Study of PPV infection on stone fruits

Thesis of Ph.D. dissertation

JÁNOS ÁDÁM

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Ph.D. School

Name: Doctoral School of Horticultural Sciences

Field: Crop Sciences and Horticulture

Head of School: Prof. Dr. Éva Németh Zámboriné
Doctor of the Hungarian Academy of Sciences
Head of Department of Medicinal and Aromatic Plants
SZENT ISTVÁN UNIVERSITY
Faculty of Horticultural Sciences

Supervisor: Prof. Dr. László Palkovics
Doctor of the Hungarian Academy of Sciences
Head of Department of Plant Pathology
SZENT ISTVÁN UNIVERSITY
Faculty of Horticultural Sciences

The applicant met the requirement of the Ph.D. regulations of the Szent István University and the thesis is accepted for the defence process.

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Head of Ph.D. School Supervisor

1. INTRODUCTION

In Hungary stone fruits are grown in the largest quantity and area after the apple. The domestic environmental conditions are suitable for the cultivation of all temperate fruit species, but many factors influence their ability of growing. In addition to climate change and the increasing incidence of extreme weather phenomena, the already known and new plant protection problems causing difficulties, including the major damage caused by viral infections.

Thanks to the versatile processing and high ecological tolerance, the plum (*Prunus domestica* L.) still grown in the highest quantities among the stone fruits. In our country plum has a high degree of crop safety, it grows well and regularly at low cost and is suitable for machine harvesting. Our country lies on the northern boundary of the economical cultivation of peaches (*Prunus persica* L.) and apricot (*Prunus armeniaca* L.). Both species are very popular and sought-after fruit, so they are grown on a relatively large area in Hungary, peach is grown on the third largest area after sour cherry and plum. Apricot have a wide variety of cultivars, but in Hungary it is worthwhile to grow cultivars that meet local (cooler) environmental conditions, because these varieties can only compete with import apricots from the Mediterranean region.

Plum pox virus (PPV, Sharka virus) is a dominant pathogen in the cultivation of stone fruits. Severe damage is mainly caused in plum, peach and apricot orchards. The resulting yield loss and economic damage can be estimated for billions of euros to the world nowadays. Its danger is enhanced by the widespread presence of international reproductive material trade and is demonstrably present throughout the world in all major stone fruit growing countries. The incidence of

PPV in Hungary has been known since 1948 (Szirmai, 1948), and is considered among the most dangerous pathogens causing devastating damage in stone fruit orchards. As a result of the disease, the quantity and quality of the fruits is reduced, the aesthetic and the content of the product decreasing, the fruit is more easily damaged at the time of delivery, it deteriorates during storage. Protection strategies against PPV are the control of aphids, the use of healthy reproductive material and the breeding of resistant cultivars. Breeding resistant cultivars to the plum pox is an important way of protection. Since the emergence of PPV it has been a major economic significance, until today nine virus strains have been differentiated, and in the future it may be epidemic on sour cherry and sweet cherry as well. In my work my main aims were to survey the spread of the virus, to identify the dominant virus strains in Hungary and to study the host preference of these strains. The continuous development of diagnostic methods contributes to the fight against the virus, so in addition to molecular testing, clarifying the existing techniques and testing new techniques are part of my studies. During my work I also examined the effect of commonly used rootstock-scion combinations on the infection rate. With these studies I would like to contribute to the success of cultivation.

The main goals of my thesis and my work are:

- Preparation of a survey on the PPV contamination of stone fruit orchards and home gardens in the northern counties of Hungary, identification of the strains of virus isolates from positive samples, their molecular characterization and survey the presence of the different strains in Hungary.

- Examination of peach grafts, during which we examined whether the effect of the rootstock cv. on viral contamination is justified, whether the effects of the scion cv. on viral contamination is justified and whether the effect of the rootstock-scion combinations on virus contamination is justified.
- Conducting virological testing of plum, apricot and peach trees adjacent to a plantation, detection of PPV by molecular methods, determination of virus strains, study the host preference of PPV strains.
- Improving the previously known RFLP method in the genomic region P3—CI to identify the most commonly occurring PPV strains (M, D, Rec).
- Examining the application capabilities of the Whatman FTA (Flinders Technology Associates) membrane in the area of sample collection, storage and virus diagnostics.

2. MATERIALS AND METHODS

Collection and storage of test samples

During the Hungarian survey we collected 105 samples from the northern counties of Hungary from plantations and home gardens. The survey included the following counties: Pest, Borsod-Abaúj-Zemplén, Nógrád, Szabolcs-Szatmár-Bereg, Komárom-Esztergom and Győr-Moson-Sopron counties. In the study on peach rootstock-scion combinations the rootstocks were the almond seedlings, the 'Cadaman', the 'GF677' and the 'Pe-Ma' rootstocks, and the scions were the 'Babygold 6', 'Cresthaven' and 'Michelini'. A total of 91 samples were studied in these combinations. The plantations selected for the study of the host plant preference of PPV strains were:

‘Čačanska lepotica’ plums on myrabolan rootstock, 'Redhaven' peach on bitter almond rootstock, 'Tomcot' apricot on myrabolan rootstock. To carry out laboratory tests, we collected leaf samples from 20-20 peach, apricot and plum trees. The samples in the abovementioned tests are stored at a temperature of minus 70 °C after collection. The samples included in the FTA Card study were derived from plum blossom fabrics from Bulgaria. 32 samples were examined, which were collected in 2013 and 2014 and these samples were stored at room temperature.

Molecular methods, RT-PCR and Nested-PCR

For TRNS extraction we used 100 mg tissue per sample. The equipment and chemicals for the study were included in the Kits. Total ribonucleic acid extraction was carried out based on the manufacturers' instructions. The TRNS extracted from the samples were used for further steps. The remaining volume was stored frozen at minus 70 °C for further use. For the PCR process deoxyribonucleic acid is required, so it is necessary to prepare cDNA, i.e. a complementary DNA from the single-stranded virus nucleic acid (+ssRNA). We used roughly 1500 ng/µl of TRNA per sample or 2 mm diameter purified FTA membrane with 1 µl of 100 pmol antisens primer and then 12.5 µl of the final volume with nuclease-free water. The mixture is incubated at 65 °C for five minutes then cooled on ice for ten minutes, during which the following mixture was assembled and measured to the samples: 4 µl 5x RT buffer, 2 µl 10 mM dNTPs, 1 µl (200 u) RevertAid reverse transcriptase enzyme, 0.5 µl 20 u RiboLock ribonuclease Inhibitor. Reverse transcriptions were carried out for one hour at 42 °C and then incubated for 10 minutes at 70 °C to eliminate the enzyme. The resulting cDNA is stored at minus 20 °C until further use. During the polymerase chain reaction the two investigated genomic regions were the 3'NIb-CP genes close to the 3'

end of the virus and the 3'P3–6K1–5'CI genes. PCR and Nested-PCR were carried out in 20 μl of final volume by choosing the right annealing temperature for the primers and the correct time for the chain construction of the PCR product. The primers used in the PCR were Sprimer, M4T and M4 (Chen and Adams, 2001), PP3 and PCI (Glasa *et al*., 2002) and mM5, mM3, mD5 and mD3 (Šubr *et al*., 2004).

Strain identification by RFLP and RT-PCR methods

To determine the strains of PPV, PCR products have been digested with 3 types of restriction endonucleases, *Dde*I, *Eco*RI and *Eco*RV. Based on the resulting digestion pattern the three most common strains can be differentiated. To detect the strains of the virus with PCR strain-specific forward and reverse primers are required, which are attached to both ends of the PCR product during the bonding (anellation). For the strain D the D specific forward and reverse primers bind (mD5 and mD3), and for the strain M the specific forward and reverse primers (mM5, mM3). The PCR used by us is suitable for Rec strain detection because the investigated genomic region contains the recombination point. The genome of the recombinant strain in the resulting NIb gene cDNA contains the binding location of the strain D forward primers, but not the binding location of M, so it has a 5' end with the D strain specific forward primer (mD5). The genome of the Rec strain in the cDNA of the CP gene contains the binding location of the reverse primer of the M strain, but not the binding site of D, so that the M strain specific reverse primer (mM3) will bind from the end of 3'. The result of the PCR is visualised by applying gel-electrophoresis on 1% agarose gel. The PCR products displayed during the gel process are sorted into strains based on their length. The shortest (459 bp) product is created for the M strain and the longest (664 bp) PCR product is obtained in

case of strain D. In case of the Rec strain, the cDNS section will fall between the two (605 bp).

Determination and analysis of nucleotide sequences

PCR products have been sent to the Netherlands after the PCR product has been cleaned, where we ordered the Barcode Sequencing service of the BaseClear company. The comparison and phylogenetic tree were conducted with the CLC Sequence Viewer version 7.0. software. During the creation of the phylogenetic tree, a 1000 repetition bootstrap analysis was used to draw the phylogenetic tree.

Statistical methods

We used the IBM SPSS Statistics 20 Suite for statistical analysis of the results. Independence test with Chi-squared test: This test has been used to test the independence of two variables (Harnos and Ladányi, 2005). The null hypothesis declares the independence of the variables. In our case we studied the following questions with this independence test:

- Is it justifiable that the rootstock cultivars have an effect on viral infection?
- Is it justifiable that the scion cultivars have an effect on viral infection?
- Is it justified that the rootstock-scion combinations have an effect on viral contamination?

If the significance level was lower than the first-race error (p<) the H0 hypothesis was rejected, i.e. a significant correlation was found. If a significant effect was detected, it means that there is a difference in the resistance to infection between the rootstocks, the scions or their combinations. In our studies α = 0, 05 significance, α = 0,01 is a strong significance.

Binary Logistic regression: An examination of the factors influencing the contamination of trees has also been used to estimate the probability of occurrence of the investigated event (infection during the period). In addition to the significance of the logistic regression model the differences between the rootstocks, the scions and their combinations were tested with the odds ratio. When comparing the scion cultivars, a reference level of 'Michelini' was chosen as the basis of resistance due to literary data. When comparing the combinations, we chose the combination of almond seedling x 'Michelini' for reference because of the PPV susceptibility of almond seedling and the resistance of 'Michelini'.

3. RESULTS AND DISCUSSION

During the survey conducted in the northern counties 105 showing symptoms of PPV infection and symptomless samples were collected. The targeted genomic region by the PP3 and PCI primers – 3'P3–$6K_1$–5'CI – resulted in an 836 bp long PCR product. Based on the results 42 samples were positive for PPV. Some of these positive samples were categorized into strains by RFLP method and sequence analysis. From 23 samples 12 were found to be D, 10 were Rec and only 1 was M. According to our results D and Rec strains are the most widespread in the northern counties, but M is present as well. D is dominant in Szabolcs-Szatmár-Bereg and in Komárom-Esztergom counties, Rec is dominant in Pest, Nógrád and Borsod-Abaúj-Zemplén counties. The single M isolate also originated from Borsod-Abaúj-Zemplén county.

In our study conducted on rootstock-scion combinations 91 symptomless and visually infected leaf samples were collected from one orchard located in Sóskút. From 91 samples we were able to

detect the PPV in 45 samples. From the 45 positive samples originated from rootstocks and scions 19 were infected by M and 12 were infected by D strains. In 14 cases M+D mixed infections were also detected. Based on the statistical analysis conducted on the results the rootstocks had no effect on the severity of the infection. During the study of scions we found that the cv. ‘Michelini’ showed significantly lower chance of infection compared to other scion cultivars.

In case of the ’Cadaman’ x ’Michelini’, ’GF677’ x ’Cresthaven’, and ’GF677’ x ’Michelini’ combinations the infection rate was lower than in other cases based on the chi-square test. In contrast the ‘Cadaman’ x ‘Cresthaven’ combination was significantly more susceptible to the virus. In the logistic regression model, the reference combination used was the almond seedlings x ‘Michelini’ based on former studies, because of the susceptibility of the almond seedlings and the tolerance of cv. ‘Michelini’. In case of ’GF667’ x ’Cresthaven’, ’GF677’ x ’Michelini’ and ’Cadaman’ x ’Michelini’ combinations the odds ratio (OR) was lower than 1 which shows higher tolerance, but the results are not statistically significant. In case of ‘Cadaman’ x ‘Cresthaven’ the OR is higher than one (Exp(B)=13,000; p=0,013), hence the chance of infection is significantly higher compared to the reference.

In the host preference study the results in plum samples are the following: 53% D, 18% Rec, 18% mixed Rec+M and 11% M infection. In peach samples: 50% M, 28% mixed M+D and 22% D infection. In apricot samples 57% mixed M+D, 28% M and 15% D infection was detected. During the analysis of the results host preference was detected. The number of D, M or Rec isolates in the three species shows significant differences. We can conclude that M were mostly present in peach (56%) meanwhile D were equally detected in plum and apricot samples – however in peach half of the

D infection was in mixed infection with the M strain. Recombinant isolates were only detected in plum samples, and half of these infections were in mixed infection with the M strain.

Based on the results of the experiments aiming at improvement of the PPV diagnostic methods it can be concluded that the RFLP in the 3'P3–6K_1–5'CI genomic region with the *Eco*RV restriction endonuclease effectively detects the recombinant strain, and the Whatman FTA Card is suitable for collecting PPV samples, storing them at room temperature and it is applicable to former RT-PCR analysis.

4. NEW SCIENTIFIC ACHIEVEMENTS

1. I have been surveyed the presence of PPV in home gardens and orchards. I determined the phylogenetic location of 20 isolates and classified them into strains based on the molecular tests.
2. In examining the PPV susceptibility of four rootstock and three peach scion combinations I confirmed that the tested rootstocks had no significant effect on PPV infection. Furthermore, I proved that the 'Michelini' scion was significantly less susceptible compared to the other investigated scion cultivars.
3. I studied the host preference of the PPV strains on plum, apricot and peach, the M strain dominant on peaches, the D and Rec strains are the most common on plum.
4. The RFLP test used in the P3-CI genomic region was further developed with the application of the *Eco*RV restriction endonuclease. *Eco*RV's digesting site is between GAT|ATC

nucleotides, so based on RFLP in the P3-CI genomic region the recombinant isolates can be identified.

5. REFERENCES

1. CHEN, J. és ADAMS, M. 2001. A universal PCR primer to detect members of the *Potyviridae* and its use to examine the taxonomic status of several members of the family. *Archives of virology*, 146, 757-766.
2. GLASA, M., MARIE-JEANNE, V., MOURY, B., KÚDELA, O. és QUIOT, J.-B. 2002. Molecular variability of the P3-6K 1 genomic region among geographically and biologically distinct isolates of *Plum pox virus*. *Archives of virology*, 147, 563-575.
3. HARNOS, Z. és LADÁNYI, M. 2005. Biometria agrártudományi alkalmazásokkal. Aula Kiadó, Budapest. 274-324.
4. ŠUBR, Z., PITTNEROVA, S. és GLASA, M. 2004. A simplified RT-PCR-based detection of recombinant *Plum pox virus* isolates. *Acta virologica*, 48, 173-176.
5. SZIRMAI, J. 1948. A kajszi vírusbetegsége. *Magyar Bor és Gyümölcs*, 3, 7-8.

6. LIST OF PUBLICATIONS

In the topic of the dissertation

Articles published in journals with impact factor

J, Ádám; Á, Borsos; I, Balla; A, Ittzés; L, Palkovics (2015): PPV Susceptibility of commonly used peach rootstock-scion combination. *Acta virologica* 59. 429-433. IF:1.222

Articles published in scientific journals

Ádám János; Sáray Réka; Palkovics László (2018): Hazai Plum pox virus törzsek gazdanövény preferenciájának vizsgálata. *Növényvédelem* 79.(54): 285-292.

J, Ádám; L, Palkovics; I, Tóbiás; A, Almási (2015): Presence of sharka disease in the North-Hungarian counties. *Acta Horticulturae*: Technical Communications of ISHS. 55-60.

Other scientific articles

L, Predajňa; Z, Šubr; **J, Ádám**; L, Palkovics; M, Glasa (2012): Use of FTA (WHATMAN) membranes for efficient and easy-to-use sampling and molecular detection of stone fruit and grapevine RNA viruses. *Acta Fytotechnica et Zootechnica* Special Number. 44-45.

Conference proceedings (full paper)

J, Ádám; R, Sáray; B, Stefanova; I, Tóbiás; L, Palkovics (2016): The host preference of PPV isolates in a Hungarian orchard. *Journal of Mountain Agriculture in the Balkans.* 19: 124-133.

Ádám, J.; Stefanova, B.; Dragoyski, K.; Tóbiás, I.; Palkovics, L. (2015): Use of FTA® (Whatman) membrane for collection and detection of Bulgarian PPV isolates. *Journal of Mountain Agriculture in the Balkans.* 18: 334-343.

I, Tóbiás; É, Pájtli; **J, Ádám**; A, Almási; L, Palkovics (2015): Potyviruses In Hungary. *Journal of Mountain Agriculture in the Balkans.* 18: 887-901.

Ádám, J.; Tóbiás, I.; Almási, A.; Šubr, Z.; Nagyová, A.; Glasa, M.; Palkovics, L. (2013): The presence of Sharka disease on stone fruit trees in the Hungarian-Slovak cross-border region. *Episteme Czasopiśmie Naukowo-Kulturalnym.* 3: 429-436.

László, Palkovics; Asztéria, Almási; **János, Ádám**; Boglárka, Balotai; István, Tóbiás (2012): Survey of sharka disease (*Plum pox virus*) on stone fruit trees in Northern Hungary. *Petria*. 22: 251-255.

Conference abstracts

Ádám, J.; Sáray, R. A.; Palkovics, L. (2018): *Plum pox virus* törzsek gazdanövény preferenciájának indirekt vizsgálata. In: Haltrich, Attila; Varga, Ákos (szerk.) 64. Növényvédelmi Tudományos Napok. Budapest, Magyarország: Magyar Növényvédelmi Társaság, 46.

Ádám, János; Borsos, Árpád; Balla, Ildikó; Ittzés, András; Palkovics, László (2015): Gyakorlatban használt őszibarack alanyok hatása a *plum pox virus* fogékonyságra. In: Horváth, József; Haltrich, Attila; Molnár, János (szerk.) 61. Növényvédelmi Tudományos Napok. Budapest, Magyarország: MAE Növényvédelmi Társaság. 59.

Ádám, J.; Almási, A.; Subr, Z.; Nagyova, A.; Glasa, M.; Palkovics, L.; Tóbiás, I. (2014): Sharka in the Hungarian-Slovak cross-border region. In: Seventeenth International Scientific Conference, 'EcoMontain – 2014': Summeries in Journal of Mountain Agriculture on the Balkans. 157-158.

Ádám, János; Almási, Asztéria; Tóbiás, István; Balotai, Boglárka; Palkovics, László (2013): Szilva himlő vírus (*Plum pox virus*) jelenlétének felmérése hazánk északi megyéinek csonthéjas ültetvényeiben. In: Horváth, József; Haltrich, Attila; Molnár, János (szerk.) 59. Növényvédelmi Tudományos Napok, Összefoglalók. 65.

Ádám, J.; Almási, A.; Tóbiás, I.; Subr, Z.; Nagyova, A.; Glasa, M.; Palkovics, L. (2013): Sharka in the Hungarian-Slovak Cross-Border Region. In: VII International Conference Bioresources and Viruses. 19.

Almási, Asztéria; Balotai, Boglárka; Tóbiás, István; **Ádám, János**; Palkovics, László (2012): Szilva himlő vírus (plum pox virus, ppv) fertőzöttség felmérése a magyar-szlovák határ menti régióban. In: Kőmíves, T; Haltrich, A; Molnár, J (szerk.) 58. Növényvédelmi Tudományos Napok. Budapest, Magyarország: MAE Növényvédelmi Társaság. 41.

Almási, A.; Balotai, B.; Tóbiás, I.; **Ádám, J.**; Palkovics, L. (2012): A csonthéjasok vírusfertőzöttségének felmérése a magyar-szlovák határmenti régióban. In: XXII. Keszthelyi Növényvédelmi Fórum. Keszthely, Magyarország: Pannon Egyetem Georgikon Kar. 14.