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SHORT COMMUNICATION

Evaluation of cryotherapy and meristem isolation from stolons to eliminate viruses in *Fragaria* germplasm

Thomas Wöhner[1] · **Monika Höfer**[1]

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Abstract

Viral infections pose significant threats to strawberry production. This study explored the efficacy of cryopreservation of shoot tips and meristem isolated from stolons to eradicate strawberry viruses, namely strawberry mottle virus (SMoV), strawberry mild yellow edge virus (SMYEV), strawberry crinkle virus (SCV), and strawberry vein banding virus (SVBV). The occurrence of viruses was verified by RT-PCR with specific primers. Results documented the elimination of SMoV and SCV but not of SMYEV and SVBV by cryotherapy and meristem isolation, showing that both methods can lead to the elimination, in a virus specific manner, of viruses in strawberry.

Keywords Strawberry mottle virus (SMoV) · Strawberry mild yellow edge virus (SMYEV) · Strawberry crinkle virus (SCV) and strawberry vein banding virus (SVBV) · RT-PCR · Collection

Strawberries are one of the most economically important temperate fruit crops, with an annual production of 9.157,127.5 t on an area of 389,665 ha worldwide in 2021 (FAO stat, https://www.fao.org/faostat/en). The main producing countries are the USA, the Netherlands, Morocco, Spain and Albania. For the successful cultivation of strawberries, virus-tested plant material is essential because virus infections can cause severe degenerations, deformation of leaves and other symptoms, and economic losses due to bad fruit quality (Martin and Tzanetakis 2006). More than 25 viruses have been described for strawberries to date (Fránová et al. 2019; Koloniuk et al. 2022). Strawberry mottle virus (SMoV), strawberry mild yellow edge virus (SMYEV), strawberry crinkle virus (SCV) and strawberry vein banding virus (SVBV) affect strawberry cultivation worldwide and are transmitted by aphids (Martin and Tzanetakis 2006). Although control of the vector *Chaetosiphon fragaefolii* (strawberry aphid) is possible (reviewed in CABI 2022), once a plant is infected with a virus, the only way to stop virus dissemination is an eradication of infected plants (Greber 1979; Boxus 1989; Nazarov et al. 2020; Rubio et al. 2020). The conservation of strawberry genetic resources is labour- and cost-intensive. The collection of the Julius Kühn Institute consists of 275 *Fragaria* wild species accessions and 186 *Fragaria* cultivars accessions where each accession represents an unique genotype. A majority of the collection is conserved as potted plants (three per genotype) with insecticides applied to control aphids and prevent the spread of viruses (Fig. 1a-c). These plants are multiplied during a two-year cycle via stolon propagation. Consequently, a virus will likely be perpetuated in the collection material if potted plants are infected. This makes it necessary to use virus eradication techniques like chemotherapy (Faccioli 2001; Modarresi Chahardehi et al. 2016; AlMaarri et al. 2012), thermotherapy (Faccioli 2001; Wang et al. 2006; AlMaarri et al. 2012; Zhao et al. 2018), electrotherapy (AlMaarri et al. 2012), cryotherapy (Zhao et al. 2018) or meristem culture (Faccioli 2001; Wang et al. 2006) as described for several other cultivated plant species. For strawberries, cryotherapy, thermotherapy and *in vitro* culture techniques were described for single virus eradication (Boxus 1976; McGrew 1965). However, cryotherapy has not been investigated for the eradication of multiple strawberry viruses. This study investigated the occurrence of strawberry viruses in the germplasm repository at the Fruit Genebank of the Julius Kühn Institute (JKI) Dresden-Pillnitz and tested the efficacy of cryopreservation (Höfer 2016) and meristem isolation via stolons for the eradication of strawberry viruses.

✉ Thomas Wöhner
thomas.woehner@julius-kuehn.de

[1] Institute for Breeding Research on Fruit Crops, Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Pillnitzer Platz 3a, D-01326 Dresden, Germany

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The plant material was obtained from the *Fragaria* collection of the Fruit Genebank of the Julius Kühn Institute (JKI). Seventy-seven cultivars and seven unassigned accessions of *Fragaria* ×*ananassa*, as well as 164 accession of *Fragaria* wild species and hybrids, were tested for four viruses, namely SMoV, SCV, SMYEV and SVBV by RT-PCR (Table S1) using three to four leaves of single plants for each cultivar (Fig. S1). For initial virus detection in the wild species collection and to identify possible virus free genotypes, a composite of different leaves from up to three plants per accession was collected and tested as one sample (n = 1).

Virus tests were performed by isolating total RNA from leaf material (40 mg) using the Invitrap Spin RNA Mini kit (Invitec Molecular GmbH, Berlin, Germnay) according the manufacturer's protocol. Total RNA was eluted in 50 µl dd H_2O and quantified using a NanoDrop 2000c. Synthesis of cDNA was performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher) according to the manufacturer's protocol using random hexamer oligonucleotides and oligo$(dT)_{18}$. Successful cDNA synthesis was evaluated using a standard PCR using elongation factor EF specific primers EF_F and EF_R (Flachowsky et al. 2007). The primer sequences to proof strawberry leaf material on the occurrence of strawberry viruses was obtained from the publication listed in Table S2 and RT-PCR (Thompson et al. 2003) was performed according to the master mix and conditions in Table S3 (Zhang et al. 2014). Virus specific primers (Table S2) were used in RT-PCR (Table S3). Amplicons were separated by agarose gel electrophoresis and visualized with the Molecular Imager® Gel Doc™ XR System (Bio-Rad).

The efficacy of cryotherapy and meristem isolation for virus elimination (Fig. 2) was tested on 19 cultivars (Coral, Dukat, Florika, Fraginetta, Gloria, Mieze Nova, Mrak, Pantagruella, Papa Lange, Pegasus, Pervagata, Polka, Rosa Perle, Rubia, Senga Dulcita, Senga Gigana, Symphony, Talisman, Triscana). Samples of the cultivars in the collection were first tested for viruses (test A). Stolons of plants that tested positive for a virus were collected and shoot tips were isolated in the laboratory according to the experimental procedures described in Höfer (2011). Up to three shoot tips of virus positive plants (n = 2–3) were dissected and established *in vitro*. After the establishment of plants *in vitro*, composite leaf samples were tested for viruses (test B). Plants from *in vitro* cultures that tested negative for viruses were transferred to the greenhouse for virus testing (test C) to study the effect of shoot tip dissection on virus elimination. *In vitro* apical shoot tips from cultivars that tested positive for viruses were dissected from up to four-week-old *in vitro* plants and the method described in Höfer (2016) was performed for cryotherapy and recovery of plant shoot tips. Each shoot tip was propagated individually *in vitro* and leaf samples (n = 3–10) were separately tested for viruses (test D). After the transfer of recovered plants to the greenhouse, plants were tested again for viruses (test D) to determine the efficacy of cryotherapy for virus elimination. The infection rate was calculated for each cultivar and test phase (Table S4).

A total of 84 *Fragaria* ×*ananassa* accessions and 164 accessions of 22 *Fragaria* wild species and hybrids were tested for the occurrence of four strawberry viruses. Single virus infections were determined for each *Fragaria* species (Table 1). The highest virus infection rate in *Fragaria* ×*ananassa* was with SCV (73.2%) and SMYEV (72.1%). A lower infection rate was obtained for SMoV (57.5%) and SVBV (4.3%).

A total of 22 accessions (10 accessions from *Fragaria* sp.; three accessions from *F. moschata*, two accessions from *F. bucharia and F. gracilis;* one accession for *F. cormybosa, F. mandshurica, F. moupinensis, F. virginiana, F. pentaphylla*) and two cultivars ('Linné', 'Ulrichsberg') were negative for the four viruses tested in this study. Eighty-four accession tested positive for one virus, 56 accessions for two viruses, 75 accession for three viruses, and 11 accession for four (Table S1).

A minimum frequency of 6.8% for SVBV and a maximum frequency of 82.9% for SMYEV was observed in the samples collected from 19 cultivars. *In vitro* shoot tips derived from meristems of stolons resulted in 26% (SMYEV), 68% (SMoV), 94% (SCV) and 98% (SVBV) virus-free plants. Testing of the plants in the greenhouse showed infection rates of 3% (SCV), 4% (SVBV), 18% (SMoV) and 76% (SMYEV) (Table 2). Cryotherapy resulted in 15% (SMYEV), 91% (SMoV), 99% (SVBV), and 100% (SCV) *in vitro* plants that tested negative. Greenhouse testing for viruses revealed infection rates of 0% (SCV), 0.4% (SVBV), 9% (SMoV) and 77% (SMYEV) (Table 2).

Sources of resistance to viruses and vectors have not been investigated in strawberry germplasm so far (Shanks and Barrit 1974; Barrit and Shanks 1980). Chemical control against the vectors is possible to reduce the impact of aphid-transmissible viruses. However, once a plant is infected, it should be eradicated and replaced by a new virus-tested plant. Providing virus-tested plant material for new plantings is therefore the best strategy to mitigate strawberry viruses so far (Bettoni et al. 2022). In the first part of this study, we investigated the occurrence of strawberry viruses in our collection material and found a high SMYEV infection (75%) compared with SCV, SVBV and SMoV (< 36% for all three viruses). SMYEV can cause between 30 and 100% yield losses depending on the virus strain, cultivar, environmental conditions and co-infections with other viruses (Samtani

Table 1 Frequency of four strawberry viruses in *Fragaria* germplasm

	No. of plants	% positive tested samples* / accessions & number of plants in brackets							
Species		SMoV		SMYEV		SCV		SVBV	
Fragaria ×ananassa	280	57.5	(161)	72.1	(202)	73.2	(205)	4.3	(12)
Fragaria ×bifera	3	100.0	(3)	100.0	(3)	100.0	(3)	66.7	(2)
Fragaria ×bringhurstii	1	0.0	(0)	100.0	(1)	100.0	(1)	0.0	(0)
Fragaria bucharica	8	12.5	(1)	75.0	(6)	37.5	(3)	0.0	(0)
Fragaria chiloensis	19	36.8	(7)	100.0	(19)	78.9	(15)	15.8	(3)
Fragaria corymbosa	9	22.2	(2)	44.4	(4)	0.0	(0)	33.3	(3)
Fragaria gracilis	4	50.0	(2)	0.0	(0)	0.0	(0)	0.0	(0)
Fragaria hybr.	2	50.0	(1)	100.0	(2)	50.0	(1)	0.0	(0)
Fragaria iinumae	1	0.0	(0)	100.0	(1)	0.0	(0)	100.0	(1)
Fragaria mandshurica	8	25.0	(2)	75.0	(6)	25.0	(2)	0.0	(0)
Fragaria moschata	10	30.0	(3)	10.0	(1)	40.0	(4)	10.0	(1)
Fragaria moupinensis	1	0.0	(0)	0.0	(0)	0.0	(0)	0.0	(0)
Fragaria nilgerrensis	5	0.0	(0)	100.0	(5)	0.0	(0)	20.0	(1)
Fragaria nipponica	7	14.3	(1)	100.0	(7)	42.9	(3)	0.0	(0)
Fragaria nubicola	6	0.0	(0)	100.0	(6)	33.3	(2)	0.0	(0)
Fragaria orientalis	7	28.6	(2)	100.0	(7)	28.6	(2)	0.0	(0)
Fragaria pentaphylla	3	66.7	(2)	0.0	(0)	0.0	(0)	0.0	(0)
Fragaria sp.	19	5.3	(1)	42.1	(8)	5.3	(1)	0.0	(0)
Fragaria tibetica	4	0.0	(0)	100.0	(4)	0.0	(0)	0.0	(0)
Fragaria vesca	18	38.9	(7)	100.0	(18)	83.3	(15)	5.6	(1)
Fragaria virginiana	16	43.8	(7)	93.8	(15)	50.0	(8)	0.0	(0)
Fragaria viridis	6	100.0	(6)	100.0	(6)	50.0	(3)	16.7	(1)
Fragaria yezoensis	7	28.6	(2)	100.0	(7)	28.6	(2)	0.0	(0)

* in case of *Fragaria ×ananassa* more then one sample per accession were tested

et al. 2019). The virus was absent in accessions of *F. gracilis, F. pentaphylla* and *F. moupinensis.* Whether these accessions are resistant to the virus is not known but further work could evaluate the potential use of such genetic resources towards plant resistance breeding.

Furthermore, we investigated the effect of meristem isolation and cryopreservation on virus elimination in strawberry. All viruses were eliminated by both therapeutics techniques (Table 2), although higher elimination rates were obtained for all viruses with cryotherapy (Table 2, phases B and D). Meristem isolation eliminated viruses with results consistent with previous reports (Boxus 1976). When comparing virus test results of phases B and C, higher rates of SMoV and SCV elimination were obtained, whereas no substantial difference was noticed for SVBV. The cryopreservation method showed a slight increase in virus elimination between phase B, D to E for SVBV, SCV and SMoV. However, the eradication effect was minimal for SMYEV compared to the other viruses. Bettoni et al. (2022) discussed the phenomenon of virus persistence in plants, especially potato virus S and potato virus M following cryotherapy. Another study reported the same issue for raspberry bushy dwarf virus and apple hammerhead viroid (Mathew et al. 2021). One reason could be survival of this virus in meristematic tissues as previously mentioned for other plant viruses (Bettoni et al. 2022). Furthermore, Cai et al. (2008) reported the successful elimination of SMYEV by freezing, which is contradictory to the results of this study. This virus showed the lowest SMYEV eradication rates by cryotherapy and meristem isolation in our study, suggesting that this virus is potentially able to infect meristematic tissue, or persists at very low levels below the detection threshold of RT-PCR. Whether this virus can be successfully eliminated in combination with heat- or chemotherapy, is a question for future research.

Table 2 Results from the evaluation of strawberry virus eradication via stolon meristem explant isolation and cryotherapy

Test phase	Virus	samples tested	no. positive	no. negative	%-pos.	%-difference from 100% pos. tested plant material
A	SMoV	76	51	25	69.8	-
	SMYEV		58	18	82.9	-
	SCV		58	18	73.3	-
	SVBV		5	71	6.8	-
↓ only positive plants from phase (A) were used in phase (B)						
B	SMoV	47	15	32	31.6	68.4
	SMYEV		35	12	73.7	26.3
	SCV		3	44	6.1	93.9
	SVBV		1	46	1.8	98.2
↓ only negative plants from phase (B) were used in phase (C)						
C	SMoV	49	8	41	17.5	82.5
	SMYEV		38	11	76.3	23.7
	SCV		2	47	2.6	97.4
	SVBV		2	47	4.4	95.6
↓ only positive plants from phase (B) were used in phase (D)						
D	SMoV	111	12	99	9.0	91.0
	SMYEV		99	12	85.1	14.9
	SCV		0	111	0	100.0
	SVBV		2	109	1.1	98.9
↓ only negative plants from phase (D) were used in phase (E)						
E	SMoV	108	10	98	9.1	90.9
	SMYEV		84	24	77.4	22.6
	SCV		0	108	0	100.0
	SVBV		1	107	0.4	99.6

Fig. 1 Preservation of strawberry genetic resources at Julius Kühn Institute in Dresden-Pillnitz. **a** three plants per cultivar and wild species are planted in pots, **b** healthy strawberry plants from the accession ERB0240 ('Orion'). **c** SMoV, SMYEV and SCV virus infected strawberry *Fragaria vesca* f. alba – accession FRA0185. **d** virus free stock in an insect protected site at JKI, **e** strawberries on racks in the insect protection house at the JKI

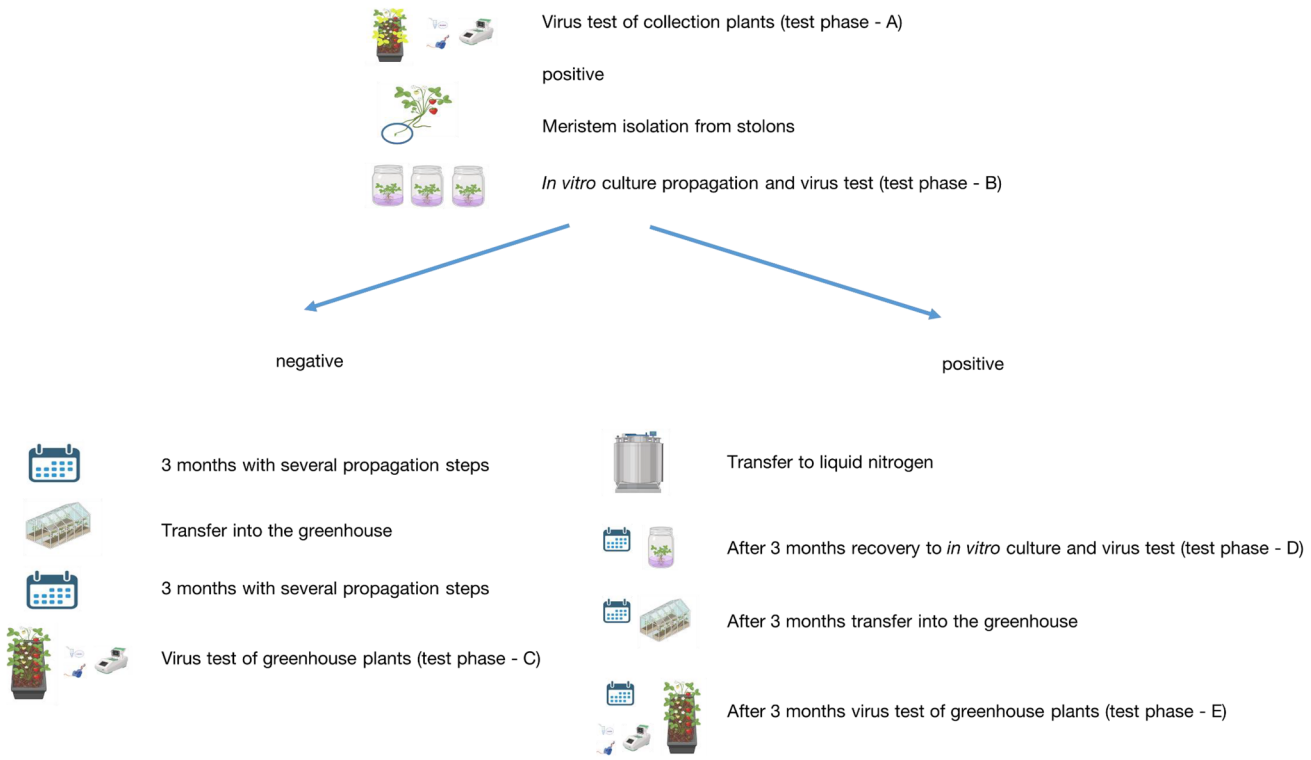

Fig. 2 Virus testing scheme for comparative evaluation of cryotherapy and meristem isolation from stolons as an eradication method for strawberry viruses with five test phases: **A** – testing material obtained from the collection site, **B** - testing material after meristem isolation, **C** - testing material in the green house, **D** - testing *in vitro* plants after cryotherapy, **E** - testing material obtained from cryotherapy in the green house (Created with BioRender.com)

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-023-01570-3.

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