Biological Evidence for Practical Immunity of Apricot Cultivar Harlayne to *Plum Pox Virus*

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Abstract

Polák J., Komínek P. (2010): **Biological evidence for practical immunity of apricot cultivar Harlayne to *Plum pox virus*.** Plant Protect. Sci., **48**: 143–148.

Ten-year results of the practical immunity investigation of apricot cv. Harlayne are presented. Two-year-old trees of cv. Harlayne were inoculated by chip-budding with six different strains and isolates of *Plum pox virus* (PPV). PPV inoculated trees grew in the field and were evaluated from 2001 to 2011. No PPV symptoms appeared in the leaves of cv. Harlayne within ten years (2002–2011), and within eight years (2004–2011) in the fruits and stones. None of the six isolates of three different PPV strains was detected in the leaves and fruits by ELISA. Suckers of the rootstock *Prunus myrobalana* developed around cv. Harlayne trees in 2005–2011 were symptomless and ELISA was negative within seven years. New trees of cv. Harlayne obtained from tested trees by budding on PPV susceptible apricot rootstock MVA-2 in 2007 were PPV free from 2008 through 2011. The presence of PPV was proved by ELISA neither in leaves of cv. Harlayne nor in rootstock MVA-2.

Keywords: stone fruits; immunity; PPV strains; symptoms; bud transmission; susceptible rootstock, runners

The best protection against quarantine *Plum pox virus* (PPV) is to grow immune or resistant cultivars of stone fruits. Dosba *et al.* (1992) proved apricot cv. Harlayne to be immune to PPV. We have confirmed the immunity of apricot cv. Harlayne to PPV by grafting onto five years old trees of apricot cv. Vegama, susceptible and infected with PPV-M. Cultivar Harlayne was certified as immune to PPV-M strain. No virus was detected either by ELISA or IC-RT-PCR, nor in the grafted indicator *Prunus tomentosa* (Polák *et al*. 1997). Biological, serological, and molecular demonstrations confirmed the immunity of cv. Harlayne to the most pathogenic PPV-M strain. There are seven recognised strains of PPV: M, D, Rec, EA, C, W, and T. Three of them M, D, and Rec are present in the territory of the Czech Republic. Since the resistance or immunity of cultivars to plant viruses can be strain-specific, the behaviour of cv. Harlayne against six different isolates of three PPV strains present in the CR was studied in the field trial. Preliminary results of three-year testing proved that no symptoms appeared on the leaves of apricot cv. Harlayne, and ELISA did not prove the presence of PPV strains in leaves (Polák *et al.* 2005). Fruits and leaves of apricot cv. Harlayne were tested by ELISA and RT-PCR and symptoms observed in another three years. Six-year evaluation carried out in the Czech Republic proved the immunity of apricot cv. Harlayne to the six different PPV strains of groups PPV-D, PPV-Rec, and PPV-M. No symptoms appeared in the leaves and fruits,

Supported by the Ministry of Agriculture of the Czech Republic, Project No. MZE 0002700604.

and ELISA did not detect the presence of PPV strains in the leaves of individual trees (Polák *et al*. 2008). Ion-Nagy *et al.* (2006) recently reported restricted PPV presence in main veins of leaves of apricot cv. Harlayne. Based on this observation apricot cv. Harlayne was included by Ion-Nagy *et al.* (2006) in the group of PPV resistant cultivars, the immunity was negated. The biological evidence for this statement is missing. Therefore our research on the immunity of cv. Harlayne to PPV continued with the same plant material (Polák *et al.* 2005, 2008). Results of the first biological test on *Prunus tomentosa* were published (Polák *et al.*1997). Trees of apricot cv. Harlayne and control trees of PPV susceptible cvs Velkopavlovická and Karola infected with PPV strains were grown in the field for eleven years. Moreover, runners of the rootstock *P. myrobalana* and new trees of cv. Harlayne on apricot rootstock MVA-2 susceptible to PPV were evaluated in technical isolation in 2008–2011. The results of ten-year investigation are presented in this contribution.

MATERIAL AND METHODS

Plant material, inoculation with PPV strains. Virus-free, two-year-old trees of cv. Harlayne and the PPV susceptible control cultivars Karola (3 trees) and Velkopavlovická (3 trees) grafted on the PPV susceptible rootstock *Prunus myrobalana* were inoculated by chip budding with six different strains and isolates of PPV in 2001: PPV-D original strain; PPV-D isolated from *P. insititia* in the Czech Republic (CR); PPV-Rec isolated from *P. domestica* in the CR, PPV-Rec isolated from *P. insititia* in the CR (Poncarová & Komínek 1998; Glasa *et al.* 2004); PPV-M original strain; PPV-M isolated from apricot cv. Vegama in the CR; PPV-M isolated from peach cv. Catherina in the CR. Buds from *P. insititia*, apricot, and peach GF-305 infected with the different PPV strains and isolates were used for the inoculation of PPV. Three trees of cv. Harlayne were used for the inoculation of each PPV isolate. Each tree was inoculated with two infected buds. The growth of PPV infected buds was checked visually. At least one bud was growing on each tree in the next year. Control trees were planted among the studied trees of cv. Harlayne infected with different PPV strains and isolates. A small orchard of apricot cvs Harlayne, Betinka, Velkopavlovická, and Karola was located on the experimental field of Crop Research Institute in Prague-Ruzyně, Prague-Ruzyně International Airport, Czech Republic.

Two biological tests of immunity of apricot cv. Harlayne to PPV strains. (1) Grafts from apricot trees of cv. Harlayne inoculated with different PPV strains were used in 2007 for budding virus-free apricot rootstocks MVA-2, susceptible to PPV. New cv. Harlayne trees growing on apricot rootstocks MVA-2 were obtained. Obtained trees with shoots of the rootstock were grown in technical isolation (screenhouse) and inspected for PPV symptoms and tested for PPV presence in 2008–2011.

(2) Runners of the rootstock *P. myrobalana* started to appear under the original (inoculated with PPV strains in 2001) cv. Harlayne trees from 2006, usually close to the trunk, but some of them at the distance up to three meters from the trunk. Runners of the rootstocks *P. myrobalana* were visually inspected for PPV symptoms and their leaves were tested for PPV presence.

Visual inspection and evaluation. *Plum pox virus* inoculated trees of apricot cv. Harlayne, and control trees of cvs Karola and Velkopavlovická were inspected monthly from May to September 2002 through 2011 for the presence of PPV symptoms in the leaves, and in June and July in the time of ripening and harvesting for the presence of PPV symptoms in the fruits and stones. Symptoms in the fruits and stones were evaluated in 2004 through 2011.

Leaves of new apricot trees of cv. Harlayne and their rootstocks MVA-2 were inspected for the presence of PPV symptoms in 2008 through 2011. Runners of the rootstocks *P. myrobalana* growing under the trees of apricot cv. Harlayne were evaluated for the presence of PPV symptoms in 2006 through 2011.

Serological evaluation by ELISA. Samples of cvs Harlayne, Karola, and Velkopavlovická leaves were tested serologically every June in 2002 through 2011. Double antibody sandwich of enzyme-linked immunosorbent assay (DAS-ELISA) procedure was used (Clark & Adams 1977).

New trees of cv. Harlayne were obtained in 2007 (see Two biological tests – 1) by budding virus-free rootstocks MVA-2 with cv. Harlayne inoculated with different PPV strains in 2001, and samples of the leaves of new Harlayne trees and its rootstocks MVA-2 were tested by DAS-ELISA in 2008 through 2011.

Samples of the leaves of *P. myrobalana* suckers growing under the cv. Harlayne trees planted in

2001 were tested by DAS-ELISA every June in 2006 through 2011.

Polyclonal PPV antibodies (Bioreba AG, Reinach, Switzerland) were used for detection of PPV strains and isolates by DAS-ELISA. Samples for ELISA were prepared by grinding 0.2 g of leaves in phosphate buffered saline, pH 7.4 with 2% polyvinylpyrrolidone and 0.2% of egg albumin at the ratio 1:20. Microplates were rated using a Dynatex MR5000 (Dynex Technologies, Chantilly, USA) reader at 405 nm. The reading of A_{405} was performed after one-hour incubation of the substrate at room temperature. Samples with $A_{405} > 0.10$ were considered as positive, and samples with $A_{405} < 0.03$ were rated as negative.

Evaluation by RT-PCR. Total RNA was isolated from flowers and leaves of apricots using an RNeasy Plant Mini Kit (Qiagen). Primers P1 and P2 were used for PPV detection (Candresse *et al*. 1995), targeted to a 243 base pair long fragment of a coat protein gene of PPV. One-step RT-PCR kit (Qiagen) was used for RT-PCR detection. Reactions were done in a PTC200 thermocycler (Bio-Rad, Hercules, USA). Products of PCR were separated by electrophoresis on a 2% agarose gel and visualised by UV light. In selected samples, strain-specific primers according to Šubr *et al*. (2004) were used for the determination of PPV strain.

Trees of cv. Harlayne were tested by RT-PCR with the above-mentioned primers in 2004, 2006, 2007, 2009, 2010, and 2011. Several trees were tested in parallel by Dr. A. Minafra at the University of Bari in 2011. Leaves of new apricot trees of cv. Harlayne and runners of *P. myrobalana* rootstocks growing under the original trees of apricot cv. Harlayne were tested by RT-PCR in 2011.

RESULTS

No symptoms appeared in the leaves of trees of cv. Harlayne after the bud inoculation with PPV-D (original), PPV-D from *P. insititia* (Czech Republic – CR), PPV-Rec from *P. domestica* (CR), PPV-Rec from *P. insititia* (CR), PPV-M from apricot (CR), PPV-M from peach (CR), and PPV-M (original) within the ten years (2002–2011). Diffuse spots and rings appeared every year in leaves of all the control trees of cvs Karola (Figure 1) and Velkopavlovická inoculated with six different PPV strains and isolates. The presence of PPV was proved by ELISA only in leaves of cvs Karola and Velkopavlovická. First fruits appeared in 2004. No symptoms of PPV appeared in the fruits (Figure 2) and stones of cv. Harlayne trees inoculated with PPV strains within eight years (2004–2011). None of the six different viral strains and isolates was detected in the leaves or fruits of PPV inoculated trees by ELISA within ten years (2002–2011). Severe PPV symptoms, diffuse spots and rings, malformations of fruits appeared every year in fruits of the control trees of cvs Karola (Figure 3) and Velkopavlovická (Figure 4). The presence of PPV was confirmed by ELISA.

Leaves of cv. Harlayne trees were also tested by RT-PCR. Results were negative in most trees every year, one tree was positive in several years, in some years (e.g. 2004) completely negative. Harlayne tree No. 6 inoculated with PPV-Rec was found to be positive in 2006, in tree No. 3 (PPV-M) the result of RT-PCR was positive in 2007, tree No. 15 (PPV-D orig.) positive in 2009, tree No. 8 (PPV-D *P. insit.*) positive in 2010, and tree No. 5 (PPV-Rec *P. insit.*) positive in 2011. Positive RT-PCR reactions were

Figure 1. Rings and diffuse spots in the leaves of apricot trees of cv. Karola infected with PPV

Figure 2. Fruits of apricot cv. Harlayne harvested from the tree inoculated with PPV-recombinant strain. No PPV symptoms

Figure 3. Malformations and diffuse spots in fruits of cv. Karola infected with PPV-Rec

Figure 4. Ring spots and mild malformation in fruits of apricot cv. Velkopavlovická infected with PPV-M (Catherina)

weak. Dr. A. Minafra obtained only the negative results in 2011. Results obtained by the testing of PPV infected control trees of cvs Velkopavlovická and Karola were strong bands. Individual positive results of RT-PCR, weak reactions were obtained from one tree in one year only, and they were never confirmed in another year(s), and they were not confirmed in the laboratory abroad. Our conclusion is that we received several false positive reactions in the course of eight years.

Suckers of *Prunus myrobalana* rootstocks susceptible to PPV developed around cv. Harlayne trees every year in 2006–2011 were symptomless and ELISA was negative during the 2006–2011 years. Suckers of *P. myrobalana* were cut every year to prevent possible PPV infection by aphids. On the other hand, severe PPV symptoms appeared every year (2002–2011) in the leaves of PPV infection sources, growing shoots of *P. insititia, P. domestica* and apricot developed from buds used for infection, infected with different PPV strains and growing in cv. Harlayne trees.

New trees of apricot cv. Harlayne obtained from tested cv. Harlayne trees inoculated by budding on the PPV susceptible apricot rootstock MVA-2 were regularly inspected for PPV symptoms and tested by ELISA. No PPV symptoms appeared either in leaves of cv. Harlayne or in apricot rootstock MVA-2. The presence of PPV was not proved by ELISA either in leaves of cv. Harlayne or in apricot rootstock MVA-2 susceptible to PPV. Trees were PPV free in 2008 through 2011. Six isolates of three different PPV strains including the most pathogenic original PPV-M were used in the study. Neither short-distance nor long-distance movement of PPV was proved in trees of apricot cv. Harlayne. Three independent biological tests proved incompatibility and cv. Harlayne as the non-host of PPV.

In the course of ten-year trial apricot cv. Harlayne was proved to be practically immune to the six different *Plum pox virus* strains and isolates.

DISCUSSION

The results of the long-term experiment of biological detection (2001–2011) proved the practical immunity of apricot cv. Harlayne to the six isolates of three different strains of *Plum pox virus*, namely: PPV-D – original, PPV-D from *P. insititia* (CR), PPV-Rec from *P. domestica* (CR), PPV-Rec from *P. insititia* (CR), PPV-M from apricot (CR), PPV-M from peach (CR), and PPV-M (original). The term "immunity" is used in a different meaning (e.g. Kůdela & Braunová 2007). In agreement with our results the immunity is incompatibility, the plant is the non-host of pathogen. From this point of view we used the term "practical immunity". According to the EPPO Specific Quarantine Requirements plants in the certification scheme must be tested by biological tests. ELISA and RT-PCR are recommended only in the certification schemes of EPPO for their simplicity and rapidity. Serological and molecular tests are not obligatory, because of lower susceptibility (ELISA) and the possibility of false positive reactions (RT-PCR).

The crucial feature is biological evidence for the practical immunity of cv. Harlayne to PPV proved by three independent tests:

(1) PPV free apricot trees of cv. Harlayne were obtained from Harlayne trees inoculated with six isolates of three different PPV strains by grafting.

New trees of cv. Harlayne were without any PPV symptoms for four years and/or during the whole time of investigation and all the results of ELISA and RT-PCR detection were negative.

(2) The six isolates of three different PPV strains were not transferred via cv. Harlayne to the PPV susceptible rootstock *P. myrobalana.* There were no PPV symptoms in the leaves of PPV susceptible apricot rootstock MVA-2 and all the results of ELISA and RT-PCR detection were negative.

(3) The third biological evidence for the immunity of cv. Harlayne to PPV-M and for the absence of short- and long-distance movement of PPV in cv. Harlayne was provided by the grafted indicator *Prunus tomentosa* (Polák *et al*. 1997). No PPV symptoms appeared in the leaves of *P. tomentosa* and no virus was detected by ELISA and RT-PCR.

The presence of PPV in leaves or fruits of apricot cv. Harlayne inoculated with six isolates of three different strains of the virus has never been detected by ELISA. Trees of cv. Harlayne were growing for ten years under the high and permanent infection pressure from shoots infected with PPV and showing severe PPV symptoms. No PPV symptoms appeared in leaves and fruits of cv. Harlayne. Biological and serological evidence for PPV absence excludes the latent presence of the virus in trees of cv. Harlayne. Several false positive reactions of RT-PCR were obtained in the course of several-year testing. Individual positive results of RT-PCR, weak reactions were obtained from one tree in one year only, they were never confirmed in another year(s), and they were not confirmed in the laboratory abroad. The practical immunity of apricot cv. Harlayne to the six isolates of three different strains PPV was confirmed by three independent biological tests and by ELISA. The practical immunity of cv. Harlayne and/or the defence mechanism prevent the multiplication of PPV in this apricot cultivar.

Ion-Nagy *et al.* (2006) recently reported limited PPV presence in apricot cv. Harlayne, but the biological evidence for PPV multiplication in this cultivar is missing. Some other publications and molecular studies on PPV resistance have appeared in the last years. The problem is that authors are paying no or only little attention to cv. Harlayne. In most experiments cvs Stark Early Orange (SEO) and Goldrich were used. In experiments are used less pathogenic PPV strains (PPV-D) with confusing results. Rubio et al. (2008) studied the long-distance movement of PPV-D through its vascular vessels as an alternative resistance evaluation method. They used the peach rootstock GF 305 (PPV susceptible) and apricot cv. SEO to evaluate the long-distance movement of PPV from the scion to the rootstock. The resistant apricot cv. SEO did not allow this movement and did not show any PPV symptoms. We published mild PPV symptoms in the leaves of cv. SEO after artificial infection with PPV-M strain by grafting, and we also proved a certain not negligible concentration of PPV-M by semi-quantitative ELISA in symptomatic leaves (Polák *et al*. 1997). Sicard *et al*. (2008) used apricot cvs SEO and Goldrich in their study. The French publication of Dosba *et al*. (1992) first proved the immunity of cv. Harlayne. The Czech publication of Polák *et al*. (1997) confirmed this immunity and proved that cv. Goldrich is not resistant, but medium susceptible to PPV with PPV symptoms in leaves and fruits and higher concentration of PPV in leaves. Sicard *et al*. (2008) concluded that the resistance to PPV in apricots is controlled by a major quantitative trait locus that explains up to 70% of the phenotypic variance. This conclusion is in agreement with the fact that Sicard *et al*. (2008) experimented with apricot cv. Goldrich, susceptible to PPV-M. Only Marandel *et al*. (2009) used in molecular studies of PPV resistance quantitative trait loci (QTLs) not only apricot cvs SEO and Goldrich but also cv. Harlayne. They were able to identify only in cv. Harlayne four distinct dominant resistance QTLs, three on linkage group 1 (LG 1) and one QTL on LG 3. Molecular studies of Marandel *et al*. (2009) proved different PPV behaviour of cv. Harlayne from other PPV resistant apricot cultivars. Results of our experiments proved the practical immunity of cv. Harlayne to *Plum pox virus*.

Acknowledgements. The authors are deeply grateful to Prof. Giovanni Martelli for critical reading and valuable comments on the manuscript, to Dr. Angelantonio Minafra for parallel RT-PCR testing in 2011, to Mrs. Jitka Pívalová for perfect technical assistance, and to Dr. Arben Myrta, Valenzano, Bari, Italy, for providing PPV-M (original) and PPV-D (original) strains described in France.

References

Clark M.F., Adams A.N. (1977): Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. Journal of Genetic Virology, **34**: 51–57.

Candresse T., Macquaire G., Lanneau M., Bousalem M., Quiot-Douine L., Quiot J.B., Dunez J. (1995): Analysis of plum pox virus variability and development of strain-specific PCR assay. Acta Horticulturae (ISHS) 386: 357–369.

Dosba F., Orliac S., Dutrannoy F., Maison P., Massonie G., Audergon J.M. (1992): Evaluation of resistance to *Plum pox virus* in apricot trees. Acta Horticulturae (ISHS) 309: 211–220.

Glasa M., Palkovics L., Komínek P., Labonne G., Pittnerová S., Kúdela O., Candresse T. (2004): Geografically and temporally distant natural recombinant isolates of *Plum pox virus* (PPV) are genetically very similar and form a unique PPV subgroup. Journal of General Virology, **85**: 2671–2681.

Ion-Nagy L., Lansac M., Eyquard J.P., Salvador B., Garcia J.A.., Le Gall O., Hernould M., Schurdi-Levraud V., Decroocq V. (2006): PPV long-distance movement is occasionally permited in resistant apricot hosts. Virus Research, **120**: 70–78

Kůdela V., Braunová M. (eds) (2007): Česko-anglická rostlinolékařská terminologie – Czech-English Plant Healt Terminology. Academia, Praha.

Marandel G., Salava J., Abbott A., Candresse T., Decroocq V. (2009): Qantitative trait loci meta-analysis of *Plum pox virus* resistance in apricot (*Prunus armeniaca* L.): new insights on the organization and the identification of genomic resistence factors. Molecular Plant Pathology, **10**: 347–360.

Polák J., Oukropec I., Komínek P., Krška B., Bittóová M. (1997): Detection and evaluation of resistance of apricots and peaches to *Plum pox virus.* Journal of Plant Disease and Protection, **104**: 466–473.

Polák J., Krška B., Pívalová J., Svoboda J. (2005): Apricot cultivars ´Harlayne´ and ´Betinka´ were proved to be highly resistant to the six different strains and isolates of *Plum pox virus* (PPV). Phytopatology Polonica, **36**: 53–59.

Polák J., Komínek P., Krška B., Pívalová J. (2008). Durable resistance of apricot cultivars Harlayne and Betinka to six different strains of *Plum pox virus.* Journal of Plant Pathology, **90** (Suppl. 1): S1.37–S1.40.

Poncarová Z., Komínek P. (1998): Restriction fragment length polymorphism differentiation of plum pox virus isolates. Acta Virologica, **42**: 26.

Rubio M., Ruiz D., Egea J., Martínez-Gómez P., Dicenta F. (2008): Evaluation ofapricot resistance to *Plum pox virus* in controlled greenhouse and natural field conditions. Scientia Horticulturae, **116**: 176–179.

Sicard O., Marandel G., Soriano J.M., Lalli D.A., Lambert P., Salava J., Badenes M.L., Abbott A.G., Decroocq V. (2008): Flanking the major *Plum pox virus* resistence locus in apricot with co-dominant markers (SSRs) derived from candidate resistence genes. Tree Genetetics Genomes, **4**: 359–365.

Šubr Z., Pittnerová S., Glasa M. (2004): A simplified RT-PCR-based detection of recombinant *Plum pox virus* isolates. Acta Virologica, **48**: 173–176.

Received for publication June 17, 2011
Accepted after corrections June 14, 2012

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