The thesis of the doctoral (PhD) dissertation

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Evaluation of the efficiency of *in vitro* virus elimination procedures performed on *Prunus* species

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Background of the work and objectives

Stone fruit trees are natural host plants for many pathogens that are transmitted vegetatively. Known viral diseases cause significant economic damage to stone fruit plantations. Viruses are easily transmitted from plant to plant with insect vectors and during vegetative propagation, prevention is the most important tool against them: it is essential to use healthy propagating material when creating orchards and continuous protection against vectors during maintenance.

The maintenance of healthy fruit propagating materials is a state task. In our country, pathogen-free stone fruit propagation materials are produced and maintained in isolated Prebasic plantations by the Hungarian University of Agriculture and Life Sciences, Institute of Horticultural Science, Research Centre for Fruitgrowing.

Based on European Union regulations, in Hungary the 14/2017 FM Decree (III. 23.), annex 9 contains the phytosanitary requirements for the maintenance of Prebasic plantations which prescribes obligatory annual testing for plum pox virus (PPV), Prunus necrotic ring spot virus (PNRSV) and plum dwarf virus (PDV) in case of sharka-host stone fruits and annual nested PCR tests for detection of European Stone Fruit Yellows Phytoplasma (ESFY) in case of apricot (*Prunus armeniaca*) cultivars. In addition to the previuosly mentioned pathogens, stone fruits can contain many other viruses and viroids. More and more viruses are being identified in *Prunus* species, even latently, without causing any symptoms.

Many techniques are used to produce virus-free plants around the world and traditional pot treatments have been replaced by *in vitro* methods. However, there is no universally usable, reliable, fast solution for elimination of viruses. Genotype dependence is a very significant factor, so plant species and varieties have different tolerances to the applied treatments. Stonefruits are particularly sensitive. to high temperature, and the efficiency of plant regeneration from meristem is lower compared to herbaceous plants or apple fruits. Different viruses also respond differently to treatments, so it may happen that the strength of the treatment needed to eliminate the viruses exceeds the plant's tolerance, and the plant dies during the treatment. An integral part of the elimination procedure is testing, the

reliable and quick detection of the presence of viruses, which greatly contributes to the success and shortening of the process.

Aims during my doctoral work:

- Establishment of sterile shoot cultures from plum and apricot cultivars infected with cherry virus A (CVA) and little cherry virus 1 (LChV-1) which were previously identified only from cherry and sour cherry host plants and the application of various *in vitro* virus elimination techniques on them,
- Comparison the effectiveness of the applied methods, examination of the responses of host plants and viruses to the treatments,
- Creation of healthy, virus-free shoot cultures from infected starting materials,
- Finding a virus elimination method which is more effective and can be used more widely for the virus removal of stonefruit species and varieties infected with various viruses, instead of the currently used precise handwork-and time-consuming techniques with low survival and uncertain outcome.

Materials and methods

Plant material

In the summer of 2017, samples were collected from the basic and prebasic stock plantation of the Research Centre for Fruit Growing, Hungarian University of Agriculture and Life Sciences (MATE), Érd, Hungary, to establish *in vitro* shoot cultures. Using high-throughput sequencing (HTS) of small RNAs, our research group was the first in Hungary to identify cherry viruses (CVA, LChV-1), previously known only from cherry and sour cherry host plants, in plum and apricot varieties, so we began to examine trees infected with viruses occurring on these new host plants. We worked with the following viral plant material confirmed by RT-PCR:

- two apricot cultivars (‘Magyar kajszi’ és ‘Pannónia’) infected with cherry virus A (CVA),
- plum (‘Besztercei Bt. 2’) infected with cherry virus A (CVA),
- apricot (‘Magyar kajszi’) coinfected with little cherry virus 1 (LChV-1) and plum pox virus (PPV).

After later sample collecting and virus testing (spring of 2019)

- a cherry tree (‘Éva’) infected with Prunus necrotic ring spot virus (PNRSV), causing serious problems in cherries and sour cherries, were also selected to establish *in vitro* shoot cultures.

Establishment of *in vitro* cultures and plant growing conditions

The leaves were removed from the fresh shoots and the one or two-node-long shoot segments were first soaked in 70% ethanol for 3 minutes in a sterile laminar box, then, after surface sterilization in HgCl2 solution (0.4%, 6 minutes), were rinsed in sterile distilled water three times. After that, the shoot pieces were placed on the plant species-specific medium. *In vitro* plants were kept in a growth room at 24 ± 1 °C under 16 h photoperiod with a light intensity of 60 μM m^{-2} s^{-1} provided by warm and cool white fluorescent lamps, and subcultured every four weeks. After successful shoot culture establishment, the virus presence was confirmed by RT–PCR, and virus-infected lines were selected and propagated for virus

elimination treatments. The interruption of the doctoral work due to personal reasons made it necessary to store the shoot cultures in a refrigerator for a long time. The shoot cultures were placed in their species-specific culture medium in a cold chamber (5.5°C, complete darkness) for 1.5, and after further experiments for another 1 year. During cold storage, cultures were transferred to fresh culture medium every 4-6 months, followed by 1-2 weeks of keeping in a growth room, and the cultures were returned to the refrigerator. As soon as the opportunity arose, the experiments were continued under the conditions described above.

In vitro virus elimination methods

Combination of in vitro thermotherapy and shoot tip culture

Two-week-old shoot cultures were transferred into a climate chamber (Panasonic MLR-352) for heat treatment at 38/36°C (day/night) and 12 h of light (160 μmol m− 2 s− 1). After 14, 15, 17, 18, 23 days of heat treatment in case of 'Besztercei Bt. 2', 14, 15, 16 days in case of 'Pannónia', 13, 16 days in case of CVA-infected 'Magyar kajszi' and after 15, 16, 20, 24 days in case of ('Magyar kajszi') coinfected with LChV-1 and PPV, 1–2 mm long shoot tips (containing 2–4 leaf primordia) were cut off under a stereomicroscope under sterile conditions and maintained on multiplication media in Petri dishes for regeneration. Shoot tips were subcultured to fresh medium every 4 weeks, and then maintained the successfully regenerated shoot cultures until virus testing.

Chemotherapy

After autoclaving, ribavirin (Duchefa, The Netherlands) or zidovudine (Sigma–Aldrich, USA) was added to the multiplication media by filter sterilization (Millipore, 0.22 μm, PES membrane) to a final concentration of 25 or 50 mg L-1. Three *in vitro* shoots (1 cm long) per vessel, (in 5 vessels, a total of 15 shoots per treatment) were maintained on a multiplication medium containing the antiviral agent for 2 successive subcultures each lasting four weeks (total 8 weeks). After 8 weeks, the apical parts (1 cm long) of the treated shoots were removed and cultured as separated lines on antiviral agent-free media until virus indexing. As a control, 1 cm long shoots were maintained

on multiplication media without antiviral agents for 8 weeks. For each treatment, 15 shoots were treated.

Combinations of thermotherapy and chemotherapy

After two weeks of *in vitro* thermotherapy, 1 cm long apical shoots were cut and transferred onto multiplication media supplemented with the antiviral agent zidovudine at concentrations of 25 or 50 mg L-1 for 8 weeks with one subculture. After 8 weeks, the shoots were cultured on antiviral agent-free media until virus indexing. In the control group, there were 1 cm shoots that had been heat-treated for 2 weeks and grown on normal, antiviral-free medium.

Virus detection

After treatment, whole micropropagated shoots without basal calli were collected to determine infection status. RNA was extracted from the *in vitro* plant samples via the CTAB method (Gambino et al. 2008). For virus detection, the reverse-transcription PCR method (RT–PCR) was used. cDNA synthesis was performed from the extracted RNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using random primers according to the manufacturer's instructions. PCR was performed using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific). For the reaction, 9.4 µl MQ water, 3 µl 5x Phire buffer, 0.75 µl - 0.75 µl forward and reverse primer (10 pmol/ µl), 0.3 µl dNTP (10 mM), 0.3 µl (0.6 U) Phire polymerase and 0.5 µl of cDNA were added, so the final volume was 15 µl.

The PCR reaction was performed with the following settings:

98°C - 30 sec
98°C - 10 sec
* °C - 10 sec
72°C - 20 sec
72°C - 1 min
} 40x

*Annealing temperature characteristic of the applied primer

The PCR results were evaluated via gel electrophoresis of the PCR products.

Results

Establishment of *in vitro* cultures from virus-infected trees

After sprouting the dormant buds in the non-contaminated vessels, the fresh shoots were cut from the stem and placed on a species-specific culture medium, and the propagation of the sterile shoot cultures began. We have successfully established *in vitro* cultures from each plant species or cultivar and maintained them throughout the experiments, providing experimental material.

Results of *in vitro* virus elimination experiments performed on CVA-infected plum shoot cultures

Results of shoot tip culture after thermotherapy

After 14-23 days from the start of the heat treatment, a total of 49 shoot tips 1-2 mm long were excised and placed on culture medium from the still viable shoot cultures, of which 19 (39%) died early (turned brown or callused and were unable to regenerate).

Examining the effect of heat treatment duration, we found that after 3 weeks of thermotherapy at 38/36°C, the harmful consequences of long-term heat treatment became visible, the plants were significantly weakened, turned yellow, and their shoot tips died. For the longest time, I excised shoot tips after 23 days of heat treatment, but after this long treatment period, tips died quickly and were unable to regenerate. The plants tolerated the 14-18 day heat treatment well; they remained green and vigorous, and their shoot tips were intact and suitable for preparation. The excised shoot tips could regenerate to proliferable shoots after a few months at a high level (69%). The plants regenerated the least (43%) after 17 days of thermotherapy, and the best (69%) after 15 days.

After several months of micropropagation, samples were taken from the successfully regenerated shoots for virus diagnostics. A total of 20 *in vitro* plants regenerated from shoot tips were tested for the presence of CVA by RT–PCR. Fifteen plants (75%) became CVA-free, while 25% remained infected. Our small-scale pilot test showed that the length of thermotherapy did not increase or reduce

the effectiveness of virus elimination within the time interval used (14–18 days), most of CVA-free plants (78%) resulted after 15 days, but after 14 or 18 days the elimination rates were 75%.

Results of chemotherapy alone or in combination with heat treatment

The plum cultivar ('Besztercei Bt. 2') proved to be tolerant to zidovudine and ribavirin treatments. The plant survival rate was not negatively affected by any of the antiviral agents used at concentrations of 25 or 50 mg L− 1. No significant phytotoxic effects were observed during these treatments.

Zidovudine alone was not able to efficiently eliminate CVA at any concentration; however, treatment with zidovudine (25 mg L− 1) after heat treatment increased the elimination from 7 to 40%. Ribavirin applied at a lower concentration (25 mg L− 1) was very efficient, and no CVA was detected in the tested plants. At a higher concentration (50 mg L− 1) of ribavirin, 40% of the tested plants were virus-free. Surprisingly, some of the control plants (7–25%) tested negative for CVA.

Results of *in vitro* virus elimination experiments performed on apricot cultures infected with CVA or LChV-1+PPV

Results of shoot tip culture after thermotherapy

After 13-24 days of heat treatment, a total of 74, 1-2 mm long shoot tips were excised from apricot cultivars and placed on culture medium from the still viable shoot cultures. Only 6 of the shoot tips (8%) began to regenerate, the rest (92%) died early (turned brown or callused). Although the apricot shoots tolerated the 2-3 week *in vitro* heat treatment, most of the prepared shoot tips were unable to regenerate. The regeneration capacity of 'Pannónia' (26.3%) was higher than that of 'Magyar kajszi' (1.8%). One piece of plants regenerated from the shoot tips of 'Magyar kajszi' (heat-treated for 20 days) were able to test, which was negative for PPV and LChV-1.

Results of chemotherapy alone or in combination with heat treatment

During the chemotherapy treatment of apricot shoot cultures, the survival of plants grown on medium supplemented with zidovudine was 100% in the majority of cases, only in the case of

CVA-infected ‘Magyar kajszi’ treated at a concentration of 50 mg/l, it decreased to 87%. A similar result was obtained when the zidovudine treatment was combined with heat treatment, the survival was also reduced only in the group CVA-infected ‘Magyar kajszi’ treated with at a concentration of 50 mg/l, then to 80%.

In the case of ribavirin, survival results of 0-93% were obtained on apricot cultures. 100 or 93% of the ‘Pannónia’ cultures treated with ribavirin (25 or 50 mg/l) were survived the treatment. Due to the poor survival results of the ribavirin treatment on LChV-1+PPV infected ‘Magyar kajszi’ shoot cultures, the treatment was repeated. The repeated treatment resulted in higher survival in the control group and the number of plants surviving the ribavirin 25 mg/l treatment also increased (from 27% to 93%), however, the plants treated at a higher concentration (50 mg/l) had a 40% survival decreased to 0%. Ribavirin treatment of CVA-infected ‘Magyar kajszi’ and zidovudine treatment combined with heat treatment of LChV-1+PPV-infected ‘Magyar kajszi’ were not carried out.

Zidovudine treatment on CVA-infected apricot cultures resulted in 0-13% virus-free status. No CVA-negative results were obtained from ‘Pannónia’ cultures, only in combination with heat treatment, at which time 20 or 27% of the plants became virus-free when zidovudine was used in concentrations of 50 and 25 mg/l. In the case of ‘Magyar kajszi’, combination with heat treatment also increased the efficiency of virus elimination from 13% to 80% in the case of 25 mg/l zidovudine, and from 8% to 50% in the case of 50 mg/l. When the ‘Magyar kajszi’ cultures infected with 2 different viruses were treated with zidovudine (25 and 50 mg/l), LChV-1 infection was reduced to 40 or 37%, while PPV infection was 87 or 67%.

As a result of the ribavirin treatment, 93% of the ‘Pannónia’ plants became virus-free, however, the virus was not detected in the control plants maintained simultaneously with this treatment. From the ‘Magyar kajszi’ cultures infected with 2 different viruses, it was not possible to maintain a virus-free plant with the first ribavirin treatment, however, the result of the repeated treatment, 20% of the plants became LChV-1- and 100% PPV-free applied the lower concentration of ribavirin. In the case of both viruses, the treatment with 25 mg/l zidovudine resulted in the highest elimination of 40%, ribavirin (25 mg/l) only 20%.

Results of *in vitro* virus elimination experiments performed on PNRSV infected cherry culture

During the chemotherapy treatments of PNRSV-infected ‘Éva' cherry shoot cultures, the survival of the plants was between 80-100%. Zidovudine (25 or 50 mg/l) resulted in 80-93% survival, while zidovudine treatments combined with heat treatment resulted in 100% survival. During the ribavirin treatment, we could not observe plant death at any concentration.

73-93% of cherry cultures that underwent chemotherapy became virus-free. Zidovudine (25 or 50 mg/l) resulted in 83 or 86% PNRSV-free plants, and when combined with heat treatment, a large proportion (73 and 93%) of free plants were obtained. Ribavirin at both concentrations resulted in 93% virus-free plants. It should be noted that the number of virus-free plants was also high in all control groups of the treatments carried out.

The results of virus elimination experiments on virus-infected *Prunus* shoot cultures depending on time

In connection with the negative samples obtained in the control groups, the question of spontaneously becoming virus-free arose. After the treatments and virus tests, 10 untreated control plants of each plant cultivar (4-week-old propagated, whole shoot bunch, without basal callus) were tested. As a result of RT-PCR performed 5.5 years after the initiation of the apricot and plum *in vitro* cultures, and 3.5 years after the initiation of the cherry culture, 100% negative samples were obtained, regardless of virus and plant type. The phenomenon needed an explanation, so we examined the culture conditions, what was in the background, and the time of the virus tests that followed the experiments.

In the untreated control plum (‘Besztercei Bt. 2’), the initial CVA infection did not decrease after 4 years, 25% of the shoot cultures examined in the first year and 7% after 4 years were CVA-free. The different virus elimination experiments performed in plums can therefore be compared and the greater efficacy of ribavirin compared to zidovudine can be confirmed. Heat treatment alone (heat-treated control group) had no antiviral effect, in combination with zidovudine (25 mg/l), the proportion of virus-free plants

increased 2x (from 20 to 40%) compared to zidovudine treatment. However, after 5.5 years, all the 10 tested control plants were negative.

When examining ‘Magyar kajszi’ coinfected with two viruses, we observed that the initial virus infection decreases with time. In the case of LChV-1, 0% after 1 year, 60% after 2 years, and 80% after 4 years, while in the case of PPV, the virus-free rate is 0% after 1 year, 20% after 2 years, and 93% after 4 years. After 5.5 years, all the 10 tested control plants were negative for both viruses. The results of the elimination experiments are therefore more difficult to evaluate due to the increasing proportion of virus-negative plants in the controls. However, the effect of the lower concentration of ribavirin treatment is still noticeable, in the experiment carried out after 2 years, 100% free plants were produced in addition to 20% of the control. The difference between the resistance of the two viruses can also be seen in the complex infected apricots, PPV was more effectively eliminated by ribavirin (100%) than LChV-1 (20%).

The virus-free rate in the untreated CVA-infected ‘Magyar kajszi’ was 11% after 3.5 years, in the experiment conducted after 4 years, 20% of the heat-treated controls were virus-free, so here the significant increase in the effectiveness of chemotherapy combined with heat treatment (at 25 mg/l from 13 to 80%, and at 50 mg/l from 8 to 50%) can be verified. After 5.5 years, however, all the 10 tested control plants were negative.

In apricot ‘Pannónia’, the control plants of the experiment carried out after 2 years were still 100% infected, in the heat-treated performed at the same time, the virus-free rate increased to 33%. After 4 years, CVA could not be detected in the control group, so the 93% virus-free result obtained with ribavirin treatment is doubtful. The negative test results of the 5.5-year-old controls further confirmed the observation that CVA infection in ‘Pannónia’ significantly decreased over time.

Even after 1.5 years, the degree of virus infection in the shoot cultures of the PNRSV-infected ‘Éva' cherry was low, from which we can conclude that PNRSV is an easily eliminated virus, and can spontaneously disappear from the plant, even as a result of being kept in micropropagation.

New scientific results

- I successfully established sterile shoot cultures from apricot and plum trees that were infected with viruses previously detected only from cherries in Hungary. We confirmed the presence of viruses in them using a molecular method.
- I successfully regenerated a virus-free *in vitro* plant from a CVA-infected plum cultivar using shoot tip culture following *in vitro* heat treatment.
- We showed that ribavirin (25 mg/l) treatment could be eliminate CVA from plum cultivar 'Besztercei Bt. 2' with 100% efficiency, and PPV from apricot cultivar 'Magyar kajszi' with a good survival rate and in a shorter time than with shoot tip culture combined with heat treatment.
- I eliminated CVA and LChV-1 from infected *Prunus* shoot cultures first time.
- We showed that the initial CVA infection of apricot cultivar 'Pannónia', as well as the initial virus contamination of 'Magyar kajszi' coinfected with two viruses (PPV+LChV-1) decreases with time in *in vitro* cultures. As a result of long-term (>5 years) *in vitro* maintenance, 100% of the plants became virus-free (verified by PCR), for all initially infected plant species and cultivars, as well as for all tested viruses.

Conclusions and suggestions

From the point of view of practice, the goal of a pathogen eradication program is to create a healthy *in vitro* plant, free from the initial presence of viruses, which can be multiplicated by micropropagation, and after rooting and acclimation, this plant material is ready for placing in the Prebasic stock collection. In all phases (*in vitro* propagation, rooting, acclimatization) overplus must be made, because 100% survival is not expected. Even a single certified virus-free *in vitro* shoot bunch may be enough to place the given plant variety in the Prebasic stock collection (3-4 plant/variety) after passing through the certification system and can be maintained as the base of production of healthy plant propagation material.

During the virus elimination experiments, we obtained virus-free plants from each, initially infected item. Based on the comparison of the methods, large differences can be observed in each experimental group. The small number of samples in the groups (10-15 pcs), the peculiarities of micropropagation and the time differences between the experiments do not allow or absolutely require statistical analysis. Therefore, it may be important in the future to repeat the treatments with the best results on a larger number of samples and to verify the results with statistical tools. Based on my work, chemotherapy has proven to be a suitable method for removing viruses from stone fruit species. Zidovudine and ribavirin are also recommended at a lower concentration (25 mg/l) in order to achieve a higher rate of virus-free status and to minimize possible phytotoxic effects. In combination with preliminary heat treatment, the elimination efficiency can be increased, so we can get virus-free plants from less starting material, with less testing and in a shorter time.

In case of plums, without the use of antiviral chemicals, a high percentage of virus-free plants can also be achieved by shoot tip cultivation after heat treatment. However, there is a lot of loss here, a large amount of destruction, regeneration is slow and the rate is low, but the risk to health of chemotherapy chemicals is also not negligible. It is therefore also worthwhile to develop an effective combination of the previously mentioned larger shoot tip and longer heat treatment period in case of apricot shoot cultures. I believe it is important to

mention the virus-free diagnostic results obtained during long-term *in vitro* maintenance. Long-term micropropagation as a virus-elimination method does not appear to be a practical option at first, however, there are plants that are sensitive to heat, to regeneration from shoot tips, and to antiviral chemicals, whose treatment is not feasible. In such cases the elimination procedure may take many years, but can still be effective.

Publications connected to the dissertation

Papers in impact factored journals

Szabó, L. K., Desiderio, F., Kirilla, Z., Hegedűs, A., Várallyay, É., & Preininger, É. (2024). Elimination of cherry virus A from Prunus domestica 'Besztercei Bt. 2'using in vitro techniques. Plant Cell, Tissue and Organ Culture (PCTOC), 157(2), 45. [Q1, IF: 2,3]

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Baráth, D., Jaksa-Czotter, N., Molnár, J., Varga, T., Balássy, J., Szabó, L. K., ... & Várallyay, É. (2018). Small RNA NGS revealed the presence of Cherry virus A and Little cherry virus 1 on apricots in Hungary. Viruses, 10(6), 318. [Q2, IF: 3,8]

In reviewed article

Szabó Luca Krisztina, Kirilla Zoltán, Preininger Éva (2019). Csonthéjasok vírusmentesítése in vitro technikákkal (irodalmi áttekintés) KERTGAZDASÁG 51(2)

Baráth, D., Czotter, N., Bükki, A., Oláh, B., Balássy, J., Varga, T., … Várallyay, É. (2018). Új csonthéjasokat fertőző vírusok kimutatása Magyarországon. Georgikon for Agriculture: A Multidisciplinary Journal in Agricultural Sciences, 22(1), 28–33.

Book chapters

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Dániel, B., Alexandra, B., Dávid, C., Nikoletta, C., Luca, K. S., Zoltán, K., … Éva, V. (2018). Detection of CVA, LChV1 and PLMVd in Prunus sps in Hungarian stock collections. Power of Viruses : Programme and Abstracts Zagreb, Horvátország : Croatian Microbiological Society (2018) p. 99 , 1 p.

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