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RT-PCR EVIDENCE OF THE NON-TRANSMISSION THROUGH SEED OF *PLUM POX VIRUS* STRAINS D AND M

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SUMMARY

Twelve different apricot selections and six peach varieties naturally infected with *Plum pox virus* (PPV) groups D and M, respectively, were used to investigate the role of seeds in the epidemiology of the virus. All plants were PPV-positive in IC-RT-PCR and the isolates were characterized by restriction analysis of amplicons with *Rsa*I restriction and analysis of coat protein migration in 15% SDS-polyacrylamide gel, followed by Western blotting. The presence of PPV was checked on fully ripe seeds, germinating seeds and seedlings. One half of the apricot seed stock was analyzed by ELISA and IC-RT-PCR, separating cotyledons, also containing the embryo, from seed coats. The virus, in both species, was mainly localized in seed coats, but cotyledons were also infected. Seeds of the other half of the stock were germinated and maintained in an insect-proof screenhouse for over 3-years (apricot seedlings) or over 6 months (peach seedlings). Seedlings never showed symptoms and were found PPV-negative by molecular assays. The conclusion was that seeds have no role in PPV-M and PPV-D epidemiology.

Key words: PPV-D, PPV-M, seed transmission, RT-PCR, stone fruits.

INTRODUCTION

Plum pox virus (PPV), one of the most important pathogen of stone fruit trees, causes ‘sharka’ disease of plum, apricot and peach in most European and Mediterranean countries. PPV has also recently been isolated from sour and sweet cherry (Crescenzi *et al.*, 1994; Kalashyan *et al.*, 1994). PPV is a quarantine agent subjected to official phytosanitary measures aiming at preventing entry and spread in countries where it does not occur.

Natural spread of PPV in orchards is due to several aphid species which transmit the virus in a non-persistent manner whereas infected propagative material is responsible for the development of infection foci. Seed transmission of PPV might play an important role in dissemination especially through breeding programmes, international exchange of germplasm and use of seedlings as rootstocks. Available information on this means of transmission is controversial. Seed transmission was first reported in apricot from Hungary (Szirmai, 1961) and in plum and peach from Romania (Savulescu and Macovei, 1965; Coman and Cociu, 1976). Later a percentage varying from 3.4 to 13.9% of infected apricot seedlings, originating from different diseased cultivars, was recorded from Hungary (Nemeth and Kolber, 1983).

These results were not confirmed by subsequent studies in which serological tests (ELISA) revealed the presence of the virus in the coat and cotyledons, comprising embryonic tissues, of seeds collected from infected apricot, plum and peach trees, but never in seedlings from infected seeds (Schimanski *et al.*, 1988; Eynard *et al.*, 1991; Triolo *et al.*, 1993; Dulic-Markovic and Rankovic, 1997; Myrta *et al.*, 1998; Pasquini *et al.*, 1998).

Based on serological, molecular and biological properties, PPV isolates and strains can be classified in four groups (D, M, EA and C) (Pasquini and Barba, 1997) characterized by differences in severity on the natural hosts, geographical distribution and latency period. These differences might have had a bearing on the contrasting results obtained with seed-transmission studies.

The advent of molecular techniques (*e.g.* RT-PCR) has improved PPV diagnosis, as they are more sensitive than serological tests and can detect very low virus concentrations in plant tissues (Wetzel *et al.*, 1992; Kolber *et al.*, 1997; Pasquini *et al.*, 1998; Varveri and Boutsika, 1998).

The problem of PPV seed transmission was therefore addressed once more by testing, serologically and molecularly, seeds and seedlings from apricot and peach sources infected with the two most representative PPV isolates (PPV-D, more common in apricot and plum, and PPV-M in peach).

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Seedlings were grown in an insect-proof screen-house to prevent contamination by viruliferous aphids and tested repeatedly for several years to exclude the possibility of a long latency period of PPV infections.

MATERIALS AND METHODS

Source of PPV-infected seeds. Ripe fruits were harvested from twelve apricot selections, from a germplasm collection, and six peach varieties grown in Veneto (Northern Italy). The presence of PPV in the mother plants was ascertained by IC-RT-PCR. Most of the apricot seeds showed yellow rings typically induced by PPV.

Characterization of PPV isolates. The PPV isolates were characterized by restriction analysis of amplicons, using *Rsa*I (Wetzel *et al.*, 1992) and by 15% SDS-polyacrilamide gel electrophoresis, followed by Western blotting with the monoclonal antibody Ispave MAb 11B7C4 (Pasquini and Barba, 1994).

Seed stratification and growing conditions. All collected seeds (more than 1000) where split into two groups; the first was immediately analyzed for the presence of PPV in cotyledons (also containing the embryo) and in seed coats, whereas the second group was stored in sand at 4°C for two months, prior to germination.

Half germinated seeds were tested, sampling separately cotyledons, plumule and radicle. The remaining germinated seeds where potted, maintained under an insect-proof screen-house for 3-years, periodically checked for the presence of leaf symptoms, and tested by IC-RT-PCR every six months. Peach seedlings were analyzed until 6-months after germination.

PPV detection. All apricot seeds were analyzed serologically (ELISA) and molecularly (IC-RT-PCR), whereas peach seeds, germinated seeds and seedlings were checked only by molecular assays.

Indirect ELISA was performed using, as second antibody, a monoclonal (Ispave MAb 11B7C4) that recognizes PPV-D and PPV-M (Pasquini and Barba, 1994).

IC-RT-PCR was done by capturing the virus from fresh leaf samples, ground 1:10 (w:v) in extraction buffer (PBS-T containing 2% PVP K25 and 20 mM sodium diethyl dithiocarbamate), in 0.5 ml microfuge tubes pre-coated with a polyclonal anti-PPV antiserum (Ispave 21). Viral RNA, released into the tubes, was submitted to a 'one tube-one step' RT-PCR protocol using universal PPV primers directed against the C-terminal region of the coat protein gene region (Candresse *et al.*, 1995). Briefly, 50 µl of RT-PCR mixture containing: 67 mM Tris-HCl pH 8.8, 17 mM $(NH_4)_2SO_4$, 0.2 mg ml^{-1} gelatin, 6 µM EDTA, 16 mM mercaptoethanol, 0.1 µg of each primer, 1.5 mM $MgCl_2$, 125 µM dNTPs (each), 2.5 U AMV-RT (Amersham), 1.5 U Taq (Promega) and 20 U of RNase OUT (Gibco) was added to washed capture tubes. cDNA synthesis and amplification were carried out in a Perkin Elmer thermal cycler at 46°C for 30 min, followed by denaturation at 95°C for 3 min and 35 cycles of amplification (94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec). After amplification, 10 µl of PCR products was analyzed by electrophoresis on a 1% agarose gel in TBE buffer (89 mM Tris; 89 mM boric acid and 2 mM EDTA), stained with ethidium bromide and finally visualized under UV light.

RESULTS

Characterization of PPV isolates. The characterization of PPV isolates from infected trees by molecular and serological methods showed that all isolates infecting apricot belonged to PPV-D, whereas all peach samples were infected by PPV-M (Table 1). Amplicons of PPV isolates from apricot contained the recognition site *Rsa*I (GTAC) specific to PPV-D, which did not occur in amplicons from peach isolates.

This strain classification was confirmed by a different coat protein (cp) migration rate in SDS-PAGE; the cp of all apricot isolates showed an apparent M_r of 36 kDa whereas the cp of peach isolates showed an apparent M_r of 38 kDa, corresponding, respectively, to PPV-D and PPV-M groups (Adamolle, 1993; Pasquini and Barba, 1994).

Analysis of ripe seeds. About 500 ripe seeds were analyzed for the presence of PPV in teguments and cotyledons.

The possibility to detect the virus differed according to the method and the tissue examined. Serological and molecular assays revealed the presence of PPV in apricot seed coats, whereas ELISA was not able to detect PPV in cotyledons even if the presence of the virus was successfully detected in the same extracts by RT-PCR, up to a percentage of 100% infection (489-I-XXIV-66 and 491-M-XXIII-54 selections).

Because of the failure of serological detection, seeds collected from infected peach trees the following year were analyzed only by IC-RT-PCR.

The results (Tables 2 and 3) showed that PPV is mainly localized in seed coats (69.9% of PPV-D infected seeds tested and 53.2% of PPV-M infected seeds tested), but was also present in cotyledons, but with a lower rate of infection (34.8% of PPV-D infected seeds and 10.6% of PPV-M infected seeds).

Table 1. Characterization of PPV isolates from apricot and peach by RFLP analysis of amplicons and SDS-PAGE coat protein migration.

Species	Accession number	RFLP *Rsa*I site	CP size (kDa)	PPV isolate
Apricot	489-I-XXIV-66	+	36	D
Apricot	489-G-XIII-83	+	36	D
Apricot	489-G-XI-17	+	36	D
Apricot	489-I-XXIV-5	+	36	D
Apricot	491-M-XXIII-54	+	36	D
Apricot	491-M-XXIV-86	+	36	D
Apricot	489-I-XXIII-138	+	36	D
Apricot	489-G-XVI-63	+	36	D
Apricot	489-I-XXIV-6	+	36	D
Apricot	489-I-XXV-117	+	36	D
Apricot	489-G-XIII-82	+	36	D
Apricot	489-I-XXIII-130	+	36	D
Peach	Rich Lady	–	38	M
Peach	Royal Gem	–	38	M
Peach	Royal Glory	–	38	M
Peach	Elegant Lady	–	38	M
Peach	Rubycrest	–	38	M
Peach	Maria Luisa	–	38	M

Notwithstanding the presence of clear-cut symptoms in the mother trees, two groups of seeds, belonging to two different apricot selections (489-I-XXIII-138 and 489-I-XXIII-82), were PPV-negative when analyzed by PCR. All the other apricot selections tested showed different levels of infection: between 51.8% (489-G-XIII-130) and 100% (489-I-XXIV-66 and 491-M-XXIII-54) in teguments and between 10.7% (489-I-XXV-17) and 100% (489-I-XXIV-66 and 491-M-XXIII-54) in cotyledons.

Peach varieties showed a percentage of seed coat infection ranging from 25.0% ('Maria Luisa') to 61.5% ('Rubycrest') and a percentage of cotyledon infection from 0 ('Royal Gem') to 15.3% ('Royal Glory').

Analysis of germinated seeds. Cotyledons, plumule and radicle, tested separately by IC-RT-PCR, showed different percentages of PPV infection. The virus was still present in cotyledons but not in plumules and radicles. Storage at 4°C of peach and apricot seeds may have affected virus survival as indicated by the fact that the percentage of infected cotyledons decreased (Table 4) when compared with the values obtained from cotyledons of the same variety tested immediately after harvesting.

Table 2. Results of ELISA and IC-RT-PCR for PPV-D detection in apricot seeds.

Selection	Seeds no.	ELISA				IC-PCR			
		Infected seed coats		Infected cotyledons		Infected seed coats		Infected cotyledons	
		no.	%	no.	%	no.	%	no.	%
489-I-XXIV-66	25	25	100.0	0	0	25	100.0	25	100.0
489-G-XIII-83	32	24	75.0	0	0	28	87.5	8	25.0
489-G-XI-17	27	27	100.0	0	0	27	100.0	11	40.7
489-I-XXIV-5	32	21	65.6	0	0	24	75.0	7	21.8
491-M-XXIII-54	20	20	100.0	0	0	20	100.0	20	100.0
491-M-XXIV-86	52	43	82.6	0	0	44	84.6	18	34.6
489-I-XXIII-138	27	0	0.0	0	0	0	0.0	0	0.0
489-G-XVI-63	20	10	50.0	0	0	14	70.0	5	25.0
489-I-XXIV-6	35	26	74.2	0	0	31	88.5	21	60.0
489-I-XXV-117	28	19	67.8	0	0	19	67.8	3	10.7
489-G-XIII-82	27	14	51.8	0	0	14	51.8	5	18.5
489-I-XXIII-130	28	0	0.0	0	0	0	0.0	0	0.0

Table 3. Results of IC-RT-PCR for PPV-M detection in peach seeds.

Variety	Seeds no.	IC-RT-PCR			
		Infected seed coats		Infected cotyledons	
		no.	%	no.	%
Rich Lady	17	10	58.8	2	11.7
Royal Gem	16	8	50.0	0	0.0
Royal Glory	26	12	46.1	4	15.3
Elegant Lady	46	26	56.5	5	10.8
Rubycrest	13	8	61.5	1	7.6
Maria Luisa	4	1	25.0	1	25.0

Analysis of seedlings. During a 3-year period apricot seedlings never showed symptoms of infection and were PPV-negative in all IC-RT-PCR tests performed every six months. Likewise, after six months of maintenance in an insect-proof screen-house, all peach seedlings were negative for the presence of PPV by IC-RT-PCR (Table 5).

DISCUSSION

Ripe seeds collected from apricot and peach showed a high percentage of PPV infection. D and M strains did not show significant differences regarding distribution in seed tissues and percentage of infection. This may be taken as an indication that the ability to infect seeds does not depend on virus strain.

Table 4. PPV detection in germinating apricot and peach seeds.

Species	Accession number	Seedlings no.	Infected cotyledons		Plumule no.	Radicle no.
			no.	%		
Apricot	489-I-XXIV-66	15	10	66.6	0	0
Apricot	489-G-XIII-83	21	3	14.2	0	0
Apricot	489-G-XI-17	12	4	33.3	0	0
Apricot	489-I-XXIV-5	20	3	15.0	0	0
Apricot	491-M-XXIII-54	9	6	66.6	0	0
Apricot	491-M-XXIV-86	32	11	34.3	0	0
Apricot	489-I-XXIII-138	21	0	0.0	0	0
Apricot	489-G-XVI-63	15	2	13.3	0	0
Apricot	489-I-XXIV-6	18	8	44.4	0	0
Apricot	489-I-XXV-117	16	0	0.0	0	0
Apricot	489-G-XIII-82	16	2	12.5	0	0
Apricot	489-I-XXIII-130	20	0	0.0	0	0
Peach	Rich Lady	4	0	0.0	0	0
Peach	Royal Gem	4	0	0.0	0	0
Peach	Royal Glory	10	1	10.0	0	0
Peach	Elegant Lady	35	3	8.5	0	0
Peach	Rubycrest	6	1	16.6	0	0
Peach	Maria Luisa	2	1	50.0	0	0

Table 5. Results of IC-RT-PCR for PPV detection in apricot and peach seedlings.

Species	Accession number	Seedlings no.	RT-PCR positive no.
Apricot	489-I-XXIV-66	25	0
Apricot	489-G-XIII-83	32	0
Apricot	489-G-XI-17	27	0
Apricot	489-I-XXIV-5	32	0
Apricot	491-M-XXIII-54	20	0
Apricot	491-M-XXIV-86	52	0
Apricot	489-I-XXIII-138	27	0
Apricot	489-G-XVI-63	20	0
Apricot	489-I-XXIV-6	35	0
Apricot	489-I-XXV-117	28	0
Apricot	489-G-XIII-82	27	0
Apricot	489-I-XXIII-130	28	0
Peach	Rich Lady	4	0
Peach	Royal Gem	4	0
Peach	Royal Glory	10	0
Peach	Elegant Lady	35	0
Peach	Rubycrest	6	0
Peach	Maria Luisa	2	0

The percentage of infected apricot cotyledon tissue was higher than that reported from other countries (Szirmai, 1961; Savulescu and Macovei, 1965; Coman and Cociu, 1976). This may be because we used RT-PCR, almost certainly more sensitive than ELISA in revealing PPV in seed tissues (Wetzel *et al.*, 1992; Kolber *et al.*, 1997; Pasquini *et al.*, 1998; Varveri and Boutsika, 1998).

The response to infectivity seems to be related to genetic factors, since not all peach varieties and apricot selections reacted in the same way. It is interesting to note that some apricot accessions showed 100% infection both in seed coat and cotyledons, whereas two selections had no apparent seed infection.

The analysis of germinating seeds showed that, during germination, the virus remains confined to reserve tissues and does not replicate in meristem. The percentage of infected cotyledons was less than in ungerminated seeds, probably because the virus begins to suffer physical breakdown (Eynard *et al.*, 1991) and its concentration declines.

Six month-old peach seedlings were PPV-negative by molecular tests and three year-old apricot seedlings, analyzed every six months, never showed positive reactions. Since our molecular assays reveal the presence of very low amounts of virus in green tissues, especially when no symptoms are shown by the plants (Faggioli *et al.*, 1999), we can exclude the occurrence of a long latency period reported for PPV-D (Conti, 1993).

Different PPV strains did not show any difference in seedling infection, indicating that the controversial results on seed transmission of PPV are not due to biological differences between PPV-D and PPV-M.

Even though the number of peach seeds was small, we conclude that seed transmission plays no role in PPV-M and PPV-D epidemiology.

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