Available online: www.notulaebotanicae.ro
Print ISSN 0255-965X; Electronic 1842-4309
Not Bot Horti Agrobo, 2014, 42(1):115-118

Elimination of *Grapevine fleck virus* by *in vitro* Chemotherapy

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Abstract

Grapevine fleck virus produces a ubiquitous disease, latent in European grapevine varieties (*Vitis vinifera* L.) and in most American rootstocks, being found in all viticultural countries, in simple or complex infections with other more dangerous viruses. Different techniques for sanitation showed controversial results regarding fleck elimination in grapevine. *In vitro* culture of 'Tămâioasă românească' 3-2-2 with fleck virus and 'Burgund' 63 Mn with double infection (*Grapevine fleck virus* and *Grapevine virus A*), naturally infected have been submitted to chemotherapy using a combination of ribavirin and oseltamivir in three concentration variants (V1-40 mg/L ribavirin + 40 mg/L oseltamivir; V2-20 mg/L ribavirin + 40 mg/L oseltamivir; V3-20 mg/L ribavirin + 80 mg/L oseltamivir) and three consecutive subcultures. The plants regenerated after each subculture were evaluated by ELISA from the viewpoint of virus elimination and the RT-PCR was used for confirmation of the diagnostic. Due the phytotoxic effect of viricides, the multiplication rate decreased on experimental variants in the next subculture comparatively to the control, but no mortality of explants has been registered. *Grapevine fleck virus* has been 100% eliminated both from simple and mixed infections on all variants by *in vitro* chemotherapy, under the simultaneous action of two viricides. Unsatisfactory results have been achieved with *Grapevine virus A* elimination.

Keywords: ELISA, oseltamivir, ribavirin, RT-PCR, sanitation, *V. vinifera* L.

Introduction

Grapevine fleck virus (GFkV) produces a ubiquitous disease, latent in European grapevine varieties (*Vitis vinifera* L.) and in most American rootstocks, being found in all grapevine producing countries of the world (Martelli and Boudon-Padieu, 2006). The prevalence of the virus is very high in both simple and mixed infections with other more dangerous viruses (Cretazzo *et al.*, 2010; Komínek, 2008). Some comparative investigations of grapevine showed the negative influence of GFkV presence on *in vivo* and *in vitro* propagation, vigour, quality of the wood, agronomic and oenological parameters (Credi and Babini, 1996; Mannini *et al.*, 2012; Vişoiu *et al.*, 2000).

It is known that GFkV can be eliminated by heat therapy, meristem tip or fragmented shoot apex culture (Bovey and Martelli, 1992). However, while some researchers have reported its successful elimination by thermotherapy (Boulila *et al.*, 1990), others have found the heat treatment as unsatisfactory (Savino *et al.*, 1985; Panattoni and Triolo, 2010). GFkV-free plants (91%) have been obtained by meristem culture without thermotherapy and, the elimination of the virus by shoot tip culture also increased proportionally with the heat treatment period (Buciumeanu and Vişoiu, 2000). The achieving of healthy grapevine through somatic embryogenesis provided promising results, despite the high proportion of abnormal embryos and their low capacity of conversion into plants (Popescu *et al.*, 2003).

Ribavirin and oseltamivir are chemical drugs used in virus elimination in various horticultural species. Ribavirin has been successfully used in *Grapevine virus A* (GVA) (Panattoni *et al.*, 2007), *Grapevine fanleaf virus* (GFLV) (Weiland *et al.*, 2004), *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Skiada *et al.*, 2013) elimination and oseltamivir in *Grapevine leafroll-associated virus 3* (GLRaV-3) effectively removing (Panattoni *et al.*, 2006).

The use of viricides in media led to 37.5% GFkV-elimination with 10 mg/L ribavirin and 25% virus removing in the presence of 40 mg/L oseltamivir, as the highest sanitation rate achieved after one subculture in each case (Guță, 2010).

The aim of the present work was to eliminate GFkV in simple and mixed infections in two *Vitis vinifera* L. genotypes, by chemotherapy with ribavirin and oseltamivir used concomitantly, in controlled conditions of *in vitro* culture.

Materials and methods

Biological material. In vitro stabilized grapevine (*Vitis vinifera* L.) belonging to 'Tămâioasă românească' 3-2-2 proposed for approval and 'Burgund' 63 Mn approved clones of economic interest in Romanian viticulture have

been used in the experiment. These genotypes have been identified as naturally infected with GFkV, and GFkV in addition with GVA, respectively.

The explants collected from infected plants have been cultured on solidified M&S (Murashige and Skoog, 1962) basic medium supplemented with 1mg/L benzylaminopurine (BAP) and 0.5 mg/L β-indolylacetic acid (β-IAA). The multiplication and regeneration processes carried out in the growth chamber, at 22±1 °C, 16 hs photoperiod, 3000-3500 lx. Infected cultures were the source of inocula for grapevine chemical drug treatment.

Treatment with viricides. Ribavirin (1-β-D-ribofuranosyl-1, 2,4 triazone-3-carboxamide) and oseltamivir ([(3E,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid)]) as viricides belonging to inosin monophophate dehydrogenase (IMPDH) and neuraminidase inhibitors (NI) group, respectively, have been used. Ribavirin as pure substance and oseltamivir as capsules containing oseltamivir phosphate have been available. The viricide mixtures have been experienced in three variants (V1, V2, V3) of concentration during three consecutive subcultures (S1, S2, S3), as follows: V1= 40mg/L ribavirin + 40 mg/L oseltamivir; V2= 20mg/L ribavirin + 40 mg/L oseltamivir; V3 = 20mg/L ribavirin + 80 mg/L oseltamivir. The chemical drugs have been added to the medium before autoclaving.

The microshoot originated from stabilized cultures have been cut in single node fragments and subcultured on media containing viricides. Each inoculum constituted one repetition and has been individually evaluated.

The phytotoxic effect of viricides expressed by multiplication rate – number of formations specific for multiplications (adventive buds + primordia of shoots + shoots)/ initiated explants has been assessed after each subculture.

The treatment with viricides was followed by a subcultivation on proliferating medium without chemical drugs, than on rooting medium, rooted shoots being acclimatized.

Virus detection. In order to establish the efficiency of the method, expressed by virus elimination rate (no. of virus-free plants/no. of regenerated plants/initiated inoculum x 100), three months old acclimated plants, resulted from each subculture and variant, have been analyzed individually by ELISA and several ELISA negative, by RT-PCR.

ELISA was performed according to the method described by Clark and Adams (1977), using detection commercial kits.

RNA extraction and reverstranscription steps have been done using commercial kits protocols. Two pairs of primers have been used: GFkV585F (CTC AGC CTC CAC CTT GCC CCG T), GFkV 1117R (CAA TTT GGC TGG GCG AGA AGT ACA) and GFkV 6351F (CTC TCC GCC TCG TCT GAT GA), GFkV 7064R (TCG GTT CAT GAC GAG GGA GT) (Naidu et al., 2010). The initial denaturing was performed at 95°C for 10 min, followed by 40 cycles of 95 °C for 30 s, annealing (at 55 °C for the first primer set and 50 °C for the second) for 30 s, extension at 72°C for 60 s and final polymerization at 72°C for 7 min. The size of amplified DNA has been determined using the 100 bp molecular marker.

Results and discussion

The phytotoxic effect of viricides. Ribavirin has a phytotoxic effect expressed by a multiplication rate and mortality of inocula that is more pronounced comparatively to oseltamivir (Guță *et al.*, 2009). The multiplication rate decreased after the first and the second subculture with 4.30 and 3.03 respectively, in the case of GFkV-infected Caner variety growth on media containing 40 mg/L ribavirin, so that the inocula did not survive after three subcultures. On the other hand, the oseltamivir induced an intense proliferation of adventive buds after the first subculture, formations that did not totally evolve in shoot elongation and differentiation, registering no mortality of inocula on any concentration of viricide (Guță, 2010). The simultaneous use of ribavirin and oseltamivir for GFkV-elimination in 'Tămâioasă românească' 3-2-2 genotype allowed the triggering of caulogenesis processes on all variants and no mortality of explants after three consecutive subcultures has been registered. The visual observations revealed the shoot elongation and differentiation, as well as, their chlorosis depending on the viricides concentration, especially of ribavirin, whose phytotoxic effect is more evident as compared with that of oseltamivir.

The multiplication rate decreased both on experimental variants and subcultures comparatively to the control. After the first subculture, the highest concentration of ribavirin in V1 determined a higher degrease of the multiplication rate (2.2) in comparison with the control (3.0), the reducing by half of the viricide concentration, completed by the presence of oseltamivir causing an intense proliferation of adventive buds. Even a concentration of 40 mg/L ribavirin combined with 40 or 80 mg/L oseltamivir determined a decrease of the multiplication rate, but it did not induce the explants mortality, not even after three subcultures of treatment (Guță, 2010). This behaviour is due, first of all to

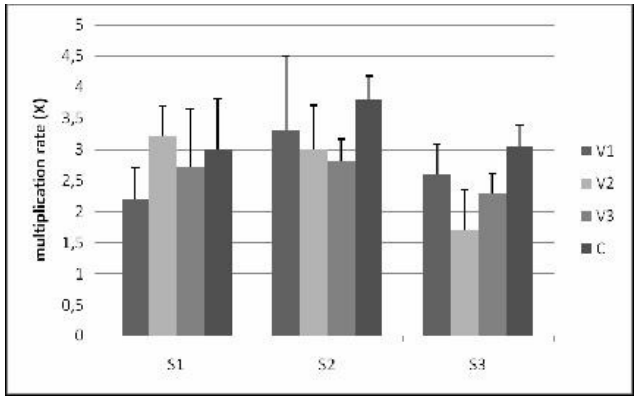

Fig. 1. The influence of viricides on grapevine multiplication rate to GFkV- infected 'Tămâioasă românească' 3-2-2 cv. The values are averages, the bars indicate the standard deviations

the different phytotoxic effect of viricides and, secondly, to the different tolerance to viricides and regenerative potential of the studied grapevine genotypes. The multiplication rate values demonstrated that the correlations regarding the influence of viricide concentrations and exposure time can hardly be made on the evolution of the cultures (Fig. 1).

The treatment with viricides applied to GFkV + GVA

naturally infected 'Burgund' 63 Mn genotype determined a striking increase of the multiplication rate after the first subculture, in all variants, as compared to the control.

The multiplication rate decreased by half in the second subculture to all variants, without differences brought about by the viricides concentration. This behaviour may be explained by the presence of viricides in media which induce a stimulation of adventive buds formation that, in the following subculture did not evolve in shoots differentiation and elongation.

Tab. 1. Grapevine plants 'Tămâioasă românească' 3-2-2 regenerated by *in vitro* chemotherapy with ribavirin and oseltamivir

Sub-cultures	Variants		
	V1	V2	V3
Regenerated plants no. (average ±S.D, n=5)			
S1	25.6±0.8944	26.6±1.1401	25.8±0.8366
S2	30.0±1.5811	29.0±1.5811	25.4±0.5477
S3	28.4±1.1401	25.8±1.3038	26.6±1.1401

Virus elimination. Following the *in vitro* chemotherapy, there have been regenerated, on average, between 25 and 32 grapevines on each variant of 'Tămâioasă românească' 3-2-2 genotype (Tab.1); the plants have been individually analyzed by ELISA and all of them identified as GFkV-free.

The elimination of GFkV by *in vitro* chemotherapy started with the separate use of the ribavirin and oseltamivir viricides at different concentrations and treatment period. The highest elimination rate has been obtained at 10 mg/L ribavirin (37.5 %) or 40 mg/L oseltamivir (25 %) (Guță, 2010). In the present work, the simultaneous use of ribavirin and oseltamivir led to the total elimination of GFkV in all experimental variants.

The management of chemotherapy efficiency with the aim of inactivation of viral multiplication led to the conclusion that a concentration of 20 mg/L ribavirin combined with 40 mg/L oseltamivir (V2) and one subculture of treatment is sufficient for a maximum effect of virus elimination.

In the viral complex infecting 'Burgund' 63 Mn genotype, GFkV has been 100% eliminated, while GVA registered the highest virus elimination rate of 9% after the first subculture of V1 (Tab. 2).

GFkV has been completely eliminated both from simple and mixed infections with GVA by the simultaneous use of ribavirin and oseltamivir viricides added in the medium before autoclaving, while some authors were successful in using the same substances ultrafiltered, added in the medim after autoclaving (Panattoni *et al.*, 2011). This has been based on the results of ribavirin FTIR (Fourier Transform Infrared Spectroscopy) spectral analysis at different temperatures, which demonstrated that at an autoclaving temperature of 120°C, the viricide is in a stable form (Topală, 2013).

Electrophoresis analysis of RT-PCR product showed a single amplified fragment of about 500 bp and 700 bp, in the infected plant used as control and no amplification product has been revealed in healthy regenerated vines.

In addition, *in vitro* chemotherapy as virus-elimination method in grapevine can be successfully applied, because the RAPD (Random Amplified Polymorphic DNA) results emphasized elsewhere that the viricide used in medium did not induce the genetic variability of virus-free regenerated plants (Guță, 2010).

Conclusions

The concomitant use of ribavirin and oseltamivir led to the effective elimination of GFkV in 'Tămâioasă românească' 3-2-2 grapevine genotype. In the complex infection together with GVA, GFkV behaved similarly, being 100% eliminated in 'Burgund' 63Mn clone. The viricides mixture allowed the evolution of the cultures and the plants regeneration on all variants. Three month old acclimated plants have been analyzed by ELISA and the GFkV- free status has been confirmed by RT-PCR.

Acknowledgements

This work was supported by the Romanian National Authority for Scientific Research, CNDI-UEFISCDI, project number 104/2012 (PN-II-PT-PCCA-2011-3.1).

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Tab. 2. Efficiency of *in vitro* chemotherapy with ribavirin and oseltamivir for GFkV+GVA elimination in grapevine plants 'Burgund' 63 Mn. Number of plants represents average±S.D. (n=5) of regenerated plants/inoculum initiated, ELISA analyzed

Subcultures	Variants								
	V1			V2			V3		
	Regenerated plants (no.)	GFkV free (%)	GVA Free (%)	Regenerated plants (no.)	GFkV Free (%)	GVA free (%)	Regenerated plants (no.)	GFkV Free (%)	GVA Free (%)
S1	19.2±1.3038	100	9	20.0±1.5811	100	3	19.8±1.4832	100	1
S2	9.4±1.5165	100	5	9.6±1.9493	100	1	10.0±2.2360	100	0
S3	9.0±1.5811	100	6	10.0±2.2360	100	2	9.8±1.9235	100	1

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