Preliminary results of *in vivo* thermotherapy of plum, apricot and peach cultivars artificially infected with PPV-M and PPV-D strains of *Plum pox virus*

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ABSTRACT: The elimination of *Plum pox virus* (PPV) in different stone fruit cultivars was verified by the method of thermotherapy *in vivo*. Trees of two plum cultivars Čačanská lepotica and Švestka domácí, apricot cultivars Leskora and Velkopavlovická, and peach cultivars Redhaven and Earliglo were used. They were infected artificially with two strains of the virus (PPV-D, PPV-M). Two cycles of thermotherapy *in vivo* were performed. During the first cycle, 16 trees of plum, apricot and peach were treated for 15 days at 37°C. In the second thermotherapy cycle, 10 trees of individual cultivars of plum, apricot and peach were treated for 22 days at 37°C. In the first thermotherapy (T1), 8 trees out of 16 died; PPV was eliminated in 2 trees of cv. Čačanská lepotica, 1 tree of cv. Švestka domácí and 2 trees of cv. Velkopavlovická. In the second thermotherapy (T2), 1 of 10 treated trees died. The virus was eliminated in 2 trees of cv. Čačanská lepotica, 1 tree of cv. Leskora, 2 trees of cv. Velkopavlovická, and 1 tree of cv. Redhaven. Nine (T1) and seven (T2) months after the thermotherapy, the presence of PPV was detected in 6 out of 11 originally recovered trees using ELISA. Out of 26 trees, 4 trees remained recovered: 2 plum trees and 2 apricot trees. One of these trees, apricot cv. Leskora was originally infected with PPV-M strain, whereas the other three with PPV-D strain. None of the 10 peach trees was treated successfully.

Keywords: *Plum pox virus* (PPV); apricot; peach; plum; fruit trees; thermotherapy *in vivo*

The *Plum pox virus* (PPV) belongs among the most devastating plant viruses and, at the present time, it occurs not only throughout most European countries but also in North and South America, Asia and Africa. Only Australia and New Zealand have not been tainted with this virus, yet. It is widely spread in the Czech Republic and the virus-free material of stone fruits for outplanting is available only due to the fact that the production of nursery material is derived from parental virus-free plants of individual cultivars which are cultured and maintained in isolation. However, the chance of random contamination of the parental plants with sharka cannot be ruled out. It can appear in the case of culturing new sharka-resistant stone fruit cultivars, or a cultivar infected with sharka can be imported and would need a treatment.

It is possible to eliminate PPV in plants using classical thermotherapy *in vivo*; other possibilities are thermotherapy or chemotherapy *in vitro* in cultures of stone fruits. Until now, only a few original reports dealing with elimination of PPV by *in vivo* thermotherapy have been reported; their treatment protocols vary and are not in one line. Also, none of the published papers specified the PPV strain infecting the trees. We therefore verified the individual methods and protocols on elimination of PPV in different stone fruits. Up to the 1980s, the protocols of *in vivo* thermotherapy were mostly used when the infected plants were exposed to higher temperature for a few weeks. Minoiu (1975, 1976) used alternating temperatures of 36°C, 46°C and 60°C for a period of 37 days to eliminate PPV from plum trees. Kegler (1967, 1968) used the temperature of 37°C for 2–3 weeks, followed with grafting the recovered green shoots on virus-free rootstocks. For the elimination of viral complexes PPV, *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) from four plum cultivars, Janečková (1993) used a combined method of *in vivo* thermotherapy and *in vitro* chemotherapy of plants in 2 cycles: 1st cycle (fall) 3 weeks 34.5°C and 1 week 37.5°C; 2nd cycle (spring) a stepwise temperature increase from 20°C to 34°C in the course of 8 days, followed by 4 weeks at 36.5°C and 2 days

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at 37.5°C. She then collected shoot-tops of grafted plants and cultured them *in vitro*, continuing with chemotherapy using ribavirin.

The other published works are based on elimination of PPV from stone fruits using *in vitro* cultures. In this way, Mosella Chancel et al. (1980) eliminated PPV and PNRSV from peach trees. Vertesy (1981) eliminated PPV and PNRSV using *in vitro* cultures from two plum rootstocks and Isac (1985) eliminated PPV from three plum cultivars. The important findings on methodology for the elimination of viruses (including PPV) from fruit trees are reported in the original paper of Knapp et al. (1995) and also in the work of Spiegel et al. (1995). Mink et al. (1998) published an overview of findings and utilization of thermotherapy for the elimination of viruses, viroids and phytoplasmas from perennial plants. Further developments towards the utilization of thermotherapy and chemotherapy in order to gain virus-free stone fruits were presented by Howell et al. (2001).

Our experiments have been designed to verify the recovering methods for the elimination of PPV from different stone fruits and the influence of PPV strain on the recovery procedure.

We used the two most widely spread strains of the virus, PPV-D and PPV-M, which infect plum, peach and apricot trees. In the preliminary report, the results of determining the conditions for *in vivo* treatments of the above-mentioned stone fruit cultivars are presented.

MATERIAL AND METHODS

Plant material

For the model experiment of thermotherapy *in vivo,* following cultivars fruit were chosen:

plum – cv. Čačanská lepotica/St. Julien, and cv. Švestka domácí/myrobalan;

apricot – cv. Leskora/St. Julien, and cv. Velkopavlovická/M-VA-3;

peach – cv. Redhaven/B-VA-2, and cv. Earliglo/B-VA-3.

Two-year-old trees were obtained from Research and Breeding Institute of Fruit Growing Ltd., Holovousy. The trees were planted into containers on October 1, 2005, and vernalized at 4°C during the period of December 1, 2005 to January 10, 2006. Afterwards, the trees were placed in a greenhouse at 10°C. Two weeks later, the temperature was increased to 20°C.

Inoculation of trees with PPV-M and PPV-D, testing the presence of virus

Two months after the placement into the greenhouse the trees were inoculated with individual PPV strains. For the inoculation of virus PPV infected grafts from apricot cv. Karola (susceptible to PPV) were used; they were taken from trees infected with PPV-D or PPV-M. Each tree was inoculated with two buds. Five till ten trees of each cultivar of plum, apricot and peach were inoculated with individual strains of PPV. In the course of the vegetative season of 2006, the presence of PPV was not detected. Trees were vernalized at 4°C again during winter time. The presence of PPV-M and PPV-D in individual trees was detected using ELISA till spring 2007.

Thermotherapy *in vivo*

Two cycles of thermotherapy *in vivo* were performed. In the first thermotherapy, the total of 16 trees of plum, apricot and peach cultivars were treated: one apricot tree of cv. Velkopavlovická and two peach trees of cv. Earliglo were infected with PPV-M; one apricot tree of cv. Leskora and two of cv. Velkopavlovická, three peach trees of cv. Redhaven and one of cv. Earliglo, two plum trees of cv. Čačanská lepotica and four of cv. Švestka domácí were infected with the strain PPV-D. The thermotherapy was running for 15 days in a thermal room with controlled light and temperature setup at 37°C. The light setup was 14h-day and 10h-night. The temperature was increased stepwise from 20°C to 37°C and left at that level for 15 days, followed by a stepwise decrease down to 24°C. After the thermotherapy *in vivo* was finished, the trees were placed in greenhouse.

Ten trees were involved in the second treatment (T2): one apricot tree of cv. Leskora and one of cv. Velkopavlovická, one peach tree of cv. Redhaven and two of cv. Earliglo, and one plum tree of cv. Čačanská lepotica were infected with PPV-M; one apricot tree of cv. Velkopavlovická, one peach tree of cv. Redhaven, one plum tree of cv. Čačanská lepotica and one of cv. Švestka domácí were infected with PPV-D. The same light and temperature setup was chosen for the second thermotherapy, but the interval at 37°C was prolonged to 22 days. After the thermotherapy was finished, the temperature was subsequently decreased down to 25°C.

The presence of PPV in trees after the treatment and their placement in a greenhouse was deter-

Table 1. The results after the finished *in vivo* thermotherapy of plum, apricot and peach trees infected with PPV-M and PPV-D

Stone fruit	Cultivar	Total number of trees				Number of died trees				ELISA positive				ELISA and RT-PCR negative			
		PPV-M		PPV-D		PPV-M		PPV-D		PPV-M		PPV-D		PPV-M		PPV-D	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Plum	Čačanská lepotica	0	1	2	1	–	0	0	0	–	0	0	0	–	1	2	1
	Švestka domácí	0	0	4	1	–	–	2	0	–	–	1	1	–	–	1	0
Apricot	Leskora	0	1	1	0	–	0	1	–	–	0	0	–	–	1	0	–
	Velkopavlovická	1	1	2	1	1	0	0	0	–	0	0	0	0	1	2	1
Peach	Redhaven	0	1	3	1	–	0	2	–	–	0	1	–	–	1	0	–
	Earliglo	2	2	1	0	1	2	1	–	1	–	0	–	0	0	0	–

mined using DAS-ELISA. The trees in which the presence of virus was not detected by ELISA were tested using RT-PCR, immediately after the treatment and 9 (T1) or 7 (T2) months after the treatment.

Detection of PPV using DAS-ELISA

For the detection of PPV with DAS-ELISA (Clark, Adams 1977), commercial polyclonal antibodies were used (IgG and IgG conjugated with alkaline phosphatase) from the Loewe Company (Germany). Six leaves were collected from different parts of the tested tree and were combined to prepare a representative sample: 1 g of leaves was homogenized in extraction buffer using manual homogenizer and 200 µl was pipetted into 2 wells of microtitre plate. Results were evaluated on the spectrophotometer Dynatech MR 5000 at 405 nm. ELISA tests were done before treatment, 2 weeks after the treatment, and 9 (T1), eventually 7 (T2), months after the treatment.

Detection of PPV by RT-PCR

RT-PCR was used for testing of the trees for which negative DAS-ELISA results were obtained immediately after the thermotherapy and 7–9 months later. The total RNA was isolated from samples of 0.1 g of leaf tissue, using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Primers P1, P2 (Wetzel et al. 1991) and GO Taq polymerase (Promega Corporation) were used for the RT-PCR amplification. Aliquots of PCR products were run on 1% agarose gel. DNA was stained with SYBR Green (Invitrogen).

RESULTS AND DISCUSSION

The results of *in vivo* thermotherapy of apricot, peach and plum cultivars from both cycles are given in Table 1. A total of 8 trees out of 16 (50%) died in the course of the first thermotherapy. One plum tree of cv. Velkopavlovická and one peach tree of cv. Earliglo, both infected with PPV-M, one apricot tree of cv. Leskora, two peach trees of cv. Redhaven, one peach tree cv. Earliglo, and two plum trees of cv. Švestka domácí, all infected with PPV-D, did not survive the therapy. DAS-ELISA and RT-PCR conducted after the thermotherapy proved the presence of PPV in one peach tree of cv. Earliglo infected with PPV-M, and in one peach tree of cv. Redhaven and one plum tree of cv. Švestka domácí, both infected with PPV-D. A total of five trees were recovered: two trees of cv. Čačanská lepotica, one tree of cv. Švestka domácí, and two trees of cv. Velkopavlovická. The results were verified using RT-PCR. In three other trees the presence of PPV was detected using ELISA after nine more months: one tree of cv. Čačanská lepotica, one tree of cv. Velkopavlovická, and one tree of cv. Švestka domácí. Only two trees were recovered, namely the cultivars Čačanská lepotica and Velkopavlovická. The results were proved by RT-PCR.

During the second thermotherapy (T2), when only 10 trees underwent the treatment, only two trees died. The presence of virus was proved by ELISA in one plum tree of cv. Švestka domácí and in one peach tree of cv. Earliglo. Total of six trees were recovered: 2 plum trees of cv. Čačanská lepo-tica (one infected with PPV-M and one with PPV-D), 1 apricot tree of cv. Leskora (PPV-M), 2 apricot trees of cv. Velkopavlovická (one infected with PPV-M and one with PPV-D), and 1 peach tree of cv. Redhaven (PPV-M). The results were verified

Table 2. Overview of results 9 (T1) or 7 (T2) months after the finished *in vivo* thermotherapy

Stone fruit	Cultivar	Total number of trees	ELISA and RT-PCR negative			
			PPV-M		PPV-D	
			T1	T2	T1	T2
Plum	Čačanská lepotica	4	0	0	1	1
	Švestka domácí	5	0	0	0	0
Apricot	Leskora	2	0	1	0	0
	Velkopavlovická	5	0	0	1	0
Peach	Redhaven	5	0	0	0	0
	Earliglo	5	0	0	0	0

using RT-PCR. After another seven months, the presence of PPV was detected using ELISA in four other trees: 1 plum tree of cv. Čačanská lepotica, 2 apricot trees of cv. Velkopavlovická, and 1 peach tree of cv. Redhaven. ELISA results were verified using RT-PCR. Only two trees were recovered: 1 plum tree of cv. Čačanská lepotica and 1 apricot tree of cv. Leskora.

An overview of the results of ELISA and RT-PCR tests in the following year is shown in Table 2. Out of the total of 26 trees, 4 trees were recovered: 2 plum trees of cv. Čačanská lepotica, 1 apricot tree of cv. Velkopavlovická and 1 apricot tree of cv. Leskora. None of the peach trees was successfully treated. Most peach trees died during the thermotherapy. As peach trees belong to thermophilic stone fruit species, it will be unavoidable to identify the reason of their high mortality rate during the thermotherapy. To consider the results as final, it will be necessary to test the recovered trees again in the next vegetative season.

Three original reports dealing with elimination of PPV with *in vivo* thermotherapy of plum cultivars have been published, whereas no paper has been published on elimination of PPV in apricot and peach using *in vivo* thermotherapy. Minoiu (1975, 1976) used alternating temperatures of 36°C, 46°C, and 60°C for a period of 37 days. In our attempts most of the peach trees died at the temperature of 37°C during 15 (T1), and 22 (T2) days. Kegler (1967) dealing with plums used the same temperature of 37°C for 2–3 weeks and obtained similar results as in our trials. Janečková (1993) applied cycles of *in vivo* thermotherapy of plum cultivars Čačanská lepotica, Gabrovská, Carská, and Wazon's Gage using the temperatures from 34.5 to 37.7°C and intervals from 8 days to 4 weeks in combination with chemotherapy. The efficiency of therapy was 90% for PPV. It is difficult to compare partial published results with complex (plum, apricot, peach, different PPV strains) and more uniform (*in vivo* thermotherapy at 37°C) investigation in our trials resulting in the 15% efficiency of *in vivo* thermotherapy of plum and apricot trees; *in vivo* thermotherapy of peach trees has not been successful, yet.

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Předběžné výsledky *in vivo* termoterapie odrůd švestky, meruňky a broskvoně uměle infikovaných kmeny PPV-M a PPV-D viru šarky švestky

ABSTRAKT: V různých odrůdách peckovin byla metodou *in vivo* termoterapie ověřována eliminace viru šarky švestky, *Plum pox virus* (PPV). Byly použity stromy dvou odrůd švestky, Čačanská lepotica a Švestka domácí, odrůd meruňky Leskora a Velkopavlovická a odrůd broskvoně Redhaven a Earliglo, uměle infikovaných dvěma kmeny viru (PPV-D, PPV-M). Byly provedeny dva cykly termoterapie *in vivo.* Během prvního cyklu bylo léčeno 16 stromů švestky, meruňky a broskvoně 15 dní při 37 °C. Ve druhé termoterapii bylo léčeno 10 stromů jednotlivých odrůd švestky, meruňky a broskvoně 22 dní při 37 °C. Během první termoterapie (T1) odumřelo osm stromů z šestnácti. PPV byl eliminován ve dvou stromech švestky Čačanská lepotica, v jednom stromu Švestky domácí a ve dvou stromech meruňky Velkopavlovická. Ve druhé termoterapii (T2) odumřel jeden strom z deseti léčených. Virus byl eliminován ve dvou stromech švestky Čačanská lepotica, v jednom stromu meruňky Leskora, ve dvou stromech meruňky Velkopavlovická a v jednom stromu broskvoně Redhaven. Devět (T1) a sedm (T2) měsíců po termoterapii byla přítomnost PPV zjištěna pomocí ELISA v šesti z jedenácti původně ozdravených stromů. Ze dvaceti šesti léčených zůstaly čtyři ozdravené stromy: dva stromy švestky a dva stromy meruňky. Jeden z těchto stromů, meruňka odrůdy Leskora, byl původně infikován PPV-M kmenem, zatímco v dalších třech případech to bylo PPV-D kmenem. Žádný z deseti stromů broskvoně se nepodařilo úspěšně vyléčit.

Klíčová slova: virus šarky švestky (PPV); meruňka; broskvoň; švestka; ovocné stromy; termoterapie *in vivo*

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