The elimination of *Plum pox virus* in plum cv. Bluefree and apricot cv. Hanita by chemotherapy of *in vitro* cultures

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Abstract

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In vitro cultures of plum cv. Bluefree and apricot cv. Hanita infected with *Plum pox virus* (PPV) were used for the virus elimination by chemotherapy. Low ribavirin concentrations of 5 and 10 mg/l in Murashige-Skoog medium were applied in the treatment. *Plum pox virus* was completely eliminated by 5 mg/l of ribavirin in plum cv. Bluefree within twenty weeks and in apricot cv. Hanita in twelve weeks of the application. *Plum pox virus* was completely eliminated by 10 mg/l of ribavirin both in plum cv. Bluefree and apricot cv. Hanita within twelve weeks. The presence of PPV was not proved by RT-PCR. Clones of plum cv. Bluefree and apricot cv. Hanita were re-tested by RT-PCR one year after the termination of the ribavirin treatment and negative results confirmed the elimination of *Plum pox virus*.

Keywords: sharka; plant virus; chemotherapy; ribavirin; apricot; plum

Plum pox virus (PPV) is the most harmful virus infecting stone fruit. PPV is distributed throughout Europe and was also found in North and South America, Asia, and Africa. It is widely spread in plums and myrobalans in the Czech Republic (Polák 2002) and present in some orchards of apricots and peaches. The availability of virus-free material of stone fruit is only due to the fact that production of nurseries is derived from mother plants maintained in technical isolation. The chance of random contamination of mother plants with PPV cannot be ruled out. Therefore, an establishment of a treatment for the elimination of PPV in mother stock is necessary. Different methods are employed for obtaining initial PPV-free planting materials, such as *in vivo* thermotherapy (Mosella Chancel et al. 1980; Janečková 1993), thermotherapy of *in vitro* cultures (Vertesy 1981; Isac 1985; Knapp et al. 1995; Spiegel et al. 1995), chemotherapy, or their combinations (Howell et al. 2001).

Spontaneous elimination of PPV from untreated *in vitro* cultures of apricots was not observed; on the other hand, a hundred percent success rate was recorded after the combined treatment of thermotherapy and meristem preparation in the apricot cultivar Ungarische Beste (Knapp et al. 1997). However, stone fruit cultivars are sensitive to heat treatment and low percentage of *in vitro* plants survive (Deogratias et al. 1989). Recently, Paunovic et al. (2007) succeeded with *in vitro* production of *Plum pox virus*-free plum by chemotherapy with ribavirin (1-β-ribofuranosyl-1,2,4,-triazole-3-carboxamide, trade name: virasol). In our attempts chemotherapy of *in vitro* cultures of plum and apricot infected with PPV was used for the virus elimination. Ribavirin was applied for several months in

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low concentrations in the treatment of plum cultivar Bluefree and apricot cultivar Hanita.

MATERIALS AND METHODS

Screening of plants by ELISA, sampling for ELISA and RT-PCR

Trees of plum cv. Bluefree and apricot cv. Hanita infected with PPV were used for the preparation of *in vitro* cultures and chemotherapy. Plants of plum cv. Bluefree and apricot cv. Hanita were screened for PPV, *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple mosaic virus* (ApMV), and *Apple chlorotic leaf spot virus* (ACLSV) by DAS-ELISA (Clark, Adams 1977). Polyclonal antisera from Loewe Biochemica GmbH (Otterfing, Germany) were used for testing. Only the PPV was found to be present in plants.

Older bottom leaves were used for the ELISA detection of viruses in trees of plum and apricot grown in greenhouse. Mixed leaf samples from one flask (four individual shoots) were used for testing by RT-PCR immediately after every period of chemotherapy.

Establishment and multiplication of plum and apricot cultivars *in vitro*

Scions of 5–7 mm in diameter with well developed axillary buds were cut in May from trees of plum cv. Bluefree and apricot cv. Hanita infected with PPV grown in a greenhouses in technical isolation. The shoots were sterilized in 20% bleach (bleach: NaClO min. 5% + NaOH min. 2%) for 15 min. After the sterilization it was necessary to wash it three times in sterile deionised water in a flow box. The scion pieces were placed onto the prepared MS medium (Murashige, Skoog 1962) in 0.7% of purified agar-agar (fine powder by Sigma-Aldrich, St. Louis, USA). The other components of the media were: phytohormones: 6-benzylaminopurine (BAP) 1.2 mg/l, 2,4-dichlorophenoxyacetic acid (2,4-D) 0,1 mg/l; myo-inositol 100 mg/l, sucrose 20 g/l. The cultures were kept for two months at 21 ± 0.5°C with a light intensity of 5,000 lx provided by cool white fluorescent tubes with 14 h photoperiod. Upon establishing the aseptic culture, regenerated shoots were multiplied in order to obtain sufficient number of shoots to set up the experiment with chemotherapy on MS medium. Seven repetitions and four shoots in one repetition were conducted in the experiment with chemotherapy. The presence of PPV was checked again with ELISA in *in vitro* cultures of plum and apricot before the application of ribavirin. Randomly chosen leaf samples were collected for the testing of untreated *in vitro* plants of plum and apricot with ELISA.

Chemotherapy

After two months the *in vitro* cultures were transferred onto fresh MS medium with ribavirin. Multiplied cultures (PPV-infected shoots) were treated with ribavirin by their subculturing on MS medium with ribavirin at concentrations of 5 and 10 mg/l. Five different periods of time of ribavirin treatment were used: 9, 12, 16, 20, and 27 weeks. For each period, twenty eight shoots (seven flasks, each with four shoots) were exposed to both ribavirin concentrations treatments. MS medium with ribavirin was changed every four weeks.

Testing of *in vitro* plants by RT-PCR

The presence of PPV was tested by RT-PCR of *in vitro* cultures of plum and apricot after the chemotherapy by ribavirin. The total RNA was isolated from samples of 0.1 g of leaf tissues, using an RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Primers P1, P2 (Wetzel et al. 1991); PPV-RR (Varga, James 2005) and F3 (Varga, James 2006) were used for the RT-PCR detection of PPV. GO Taq polymerase (Promega, Madison, USA) was used for RT-PCR amplification. The PCR products were analyzed in 1% agarose gel electrophoresis, staining was done with SYBR Green (Invitrogen, California, USA). Virus-free *in vitro* clones were then selected and cultivated in MS medium without the ribavirin. PPV-free clones were re-tested by RT-PCR one year after the chemotherapy.

Rooting

Medium used for rooting of the *in vitro* plants was after Paunovic et al. (2007). Plants were recovered in small hothouses with 100% relative humidity (RH) and kept in a cultivation room. *In vitro* plants

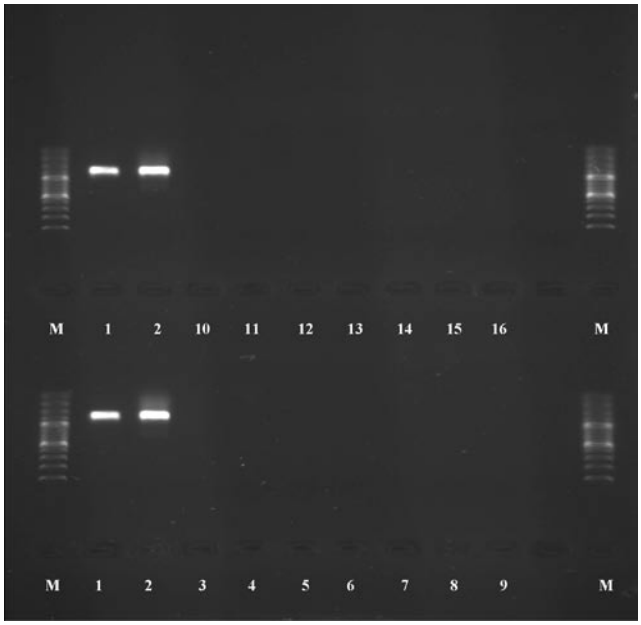

Fig. 1. RT-PCR (primers PPV-RR, F3) of samples after recovering by chemotherapy.
Position 1, 2 – positive control; 3–9 samples of *Prunus* cv. Bluefree; 10–16 samples of apricot cv. Hanita, M – Marker 100bp DNA ladder (Fermentas)

with bases of roots' primordia were transferred into peat tables to develop the further roots able to gain nutrients from substrate and synthesize life important elements. Because of physiological stress from radical changeover of conditions, plants were watered with solution of micro and macro elements (Kalinina 2007). Plants rooted *in vivo* were transferred into substrate and were habituated to greenhouse conditions where the artificial humidity and solutions of elements were already missing.

RESULTS AND DISCUSSION

Trees of plum cv. Bluefree and apricot cv. Hanita were proved to be infected with PPV only. Another viruses PNRSV, PDV, ApMV, and ACLSV were not detected by ELISA in tree exploited for elimination of PPV. The presence of PPV was confirmed again by ELISA in *in vitro* cultures of plum and apricot before the application of ribavirin. In our trials *Plum pox virus* was successfully eliminated by long time treatment of *in vitro* clones of plum cv. Bluefree and apricot cv. Hanita with low ribavirin concentrations of 5 and 10 mg/l in MS medium. The optimal time period of the ribavirin treatment varied between 12 and 20 weeks, depending on the fruit tree species and the ribavirin concentration. The negative RT-PCR results presented on Fig. 1. were obtained both, directly after the treatment and one year after chemotherapy. Results of chemotherapy of plum cv. Bluefree and apricot cv. Hanita are presented in Table 1.

All the 28 tested shoots of apricot cv. Hanita treated with ribavirin in concentration of 5 mg/l were PPV-free after the twelve weeks. RT-PCR results at the end of the ribavirin treatment period of nine weeks indicated that the 5 mg/l ribavirin therapy was effective in eliminating PPV in apricot cv. Hanita in four flasks (16 shoots) only; all the

Table 1. Results of chemotherapy of plum cv. Bluefree and apricot cv. Hanita infected with *Plum pox virus*

Plant species	Concentration of ribavirin	Number of weeks of chemotherapy				
		9	12	16	20	27
Plum cv. Bluefree	5 mg/l	0+	0	8	28	28
	10 mg/l	0	28	28	28	28
Apricot cv. Hanita	5 mg/l	16	28	28	28	28
	10 mg/l	0	28	28	28	28

+The number of PPV-free shoots from 28 tested one

Fig. 2. Plum tree cv. Bluefree after chemotherapy with roots

28 shoots of plum cv. Bluefree remained infected with PPV. With this ribavirin concentration, it was necessary to extend the treatment up to 20 weeks, when 100% success rate was recorded.

With the concentration of 10 mg/l ribavirin the virus was eliminated from all the tested shoots, independently of the species, in 12 weeks. The conclusion of results obtained is the recommendation to use the concentration of 10 mg/l of ribavirin for twelve weeks for the elimination of PPV from plum and apricot trees.

Chemotherapy treatment of four *Prunus domestica* L. cultivars: Stanley, Opal, Pozhegaca, Tetevenska infected with PPV was applied at lower rates than those necessary for virus elimination (Gabova 1995). Ribavirin was applied at 0.5, 1.0, and 10 mg/l for 30 days. The treatment with ribavirin at 10 mg/l led to high inhibition of PPV in resistant cultivars Stanley and Opal (91–75%), while in the susceptible cvs. Pozhegaca and Tetevenska the virus concentration was reduced to 46–30%. PPV was not eliminated, just reduced, even at 10 mg/l concentration of ribavirin after 30 days of *in vitro* treatment. The results obtained demonstrate the possibility to develop a system for specific determination of quantitative resistance to PPV.

Chemotherapy was recently applied for the elimination of PPV in plum cv. Cacanska Lepotica by Paunovic et al. (2007), using ribavirin in concentrations of 10 up to 100 mg/l. Ribavirin in high concentration (60–100 mg/l) was phytotoxic. When 40 mg/l of ribavirin for six weeks was applied, only 15% of plants were reported to be virus-free. Furthermore, PPV was not eliminated by 10–20 mg/l ribavirin in six weeks of application. Paunovic et al. (2007) and Gabova (1995) applied ribavirin for short period of time, six and four weeks only, respectively. This time of application was too short for elimination of PPV by 10 or 20 mg/l ribavirin. Gabova (1995) did not succeed in the elimination of PPV in four plum cultivars, she just demonstrated the possibility of using chemotherapeutic treatment to develop a system for the determination of quantitative resistance to PPV. Our results obtained with application of low ribavirin concentration for different time periods proved that the longer period of time is necessary for successful chemotherapy. In our trials, the shortest period of time of chemotherapy in which PPV-free material was obtained was recorded to be nine weeks (63 days) for the apricot and twelve weeks (84 days) for the plum. PPV was not eliminated in shoots of plum cv. Bluefree and was only partially eliminated in apricot cv. Hanita after application of ribavirin for nine weeks. Chemotherapy of plum and apricot by ribavirin applied in concentration 10 mg/l for twelve weeks of treatment was efficient for PPV elimination. No phytotoxicity was observed on the used medium. Longer periods of chemotherapy – 20, and 27 weeks by ribavirin in concentration 5 or 10 mg/l resulted in 100% elimination of PPV (Table 1). The all recovered plants were tested one year after the chemotherapy and remained PPV-free. No significant influence of low concentration of ribavirin on the multiplication ratio, growth of the shoots and inducing of roots (Fig. 2) compared to the untreated plantlets was observed.

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