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Development of virus elimination techniques in the production of grapevine propagation material

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INTRODUCTION

Grapevine can be infected by many pathogens, including viroids, viruses, phytoplasmas, bacteria and fungi. Several pathogens in these groups can infect in latent forms, which, if contaminated propagating material is used, can promote their spread to other plantations, so the use of pathogen-tested propagating material is crucial. Currently, there is no well-developed method to successfully defend against the appearance of these diseases caused by pathogens spreading with the propagating material in plantations, so in many cases the condition of infected vines is reduced and they are often cut out for plant health or economic reasons. These pathogens can cause serious economic damage, as they can also adversely affect the quantity and quality of the crop. In order to minimise economic damage, the predecessor of the Research Station of Kecskemét of the Institute of Viticulture and Viticulture of the Hungarian University of Agricultural and Life Sciences has been detecting important viruses using bioassays since the 1960s, and subsequently establishing initial and prebase stocks free from certain viruses, in which János Lehoczky played a key role. At the same place nowdays the production of high-quality pathogen-tested propagating material and the maintenance of pathogen-tested initial and prebase stocks is already carried out under isolated and field conditions. Achieving the status of being free from the quarantine pathogens is an important criterion, as cultivars/clones showing symptoms of these pathogens cannot be included in the National Register of Cultivars (87/2006 (XII.28.) FVM Decree). This work is therefore of great importance, as new cultivars selected by conventional and molecular breeding and as well as produced by cross-breeding methods can often be infected with several viruses and viroids, which can lead to the appearance of serious symptoms.

In recent decades, researchers have developed several methods to eliminate different pathogens. Simple hot water treatment can be used to reduce the concentration of fungi and phytoplasmas, while viruses and viroids can only be removed by more complex tissue culture methods, such as meristem cultures, somatic embryogenesis and chemotherapy. These procedures act by different mechanisms and can therefore be used with varying degrees of efficacy for the elimination of different grapevine viruses and viroids. There is a continuous need to improve the virus elimination protocols, both in terms of the tissue types used and the antiviral agents applied. Reducing the length of the virus elimination process is an important factor, as it increases the number of healthy plants that can be produced in a given time interval.

The regeneration time of meristems is significantly longer than that of larger shoot tips, so time can be saved by using the latter, but various additional treatments could also be required. These include heat therapy, cryotherapy or chemotherapy with antiviral agents. Antiviral chemicals also often have negative effects on plants (e.g. chlorosis, growth inhibition, mortality),

which must be taken into account when using them. The choice of the tissue type to be treated, the length of treatment and the concentration of the chemical also require great care. The virus elimination effect of the somatic embryogenesis has already been proven, but the establishment of the widely used anther and ovary-based cultures is only feasible when the inflorescences are available and if unsuccessful, other methods must be used.

The virus-free status of the plants regenerated with different methods has to be checked. For this purpose, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods are the most commonly used. In the latter case, we need to make sure of the presence and quality of the nucleic acid isolated from our plants. To do this, we need to choose an appropriately chosen reference gene, which requires preliminary experiments.

WORK BAKGROUND AND OBJECTIVES

1. One of the main goals of my work was to develop an optimal combination of tissue culture methods and other applicable technological options in pathogen elimination. Meristem culture and somatic embryogenesis have been shown to be effective virus eradication methods based on previous RT-PCR and ELISA tests. However, at the start of my work, the efficiency of somatic embryogenesis had not yet been examined using more sensitive techniques. Therefore, I have planned to evaluate the virus and viroid elimination efficiency of somatic embryogenesis using high-throughput small RNA sequencing, which could provide a more comprehensive picture of the significance of this method. Additionally, I have also aimed to compare the efficiency of the two techniques.
2. Shoot regeneration from grapevine apical meristems smaller than 0.2 mm is observed in only 0.5–10%. In contrast, based on our experiments the regeneration ability of *in vitro* shoot tips of 2 mm could be up to 100%, but this size alone is unsuitable for virus eradication. Antiviral compounds previously reported in the literature (e.g., ribavirin, zidovudine, 2-thiouracil) may offer a viable solution for this problem, as they allow the use of complicated and often difficult meristem cultures to be omitted.
3. Therefore, the next objective of my work was to investigate the virus elimination efficacy of ribavirin on a large number of plants and to attempt to use of 2-thiouracil and zidovudine for grapevine virus eradication for the first time.
4. Testing plants that have undergone virus elimination is crucial, for which RT-PCR serves as a suitable method. This requires successful cDNA (copy DNA) synthesis from sufficient quantities of high-quality nucleic acids, enabling the detection of RNA-genome pathogens. For its verification numerous reference gene primers are available, however the origin of the resulting PCR product is not clear during their appliciation. To address this, I have planned to test housekeeping gene primers targeting one or more intron-containing gene regions that are amplifies differently sized fragments from genomic DNA (gDNA) and copy DNA (cDNA) as introns are being spliced out from mRNA during its maturation, while from gDNA longer fragments - containing the introns - are amplified.

MATERIALS AND METHODS

Plant materials

Plant material of the experiments related to somatic embryogenesis and meristem cultures

In the case of this experiment three *Vitis vinifera* cultivars were used: H.7-3 and H.14-1 clones of the cultivar 'Ottonel muskotály' and with the 'Trilla' and 'Szirén' cultivars. For meristem culture shoots of *in vitro* plants were used, while for somatic embryogenesis anthers isolated from the inflorescences of field-grown plants were used.

Plant material of the chemotherapeutic experiments

In the course of chemotherapy with antiviral agents, I worked with clones of three grape cultivars, the plant lines of which were selected according to their viral infection: the A1 and ÜH2 lines of 'Furmint' P51, the A1 line of 'Kadarka' P131 and the A1 line of 'Sárfehér'. The virus infection status of the selected genotypes was first mapped by small RNA sequencing from leaf samples taken from greenhouse-grown mother plants and then checked by RT-PCR. Subsequently, shoots of the source plants were used to establish *in vitro* cultures after surface sterilization. The resulting *in vitro* plants were micropropagated and their virus infection was again checked by RT-PCR. In each case, treatments with different antiviral agents were performed using 1-2 cm shoots of *in vitro* plants of the listed clones.

Plant material of the reference gene experiments

In this experiment I worked with 24 (7 rootstocks and 17 scions) grapevine cultivars: *V. berlandieri* × *V. riparia* 'Teleki 5C', *V. berlandieri* × *V. riparia* 'Teleki-Fuhr S.O.4', *V. berlandi*eri × *V. riparia* 'Teleki-Kober 5BB', (*V. berlandieri* × *V. riparia*) × *V. vinifera* 'Georgikon 28', *V. riparia* × *V. cinerea* 'Börner', *V. berlandieri* × *V. rupestris* 'Ruggieri 140', *V. berlandieri* × *V. rupestris* 'Richter 110', 'Kövidinka', 'Sárfehér', 'Kunleány', 'Miklóstelep 7', 'Kadarka', 'Kék bakator', 'Juhfark', 'Neoplanta', 'Pintes', 'Zefír', 'Furmint', 'Esther', 'Ottonel muskotály', 'Olasz rizling', 'Vulcanus', 'Zervin' and 'Piros bakator'. The utilized tissues of the listed varieties were as follows:

- leaves of *in vitro* plants
- leaves and petioles of field-grown plants
- cambial scrapings from dormant canes of field-grown plants

Establishment of *in vitro* cultures

I started the process with the hot water treatment of the canes of the collected cultivars during which they were incubated on 51 °C for 30 minutes. Afterwards. canes were sliced into single-noded cuttings. I planted these into plastic cups filled with prelite and moistened them with tap water. I detached the green shoots developed from the buds and rinsed them in a 70% ethyl alcohol solution for 30 seconds followed by a soak in an aqueous sodium hypochlorite solution (1% NaOCl and 0,1% Tween 20) and finally they were washed three times in sterile distillated water. After this shoot tips of approximately 0.5 cm were prepared from the shoots with the naked eye and shoot elongation was induced with the application of plant growth regulators (metatopolin - mT or BA) on MS medium containing half amount of MS macroelements (Murashige & Skoog 1962). Following their growth I transferred the shoots to hormone-free MS medium to promote root formation. The *in vitro* plants were maintained in growth chamber at 25 °C with monthly subculturing.

Experiments with intron-containing gene sequence-specific reference gene primers

For the experiments related to reference genes I first performed RT-PCR using primer pair specific for *18S rRNA* on leaf samples of 24 field-grown grapvines. For this I used nucleic acid isolated from the leaves and the cDNA synthetized from it. In the next step I did the same with a primer pair specific to an intron-cointaining region of the *PEP* gene and compared the results.

After that I tested 4 additional primer pairs (*actin*, *EF1α*, *GAPDH*, *tubulin*) specific to intron-containing reference gene regions on the same 24 grape cultivars. Subsequently, reliable primer pairs were then tested on samples originating from different tissue types (*in vitro* leaves, field-grown leaves and petioles, cambium scrapings) of 12 cultivars.

Virus elimination methods used

Meristem cultures

Meristems (0.1-0.5 mm in size) were placed on half-MS media in Petri dishes containing 30g/l sucrose and 1 mg/l mT. Petri dishes were wrapped in aluminium foil and the meristems were kept in dark for 3 days. The growing yet still rootless shoots were then transferred to half-MS media of the same compositon contains only 0.2 mg/l mT for root formation. The rooted independent plant lines were then transferred and propagated on hormone-free half-strength MS medium supplemented with 10 g/l sucrose.

Somatic embryogenesis

Somatic embryogenesis was performed according to the method as described by Oláh et al. (2009). Inflorescences of the four field-grown mother plants were surface sterilized according to that earlier described method by the establishment of *in vitro* cultures. Subsequently, the flowers were dissected, and anthers attached to filaments were placed onto solid, callus-inducing MST medium containing 0.05 mg/l thidiazuron (TDZ), 1.1 mg/l 2,4-D, 20 g/l sucrose solidified with 5 g/l agar. Anther cultures were then incubated at 24°C in darkness. Later, calli exhibiting embryogenic characteristics were transferred to MSAc medium containing 1 g/l activated charcoal, 10 g/l sucrose, and 3 g/l Gelrite. Finally, independent shoots regenerating from the developed embryos were individually subcultured and propagated on the same MS medium.

Plant chemotherapy

The different antivirals were applied to the shoots of the selected cultivars/clones at the concentrations indicated in Table 1. Shoot tips of 2 mm were then separated from the treated plants and plants were regenerated from them. Using nucleic acid isolated from the leaves of the resulting plants, I tested them by RT-PCR for viruses/viroids previously detected in the starting material.

***Table 1**: Treatments of selected cultivars/clones with antiviral agents*

Cultivar/clone	Treatment	Applied concentration
'Sárfehér' A1	Ribavirin	25 mg/l
	Zidovudin	10, 20, 30, 40, 80, 120 mg/l
	2-thiouracil	5, 10, 15, 20 mg/l
	PR + 2-thiouracil	10, 20 mg/l
'Furmint' P51 A1	Ribavirin	25 mg/l
	2-thiouracil	20 mg/l
	Ribavirin + Zidovudin	25 mg/l + 50 mg/l
'Furmint' P51 ÜH2	Ribavirin	25 mg/l
	Ribavirin + Zidovudin	25 mg/l + 50 mg/l
	PR + 2-thiouracil	20 mg/l
'Kadarka' P131 A1	Ribavirin	25 mg/l
	PR + Ribavirin	25 mg/l
	Zidovudin	50 mg/l
	PR + 2-thiouracil	10 mg/l

PR – pre-rooting of the shoots designated for treatment with 0.8 mg/l IBA hormone on half-MS medium

Nucleic acid isolation

For nucleic acid isolation 50 mg of tissues of the plants to be tested were used. The process followed the CTAB-based method described by Xu et al. (2004). The purified nucleic acid was dissolved in 100 µl of sterile distilled water and stored at -80°C until use.

cDNA synthesis

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #K1622) according to the manufacturer's protocol.

Virus diagnostic methods used

RT-PCR

PCR was performed using the DreamTaq DNA Polymerase Kit (Thermo Scientific, #EP0703). The reaction mixture was prepared according to the manufacturer's instructions. For experiments related to virus elimination cDNA from plants already proven to be infected with the virus as a positive control, while for null control sterile distilled water were used. The samples were then placed in the PCR apparatus where the reaction was run through the cycles described in Table 2.

***Table 2**: Steps of the PCR programs and their duration*

Process	Temperature	Time (minute)
Pre-denaturation	94 °C	3:00
Denaturation	94 °C	0:30
Primer annealing	50-60 °C	0:30
Extension	72 °C	0:30-1:30
Final extension	72 °C	3:00

To check the quality of nucleic acid and the success of cDNA synthesis prior to the detection of pathogens, several reference gene-specific primer pairs were used in the experiments and are listed in Table 3. For the *actin*, *EF1α*, *GAPDH*, *PEP* and *tubulin* genes, primers specific for sequences containing one or two introns were used (Figure 1).

***Table 3**: The reference gene primers used in the experiments*

Gene	Primer name	Sequence (5'-3')	Reference
18S rRNA	18S rRNA_Fw	CGCATCATTCAAATTTCTGC	Gambino & Gribaudo 2006
	18S rRNA_Rev	TTCAGCCTTGCGACCATACT	
actin	act-fw1	GGCCGATACTGAAGATATCCAG	This work
	act-rev1	ACCAGAATCCAGCACAATACC	
EF1α	elf-fw1	GGGTAAGGAGAAGGTTCACATC	This work
	elf-rev1	TGCCTTGGAGTACTTTGGTG	
GAPDH	gapdh-fw2	GCAGTCAACGATCCATTCATC	This work
	gapdh-rev2	AGCCTTGTCCTTGTCAGTG	
PEP	PepS2fw	GTCCTTACAGCACATCCTACTC	This work
	PepS2rev	CCCACCCATCCAAGAAGAAA	
tubulin	tub-fw2	CACGATGCTTTCAACACCTTC	This work
	tub-rev2	CTTCATTGTCCAAGAGCACAG	

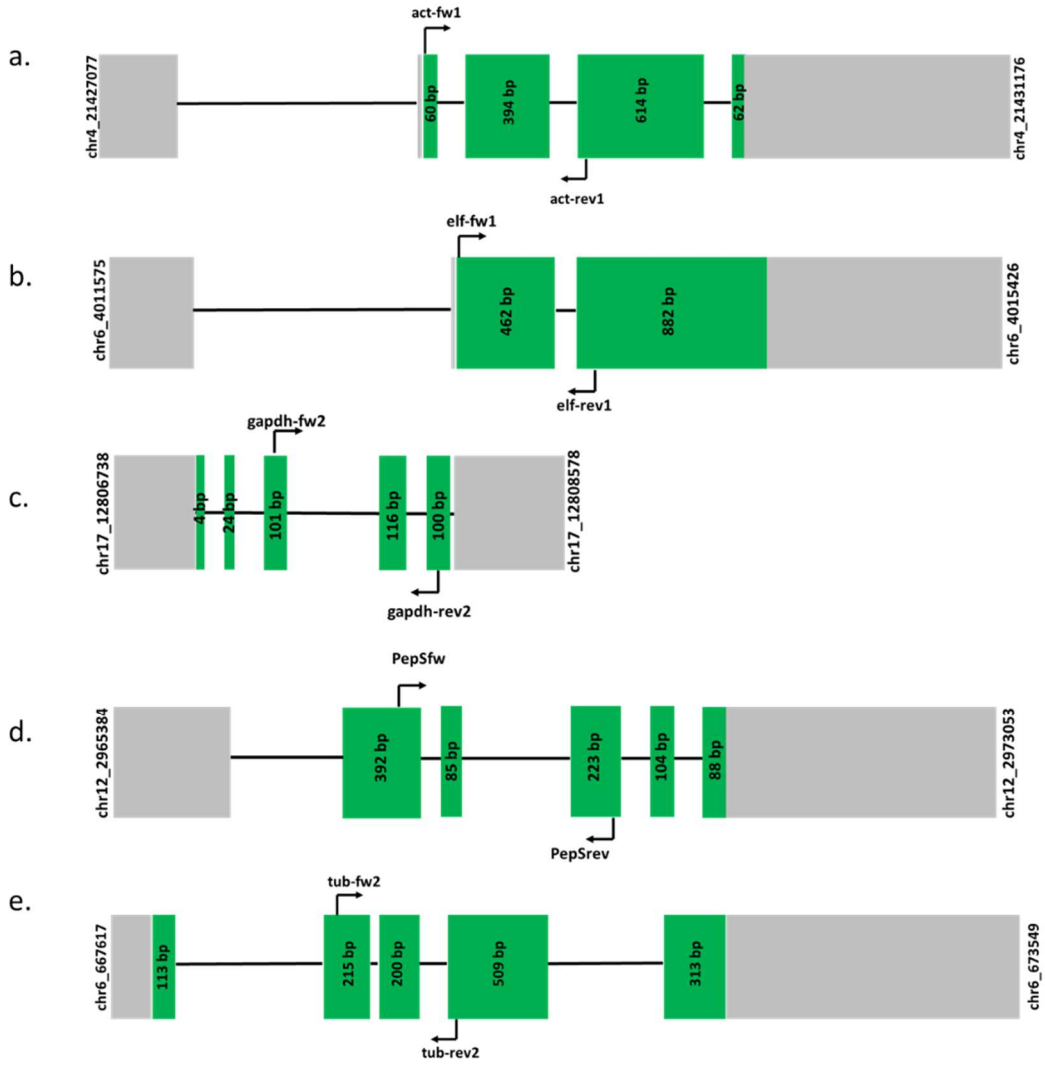

***Figure 1**: The locations of the primers designed to intron-containing regions of reference genes on the affected Vitis vinifera chromosomes*

The locations of the primer pairs specific to *actin* (a.), *EF1α* (b.), *GAPDH* (c.), *PEP* (d.) and *tubulin* (e.) genes on the affected chromosomes. The green fields indicate exons within the gene, the black lines indicate introns. The gray fields indicate the 5' upstream and 3' downstream sequences that fall outside the affected sequences.

High-throughput sequencing of small RNAs

For this sequencing method, I first needed to prepare small RNA libraries. I collected younger and older leaves, tendrils, and shoot tips from four greenhouse mother plants, then performed nucleic acid extraction according to the method described by Gambino & Gribaudo (2008).

Following nucleic acid extraction, I created RNA sample mixtures (pools) that contained an equal concentration mixture of samples from different tissues for each plant. Samples were electrophoresed on an urea-containing (8%) polyacrylamide gel, and the small RNA fractions were excised and purified. After the small RNA library preparation was performed according to the "Illumina TruSeq Small RNA Library Prep Reference Guide (# 15004197 v02)". Finally,

small RNA libraries were sent for sequencing. After sequencing, the obtained FASTQ files were analysed using the CLC Genomics Workbench (Qiagen) software.

RESULTS AND DISCUSSION

Experiments related to reference genes

Conventional and intron-containing reference gene sequence-specific primer pairs

After the RT-PCR with the widely used *18S rRNA* primer pair and the primer pair specific for the *PEP* gene sequence containing introns showed that the latter amplifies a shorter fragment of the cDNA template, as the introns are excised during the maturation of the RNA that provides the template for cDNA synthesis. However, the fragment of gDNA origin is still present in the gel, as transcription can still occur from the residual gDNA in the sample used for cDNA synthesis (Figure 2).

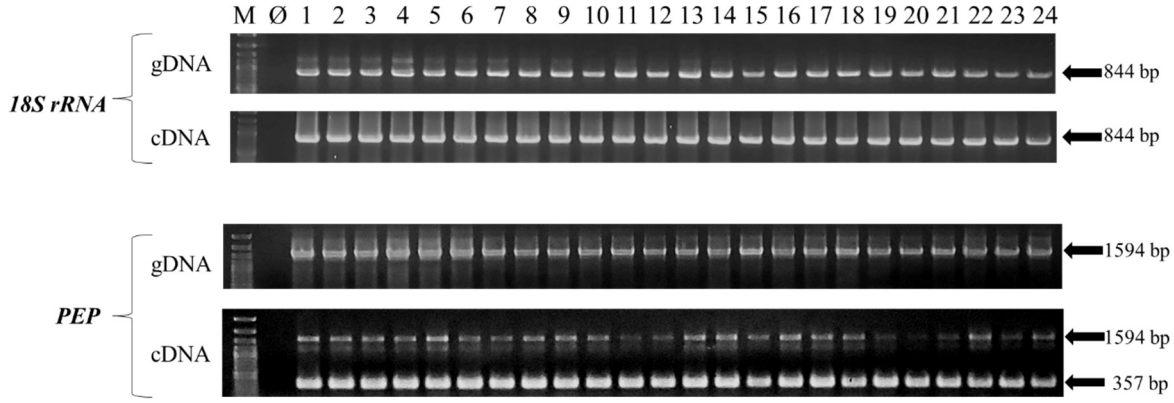

***Figure 2**: Gel images of RT-PCR reactions with 18S rRNA and PEP reference gene primers using genomic DNA and cDNA from 24 grape leaf samples as template*

Sample order: M: molecular weight marker, Ø: null control, 1.-24.: 'Teleki 5C', 'Telefi-Fuhr S.O.4', 'Teleki-Kober 5BB', 'Georgikon 28', 'Börner', 'Ruggieri 140', 'Richter 110', 'Kövidinka', 'Sárfehér', 'Kunleány','Miklóstelep 7', 'Kadarka', 'Kék bakator', 'Juhfark', 'Neoplanta', 'Pintes', 'Zefír', 'Furmint', 'Esther', 'Ottonel muskotály', 'Olasz rizling', 'Vulcanus', 'Zervin', 'Piros bakator'

The testing of intron-containing gene sequence-specific reference gene primers in tissues with different origins

Hereafter I tested the operation of 4 additional reference gene (*actin, GAPDH, EF1α, tubulin)* primer pairs using nucleic acid extracts from the leaves of 24 grape cultivars as templates. I observed that all of them amplified the appropriate gDNA origin fragment, but since a shorter, aspecific fragment appeared next to the expected gDNA origin fragment when using the GAPDH primer pair, I excluded it from further experiments.

After that the operation of the three reliable primer pairs (*aktin, EF1α, tubulin*) was investigated in samples of *in vitro* leaves, field-grown leaves and petioles and cambial scrapings derived from 12 cultivars.

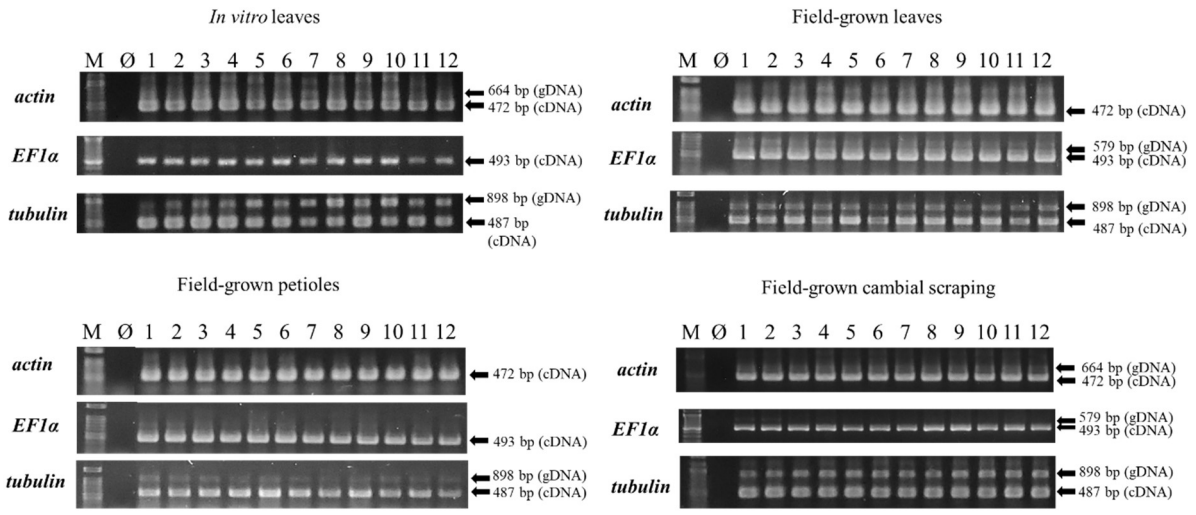

***Figure 3**: Results of RT-PCR assays on cDNAs synthesized with random hexamer primers on samples of different tissue origin*

Sample order: M: molecular weight marker, Ø: null kontroll, 1.-12.: 'Teleki 5C', 'Teleki-Fuhr S.O.4', 'Georgikon 28', 'Börner', 'Kövidinka', 'Sárfehér', 'Kadarka', 'Zefír', 'Furmint', 'Esther', 'Ottonel muskotály', 'Olasz rizling'

As it can be seen on Figure 3 all the reference gene primer pairs amplified the fragments of cDNA origins in the case of all 4 tissue types, so they are suitable for the confirming the successful cDNA synthesis. There were differences in the fragments of gDNA origin, as only the tubulin primer pair was able to amplify it from all samples. The fragment of gDNA origin occasionally amplified by the EF1α primer pair was not well distinguishable from the fragment of cDNA origin. This could be due to the fact that it is specific for only one short intron-containing (86 bp) region. It should be noted, however, that the presence of the gDNA-derived fragment is not essential to confirm the success of cDNA synthesis, since knowledge of the length of the shorter fragment is sufficient information to identify it.

Investigation of the efficiency of somatic embryogenesis and meristem cultures using a sensitive diagnostic method

Virus diagnostic of the selected mother plants

Based on the small RNA HTS and the following RT-PCR tests of the mother plants selected for this experiment ('Ottonel muskotály' H.7-3, 'Ottonel muskotály' H.14-1, 'Szirén', 'Trilla') they were infected with several viruses and viroids (GFkV, GPGV, GRSPaV, GRVFV, GSyV-1, GVT, GYSVd-1, HSVd).

Virus elimination of the selected cultivars by somatic embryogenesis and meristem culture

Somatic embryogenesis from anthers was successful for all four cultivars/clones, while meristem culturing was unsuccessful for the two 'Ottonel muskotály' clones. The regenerated plant lines were also tested by small RNA HTS and RT-PCR (Table 5).

***Table 5**: Summary of virus elimination efficiency of somatic embryogenesis and meristem cultures*

CULTIVAR_ METHOD	DIAGNOSTICAL METHODS		VIRUSES												VIROIDS			
			GFkV		GPGV		GRSPaV		GRVFV		GSyV-1		GVT		GYSVd-1		HSVd	
MO7_SE	small RNA HTS (pool)		-		n.t.		-		-		-		n.t.		+		-	
	RT-PCR	pool	-		n.t.		-		-		-		n.t.		-		-	
		individuals	7/7	100%	n.t.		7/7	100%	7/7	100%	7/7	100%	n.t.		6/7	86%	7/7	100%
MO14_SE	small RNA HTS (pool)		-		-		-		-		-		-		+		-	
	RT-PCR	pool	-		-		-		-		-		-		+		-	
		individuals	8/8	100 %	8/8	100%	8/8	100%	8/8	100%	8/8	100%	8/8	100%	6/8	75%	8/8	100%
T_SE	small RNA HTS (pool)		n.t.		-		-		n.t.		-		-		+		+	
	RT-PCR	pool	n.t.		-		-		n.t.		-		-		+		+	
		individuals	n.t.		12/12	100%	12/12	100%	n.t.		12/12	100%	12/12	100%	1/12	8%	10/12	83%
T_ME	small RNA HTS (pool)		n.t.		-		+		n.t.		-		-		+		+	
	RT-PCR	pool	n.t.		-		+		n.t.		-		-		+		+	
		individuals	n.t.		3/5	60%	2/5	40%	n.t.		5/5	100%	4/5	80%	0/5	0%	0/5	0%
SZ_SE	small RNA HTS (pool)		n.t.		-		+		n.t.		-		-		+		+	
	RT-PCR	pool	n.t.		-		+		n.t.		-		-		+		+	
		individuals	n.t.		11/11	100%	5/11	45%	n.t.		11/11	100%	11/11	100%	9/11	82%	9/11	82%
SZ_ME	small RNA HTS (pool)		n.t.		+		+		n.t.		-		+		-		+	
	RT-PCR	pool	n.t.		+		+		n.t.		-		+		-		+	
		individuals	n.t.		2/4	50%	2/4	50%	n.t.		4/4	100%	2/4	50%	4/4	100%	2/4	50%

MO7_SE: Ottonel muskotály H.7-3 somatic embryo-derived lines; MO14_SE: Ottonel muskotály H.14-1 somatic embryo-derived lines; T_SE: Trilla somatic embryo-derived lines; T_ME: Trilla meristem-derived lines; SZ_SE: Szirén somatic embryo-derived lines; SZ_ME: Szirén meristem-derived lines; n.t.: not tested as the mother plant was not infected; for the individuals, the numbers indicate infection-free/total tested plants

GFkV and GRVFV viruses were not detected in plants regenerated via somatic embryogenesis (SE). The elimination of GSyV-1 was highly efficient using both methods, as it was no longer present in either SE or meristematic (ME) plants. In addition, GPGV, GRSPaV and GVT viruses were successfully eliminated using both methods, as well as GYSVd-1 and HSVd viroids, with the exception of the Trilla ME lines.

However, there were differences in the effectiveness of the two methods. Somatic embryogenesis usually surpassed meristematic cultures in terms of virus and viroid elimination efficiency and was 100% efficient for viruses in most cases. The only exceptions to this were the Szirén SE plants, where GRSPaV elimination was less successful at 45%.

For viroids, somatic embryogenesis was also found to be a more efficient method, but its efficiency did not always approach the 100% observed for viruses. For example, 92% of the Trilla SE individuals remained infected with the GYSVd-1 viroid, which is an exceptionally high rate, but none of the ME-derived individuals were GYSVd-1-free.

Chemotherapy related experiments

Virus and viroid diagnostic analysis of the plant material used in chemotherapy experiments

The virus and viroid infection profiles of the mother plants were determined using small RNA HTS and subsequently validated by RT-PCR. The presence of GFkV, GFLV, GLRaV-1, GPGV, GRSPaV, and GVA viruses, along with GYSVd-1 and HSVd viroids were detected.

Investigation of ribavirin treatment effects on selected cultivars

Survival rate of the treated shoots and the regeneration of the 2 mm shoot tips

The survival rate of ribavirin-treated (25 mg/l) shoots ranged between 28.8% and 49.3%. The regeneration rate of the 2 mm shoot tips excised from treated plants varied between 36% and 85.2% but varied considerably among the cultivars. The most unfavorable ratios occurred with the 'Kadarka' P131 A1 clone, but by pre-rooting (0.8 mg/l IBA half-MS) the shoots selected for treatment we successfully improved the ratios (Table 6).

***Table 16**: Survival rate of the Kadarka P131 A1 shoots treated with ribavirin (25 mg/l) and the regeneration rate of the 2 mm shoot tips*

Type of the treated shoots	Survival rate of the treated shoots	Regeneration rate of the 2 mm shoot tips
 Shoots with no root	30/104 (28,8 %)	18/50 (36 %)
 Rooting shoots	40/50 (80 %) + 51,2 %	61/89 (68,5 %) + 32,5 %

Evaluation of virus and viroid elimination efficacy of ribavirin in the treated cultivars

To evaluate the virus elimination efficacy of ribavirin, I performed RT-PCR tests on the *in vitro* indvidiuals regenerated post-treatment (Table 7).

***Table 7**: Proportion of plants free from each virus or viroid following ribavirin treatment (25 mg/l)*

Virus/Viroid	Cultivar	Virus- or viroid-free/tested plants by cultivar	Virus- or viroid-free/tested plants in total
GFkV	Furmint P51 A1	36/46 (**78,3 %**)	36/46 (**78,3 %**)
GFLV	Furmint P51 ÜH2	0/41 (**0 %**)	0/41 (**0 %**)
GLRaV-1	Furmint P51 A1	2/46 (**4,3 %**)	5/122 (**4,1 %**)
	Kadarka P131 A1	3/76 (**3,9 %**)	
GPGV	Sárfehér A1	66/68 (**97,1 %**)	103/105 (**98,1 %**)
	Furmint P51 A1	37/37 (**100 %**)	
GRSPaV	Sárfehér A1	53/68 (**77,9 %**)	151/ 231 (**65,4 %**)
	Kadarka P131 A1	38/76 (**50 %**)	
	Furmint P51 ÜH2	28/41 (**68,3 %**)	
	Furmint P51 A1	32/46 (**69,6 %**)	
GVA	Furmint P51 A1	3/46 (**6,5 %**)	3/46 (**6,5 %**)
GYSVd-1	Furmint P51 A1	0/46 (**0 %**)	0/231 (**0 %**)
	Furmint P51 ÜH2	0/41 (**0 %**)	
	Kadarka P131 A1	0/76 (**0 %**)	
	Sárfehér A1	0/68 (**0 %**)	
HSVd	Furmint P51 A1	0/46 (**0 %**)	0/190 (**0 %**)
	Kadarka P131 A1	0/76 (**0 %**)	
	Sárfehér A1	0/68 (**0 %**)	

I found that the elimination of GFkV, GRSPaV and GPGV was successful and highly efficient (65-98%). For GLRaV-1 and GVA, I also regenerated virus-free plants for all cultivars, but at lower rates (4.1 and 6.5%). GFLV infection was detected in all 41 plants tested, so its eradication was unsuccessful at the end of the experiment. For GYSVd-1 and HSVd viroids, despite the high number of individuals tested, all plants remained infected after treatment with 25 mg/l ribavirin.

In the case of 'Kadarka' P131 A1, where both unrooted and pre-rooted shoots were treated, there was not a large difference in the proportion of GRSPaV- and GLRaV-1-free plants, but the number of virus-free individuals was higher in shoots treated after pre-rooting (Table 8). This further confirms the observation that shoot survival and regeneration of detached 2 mm shoot tips in cultivars that respond more sensitively to ribavirin treatment can be improved by pre-rooting without reducing the de-virulence efficiency of ribavirin treatment.

***Table 8**: Proportion of Kadarka P131 A1 plants free from each virus/viroid after ribavirin treatment (25 mg/l)*

Virus/Viroid	Type of treatment	Virus- or viroid - free/tested plant
GRSPaV	R25	9/18 (50 %)
	GYR25	29/58 (50 %)
GLRaV-1	R25	1/18 (5,5 %)
	GYR25	2/58 (3,4 %)
GYSVd-1	R25	0/9 (0 %)
	GYR25	0/58 (0 %)
HSVd	R25	0/9 (0 %)
	GYR25	0/58 (0 %)

R25: ribavirin treatment (25 mg/l); GYR25: pre-rooting with 0.8 mg/l IBA followed by ribavirin treatment (25 mg/l)

Chemotherapeutic treatment of the selected cultivars with zidovudine

Zidovudine has not been previously used to eliminate grapevine viruses, so I tested its effect at concentrations of 10, 20, 30, 40, 80 and 120 mg/l on ‘Sárfehér’ A1 shoots. All treated shoots survived the 3-month treatment as well as the control shoots, however, I found that zidovudine treatment at 120 mg/l for 3 months failed to eliminate GPGV and GRSPaV viruses. The same result was obtained after treating ‘Kadarka’ P131 A1 shoots infected with GRSPaV and GLRaV-1 viruses with 50 mg/l zidovudine.

Combined treatment of the selected cultivars with ribavirin and zidovudine

For this experiment, I chose virus-infected species that I had previously been unable to eliminate with ribavirin alone, or only very rarely. For this I treated the A1 and ÜH2 lines of clone ‘Furmint’ P51 with 25 mg/l ribavirin and 50 mg/l zidovudine. Based on the RT-PCR tests it could be said that the combined use of ribavirin and zidovudine resulted in no or no significant increase in the percentage of virus-free individuals compared to treatment with ribavirin alone. However, I was able to regenerate GFLV-free ‘Furmint’ P51 ÜH2 and GYSVd-1-free ‘Furmint’ P51 A1 plants, which I was not able to achieve previously using only ribavirin.

Chemotherapeutic treatment of the selected cultivars with 2-thiouracil

During the treatment of ‘Sárfehér’ with 2-TU I observed that concentrations as low as 5 mg/l has already caused the death of the treated shoots within a short time. Pre-rooting (0.8 mg/l IBA) of shoots designated to be treated with 2-TU also greatly improved their survival rate (<75%) in this case. However, plants grown from 2 mm shoot tips detached from treated plants remained infected with GPGV and GRSPaV viruses without exception after 2 months of

treatment with 10 and 20 mg/l 2-TU. In the experiments on ‘Furmint’ P51 ÜH2 and ‘Kadarka’ P131 A1 plants, all individuals also remained infected with the previously detected viruses.

CONCLUSIONS AND SUGGESTIONS

Based on the small RNA HTS performed in the present study, somatic embryogenesis is also a suitable virus and viroid elimination method that complements the results of previous RT-PCR and ELISA-based investigations. The efficiency of somatic embryogenesis usually exceeded that of meristem cultures and in some cases it was successfully applied to genotypes where meristem cultures were not successful (in this case 'Ottonel muskotály' H.7-3 and 'Ottonel muskotály' H.14-1 clones). The high rate of GYSVd-1 infection in 'Trilla' SE lines suggests that callus cells containing this viroid may develop embryos more easily than those infected with the virus. However, further studies on viroid infection of other genotypes may be needed to confirm this finding. Somatic embryogenesis was a successfully feasible method for all 4 genotypes in the present experiment, unlike meristem cultures, but this does not mean that it is less genotype-dependent. Genetic divergence due to somaclonal variability from the starting material may be a real and more frequent threat than in the case of other eradication methods, so that at least ampelographic examination of regenerated individuals may be recommended after virus elimination processes to allow selection of individuals with clear divergence.

The difficulty in detecting GRSPaV and GVT viruses in field plants indicates that small RNA HTS-based protocols need further optimizations, despite the fact that detection of the same viruses from greenhouse and *in vitro* plants has not encountered similar problems. At the same time, based on the studies performed here RT-PCR is a suitable method to complement small RNA HTS-based virus detection results in such cases and also indicates that the time for routine virus detection based exclusively on small RNA HTS has not yet arrived. However, it cannot be ignored that outstanding successes have been achieved by its use in the detection of new, previously unidentified pathogens.

During ribavirin treatments, the elimination of GFLV was unsuccessful, which may be explained by the fact that it is not a phloem-limited virus. Accordingly, the 2 mm shoot tips of the treated plants were more likely to remain infected. However, this is in contrast to the publications of Aiter et al. (2020) and Weiland et al. (2003), where GFLV was eliminated with efficiencies of 75% and 94%, respectively. In the case of the 'Kadarka' P131 clone, the additional pre-rooting step significantly improved the survival rate of shoots under ribavirin treatment, a method that was later also applicable in 2-thiouracil treatments. This may open new possibilities for the use of potential antiviral agents with strong phytotoxic effects. The reason of this could be that the pre-rooted shoots were slower to take up ribavirin through the root than shoots with fresh cuts on their stems, so they also got the negative effects of the chemical less intensively. It is also possible that other metabolites with a protective function (e.g. salicylic

acid, jasmonates, antioxidants) accumulated in higher amounts in these shoots, but further plant physiology studies would be needed to check this.

2-thiouracil and zidovudine alone were not effective in eliminating grapevine viruses even after several months of treatment. It is important to note, however, that the effect of the treatments used on virus titres was not investigated, so it cannot be generally stated that it were not reduced. It would therefore be reasonable to investigate the virus elimination effect of these agents using other diagnostic methods such as RT-qPCR. This may be suggested by the fact that the combined use of ribavirin and zidovudine allowed the regeneration of both a GYSVd-1 and a GFLV infection-free line, which was not previously possible with a single agent.

A fundamental conclusion from the experiments carried out is that since different virus elimination methods acts via distinct mechanisms, their effectiveness also varies among each cultivars and viruses. It may therefore be advisable to use several methods in parallel, optimising or combining them in order to obtain virus-free propagating material in the shortest possible time.

The success of cDNA synthesis prior to the detection of pathogens with RNA genomes is a key factor in nucleic acid-based diagnostic methods. However, several nucleic acid extraction protocols may result in samples containing residual genomic DNA, and thus conventional reference gene primers may give false-positive results following RT-PCR. Primers specific to gene sequence containing introns offer a good alternative for this. They prove the successful cDNS synthesis even without the use of costly RNA isolation Kits or DNase treatment. However, if a clear separation of fragments of gDNA and cDNA origin is desired after gel electrophoresis, primer pairs specific for gene sequences containing multiple and/or longer introns should be used. In the future, beyond the household genes already investigated within the framework of this doctoral thesis, testing primers designed for the intron-containing regions of the also widely targeted *UBQ*, *PP2A* (Protein Phosphatase 2A) or even *CYP* (Cyclophilin) genes under similar conditions could provide a good research basis, but testing the already investigated primer pairs on samples from stressed grapevine plants could also be a good research direction, as for example the *tubulin* gene may produce unstable results under heat and drought stress (Gu et al 2011). Considering all these factors, expanding the number of such new primers could provide a valuable toolkit for diagnostic testing under diverse conditions - whether analyzing samples collected during vegetative growth phases or dormancy period.

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NEW SCIENTIFIC FINDINGS

1. We were the first to evaluate the effectiveness of somatic embryogenesis in virus and viroid elimination using small RNA high-throughput sequencing. This method demonstrated outstanding virus elimination efficiency in the case of the 'Ottonel muskotály' clones as well as in the 'Trilla' and 'Szirén' cultivars, though it was less effective against viroids. We were also the first to publish results demonstrating that somatic embryogenesis is effective in eliminating GPGV, GSyV-1, GRVFV, and GVT viruses.

2. During antiviral treatments, we found that pre-rooting shoots selected for treatment for 1-2 weeks with 0.8 mg/l IBA could greatly increase the number of shoots surviving ribavirin (and 2-thiouracil) treatment, and the regeneration rate of the 2 mm shoot tips isolated after treament. Besides, the effect of ribavirin still maintained.

3. With the first time use of the intron-containing gene region-specific *actin*, *EF1α*, *PEP*, and *tubulin* primers, we were able to separate the genomic DNA-derived and cDNA-derived amplified fragments in leaf samples from 24 grapevine cultivars (rootstocks, scions, white and blue varieties). Subsequently, we repeated this process using *actin*, *EF1α*, and *tubulin* primer pairs with samples from different tissues (leaf, petiole, cambial scraping) of 12 grapevine cultivars. These results demonstrate the reliability of these primers and their application provides clear verification of successful cDNA synthesis.

PUBLICATIONS RELATED TO THE THESIS

Articles published in peer-reviewed journals with IF:

1. Oláh, R., Deák, T., **Turcsán, M**., Szénási, M., Bordé, Á., & Szegedi, E. (2017). Use of an intron containing grapevine gene as internal control for validation of cDNA synthesis in virus detection by RT-PCR. *Eur J Plant Pathol.* 149(3), 765-770. (**IF: 1,5**)
2. Szegedi, E., Deák, T., **Turcsán, M**., Szénási, M., Bordé, Á., & Oláh R. (2018). Evaluation of intron containing potential reference gene-specific primers to validate grapevine nucleic acid samples prepared for conventional PCR and RT-PCR. *Vitis* 57, 69–73. (**IF: 1,36**)
3. **Turcsan, M**., Demian, E., Varga, T., Jaksa-Czotter, N., Szegedi, E., Olah, R. & Varallyay, E. (2020) HTS-based monitoring of the efficiency of somatic embryogenesis and meristem cultures used for virus elimination in grapevine. *Plants* 9, 1782. (**IF: 3,93**)

Articles published in peer-reviewed journals:

1. **Turcsán, M**., Deák, T., Oláh, R., & Szegedi, E. (2019). Intron-containing housekeeping genes as useful tools in grapevine virus detection by PCR-based protocols. *Res J Plant Pathol* Vol.2 No.1: 03.
2. **Turcsán, M**., Szegedi, E., Oláh, K., Oláh, R., Jahnke, G., Varga, Zs., Deák, T., & Sárdy, D. A. N. (2023) Referenciagénekre tervezett új primerek alkalmazhatósága rutinszerű szőlő vírusdiagnosztikai vizsgálatokhoz. *Kertgazdaság* 55, 4: 33-45.

Other articles with IF not closely related to the thesis:

1. Olah, R., **Turcsan, M**., Olah, K., Farkas, E., Deak, T., Jahnke, G., & Sardy, D. A. N. (2022). Somatic embryogenesis: A tool for fast and reliable virus and viroid elimination for grapevine and other plant species. *Horticulturae*, 8(6), 508. (**IF: 3,1**)
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3. Olah, R., **Turcsán, M**., Jaksa-Czotter, N., Olah, K., Sardy, D. A. N., Mavrič Pleško, I., & Varallyay, E. (2024). First report of grapevine leafroll-associated virus 4 infecting grapevine in Hungary. *Plant disease*, *108*(7). **(IF (2023): 4,4)**

The full publication list is available here:

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