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## Influence of chemotherapy on development and production of virus free *in vitro* strawberry plants

JIŘÍ SEDLÁK\*, FRANTIŠEK PAPRŠTEIN, JANA SUCHÁ

Research and Breeding Institute of Pomology Holovousy Ltd., Hořice, Czech Republic

\*Corresponding author: [sedlak@vsuo.cz](mailto:sedlak@vsuo.cz)

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**Abstract:** The objective of the study was to determine effects of ribavirin on development and health status of *in vitro* grown strawberry cultivars ‘Honeoye’ and ‘Elkat’ infected with viruses *Strawberry mild yellow-edge virus* (SMYEV), *Tomato ringspot virus* (ToRSV) and *Arabis mosaic virus* (ArMV). Antiviral compound ribavirin was added in concentrations 20, 40, 80 and 160 mg/l to the same MS medium as for multiplication. Growth reduction was noted on medium with 160 mg/l ribavirin and to a lesser degree in the 40 and 80 mg/l treatments. At the end of chemotherapy, *in vitro* clones free of viruses detected previously in the initial plants were obtained for both selected cultivars across all ribavirin concentrations. The highest number of plants (94) with negative results of ELISA testing was noted on medium with the highest ribavirin concentration 160 mg/l and the lowest (73) on medium with the lowest concentration 20 mg/l of ribavirin. The treated plants look symptomless and appear morphologically equal to the untreated control plants. Results indicate that ribavirin treatment of *in vitro* plants is a suitable method for eliminating SMYEV, ToRSV and ArMV from strawberry.

**Keywords:** *Fragaria × ananassa*; ribavirin; phytotoxicity; SMYEV; ToRSV; ArMV

Strawberry (*Fragaria × ananassa* Duchesne) is an economically important soft fruit crop with unique taste, flavour and dietary benefits. Strawberries are also one of the most popular commercial and garden fruits in the Czech Republic.

Unfortunately, several pathogens of viral nature infect strawberries, causing a wide range of symptoms and resulting in poor quality of fruits and lower than expected yields. They may also influence and limit the choice of parental lines in strawberry breeding programmes. Aphid-borne potyvirus SMYEV (*Strawberry mild yellow-edge virus*) causing yellow edge to the younger leaves and considerable reductions in fruit yield is one of

the most frequently encountered and important viral pathogens (THOMPSON, JELKMANN 2004; MA et al. 2015). Nematode transmitted viruses *Tomato ringspot virus* (ToRSV) and *Arabis mosaic virus* (ArMV) are expected to re-emerge in strawberry cultivation all over the world due to the restricted use and phase – out of the more efficient nematocides (TZANETAKIS 2010; MARTIN, TZANETAKIS 2015). Over the past several years, strawberry production in Canada and the United States has been severely affected by strawberry acute decline symptoms. The decline disease is apparently caused by synergistic infections of several viruses, including SMYEV (MA et al. 2015).

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Virus control in berry crops starts with the development of plants free of targeted pathogens through a combination of testing and therapy (MARTIN, TZANETAKIS 2015). A synthetic nucleic acid base analogue ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was selected for our experiments because of its broad-spectrum antiviral activity and reported ability to reduce viruses in many rosaceous plant species (SIDWELL et al. 1972; HANSEN 1989; JAMES 2001, 2010; CIESLINSKA 2003, 2007; PAUNOVIC et al. 2007; HAUPTMANOVÁ, POLÁK 2011). At the time of its discovery, however, due to the price of ribavirin, its wider application for chemotherapy of plants was ruled out. Recently, ribavirin has become more readily available and utilised for broad spectrum of plant species including fruit crops. This paper reports the results of SMYEV, ToRSV and ArMV elimination from two economically important strawberry cultivars 'Honeoye' and 'Elkat' using *in vitro* chemotherapy with ribavirin and subsequent excision of newly formed shoot tips with the apical meristematic area.

## MATERIAL AND METHODS

The virus status of selected initial strawberry plants of 'Honeoye' and 'Elkat' from maintenance breeding collection was evaluated before the beginning of chemotherapy. The ELISA tests for three viral pathogens ToRSV, ArMV, SMYEV were conducted. Samples for ELISA were taken in August. Commercial kits from Bioreba were used. Absorbance readings were recorded using a Sunrise photometer at 405 nm.

In August, Infected shoot cultures were established from excised tips 4–5 mm long that had been taken from actively growing runners of multiplying adult virus infected strawberry plants (Table 1) kept in insect-proof greenhouse. The initial explants for *in vitro* culture establishment were surface-sterilised with a 0.15% solution of mercuric chloride for 1 minute. This was followed by two rinses in sterile, deionized water. The explants were grown for one month

in 100 ml Erlenmeyer flasks (5 shoot tips per flask) each with 25 ml of culture medium. The culture medium contained MS salts and vitamins (MURASHIGE, SKOOG 1962) supplemented with 100 mg/l inositol, 2 mg/l glycine, 30 g/l sucrose and 1.5 mg/l 6-benzylaminopurine (BAP). The medium was gelled with 0.7% (W/v) Difco Bacto agar.

The pH of the medium was adjusted to 5.8 before adding the agar and autoclaving at 120°C at 100 kPa for 15 minutes. Cultures were kept at 22 ± 1°C with a 16-h photoperiod provided by cool-white fluorescent tubular lamps (Sylvania F18W) positioned 30 cm above the level of cultures.

*In vitro* cultures were serially subcultured in four-week interval for three months on MS medium supplemented with 1.5 mg/l BAP. This provided a stock collection of shoots for chemotherapy treatment.

Control tests by ELISA for SMYEV, ToRSV and ArMV presence were carried out in developed *in vitro* explants at the end of multiplication phase. Then the individual explants in the form of single rosettes (8–10 mm in diameter) with at least three well developed leaves were adjusted aseptically by scalpel to the mass 0.2 ± 0.01 g in sterile conditions of a laminar flow hood. Such explants were then transferred to treatment media with the anti-viral agent. Antiviral compound ribavirin was filter sterilised (25 mm, Acrodisc Syringe Filter 0.2 µm, Pall Gelman, USA) and added in concentrations 20, 40, 80 and 160 mg/l to the same MS medium as for multiplication. *In vitro* cultures from infected strawberry plants were grown on media with ribavirin for 4 weeks in the same conditions as for multiplication. For each cultivar and ribavirin concentration, 20 *in vitro* initial explants were grown and observed during and after chemotherapy to evaluate the survival and potential phytotoxicity of ribavirin. Fresh mass of newly *in vitro* formed shoots on media with different ribavirin concentrations was determined by weighing one month after beginning of ribavirin treatment. Treatment means for each cultivar and ribavirin concentration were compared with the standard error (SE) of the mean. Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze and compare differences in increase of fresh weight in particular treatments. At the same time, the frequency of virus elimination in particular *in vitro* plants was determined based on ELISA results obtained for samples aseptically taken from previously weighed explants. Control plants of both virus infected strawberry cultivars not treated with ribavirin were also included in the experiment

Table 1. Results of ELISA testing of initial strawberry plants

Cultivar	ToRSV	ArMV	SMYEV
Elkat	+	+	+
Honeoye	+	–	+

ToRSV – *Tomato ringspot virus*; ArMV – *Arabidopsis mosaic virus*; SMYEV – *Strawberry mild yellow-edge virus*

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and weighed after 4 weeks of cultivation in the same growing conditions as for multiplication.

## RESULTS

The results of ELISA proved the presence of mixed infection of SMYEV and ToRSV in initial strawberry plants of both cultivars ‘Honeoye’ and ‘Elkat’. Cultivar ‘Elkat’ was also infected with ArMV (Table 1).

The sterilization procedure was successful. Bacterial and fungal contaminations did not occur and aseptic *in vitro* cultures of both strawberry cultivars were established. Initial *in vitro* plants were successfully micropropagated on used MS medium. Additional shoots were produced through axillary bud growth. During *in vitro* multiplication phase, strawberry explants appeared vigorous and healthy and no deleterious effects on shoot morphology at used BAP concentration were observed. Control tests by ELISA confirmed presence of SMYEV, ToRSV and ArMV in established *in vitro* plants. Obtained actively growing *in vitro* shoots with well-developed leaves and shoot tips were subsequently used for chemotherapy.

All initial *in vitro* strawberry explants survived the chemotherapy and developed new shoots. There was not a significant negative effect of ribavirin on the shoot growth of treated *in vitro* cultures on medium with 20 mg/l of ribavirin and the weight of newly formed shoot mass was similar

as compared to the not treated control (Table 2). Weight reduction was noted mainly on medium with 160 mg/l ribavirin and to a lesser degree in the 40 and 80 mg/l treatments in the case of both cultivars. Moreover, stunting of the plants, tissue yellowing and necrosis were observed in the case of the highest ribavirin concentration 160 mg/l.

ELISA results showed that *in vitro* clones free of all 3 viruses were obtained for both selected cultivars across all ribavirin concentrations (Table 3). However the frequency of virus elimination differed considerably among cultivars, viruses and ribavirin concentrations. Generally, the best results of chemotherapy were obtained with elimination of SMYEV from strawberry cultivar ‘Honeoye’, where all plants in all ribavirin concentrations tested negative after one month of *in vitro* chemotherapy. In contrast, the lowest number of plants (4) with negative results of ELISA was obtained with ArMV infected ‘Elkat’ on medium with 40 mg/l of ribavirin.

Concerning ribavirin concentration, the highest concentration of ribavirin 160 mg/l yielded the highest number of plants (94) with negative results of ELISA testing in the total sum. In contrast, the lowest number of ELISA negative plants (73) was noted on medium with the lowest concentration 20 mg/l of antiviral.

Selected ELISA negative plants were multiplied on MS medium with 1.5 mg/l BAP, rooted and transferred to *ex vitro* conditions. The treated plants look symptomless and appear morphologically equal to the untreated control plants.

Table 2. Average weight (g) and SE of newly formed shoots on media with different ribavirin concentration after one month of cultivation

Cultivar	Ribavirin concentration (mg/l)				
	control	20	40	80	160
Elkat	2.3 ± 0.1 <sup>a</sup>	2.2 ± 0.1 <sup>a</sup>	1.9 ± 0.1 <sup>ab</sup>	1.8 ± 0.1 <sup>ab</sup>	0.9 ± 0.1 <sup>c</sup>
Honeoye	1.7 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>a</sup>	1.4 ± 0.1 <sup>ab</sup>	1.3 ± 0.1 <sup>ab</sup>	0.6 ± 0.0 <sup>c</sup>

<sup>a</sup> mean ± SE followed by the same letters are not significantly different at the 0.05 level of significance (Duncan’s multiple range test)

Table 3. Number of *in vitro* plants with negative results of ELISA testing

Ribavirin concentration (mg/l)	Cultivars and viruses					Totally
	Elkat			Honeoye		
	ToRSV	SMYEV	ArMV	ToRSV	SMYEV	
20	15	9	12	17	20	73
40	20	18	4	19	20	81
80	19	17	12	15	20	83
160	20	19	17	18	20	94

## DISCUSSION

The results presented in this study demonstrate success in the whole system of sanitation from plant viruses by a combination of *in vitro* cultures and chemotherapy with ribavirin. This is in agreement with results reported by several authors (JAMES et al. 1997; JAMES 2001; CIESLINSKA 2002, 2003; CIESLINSKA 2007; JAMES 2010; HAUPTMANOVÁ, POLÁK 2011), who noted that antiviral-compound ribavirin has the potential to enhance virus elimination.

Ribavirin was detrimental to tissues of *in vitro* grown strawberry plants mainly in medium with concentration 160 mg/l and to a lesser degree in the 40 and 80 mg/l treatments. Our results are in accordance with other authors (FACCIOLI, MARANI 1998; CIESLINSKA 2002, 2003, 2007), who reported that ribavirin starts to be phytotoxic, when used at concentrations higher than 40 mg/l.

ArMV in cultivar ‘Elkat’ was the most difficult to eliminate. It seems that success with chemotherapy depends not only on the virus and method used, but there is also the possibility of specific interaction between pathogen and particular genotype as a host.

## CONCLUSION

Results indicate that ribavirin treatment of *in vitro* plants is a suitable method for eliminating SMYEV, ToRSV and ArMV from strawberry. Monitoring of obtained plant material continues to determine, if any phenotypic changes or abnormalities emerge. Subsequent-retesting of treated cultures as plants in standard field 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. After verification of its health status, material resulting from this study will be kept under technical insulation and potentially used in established system of certification for production of virus-free plant material.

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