Bulgarian Journal of Agricultural Science, 22 (No 2) 2016, 267–271
Agricultural Academy

NON-TRANSMISSION OF *PLUM POX VIRUS* THROUGH SEEDS OF MYROBALAN AND APRICOT

I. KAMENOVA
Agricultural Academy, AgroBioInstitute, BG-1164 Sofia, Bulgaria

Abstract

KAMENOVA, I., 2016. Non-transmission of *plum pox virus* through seeds of myrobalan and apricot. *Bulg. J. Agric. Sci.*, 22: 267–271

Seeds from wild-grown myrobalan (*Prunus cerasifera* Ehrh.) infected with PPV-D strain and from apricot (*P. armeniaca*) infected with PPV-Rec strain were investigated for their potential role in virus transmission. The presence of PPV was checked in fully ripe seeds, geminated seeds and seedlings. While the results obtained by DASI-ELISA tests of whole seeds and separated seed coats from myrobalan were negative for PPV, IC-RT-PCR showed virus presence in forty six and forty seven samples out of 50 tested, respectively. Both DASI-ELISA and IC-RT-PCR detected PPV in the whole seeds and seed coats from apricot displaying pale spots and/or rings on the stones and did not detect the virus in the seeds coming from stones without symptoms. The virus was identified in the cotyledons containing the embryo only by IC-RT-PCR and only in apricot seeds showing symptoms on the stone. Seedlings investigated for a period of two years never showed symptoms and were found PPV-negative, both by DASI-ELISA and IC-RT-PCR. On the base of the results obtained it can be concluded that the seeds from *P. cerasifera* Ehth. infected with PPV-D and from apricot infected with PPV-Rec strain do not transmit the virus.

Key words: serological, molecular, assays, seed coats, embryo

Introduction

Plum pox virus (PPV) the causal agent of Sharka disease (Atanasoff, 1932/1933) belongs in the Potyvirus group (Anonymous, 1974; Nemeth, 1986). It is spread in a non-persistent manner by more than twenty seven aphid species (Homoptera: Aphididae) and by vegetative propagation of infected material. Seed transmission could provide another means of spread and it is important from epidemiological point of view.

Up to now the reports on the seed transmissibility of PPV are contradictory. The earliest assays performed in Hungary (Szirmai, 1961) and Romania (Coman and Cocio, 1976; Savulesku and Mackovei, 1965) by the use of indicator plants have shown transmission of PPV by seeds. Based on the results obtained by ELISA method Nemeth and Kölber (1983) have shown from 3.4% to 13.9% infection of apricot seedlings obtained from different diseased cultivars. These results have not been confirmed later when ELISA method with the use of polyclonal and monoclonal antibodies and RT-PCR technique have been applied. Presently the reports for non-transmission of PPV through seeds are prevalent (Dulic-Markovic and Rankovoc, 1996; Glasa et al., 1999; Milusheva et al., 2008; Myrta et al., 1998; Pasquini et al., 1998, 2000; Schimanski et al., 1988; Triolo et al., 1993; Zagrai and Zagrai, 2008).

In this study, DASI-ELISA and IC-RT-PCR techniques were used to test the presence of PPV in seeds and seedlings from wild-grown myrobalan (*P. cerasifera* Ehth.) and apricot (P. *armeniaca*) trees infected with PPV-D and PPV-Rec strains, respectively.

**E-mail:* vkamenova@abi.bg; ivanka.kamenova@yahoo.com

Materials and Methods

Source of PPV-infected seeds

One hundred and fifty seeds from wild-grown myrobalan (*P. cerasifera* Ehth.) and seventy seeds from apricot tree grown in a courtyard in the region of Sofia were collected from fully ripe fruits. No symptoms on the stones of myrobalan were observed and the symptoms on the leaves consisted of slight light green lines, rings and spots. Sixty stones out of collected seventy from apricot showed pale rings typically induced by PPV, while the other 10 stones were without any symptoms.

The presence of PPV infection in mother plants was ascertained by double antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) test and the use of universal monoclonal antibody (MAb) 5B according Cambra et al. (1994). Strain identification of the isolates was performed by DASI-ELISA test and MAbs AL and 4DG5 specific for PPV-M and PPV-D strains, respectively followed by immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) and mD5/mM3, mM5/mM3 and mD5/mD3 primers specific for PPV-Rec PPV-M and PPV-D strains against (Cter)NIb-(Nter)CP region (Šubr et al., 2004).

Seed stratification and growing conditions

Fifty whole seeds from myrobalan and twenty from apricot were analyzed immediately after their separation from the ripe fruits.

The other seeds (100 from myrobalan and 50 from apricot) were stratified in humid sand at 4°C for 4 months (Stokes, 1965). After this period the seeds were immersed in distilled water (dH_20) for 1 night and than in a solution of hydrogen peroxide diluted in dH_20 at a ratio of 1:9 (v/v). Following break of the stones the nuts were placed on wet filter paper at 4°C (refrigerator) for about 20–25 days. After the germination of the seeds they were processed as follows:

- The coats of fifty seeds from myrobalan were separated for analyses and these seeds (without coats) together with other fifty geminated seeds were planted separately in pots in an isolated room (protected from insects) in a greenhouse.
- Twenty apricot seeds with typical PPV symptoms and 10 seeds without symptoms on the stone were tested, sampling separately seed coats and cotyledons containing the embryo. Additionally only the coats of 10 more seeds (with symptoms on the stone) were also tested.
- Twenty germinated seeds from apricot were planted directly in pots and grown for two years in a greenhouse.

All seedlings obtained (80 from myrobalan and 20 from apricot) were observed visually for the presence of symptoms in the course of two years. Two to three leaves (depending on the development of the respective seedling) from the top, middle, and bottom part of each one were collected. In total 100 samples were tested serologically and molecularly during the first year. In the second year of the experiment each sample was serologically analyzed again individually, while for IC-RT-PCR analyses five samples were pooled together.

PPV detection

One and same sample from the whole seeds, the different parts (seed coats, cotyledons with the embryo) and seedlings was tested serologically (DASI-ELISA with MAb5B) and molecularly (IC-RT-PCR) with the universal primers P1/P2 (Wetzel et al., 1992).

Controls of sample buffer only (water in IC-RT-PCR), leaves from infected and uninfected with PPV *N. benthamiana* plants were included in the assays. In DASI-ELISA test absorbance at 405 nm was measured with a Micro Plate reader (LKB) about 60 to 90 min after the addition of the substrate (*p*-nitrophenyl phosphate). A threshold value for positive samples was set at two to three times the value of uninfected control.

cDNA synthesis and amplification were carried out on a thermal cycler (Quanta Biotech) at 50°C for 30 min, followed by denaturation at 94°C for 5 min and 35 cycles of amplification (94°C for 30 s, 65°C for 30 s, 72°C for 30 s). After amplification, 10µl of PCR products was analyzed by electrophoresis on 1.5% agarose gel in TAE buffer (40 mM Tris, 2 mM EDTA и 20 mM acetic acid, pH 8.4) in the presence of GelRed™ dye (stock solution x10 000, Biotium). DNA bands were visualized using UV transllluminator (EC 3 Imaging System).

Results and Discussion

The characterization of PPV isolates from infected myrobalan and apricot trees by DASI-ELISA method showed PPV-D strain in myrobalan samples (leaves and fruits) and PPV-M strain in apricot (leaves and fruits), while IC-RT-PCR analyses confirmed PPV-D in myrobalan but determined the presence of PPV-Rec strain in apricot (PPV-M and PPV-Rec strains are serologically identical).

Virus detection in the whole seeds and/or its different parts (seed coats, cotyledons with the embryo) was dependent on the method applied and tested tissues. The results from serological and molecular assays are summarized in Table 1.

Table 1

Results from serological and molecular analyses of seeds

Fruit specie/Tissue	Detection method	
	DASI-ELISA	IC-RT-PCR
Myrobalan (*P. cerasifera* Ehth).		
whole seeds	0[a]/50[b]	46[a]/50[b]
seed coats	4/50	47/50
seedlings	0/80	0/80
Apricot (*P. armeniaca*)		
whole seeds[c]	17/20	20/20
seed coats[c]	10/10	10/10
seed coats[c]	18/20	18/20
cotyledons with embryo[c]	0/20	18/20
seed coatsd	0/10	0/10
cotyledons with embryo[d]	0/10	0/10
seedlings	0/20	0/20

Legend: a = number infected, b = total number, c = stones with symptoms, d = stones without symptoms

Notwithstanding the absence of any symptoms on myrobalan stones and negative extinction values of DASI-ELISA test (0.026–0.061 at 1.416 and 0.053 values for positive and negative controls, respectively) IC-RT-PCR analysis demonstrated virus presence in 46 out of tested 50 whole seeds.

Unlike myrobalan seeds, seventeen whole apricot seed out of tested 20 ones, all with the typical for PPV rings reacted positive in DASI-ELISA test (extinction values between 0.113 and 0.971). IC-RT-PCR, however, showed virus presence in all tested 20 apricot seeds.

Using DASI-ELISA PPV was detected in the coats of only four seeds from myrobalan, while IC-RT-PCR analyses proved its presence in forty seven out of fifty tested. Both DASI-ELISA and IC-RT-PCR showed infection in the coats of 28 out 30 tested apricot seeds with pale rings on the stone, while no virus was detected in the coats of the apricot seeds without any symptoms on the stone.

DASI-ELISA results showed no PPV presence in the cotyledons with the embryo, both in all testes 30 apricot seeds (20 with rings on the stone and 10 without rings). Subsequent IC-RT-PCR analysis, however, found PPV in 18 out of 20 tested cotyledons with the embryo in the seeds coming from stones with symptoms on them and proved its absence in the cotyledons in seeds without symptoms on the stone.

Not even a single seedling showed any symptoms of PPV during the two years of experimental period. In all 80 myrobalan and 20 apricot seedlings DASI-ELISA and IC-RT-PCR did not detect PPV, although the analyses were performed twice in the period of two years.

In this study seeds from myrobalan infected with PPV-D strain and from apricot infected with PPV-Rec strain were analyzed serologically and molecularly to check their ability to transfer the virus into the progeny seedlings. Nevertheless, PPV was present in the whole seeds and in the coats, both of myrobalan and apricot, as well as in the cotyledons with the embryo in apricot seeds the infection was not transferred into the seedlings thus indication non-transmission of PPV through the seeds. Similarly, evidence for non-transmission of PPV by seed in infected peach, apricot, plum and myrobalan trees has been reported by Jordovoc (1963), Dulic-Markovoc and Rankovic (1997), Eynard et al. (1991), Glasa et al. (1999), Milusheva et al. (2008), Myrta et al. (1998), Pasquini et al. (2000), Schimanski et al. (1988), Thomidis and Karajiannis (2003), Triolo et al. (1993), Zagrai and Zagrai (2008). By the use of immunosobent electron microscopy Eynard et al. (1991) have observed the absence of intact PPV particles in the seeds of two apricot cultivars and on that base have raised the hypothesis for a particle-breakdown process in the mature seeds. Like the above mentioned authors Triolo et al. (1993) have suggested the existence of a natural process of inactivation of virus particles during seed germination. Proteolytic activity preventing virus spread and multiplication in the meristems and embryonic tissues (Solomon, 1989) or the physiological changes during seed maturation (Johanses et al., 1994) are the other possible virus inactivation mechanisms.

PPV presence in the whole seeds and in the coats of myrobalan and also in the cotyledons with the embryo in apricot seeds was proved molecularly but not serologically reflecting the higher sensitivity of IC-RT-PCR for virus detection in the cases of very low virus concentration. This finding is in agreement with the reported by Glasa et al. (1999) results for PPV absence in any part of seeds of several plum cultivars and myrobalan (*P. cerasifera* Ehth.) when tested by DAS-ELISA and its presence in the coats of the seeds when tested by RT-PCR. Analyzing a large number (500) of apricot seeds Pasquini et al. (2000) have reported that while in the seed coat PPV has been identified successfully both by DASI-ELISA and IC-RT-PCR methods its detection in cotyledons with the embryo has been possible only by IC-RT-PCR (up to 100%). Using ELISA method Myrta et al. (1998) were not able to detect the virus in the cotyledons of three apricot and one plum cultivars, while only by ELISA test Milusheva et al. (2008) have reported 5% and 23% infection in the cotyledons in one apricot and one plum cultivars, respectively. In investigations for transmission of PPV through the seeds of twelve in-

fected apricot and six peach cultivars Pasquini et al. (2000) have recorded different levels of infection in the cotyledons ranging from 0 to 15.5% and from 10.7% to 100% of peach and apricot cultivars, respectively, indicating that the different response of the seeds to infectivity could be related to some genetic factors.

The results obtained do not allow drawing of any distinction in the distribution of PPV-D and PPV-Rec strains in the different part of the seeds, since the experiments with myrobalan seeds were limited to virus detection in only whole seeds and the coats without tests of the cotyledons. In experiments with seeds of apricot infected with PPV-D strain and seeds of peach infected with PPV-M strain Pasquini et al. (1998, 2000) did not establish differences between these two strains, both in the percentage of contamination of the seeds and in their presence in the various tissues of the seed. Based on that, the authors have suggested that the possibility of infection of the seeds is not dependent on the virus strain and could be connected with some genetic factors associated with the host.

Despite the vast number of reports encompassing different hosts, virus strains and geographical areas proving non-transmission of PPV from mother plants to progeny seedlings, recently Slovakova et al. (2002) have recorded PPV infection (detected only by DAS-ELISA with polyclonal antibodies) in several apricot seedlings of cv. V-66052 grown from seeds infected with PPV-M strain. This latest report for seed transmission of PPV still require confirmation as it has been indicated by the authors due to the frequent cross-reaction of some polyclonal antibodies in ELISA tests. According to Pasquini and Barba (2006) hypothetically, the only possibility of seed transmission of PPV would arise from a mutation in the helper component of the virus, associated with high susceptibility of the infected *Prunus* cultivar.

Conclusion

There is no evidence for virus transmission through the seeds of myrobalan and apricot infected with PPV-D and PPV-Rec strains, respectively. The finding of non-transmission of PPV through myrobalan seeds is important from a practical point of view since it allows the use of seed rootstocks of this specie. The well known uneven distribution of PPV in infected trees together with its frequent concentration at very low levels, require the use of PCR analyses when the potential spread of this pathogen by seeds is studied. The results obtained proved also Sharka diagnostic value of the pale spots/rings observed on the stones of infected apricot fruits.

References

Anonymous, 1994**.** Progrès rèalizès dans la connaissance de la Sharka. *EPPO Bulletin***, 4:** 125.

Atanasoff, D., 1932/1933. Plum pox. A new virus disease. In: Yearbook Faculty Agricultural University, Sofia, **11**: 49–69.

Cambra, M., M. Asensio, M. T. Corris, E. Perez, E. Camarasa, J. A. Garcia, J. J. Moya, D. Lopes-Abella, C. Vela and A. Sanz, 1994. Detection of *plum pox* potyvirus using monoclonal antibodies to structural and non-structural proteins. *Bulletin OEPP/EPPO,* **24:** 569–577.

Coman, T. and V. Cociu, 1976. Transmission de la sharka par le pollen et par les graines. *Bulletin d'Information Sharka*, **2**: 15–21.

Dulic-Markovic, I. and M. Rankovic, 1997. An experiment with *plum pox viru*s transmission by apricot and peach seeds. In Proceedings of Middle European Meeting 96'on Plum Pox, Budapest, pp: 117–119.

Eynard, A., P. Roggero, R., Lenzi, M. Conti and R. G. Milne, 1991. Test for pollen and seed transmission of *plum pox virus* (Sharka) in two apricot cultivars. *Advances in Horticultural Science,* **3**: 104–106.

Glasa, M., I. Hrinovsky and O. Kudela, 1999. Evidence for non-transmission of *plum pox virus* by seeds in infected plum and myrobalan. *Biologia,* **54**: 481–484.

Johansen, E., C. M. Edwards and O. R. Hampton, 1994. Seed transmission of viruses. Current perspectives. *Annual Review of Phytopathology***, 32:** 363–368.

Jordović, M.,1963. Investigation of the spead and some factors of spreading *plum pox virus*. *Phytopathologia Mediterranea*, **2** (3): 167–170.

Milusheva, S., P. Gercheva, V. Bozhkova and I. Kamenova, 2008. Experiments on transmission of *plum pox virus* through *Prunus* seeds. *Journal of Plant Pathology,* **90** (Suppl.): S1.23 – S1.26.

Myrta, A., B. Di Terlizzi and V. Savino, 1998. Study of the transmission of *plum pox* potyvirus through seeds. *Phytopathology Mediterranea,* **37**: 41–44.

Nemeth, M., 1986. Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees. *Akademiai Kiado*, Budapest, Hungary.

Nemeth, M. and N. Kölber, 1983: Additional evidence on seed transmission of *plum pox virus* in apricot, peach and plum proved by ELISA. *Acta Horticulturae,* **130:** 293–300.

Pasquini, G. and M. Barba, 2006. The question of seed transmissibility of *plum pox virus. Bulletin OEPP/EPPO,* **36**: 287–292.

Pasquini, G., A. M. Simeone, L. Conte and M. Barba, 1998. Detection of *plum pox virus* in apricot seeds. *Acta Virologica,* **42**: 260–263.

Pasquini, G., A. M. Simeone, L. Conte and M. Barba, 2000. RT-PCR evidence of non-transmission through seeds of *plum pox virus* strains D and M. *Journal of Plant Pathology,* **82**: 221–226.

Savulescu, A. and A. Makovei, 1965. Studies on the sharka (plum pox) virus and related line pattern virus. *Šastita Bilja,* 1**6**: 357–366.

Schimanski, H. H., M. Gruntzig and E. Fuchs, 1988. No transmission of the *plum pox virus* in plum and apricot seed source clones. *Zentalbl. Microbiol*., **143**: 121–123.

Slovakova, L., A. Danadova and O. Vanavicova, 2002. Possibility of *plum pox* transmission by seeds of some stone fruit trees in Slovakia. *Plant's Health,* **6**: 67–70.

Solomon, R., 1989. A possible mechanism for exclusion of plant viruses from embryonic and meristematic tissues**.** *Research Virology***, 140:** 453–460.

Stokes, P., 1965. Temparature and seed dormancy. In W. Ruhland (Ed.) Encyclopedia of Plant Physiology, Vol. 15, part 2, *Spinger, Berlin*, Germany, pp: 746–803.

Šubr, Z., S. Pittnerova and M. Glasa, 2004. A simplified RT-PCR-based detection of recombinant *plum pox virus* islates. *Acta Virologica*, **48**: 173–176.

Szirmai, J., 1961. Report on fruit tree virus disease in Hungary. *Tijscbrift Planteavl*, **65**: 220–229.

Thomidis, T. and I., Karajiannis, 2003. Using ELISA and PCR to test the the potential for spread of *plum pox virus* by seeds of different stone fruit cultivars. *New Zealand Journal of Crop and Horticultural Sciernce,* **31**: 69–72.

Triolo, E., M. Ginanni, A. Materazzi and A. Paolucci, 1993. Further evidence of the non-transmission through seed of *plum pox virus* in apricot. *Advances in Horticultural Science,* **7:** 109–111.

Wetzel, T., T. Candresse, G. Macquaire, M. Ravelonandro and J. Dunez, 1992. A highly sensitive immunocaptire polymerase chain reaction method *for plum pox potyvirus* detection. *Journal of Virological Methods,* **39**: 27–37.

Zagrai, L. and I. Zagrai, 2008. Non-transmission of Plum pox virus D and Rec strain trhough the seeds in plum*. Bulletin UASVM, Horticulture*, **65** (1): 523.

Received October, 20, 2015; accepted for printing February, 19, 2016