)	41 (56.9)	26 (36.1)	5 (6.9)
5	6 / 22	22 (100)	7 (31.8)	14 (63.6)	1 (4.5)	22 (100)	7 (31.8)	14 (63.6)	1 (4.5)
	TOTAL	200 (100)	105 (52,5)	88 (44,0)	7 (3,5)	200 (100)	105 (52,5)	86 (43,0)	9 (4,5)

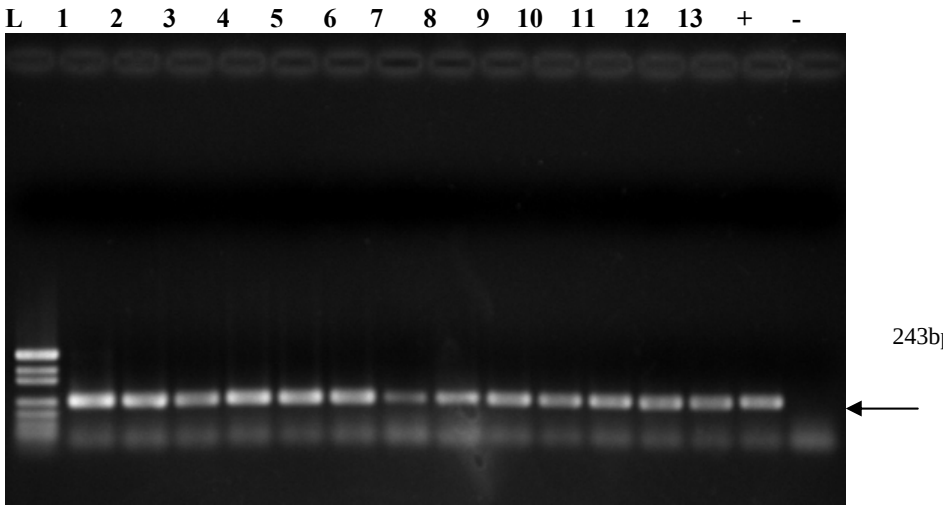
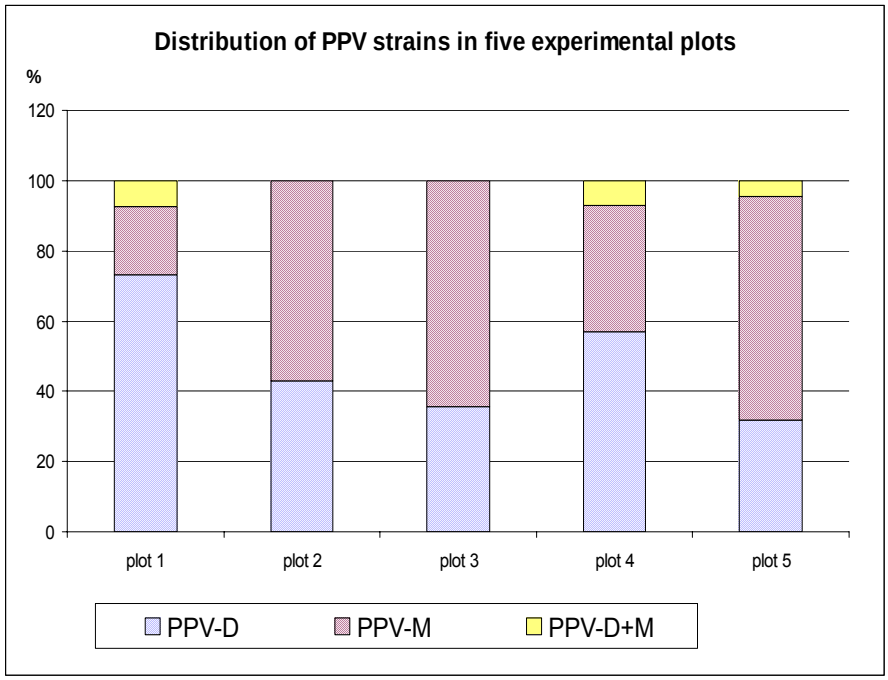


Fig.1. Agarose gel electrophoresis (1.5%) of PCR products: L- DNA Ladder, 1-13 positive samples, + positive control, - negative control

The distribution of the both strains and mixed infections, also, was different from one plot to another figure 2.



We can observe that in the some plots the chlorotic strain is prevalent while in another predominant is necrotic strain. Also, the mixed infections can be present or not. Thus, whether in the 1st and 4th experimental plot the singular infections with PPV-D strain are predominant the situation is vice versa in the 3rd and 5th plot where the prevalent strain is represented by PPV-M. An equilibrium between the two serogroups was registered in the 2nd experimental plot.

The mixed infections involving chlorotic and necrotic strains were found in the 1st, 4th and 5th experimental plot but the frequency was quite reduced.

The results revealed that PPV strains D and M are naturally present in our experimental plots and their association in a mixed infection can also occur.

CONCLUSIONS

- PPV strains D and M are naturally present in Bistrita area and their association in a mixed infection can also occur.
- The distribution of the both strains and mixed infections, also, was different from one plot to another.

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