Results of *in vitro* chemotherapy of apple cv. Fragrance – Short communication

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Abstract

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The effect of the chemotherapy with ribavirin on the elimination of the pome fruit viruses from *in vitro* grown plants of infected apple cv. Fragnance has been investigated. The results of ELISA and RT-PCR testing proved the presence of mixed infection of *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) in the initial field-grown tree of this apple cultivar. Obtained actively growing *in vitro* shoots with well-developed leaves and shoot tips were subsequently used for chemotherapy with ribavirin. Attempts to fully eliminate viruses by ribavirin in lower concentration 20 mg/l were not successful. However *in vitro* plants of one mericlone (FR1R20) sanitated from ASPV and ASGV, which were infected with ACLSV only after the first chemotherapy cycle, were subjected to repeated treatment on medium with higher ribavirin concentration 100 mg/l. The success of chemotherapy with ribavirin at 100 mg/l was 76% for ACLSV elimination after the second round. In the course of both chemotherapy cycles (20 mg/l and 100 mg/l), *in vitro* plants did not display symptoms of phytotoxicity.

Keywords: ribavirin; PCR; ELISA; virus detection; virus elimination; virus infection

Apple (*Malus domestica* L.) is a globally important horticultural species. Unfortunately this species is threatened by the mechanically sap-transmissible pathogens of viral nature, which cause major losses in apple plantations (Desvignes et al. 1999). Some of these widely distributed pathogens are well identified and characterized. *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV) are common viruses affecting different species of *Malus* genus (Nemeth 1986; Knapp et al. 1998; Desvignes et al. 1999; Kundu 2001). Apple viruses ASGV, ACLSV and ASPV are usually latent on most apple cultivars used in commercial plantations (Mink et al. 1998; Desvignes et al. 1999).

Attempts to eliminate certain viruses from infected apple cultivars by a combination of *in vitro* cultures and heat therapy resulted in only partial success in our previous experiments (Paprstein et al. 2008, 2009). Some authors also reported that especially mixed infections of more than one virus are more difficult to eliminate (Knapp et al. 1995; Da Câmara Machado et al. 1998; Wang et al. 2006; Paprstein et al. 2008).

The objective of the present study was to develop a virucide-based method to eliminate ASPV, ACLSV and ASGV from infected newly bred apple cv. Fragrance during the micropropagation. Ribavirin (Virazole; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) as a synthetic nucleic acid base analogue with antiviral activity against several plant

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viruses (Hansen 1989; James 2001) was selected for our experiments. Apple cv. Fragrance was used as an experimental model for chemotherapy, because this cultivar is considered to be prospective for Czech fruit growing industry. Healthy plant material resulting from our sanitation procedures should enter the scheme for the production of certified propagative material in the Czech Republic.

MATERIAL AND METHODS

The virus status of selected initial tree of cv. Fragrance from maintenance breeding collection was evaluated by ELISA and RT-PCR before the beginning of chemotherapy. Samples for ELISA and RT-PCR testing (buds, leaves) were taken in April, 2011. Commercial antiserums from Bioreba (Reinach, Switzerland) were used for ELISA. The protocol according to Menzel et al. (2002) including primers was used for RT-PCR detection of viruses. RNA was extracted from initial plant material using RNeasy Plant Mini Kit (Qiagen, Venlo, the Netherlands).

Virus-infected *in vitro* shoots were established from excised cv. Fragrance tips (5–15 mm in length) that were forced for 10 days in laboratory conditions. The donor shoots were collected from 10-year-old field-grown trees in April, 2011. Initial explants were surface disinfested with a 0.15% solution of mercuric chloride for 1 min, followed by rinsing in a sterile, deionized and demineralized water. Cultures were grown in Erlenmeyer flasks (5 shoots per flask) each with 25 ml of MS medium (Murashige, Skoog 1962), to which 1.5 mg/l BAP (6-benzylaminopurine) was added. The pH of the medium was adjusted to 5.8 with NaOH prior to dispensing and autoclaving at 120°C at 100 kPa for 15 minutes. All cultures were cultivated in controlled environment chambers at 22 ± 1°C under cool-white fluorescent tubular lamps (Sylvania F18W; Havells Sylvania, Erlangen, Germany) and 16-h photoperiod. As soon as enough shoots had developed (after 6 four-week subcultures), individual shoots with leaves (5–10 mm in length) were excised from stock collections and transferred to treatment media with ribavirin.

The antiviral compound ribavirin, obtained from Sigma-Aldrich (St. Louis, USA), was filter-sterilized (Supor Acrodisc 0.2 μm; Pall Corporation, Port Washington, USA) and added after autoclaving into the same MS medium as for multiplication.

Concentration of 20 mg/l was used for the first chemotherapy cycle. *In vitro* plants of one mericlone sanitated from ASPV and ASGV, which were infected with ACLSV only, were subjected to repeated treatment on medium with higher ribavirin concentration 100 mg/l. For the first treatment, 20 individual *in vitro* plants with well-developed shoot tips were grown. Twenty-one *in vitro* plants were used for the second treatment.

Treated *in vitro* cultures were proliferated in medium containing virucide ribavirin for 4 weeks. Then the apical parts of the axis about 3 mm in length comprising the apical meristem plus two to three leaf primordia, which developed during chemotherapy, were dissected under a laminar flow hood and transferred to a fresh multiplication MS medium with 1.5 mg/l 6-benzylaminopurine (BAP).

These explants were returned to standard growth conditions in a growth chamber. Treated shoot cultures of apples were observed during and after chemotherapy to evaluate the survival of apices and potential phytotoxicity of ribavirin.

Vegetatively *in vitro* propagated progeny of each particular dissected explant after chemotherapy was described as a mericlone. A period of about 5 months was necessary to obtain well-established actively growing cultures of particular mericlones.

The frequency of virus elimination was determined by a highly sensitive immunocapture RT-PCR procedure for the viruses ACLSV, ASGV and ASPV detected in the initial field-grown tree. ApMV, which was not detected in this initial tree, was not tested after chemotherapy.

RESULTS AND DISCUSSION

The results of ELISA and RT-PCR testing proved the presence of mixed infection of ASPV, ACLSV and ASGV in the initial field grown tree of apple cv. Fragrance (Table 1). ApMV was not found.

After sterilization procedure, the cultivar was successfully multiplied in *in vitro* cultures on MS medium with 1.5 mg/l BAP. Obtained actively growing *in vitro* shoots with well-developed leaves and shoot tips were subsequently used for chemotherapy.

In the course of both chemotherapy cycles, at ribavirin concentrations 20 and 100 mg/l used, plants did not display symptoms of phytotoxicity and appeared vigorous and healthy. There was not a significant negative effect of ribavirin on the shoot

Table 1. Results of ELISA and RT-PCR testing of initial field-grown tree of cv. Fragrance

Pathogen	ApMV		ACLSV		ASGV		ASPV	
Test	ELISA	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR
Result	–	–	+	+	+	+	+	+

ApMV – *Apple mosaic virus*; ACLSV – *Apple chlorotic leaf spot virus*; ASGV – *Apple stem grooving virus*; ASPV – *Apple stem pitting virus*

growth of treated *in vitro* cultures as compared to the control stock collections. After chemotherapy, 100% of isolated apices developed vigorous shoots with leaves about 1 cm and multiplied in standard growth conditions. This is contradictory to findings of other authors (Hansen, Lane 1985; Deogratias et al. 1989; Cieslinska 2002, 2007), who reported that ribavirin starts to be phytotoxic, when used at concentrations higher than 40 mg/l. It seems that there is a species- and genotype-specific difference in the susceptibility of *in vitro* grown plants to higher ribavirin concentrations.

Table 2. Effect of ribavirin (20 mg/l) on elimination of viruses from cv. Fragrance

Mericlone	RT-PCR testing after chemotherapy		
	ACLSV	ASGV	ASPV
FR 1R20	+	–	–
FR 2R20	+	+	–
FR 3R20	+	+	–
FR 4R20	–	–	+
FR 5R20	–	+	+
FR 6R20	–	–	+
FR 7R20	–	+	–
FR 8R20	–	–	+
FR 10R20	+	–	+
FR 11R20	–	–	+
FR 13R20	+	+	–
FR 14R20	–	–	–
FR 15R20	+	+	–
FR 16R20	–	–	+
FR 17R20	+	–	+
FR 18R20	–	–	+
FR 19R20	–	+	+
FR 20R20	–	–	+
FR 21R20	+	–	+
FR 23R20	+	–	+

ACLSV – *Apple chlorotic leaf spot virus*; ASGV – *Apple stem grooving virus*; ASPV – *Apple stem pitting virus*

Attempts to fully eliminate viruses by ribavirin in lower concentration (20 mg/l) were not successful. After the end of the first chemotherapy cycle, this ribavirin treatment showed a certain inhibitory activity against viruses present in initial plant material, but no mericlone derived from treated *in vitro* shoots was free of all tested viruses (Table 2). ASPV was the most prevalent virus in 65% of *in vitro* plants after the first chemotherapy cycle and this pathogen seemed to be more resistant to ribavirin in our experiment. On the contrary, we obtained only 35% infection rate for ASGV. In accordance with our results, high antiviral activity of ribavirin against ASGV was also reported by James (2001). However, it must be pointed out that the success of chemotherapy can also be related to the concentration of particular virus in initial plant material.

In vitro plants of one mericlone (FR 1R20) sanitated from ASPV and ASGV, which were infected with ACLSV only, were subjected to repeated treatment on medium with higher ribavirin concentration 100 mg/l. After the end of the second chemotherapy cycle, 16 *in vitro* mericlones free of all viruses discovered previously in the initial field-grown tree were obtained for cv. Fragrance (Table 3). The success of chemotherapy with ribavirin at 100 mg/l was 76% for ACLSV elimination after the second round of chemotherapy.

In accordance with our results, several studies indicated the inhibition of virus ACLSV by the use of ribavirin. Hansen and Lane (1985) obtained virus-free shoots of apple cv. Winter Banana after two subculture periods of 3–4 weeks on MS medium with ribavirin added at 10–80μM. Cieslinska (2002) was able to eliminate ACLSV from 78–88% of pear shoots treated with ribavirin in concentrations 25 and 50 mg/l. The same author reported that ribavirin at concentration 25–100 mg/l was effective in eliminating ACLSV from myrobalan (Cieslinska 2007).

Table 3. Effect of ribavirin (100 mg/l) on elimination of viruses from cv. Fragrance

Mericlone	RT-PCR testing after chemotherapy		
	ACLSV	ASGV	ASPV
FR 1R20/1R100	–	–	–
FR 1R20/2R100	–	–	–
FR 1R20/6R100	–	–	–
FR 1R20/7R100	–	–	–
FR 1R20/9R100	–	–	–
FR 1R20/10R100	+	–	–
FR 1R20/11R100	–	–	–
FR 1R20/12R100	–	–	–
FR 1R20/15R100	–	–	–
FR 1R20/16R100	–	–	–
FR 1R20/17R100	–	–	–
FR 1R20/19R100	–	–	–
FR 1R20/20R100	+	–	–
FR 1R20/21R100	+	–	–
FR 1R20/22R100	–	–	–
FR 1R20/23R100	–	–	–
FR 1R20/24R100	+	–	–
FR 1R20/25R100	–	–	–
FR 1R20/26R100	+	–	–
FR 1R20/28R100	–	–	–
FR 1R20/31R100	–	–	–

ACLSV – *Apple chlorotic leaf spot virus*; ASGV – *Apple stem grooving virus*; ASPV – *Apple stem pitting virus*

CONCLUSION

Based on the first results of testing, repeated treatment and higher concentration of ribavirin seem to be an applicable technique to eliminate viruses from apple. From a practical point of view and mainly in comparison with thermotherapy, it does not require expensive equipment for precise heating and cultures can be grown in standard controlled-environment chambers at a standard growth temperature (22–25°C). Achieved results are preliminary. Subsequent-retesting of the chemotherapy treated apple plants after transfer to *ex vitro* conditions is necessary to confirm virus elimination and intercept possible re-accumulation of virus particles from low levels of infection to a level of detection. Virus indexing will continue for a minimum of three years. Morphological assessment of ribavirin treated plants will also be carried out.

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