

Quality improvement of the grapevine planting stock through virus elimination by *in vitro* chemotherapy

I.C. Guță^{1*} and E.C. Buciumeanu¹

¹National Research and Development Institute for Biotechnology in Horticulture, Ștefanesti - Arges, Romania.

*Corresponding author email: gutaionelacatalina@yahoo.com

ABSTRACT

In the context of durable viticulture, the initial propagating material is primarily responsible for the health of vineyards from the virological point of view, also this having an important role in controlling the spread of virus diseases. This study analyzed the behavior of grapevine genotype infected with *Grapevine fleck virus* ('Regina viilor'), and grapevine genotypes infected with GFkV and *Grapevine Pinot gris virus* ('Pinot gris 14 Șt.', 'Merlot 146 Mn.', 'Balaban alb') when applying *in vitro* chemotherapy with ribavirin and oseltamivir used simultaneously, with the aim of new virus-free plants regeneration. Regarding the phytotoxic effect of antivirals, the apices and axillary buds of 'Pinot gris 14 Șt.' and 'Regina viilor' showed good tolerance to treatment, while the explants of 'Balaban alb' and 'Merlot 146 Mn.' were seriously affected, especially the axillary buds. The chemotherapy led to the effective elimination of *Grapevine fleck virus* in all studied grapevine genotypes, while *Grapevine Pinot gris virus* was eliminated in a rate of 75-100%, depending on the genotype and type of explant. The phytotoxic effect induced by antivirals, the tolerance of genotypes to certain treatment and the *in vitro* regenerative potential of each genotype proved to be the main factors influence the efficiency of the applied treatment and the number of virus-free plants obtained.

Keywords: *Vitis*, germplasm, GFkV, GPGV, ribavirin, oseltamivir, phytotoxic effect

INTRODUCTION

A sustainable strategy for the biodiversity protection of genetic resources from germplasm collections for *Vitis*, requires the application of appropriate methods of diagnosis and control of the diseases, as well as pathogens such as viruses. Constituting the initial propagating material, the germplasm collections provide the propagation viticultural material with high biological value, free of the main viruses (*Grapevine fanleaf virus* - GFLV, *Grapevine leafroll-associated virus 1* - GLRaV-1, *Grapevine leafroll-associated virus 3* - GLRaV-3, *Grapevine fleck virus* - GFkV). Worldwide, regulations and programs are applied in order to detect viral diseases through specific testing methods, methods to prevent the spread of viruses, rules for the circulation and exchange of biological material (Kress, 2016; Al Rwahnih, 2018). The identification of simple or multiple infections involves the application of viral elimination methods. The grapevine viruses behave differently to the sanitation methods both in terms of virus elimination rates and the possibility of recurrence of the infection. It seems that *in vitro* chemotherapy offers increased safety as compared to other applied methods (Guță *et al.*, 2018).

Starting from 2006, at NRDIBH Stefanesti - Arges were performed studies on grapevine viruses' elimination by *in vitro* chemotherapy. The method was applied for the following viruses: GFLV, GFkV, GLRaV-1, GLRaV-3 and grapevine virus A (GVA), both in simple infections and in viral complexes. Among the chemotherapeutic substances were tested ribavirin (Guță *et al.*, 2017a), oseltamivir (Guță *et al.*, 2010), their combinations (Guță *et al.* 2014, 2017b), and darunavir and isoprenosin (Guță and Buciumeanu, 2018).

This study aimed to evaluate the behavior of some grapevine genotypes under the *in vitro* chemotherapeutic treatments to regenerate new virus-free plants, stored in the germplasm collection.

MATERIALS AND METHODS

Four grapevine genotypes stored in the germplasm collection of NRDIBH Stefanesti - Arges, 'Pinot gris 14 Șt.' - since 2008, 'Merlot 146 Mn'. – since 2011, 'Balaban alb' – since 2016 and 'Regina viilor' - since 2019 were found positive ELISA with GFkV. Also, to the first three was identified the presence of *Grapevine Pinot gris virus* (GPGV), a virus that is not mentioned in the list of pathogens affecting the quality of viticultural propagating material (Order 1267/2005), but increasingly present in plantations around the world. As a result, they were introduced in the virus elimination program, the method applied being *in vitro* chemotherapy with ribavirin and oseltamivir (patented procedure).

The treatment consisted in the *in vitro* cultivation of explants (apices and axillary buds) collected from virus-infected plants, on M&S aseptic medium (Murashige and Skoog, 1962) specific to grapevine (1 mg/L benzylaminopurine - BAP) and 0.5 mg/L β -indolylacetic acid - β -AIA), supplemented with ribavirin and oseltamivir, for one subculture (30-45 days), followed by 2-3 subcultures on basic medium, without viricides, until obtaining the *in vitro* rooted plants. The multiplication and regeneration processes occurred in the growth chamber, under controlled conditions.

The phytotoxic effect of chemotherapeutic substances on the evolution of explants (apices and axillary buds) was evaluated, expressed by the multiplication rate (number of adventive buds + shoots primordia + shoots, resulting from an initiated explant) and regeneration rate (number of *in vitro* regenerated plants/initiated explant).

In vitro rooted plants were subsequently acclimatized and virologically tested by ELISA (Clark and Adams, 1977), using commercial reagents from Bioreba, Switzerland, according to the instructions in the reagent kits.

Multiplication and regeneration rates were statistically analyzed with SPSS 10 for Windows, each initiated explant being a repetition, the differences being considered significant at $P < 0.05$, according to one-way ANOVA, LSD test.

RESULTS AND DISCUSSIONS

Chemotherapy with ribavirin and oseltamivir applies to the main grapevine viruses (GFLV, GLRaV-1+3, GFkV) both in simple infections and viral complexes. The establishment of treatment parameters aimed to obtain the highest virus elimination rates, taking into account the phytotoxic effect induced by the used antiviral, virus infection, the regenerative potential of various genotypes propagated by *in vitro* cultures and their tolerance to applied treatment.

Ribavirin has a pronounced phytotoxic effect as compared to oseltamivir, expressed by the multiplication rate and inoculum mortality (Guță *et al.*, 2009). In a previous study, at 40 mg/L ribavirin, the GFkV-infected Caner cultivar behaved by significantly decreasing the multiplication rate after the first and second subcultures, so that the inocula did not survive

after three subcultures of treatment. On the other hand, in the same genotype, oseltamivir induced intense proliferation of adventive buds after the first subculture, which did not fully evolve in the shoots differentiation and elongation and no registration of inocula mortality at any concentration. The simultaneous use of the viricides ribavirin and oseltamivir, in order to increase the GFkV elimination rate in a 'Tămâioasă românească' selection, allowed the initiation of caullogenesis processes on all experimental variants, the mortality of explants being not registered even after three consecutive subcultures of treatment (Guță *et al.*, 2010).

Because in this study GPGV infects three genotypes, the application of chemotherapy with ribavirin and oseltamivir can bring information on the possibility of eliminating this virus.

The response of the studied genotypes to the phytotoxic effects of the drugs was different. The explants, both the apices and the axillary buds of 'Pinot gris 14 Șt.' and 'Regina viilor' genotypes showed good tolerance to medium supplemented with viricides (Figure 1), while at 'Balaban alb' and 'Merlot 146 Mn.' genotypes were recorded necrosis until the loss of viability, especially of the axillary buds (Figure 2).



Figure 1. Aspect of explants, apices (left) and axillary buds (right), belonging to 'Pinot gris 14 Șt.' genotype after a subculture on medium with viricides

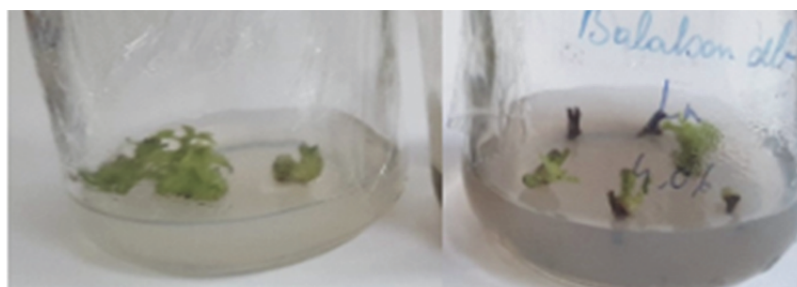


Figure 2. Aspect of explants, apices (left) and axillary buds (right), belonging to 'Balaban alb' genotype after a subculture on medium with viricides

As expected, the presence of chemotherapeutic compounds in the medium (subculture 1) determined a weak caullogenesis, the multiplication rates recorded being quite low in both types of explants, in all studied genotypes. The subcultivation on medium without viricides (subculture 2) determined sometimes a significantly growth of apices from 'Pinot gris 14 Șt.' and 'Balaban alb' or of axillary buds from 'Merlot 146 Mn.' and 'Balaban alb' genotypes, as compared to the subculture 1. Also, after the next viricide-free subculture (subculture 3), the genotypes behaved differently: some continued their adventive bud formation processes, thus increasing the multiplication rates (apex cultures of 'Balaban alb' and 'Regina viilor', axillary buds cultures of 'Pinot gris 14 Șt.', 'Merlot 146 Mn.' and 'Regina viilor'), to others started the differentiation and elongation of the shoots (apex cultures of 'Pinot gris 14 Șt.'

and axillary buds culture of 'Balaban alb'), which led to the decreasing of multiplication rate (Figure 3).

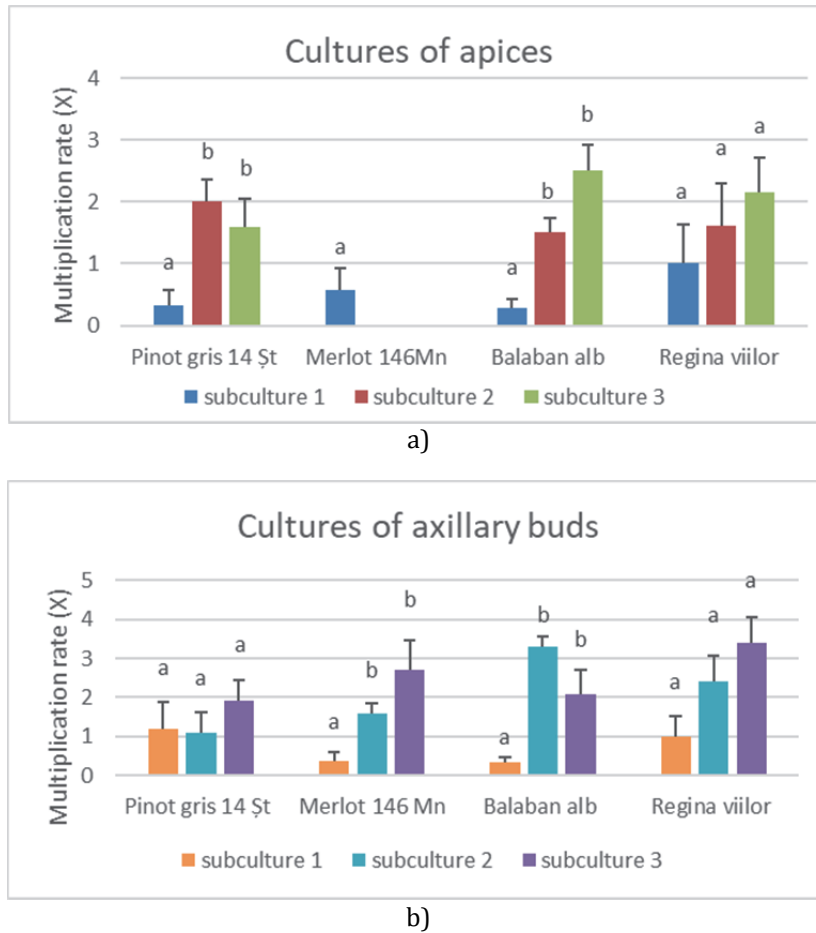


Figure 3. The effect of chemotherapy during a subculture, on the development of apices (a), (upper) and axillary buds (b), (bottom) collected from virus-infected grapevine genotypes ('Pinot gris 14 Șt.', 'Merlot 146 Mn'. and 'Balaban alb' infected with GFkV + GPGV; Regina viilor infected with GFkV). Values are mean $\pm$ SD, the bars indicate the standard deviations, the lowercase letters represent significant differences at $P < 0.05$ comparing the cultures on media with viricides (subculture 1) with those after treatment (subcultures 2 and 3)

Although micropropagation leads to a very large number of new plants as compared to classical propagation methods, chemotherapeutic treatments reduced the progressive development of initiated explants, so that quite a few *in vitro* rooted plants were obtained to all studied genotypes. It is taken into account that the acclimatization of *in vitro* rooted plants requires some processes of adaptation to *ex vitro* conditions, the rates of new plants obtaining are often sub-unitary (Table 1).

These quantifications are useful for a relative assessment of the number of explants required to be treated with chemotherapeutics leading to a sufficiently large batch to identify virus-free plants. It should be borne in mind, however, that these indicators depend on grapevine genotype and virus infection.

The new regenerated plants were ELISA- tested individually to assess the efficiency of GFkV and GPGV elimination. In all studied genotypes, GFkV was total eliminated, which once again confirmed the effectiveness of chemotherapeutic treatments in the regeneration of new

GFkV-free plants (Table 2). Similarly, results were previously obtained to other grapevine genotypes (Guță *et al.*, 2014, 2017a).

Table 1. Evaluation of *in vitro* rhizogenesis and regeneration of new grapevine plants, after *in vitro* chemotherapy (mean values $\pm$ SD)

Grapevine genotype	Explant	<i>In vitro</i> rooted plants (rate*)	Regenerated plants (rate**)
'Pinot gris 14 Șt.'	apex	2.11 $\pm$ 0.1753	0.70 $\pm$ 0.4358
	axillary bud	3.30 $\pm$ 0.5774	1.92 $\pm$ 0.3572
'Merlot 146 Mn.'	apex	0	0
	axillary bud	2.27 $\pm$ 0.4536	0.33 $\pm$ 0.7835
'Balaban alb'	apex	0.71 $\pm$ 0.3573	1.42 $\pm$ 0.7389
	axillary bud	0.33 $\pm$ 0.5627	0.20 $\pm$ 0.1536
'Regina viilor'	apex	1.00 $\pm$ 0.7652	1.62 $\pm$ 0.9824
	axillary bud	1.16 $\pm$ 0.5879	0.08 $\pm$ 0.1439

* *in vitro* rooting rate = no. of *in vitro* rooted plants/initiated explant

** regeneration rate = no. of *ex vitro* regenerated plants/initiated explant

Regarding the GPGV- elimination by *in vitro* chemotherapy, the only one available reference indicates the use of ribavirin. After 8 weeks of treatment in the first stage, the efficiency of the method was 66.7% at a concentration of 10 mg/L ribavirin and 86.7% at 20 mg/L ribavirin. Application of the same parameters of treatment in a new stage led to the complete sanitation of two genotypes out of three treated with both concentrations of viricide (Komínek *et al.*, 2016).

In our study (with one subculture of treatment with the chemotherapeutic mixture ribavirin and oseltamivir), GPGV was eliminated in different rates, ranging from 75 to 100%, depending on grapevine genotype and type of *in vitro* initiated explant culture (Table 2).

Table 2. Efficiency of virus complex GFkV+GPGV elimination by *in vitro* chemotherapy

Genotype	Explant	Tested plants (No.)	GFkV-ELISA negative plants (No.)	GFkV elimination efficiency (%)	GPGV- ELISA negative plants (No.)	GPGV elimination efficiency (%)
'Pinot gris 14 Șt. '	apex	6	6	100	6	100
	axillary bud	23	23	100	20	86.9
'Merlot 146 Mn. '	apex	0	0	0	0	0
	axillary bud	4	4	100	3	75
'Balaban alb'	apex	2	2	100	2	100
	axillary bud	3	3	100	3	100
'Regina viilor'	apex	8	8	100	-	-
	axillary bud	4	4	100	-	-

If the efficiency of virus elimination is assessed according to the types of explants used to initiate chemotherapeutic cultures, it is observed that GFkV was total eliminated in both inoculum types, while at GPGV, the apices culture led to its total elimination in 'Pinot gris 14 Șt.' and 'Balaban alb' genotypes (to 'Merlot 146 Mn.' genotype recorded the mortality of explants under the influence of chemotherapeutics), and from the axillary bud were regenerated virus-free plants of 75, 86.9 and 100% (Table 2).

CONCLUSIONS

The present study showed that *in vitro* chemotherapy based on the use of the mixture of ribavirin and oseltamivir antivirals was useful for GFkV-elimination both in simple infection at 'Regina viilor' genotype, and in complex with GPGV at 'Pinot gris 14 Șt.', 'Merlot 146 Mn.' and 'Balaban alb' genotypes.

GFkV was effectively eliminated in all studied genotypes.

The GPGV-elimination rate proved to be dependent on the grapevine genotype and type of explant: 75% to axillary bud culture of 'Merlot 146 Mn.', 86.9% to axillary bud culture of 'Pinot gris 14 Șt.' and 100% to both types of explants of 'Balaban alb'.

The phytotoxic effect induced by antivirals, the tolerance of genotypes to certain treatment and the *in vitro* regenerative potential of each genotype proved to be the main factors influence the efficiency of the applied treatment and the number of virus-free plants obtained.

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