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REGULAR ARTICLE

DIAGNOSTICS OF VIRUS PHYTOPATHOGENS FRUIT TREE *PLUM POX VIRUS, PRUNUS NECROTIC RINGSPOT VIRUS* AND *PRUNUS DWARF VIRUS* BY BIOLOGICAL AND MOLECULAR DIAGNOSTICS

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ABSTRACT

The aim of this study was to determine the incidence of viral phytopathogen *Plum pox virus, Prunus necrotic ringspot virus* and *Prunus dwarf virus* in selected localities of Slovakia and diagnose them using a molecular and biological methods. Forty samples of fruit trees of the genus *Prunus*, twenty samples from intensive plantings and twenty samples from wild subject were analysed. Biological diagnostic by using biological indicators *Prunus persica cv. GF 305, Prunus serrulata cv. Schirofugen* and molecular diagnostic by mRT-PCR were applied. Five samples with *Plum pox virus* were infected. The two samples positive for *Prunus necrotic ringspot virus* and one sample for *Prunus dwarf virus* were confirmed. The two samples were found to be infected with two viruses *Prunus necrotic ringspot virus* and *Prunus dwarf virus*. This work focuses on two techniques, their application to the diagnosis of stone fruit viruses and their routinely used for sanitary and certification programmes.

Keywords: viruses PPV, PNRSV, PDV, biological diagnostics, molecular diagnostics, RT-PCR

INTRODUCTION

The most widely used fruits as domestic plum (*Prunus domestica*), Apricot (*Prunus armeniaca*), peach (*Prunus persica*), cherry (*Prunus avium*) and sour cherry (*Prunus cerasus*) systematically belong to the genus *Prunus*. The fruits of these fruit species are widely used in manufacturing industry and they are very economically attractive. Vitality of fruit trees, quantity and quality of fruits significantly reduces viral disease.

The most serious viruses attacking the fruit trees of the genus *Prunus* includes *Plum pox virus* (PPV), *Prunus necrotic ringspot virus* (PNRSV), and *Prunus dwarf virus* (PDV), *Plum pox virus* causes a disease known as Sharka plums (**Németh, 1986**). Systematically belongs to the genus potyvirus and the family *Potyvirideae*. Presence of the virus was found in all kinds of stone-fruit except cherries. The natural hosts include blackthorn, which were infected aphids from plums and myrobalan and become secondary sources of infection (**Polák, 2006**). This virus a negative affects the ripening of the fruit, harvest and total growth of trees. Causes significant losses in yield per hectare, which can in susceptible varieties reach up to the 95 %.

Prunus necrotic ringspot virus causes a disease called cherry necrotic ring rot. Systematically belongs to the family ilarviruses and the families *Bromovidae*. *Prunus necrotic ringspot virus* is transmission by vegetative propagation of fruit trees and by vectors, mostly aphids. In cherry, sour cherry and Mahaleb cherry is frequent transfer by pollen and seed. From infected seeds which are growth from infected pollen can contain a high percentage of *Prunus necrotic ringspot virus* (**Hluchý *et al.*, 1997**). **Polák** (**2006**) reported that the disease affects especially cherries, but can also affect peaches, plums and apricots. **Németh** (**1986**) reported that the orchards may decrease the yield by more than 30%.

Prunus dwarf virus belongs to the family ilarviruses family *Bromoviridae*. Most often is found in sour cherry *Prunus cerasus* (**Németh, 1986**). *Prunus dwarf virus* causes death of buds and fruit bearing branches, weakening growth and, consequently, reduces the yield. Due to the possibility of a transmission by pollen mother, is important isolation mother plantation from the source of infection (**Hluchý *et al.*, 1997**).

The main objective of this work was to optimalize methods molecular and biological diagnostics of *Plum pox virus*, *Prunus necrotic ringspot virus*, *Prunus dwarf virus* and to make monitoring of these viruses in selected fruit nurseries and natural areas located in intensive cultivation of fruit trees. The most important strategy to control viral diseases in fruit trees is growing virusfree propagation material (buds, grafts, rootstocks), which is used

in the manufacturing process in fruit nurseries. The main benefit of diagnosis is testing of mother plantation from which ensure virusfree propaganting material for fruit nurseries and eliminates the main source of infection from mother plants.

MATERIAL AND METHODS

Methods of testing

Forty samples of fruit species in the genus *Prunus*, twenty samples from a fruit tree nurseries, fruit orchards and twenty samples from wild fruit trees were collected in 2012. The polyvalent indicator *Prunus persica GF-30* in the biological diagnostics of PPV and PDV samples were inoculated. For PNRSV samples were inoculated to one-year shoots indicator *Prunus serrulata cv. Schirofugen* which strongly react after inoculation from virus-infected plants. For each sample was inoculated five buds. Indicators are regularly inspected and on the basis of symptomatology were assessed positive samples. Samples were then tested by molecular diagnostics using RNzol Protocol: Total RNA Isolation Reagent (from Ecoli Ltd.), which was isolated using RNA from plant tissues. Multiplex RT-PCR (mRT-PCR) have been developed for detection of several viruses in a single reaction. This method is very quick, reliable and cost-effective routine diagnosis. The RT-PCR reaction was performed according to the methodology of molecular detection of stone-fruit viruses by multiplex RT-PCR (**Jarošová, Polák, Kumar, 2008**).

Isolation RNA and RT-PCR

RNA isolation was performed on 50 mg samples positive symptomatic leaves, to which was added 1 ml RNzol and sterile micropistons were leaves homogenized on ice. Samples were placed in a heating block at 30 °C for 5 minutes. After this the time has been added to the samples 200 µl of chloroform, and then samples were mixed, incubated for 3 minutes at 30 °C in a heating block, and centrifuged for 15 minutes at 4 °C and 12000 rotation.min^{-1}. The supernatant removed 700 µl to new microvials, was added isopropyl alcohol, mix the solution and the samples were incubated in a heating block 1 minutes at 30 °C. Samples were then centrifuged for 10 minutes at 4 °C and 12000 rotation.min^{-1}. The supernatant was removed and into sediment was added 500 µl of 70 % ethanol. Subsequently, the samples were mixed, centrifuged and into sediment was added 50 ml of RNase-free H_2O. Next, the samples were

incubated 10 minutes at 60 °C in a heating block. From this isolated RNA was pipetted into 50 µl of premix, 3 µl primers and 12 µl of DEPC water. Subsequently, the samples were placed into termocycler for PCR. The conditions for PCR reactions were follows: 45 °C for 30 minutes (reverse transcription), 94 °C for 5 minutes (Activation HotStarTaq polymerase), 40 cycles of three steps: 94 °C for 20 seconds (denaturation), 51 °C for 30 seconds (hybridization) and 72 °C for 1 minute (polymerization), 72 °C for 10 minutes (final phase), 12 °C storage. Visualization of PCR product was carried out by dividing the electrophoretic fragments in 1.5 % agarose gel with ethidium bromide labeled DNA. To detect the size of PCR products was used linear 100 bp DNA marker.

Table 1 List of primers

Primer	Sequence 5´- 3´	T_m	Virus
PPV-RR	CTCTTCTTGTGTTCCGACGTTTC	59 °C	PPV
F3	GGAATGTGGGTGATGATGG	57 °C	PPV
PDVdpR	CCT TTA ATG AGT CCG T	56 °C	PDV
PDVdpuF	CCG AGT GGA TGC TTC ACG	58 °C	PDV
PNcpR	CTTTCCATTCGGAGAAATTCG	54 °C	PNRSV
PNcpinF	GAGTATTGACTTCACGACCAC	57 °C	PNRSV

RESULTS AND DISCUSSION

Forty samples of fruit plants of the genus *Prunu* were tested. Samples were obtained from fruit nurseries, fruit plantation and natural locations. According to the biological and molecular diagnostics were identified five samples infected with *Plum pox virus*. Two samples were infected with *Prunus necrotic ringspot virus* and one with virus *Prunus dwarf virus*. The two samples were demonstrated mixed-infection *Prunus necrotic ringspot virus* and *Prunus dwarf virus*. Infected samples were collected from fruit orchards and the wild fruit plants of the genus *Prunus*.

The following six primers for molecular analysis were used: PPV-RR and F3 for detection of *Plum pox virus*, PNcpR and PNcpinF for *Prunus necrotic ringspot virus* and PDVdpR and PDVdpuF for *Prunus dwarf virus*. Positive samples were identified by a visible

lane of size 345 bp for *Plum pox virus*, *Prunus necrotic ringspot virus* 425 bp and 220 bp for *Prunus dwarf virus*.

The indicators *Prunus serrulata cv. Schirofugen* and *Prunus persica GF 305* for biological diagnosis were used are regularly checked and was assessed symptomatology viral infections. Results of biological and molecular diagnostics were confronted with each other. The positive samples by both diagnostics techniques were determined. The positive controls for the diagnosis of viruses were used. For all analysed plants and all detected viruses, no discrepancy was observed between results of biological and molecular diagnostics. Five samples with *Plum pox virus* were infected. The two samples were confirmed positive for *Prunus necrotic ringspot virus* and one sample for *Prunus dwarf virus*. The two samples were found to be infected with two viruses *Prunus necrotic ringspot virus* and *Prunus dwarf virus*. This work focuses on two techniques, their application to the diagnosis of stone fruit viruses and their routinely used for sanitary and certification programmes. Results are shown in the figure 1.

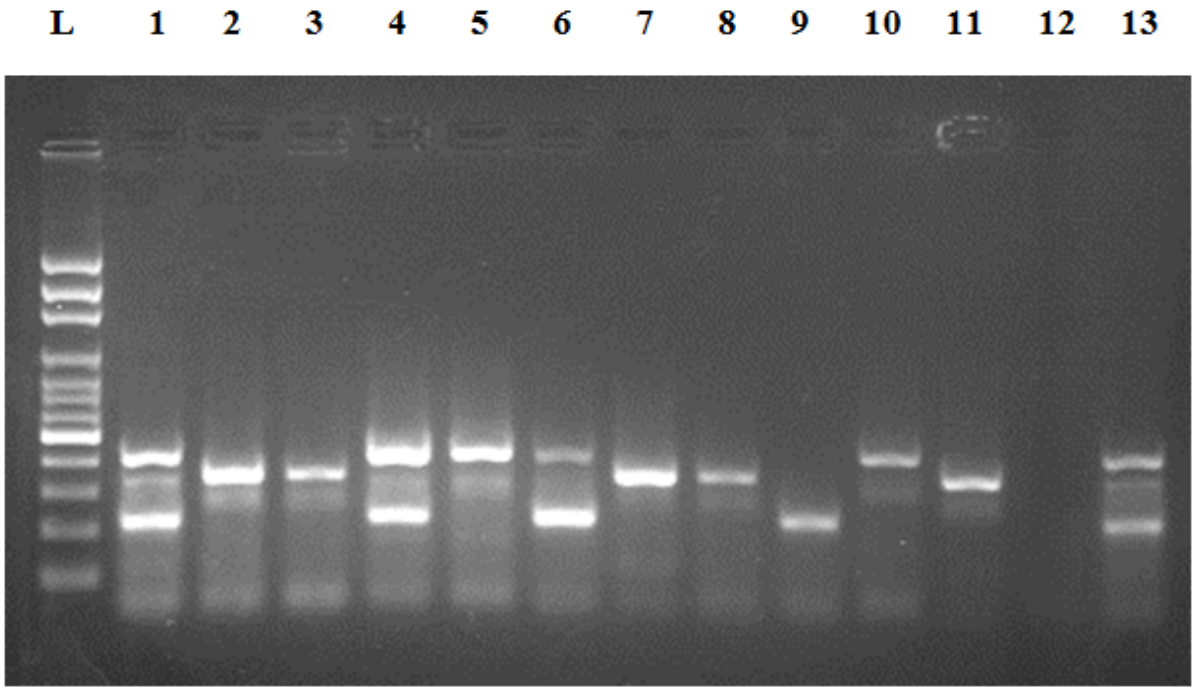

Figure 1 Agarose gel electrophoretic analysis of DNA fragments amplified from infected and healthy fruit trees by mRT-PCR

Legend: L – 100-bp DNA ladder, 1, 13 – positive controls, 2, 3, 7, 8, 11 – samples infected by *Plum pox virus*, 4, 6 – samples infected by *Prunus necrotic ringspot virus* and *Prunus dwarf virus*, 5, 10 – samples infected by *Prunus necrotic ringspot virus*, 9 – sample infected by *Prunus dwarf virus*, 12 – negative control

Stone fruit trees are affected by viruses that cause important economic losses. No chemical exist to be applied directly to control viral diseases. So, the early detection by sensitive diagnostic methods is the main way to control them. They often occur in latent form, infested plants show no symptoms. Such individuals become infected with a dangerous virus source in the production process of propagation of fruit plants. **Shors** (**2009**) reported as an important protection before infecting plant material in nurseries and fruit orchards maintaining isolation distances from potential sources of infection. It is important liquidate of sources of virus infections. Even **Polák** (**2006**) states that the most important sources of viruses plums, myrobalan shrubs and trees growing along roads and in the nature.

The main importance of diagnosis is identify infected individuals in fruit nursery and fruit orchards and immediately remove this sources of viral infection. The methods used in this work demonstrated sufficient sensitivity for the studied viruses. **Kawasaki** (**1990**) reported that the method of RT-PCR is very sensitive for the detection of plant viruses during the summer, when high temperatures is causing the low concentration of viruses in trees. Finally, it is also important monitoring for occurence of viruses in the neighborhood fruit tree nurseries and intensive fruit orchards. After removing of infected individuals will prevent to avoid the transmission of the viruses from wild fruit trees in these fruit plantations.

CONCLUSION

The aim of this work was to optimalize the conditions for molecular and biological diagnostics for these viruses: *Plum pox virus, Prunus necrotic ringspot virus, Prunus dwarf virus* and to make mapping their occurrence in the study sites for fruit species of the genus *Prunus*. From the results it can be concluded that none of the samples collected from fruit nurseries or intensive plantings was not infected with analysed viruses. Ten positive samples from fruit plants of the genus *Prunus* in the area of the village Sedliská in district Vranov nad Topľou and the surrounding cities Sabinov and Vranov nad Topľov were detected.

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