Virus Elimination in Peach cv. 'Red Haven' by Chemotherapy

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ABSTRACT
Stone fruit, as one of the most growing fruit trees in the Czech Republic, are very often attacked by the virus pathogens, which can finally cause great decrease in total yield. There are several ways to eliminate virus infection in the plant body, including in vivo and in vitro thermotherapy and chemotherapy. In a study, Prune dwarf virus, Plum pox virus and Prune necrotic ringspot virus were detected in peach trees Prunus persica cv. 'Red Haven' cultured in Czech Republic by two-step RT-PCR. Therefore in this study virus elimination was conducted by applying in vitro chemotherapy method in which the antiviral zidovudine was used at concentrations of 25 mg/l and 50 mg/l. The nodal culture was established from the one budded herbaceous shoots and cultivated on the Quoirin and Lepoivre medium with suitable stone fruit in vitro growth regulators for several weeks. The plantlets were then treated on Murashige and Skoog medium with the mentioned antivirals for three weeks. Then plantlets were transferred back to the Quoirin and Lepoivre medium without zidovudine. After one month, young plants were tested again by RT-PCR. Results were compared with two control variant; the first one without antivirals and the second one, which was previously treated by ribavirin. These results showed that zidovudine was very effective in the elimination of all the viruses. Moreover, the application of this antiviral was more effective than the application of ribavirin, because zidovudine had not a negative effect on the health condition of the plant or on its growth unlike the ribavirin treatment.

Keywords: PDV, PNRSV, PPV, Prunus persica, ribavirin, RT-PCR, zidovudine.

Abbreviations:
ELISA: Enzyme-Linked Immunosorbent Assay; PDV: Prune Dwarf Virus; PNRSV: Prune Necrotic Ringspot Virus; PPV: Plum Pox Virus; RT-PCR: Reverse Transcription Polymerase Chain Reaction; Z: Zidovudine.

INTRODUCTION
Stone fruit is often attacked by the virus pathogens. The most important virus diseases are Prune Dwarf Virus (PDV), Plum Pox Virus (PPV) and Prune Necrotic Ringspot Virus (PNRSV). These diseases are very problematic, because they cause growth and leaf deformations, chlorosis, bud necrosis, fruit deformations and finally the decrease of the total yield (Mink, 1992; Lang et al., 1997). As the most widespread and the most damaging virus pathogen of the stone fruit is considered PPV. In the Czech Republic, it is spread over most of the orchards with prune-, myrobalan-, peach- and apricot trees (Polak, 2002). PPV was at first described in 1917 in plum-trees and in 1933 in apricot-trees in the Eastern Europe, America and Asia (Boscia and Savino, 2013).

The most using methods for virus pathogen detection are the serological test ELISA (Enzyme-Linked Immunosorbent Assay), the molecular test RT-PCR (Reverse Transcription Polymerase Chain Reaction) and the biological indexing, which is the testing on the plant indicator (Nolasco, 2003).

The standard method for the virus elimination in fruit trees and obtaining the virus-free plants is the thermotherapy followed by the micro-propagation (Mink et al., 1998). It is possible to use in vivo thermotherapy (Janekova, 1993), in vitro thermotherapy (Spiegel et al., 1995), chemotherapy or the combination of both of them (Howell and Eastwell, 2001).

Lots of publications for the virus elimination and treating fruit trees and grapevine by the method of thermotherapy has been written. The results of in vivo and in vitro thermotherapy were often very problematic, because some types of the species Prunus, especially prune-trees, apricot-trees, peach-trees and cherry-trees, died during the process of thermotherapy. Stone fruit are very sensitive to the higher temperatures, so...
the big percentage of the plants have not survived the treating (Spiegel et al., 1995; Krizan et al., 2008).

The results of the method chemotherapy for virus elimination are much more perspective. Chemotherapy is one of the way to eliminate the virus in the plants. Different antivirals can be used for the chemotherapy in different plants (Matthews, 1953), e.g. Citrus (Sanjeev et al., 2007), raspberry (Pupola et al., 2009), Vitis vinifera (Skiada et al., 2013) and Cynara scolimous (Navacchi et al., 2005). Acyclovir (Weiland et al., 2004; Navacchi, 2005), ribavirin (Cieslinska, 2007), azidothymidine (5’-TCCTTGTCAAGCCAA; Nassuth et al., 2000) have been used for a chemotherapy in plants. Ribavirine (or azidothymidine) is an antiviral drug used for the treatment of HIV/AIDS infection. It is the most used antiretroviral. Ribavirine inhibits the reverse transcriptase factor, blocks the production of DNA and new viruses (Dyer et al., 1988; Carvalho et al., 2013).

The effectiveness of chemotherapy, or the combination with the thermotherapy in some cases, was up to 100% and the percentage of the survived and healthy plants was significantly higher than it was in the classic method of thermotherapy (Cieslinska, 2007).

The main goal of this study is to find out and check the effectiveness of the new antiviral ribavirine and compare it with the commonly used ribavirin. These results could be very useful for the certification system of the plant material health condition.

MATERIALS AND METHODS

Plant Material:

The basic material for this experiment were the peach trees Prunus persica cv. ‘Red Haven’ cultivated in the quarantine rooms of the technical isolate of the Faculty of Horticulture in Lednice (Mendel University in Brno), Mendeleum-Institute of Genetics, Czech Republic. The material was infected by PDV, PPV and PNRV. One budded herbaceous shoots were taken and their surface was disinfected with 0.2 % mercuric chloride for 10 minutes and cleaned with sterilized distilled water. The nodal segment culture was established from these samples by transferring into the test-tubes on the Quoirin and Lepoivre (1977) medium for stone fruit, and cultivating in a growth cabinet. Plants were growing for couple of weeks until they were strong enough to be capable of treating by chemotherapy.

Culture Conditions:

Cultures were maintained at the temperature of 21 ± 1 °C with the photoperiod of 16/8 (day/night). For lighting, the fluorescent tubes with light intensity 20.25 μmol m⁻² s⁻¹ were used.

Chemotherapy:

In vitro chemotherapy using ribavirine (Z) was applied to eliminate viruses, at concentrations 25 mg l⁻¹ (Z 25) and 50 mg l⁻¹ (Z 50). Two types of control variant were used - the first one contained no antiviral (C0) and the second one was previously treated by the antiviral ribavirin at concentration 20 mg l⁻¹ (C1). Each variant included 15 plants in duplicate. Antivirals were added by sterile filters with the diameter of pores 0.22 μm into media right after autoclaving. Small samples of plants were cultivated on MS medium [Murashige, Skoog (1962)] with antivirals for three weeks. After this time period, samples were transformed on a new medium (Quoirin and Lepoivre, 1977) with the plant regulators (0.4 mg l⁻¹ BA, 0.01 mg l⁻¹ NAA) and cultivated without antivirals.

RT-PCR:

In order to find out if plantlets are still infected, young plant leaves were tested by a molecular detecting method named RT-PCR. For RNA isolation, Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, USA) was used. Primer pair H968 (5’-GTCTGTGGTTCTTGCAGG) and C1163 (5’-CCTTTGAGTCCACAAGCCAA; Nassuth et al., 2000) was used as the internal positive control to avoid false results. The samples were transcribed to cDNA using M-MLV RT (Reverse Aid™ Reverse Transcriptase, Fermentas, Vilnius, Lithuania) during the reverse transcription. The first step was denaturation (5 min, 95 °C), where the prepared samples contained 12 μl of HPLC water, 2.5 μl of RNA template and 0.5 μl of random primer p(dN)6 (Roche, Manheim, Germany). The next step was the synthesis of cDNA (10 min, 25 °C + 60 min, 42 °C), in which 10 μl of reaction mixture was added to each sample. The reaction mixture included 3.25 μl of HPLC water, 5 μl of RT buffer (5x, Fermentas, Vilnius, Lithuania), 1.25 μl of (10 mM) dNTPs (deoxynucleotide triphosphates) (Invitek, Berlin, Germany) and 0.5 μl of reverse transcriptase (200 Uμl⁻¹, Fermentas, Vilnius, Lithuania).

Viral cDNA was amplified in the total volume of 20.8 μl. The reaction mix for PCR consisted of 10.5 μl of water (HPLC purity), 4 μl of 5x Colorless GoTaq®Flexi Buffer for polymerase (Promega, Madison, USA), 1.2 μl of 25mM MgCl₂ (Promega, Madison, USA), 0.2 μl of 10 μM dNTP mixture (Invitek, Berlin, Germany), 0.2 μl of GoTaq® G2 Flexi DNA polymerase (5μl⁻¹) (Promega, Madison, USA), 1 μl of both primers (10μM), 0.7 μl of Flexi 5x GoTaq® Flexi Buffer for polymerase (Promega, Madison, USA), 1 μl of both primers (10μM), 0.7 μl of Flexi 5x
Green GoTaq® Flexi Buffer (Promega, Madison, USA) and 2 µl of DNA template.

The PCR was carried out as follows. After an initial denaturation by 3 min at 94 °C, were 40 cycles of 94 °C 30 s, 56 °C 30 s (depends on primer combination), 72°C 45 s and 72°C 7 min. PCR products were analyzed by electrophoresis in 1.2 % agarose gel coloured by GelRed (Biotium, Hayward, USA) and visualised with a UV transilluminator.

**Data Analysis:**

The results were analysed according to Analysis of Variance, the separation of products was tested using Tukey’s HSD (Honestly Significant Difference) test, at the level P <0.01. Results were analysed by the program package Statistica 10 (Stat Soft., USA).

**RESULTS AND DISCUSSION**

The antiviral zidovudine has been used for the method chemotherapy at two concentrations (25 and 50 mg l⁻¹) to eliminate *Prune dwarf virus*, *Plum pox virus* and *Prune necrotic ringspot virus in Prunus persica* trees cv. ‘Red Haven’. Two controls were chosen for comparing the results, ribavirin at the concentration 20 mg l⁻¹ and standard Quoirin and Lepoivre (1977) medium without antivirals. All plants were tested after the treatment and results were statistically evaluated. Figures 1, 2, 3 show the PCR products of PPV, PDV and PNRSV on agarose gel.

The first control variant without the use of antiviral was 100 % positive for PDV, PPV and PNRSV. In the second control variant including ribavirin treatment was a low number of positive plants as well (6 % PPV, 30 % PDV and 10 % PNRSV of positive plants). The most effective method for PDV, PPV and PNRSV elimination by the method of chemotherapy was the application of zidovudine (Fig. 1, 2 and 3). 100 % of virus free plants were achieved at both concentrations and there has been no damage on the plant material.

![Fig. 1. Agarose gel of Plum pox virus detection by RT-PCR. Size of the PCR products is approximately 220 base pairs. Products with the size under 100 base pairs are primer dimers.](image1)

1-6: peach samples; (-): negative plants; M: 1 Kb Plus DNA Ladder; N: negative control; P: positive control; Bl: blank sample.

![Fig. 2. Agarose gel of Prune Dwarf Virus detection by RT-PCR. Size of the PCR products is approximately 220 base pairs. Products with the size under 100 base pairs are primer dimers.](image2)

1-6: peach samples; (-): negative plants; M: 1 Kb Plus DNA Ladder; N: negative control; P: positive control; Bl: blank sample.
Fig. 3. Agarose gel of Prune necrotic ringspot virus detection by RT-PCR. Size of the PCR products is approximately 200 base pairs. Products with the size under 100 base pairs are primer dimers. 1-6: peach samples; [−]: negative plants; M: 1 Kb Plus DNA Ladder; N: negative control; P: positive control; Bl: blank sample.

Krizan et al. (2013) used ribavirin at the concentration 20 mg l⁻¹ and they observed no PPV positive plants of peach and apricot varieties. Usage of ribavirin is also recommended by the authors Jakab-Illyefalvi and Pamfil (2011) and El-Dougdoug (2010). However, higher concentrations of ribavirin (50 mg l⁻¹ or 100 mg l⁻¹) can inhibit the growth of the shoots and roots (Cieslinska, 2007, Vescan et al., 2011). Hauptmanova and Polak (2011) obtained virus free plants (plums and apricots) at lower ribavirin concentrations (5-10 mg l⁻¹).

Except ribavirin, different chemicals with antiviral effect could be used for the plant virus elimination. Matthews (1953) was interested in Lucerne mosaic virus elimination in Nicotiana glutinosa, tobacco and beans by using triazolopyrimidine analogues of quinine (guanazole), adenine analogue and hypoxanthine analogues and others. Only the named chemicals had any effect to Lucerne mosaic virus. Guanazole was the most effective in N. glutinosa and tobacco at 0.01 M. Sanjeev et al. (2007) used acyclovir, zidovudine, DHT (2,4-dioxohexahydro-1,2,5-triazine) or 2-thiouracil at 5-25 mg l⁻¹ for Indian citrus ringspot virus in Kinnow (Citrus nobilis Lour × C. deliciosa Tenora). The highest number of virus free plants was 20.8 % using acyclovir at 25 mg l⁻¹ and 21.4 % using 2-thiouracil at 25 mg l⁻¹. The maximum phytotoxicity was observed with 2-thiouracil at 25 mg l⁻¹, where percent grafting success rate was 12.5 %. Weiland et al. (2004) have found, that the treatment by acyclovir at 20 mg l⁻¹ did not produce any Grapevine fanleaf virus free plants of Vitis vinifera. Moreover, it produced higher percentage of apical necrosis in infected plants (80.4 %). Pupola et al. (2009) used azacytidine or dicyanamide at 25 mg l⁻¹ in raspberry for Raspberry bushy dwarf idaeovirus elimination. The elimination was successful in the one out of three raspberry varieties. Both chemicals did not damage the plants.

CONCLUSION

Results show that using of zidovudine and ribavirin is suitable for the Prune dwarf virus, Plum pox virus and Prune necrotic ringspot virus elimination in ‘Red Haven’ peach cultivar. 100 % of virus free plants have been observed after the chemotherapy treatment using zidovudine as the antiviral and the high number of virus free plants after using ribavirine, as well. The next step for virus elimination study will be the serological test ELISA after the chemotherapy treatment, the decrease of the concentration of the antiviral up to 10 mg l⁻¹ and the extension of the range of species and genus to other cultivars of peach, apricot, garlic and grapevine.

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REFERENCES


