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REVIEW

Grapevine chemotherapy for elimination of multiple virus infection

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Abstract

Shoot apices and first axillary buds double infected by leafroll associated virus serotype 1 and vitivirus A, collected from grapevine plants variety Servant, were subjected to in vitro chemotherapy technique. Ribavirin of 80 μmol L^{-1} added in solid medium was used for 30-90 days followed by 1-3 subcultures on free-drug proliferating medium. The phytotoxic effect evaluated by micropropagation rate, microshoots differentiation and elongation process was observed in the exposure period, but its intensity decreased along with transfer to medium without viricide. Virus-free plants regeneration was assessed repeatedly by enzyme-linked immunosorbent assay, considering the type of ribavirin treated explant from which the new grapevine derived. The plants developed from shoot apices were found 33% negative for GVA but no GLRaV-1- free plant was identified. The plants originated from axillary buds were 100% GVA–free and 11% were GLRaV-1-free. In this case, ribavirin was not appropriate to eliminate the virus complex; low obtained percentage of healthy plants is due to synergistic activity of two viruses and, perhaps, to plant adaptation to multiple viral infection. Healthy plants were further investigated on the beneficial effect of viruses elimination, in terms of dynamics of shoots growth and wood maturation.

Keywords: *Vitis*, GLRaV-1, GVA, ribavirin, phytotoxicity, ELISA

Introduction

The *Vitis* genus with its numerous species is one of the most affected by various types of viruses, but only some of 60 viruses [1] found in this crop have practical importance [2, 3, 4]. Grapevine leafroll and rugose wood, for their economical impact, are undoubtedly the most important infectious diseases of grapevine worldwide. They are induced by a complex of different viruses, member of *Ampelovirus*, *Closterovirus* and *Vitivirus* genus [5].

The major way to control grapevine virus diseases is the production of virus-free propagation material. Thermotherapy and meristem culture are the most common methods of virus elimination, rather than chemotherapy, electrotherapy, somatic embryogenesis or cryopreservation.

Until now, chemotherapy combined with *in vitro* culture used especially ribavirin (1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide), with commercial name – Virazole, as chemical drug for few single virus elimination in grapevine, with various degrees of success [6, 7, 8, 9]. In addition, the virus elimination process often involved the evaluation of phytotoxicity of viricides in various concentrations and periods of exposure [10].

Another problem is represented by the fact that the grapevine viruses are often found in combinations difficult to eliminate, due to their different behaviour to a certain antiviral treatment.

The experiment described in this study was aimed to eliminate a complex composed of two viruses (one *Ampelovirus* and one *Vitivirus*), grapevine leafroll associated virus serotype 1 (GLRaV-1) and grapevine virus A (GVA) from *V. vinifera* L, cv. Servant, by *in vitro*

chemotherapy with ribavirin and, also, to investigate the phytotoxic effect of viricide on the virus-free recovered vines after the antiviral treatment.

Materials and Methods

Source of virus- infected material: The study regarding GVA and GLRaV-1 elimination has been done on *V. vinifera* L., cv. Servant, maintained in the grapevine virus infected collection of our laboratory. The infected plants showing no virus disease symptoms cultivated on their own roots have been confirmed by enzyme-linked immunosorbent assay (ELISA) testing as having double infection with GVA and GLRaV-1. This material has not showed positive results for other dangerous viruses of this crop: fanleaf + arabis mosaic (GFLV+ArMV), GLRaV-2, GLRaV-3 or fleck virus (GFkV).

In vitro chemotherapy: Grapevine apices (0.2 – 0.3 cm) and axillary buds (first axillary bud from the distal growth of the shoots), collected from double infected mature plants over the growing season were grown on M&S (1962) basic medium [11] containing growth regulators [12] and supplemented with one antiviral chemical for virus elimination. Ribavirin at 80 μmol L^{-1} was added to the proliferating medium for 30-60-90 days (1R-2R-3R). Ribavirin was purchased from SIGMA, USA. In the end of treatment period, the groups of adventitious buds formed during the subcultures on ribavirin medium were transferred on viricid-free multiplication medium, for 1-3 subcultures (1Rf-3Rf). As the microshoots differentiated, they were cultivated on rooting medium. In the same time, few explants collected from infected mother plants followed the *in vitro* regeneration stages (1Rf – 4Rf), on free-drug medium, being the control. The expants were maintained in a controlled environment chamber with a temperature regime of 24±1°C, 16 h photoperiod, 3000 lx. Rooted vitroplants went through acclimatization stages.

Assesment of ribavirin phytotoxicity: The behaviour of infected explants on viricid medium was evaluated by comparison of average multiplication rate and microshoots elongation with control. Statistical significance was analyzed by SPSS 10 for Windows, taking $P<0.05$ as significant according to one-way ANOVA.

Virus detection by ELISA: The ELISA test was performed according to the method described by Clark and Adams [13] with commercial reagents produced by BIOREBA, Switzerland. OD405/492 nm absorbance was recorded by PR 3100 photometer. For a correct discrimination of negative and positive results, a cut-off value as three times the average values of negative control was calculated. The evaluation of virus elimination efficiency has been done two times, first using mature leaves, and next in dormant period using phloem tissue of acclimated plants.

Observation on virus-free vine: Healthy plants were further investigated on the beneficial effect of viruses elimination, in terms of dynamics of shoots growth (three measurements in the active growth period) and wood maturation.

Results and Discussions

Assesment of ribavirin phytotoxicity. Progressively increasing multiplication rates of apex and axilary buds were observed at their cultivation for 1-3 subcultures on ribavirin - containing medium followed by subcultures on viricid-free medium, as a specific behavior of the grapevine *in vitro* propagation. In the third subculture, regardless of ribavirin treatment period, the multiplication rate increased as compared to control, resulting in formation of adventitious buds from which differentiated the microshoots in the next subculture. Blocking the virus multiplication under the influence of ribavirin and also the different concentrations

of viruses in the explants used to initiate viral cultures could be the causes of this behavior. It was also noted that the vitrification phenomena decreased gradually after transferring explants on free-drug medium.

Microshooots of 2-4 cm in length, appropriate for rooting stage, were differentiated in the fourth subculture regardless the number of subcultures in the presence or without viricide (1R+3Rf; 2R+2Rf; 3R+1Rf). Most rooted shoots were obtained from one subculture with ribavirin in both types of explants (apex: 16 ± 0.360, axillary bud: 49 ± 0.513) (Table 1). These results must be correlated with the GVA + GLRaV-1 elimination.

Table 1. Multiplication rate and microshoots elongation in the presence of ribavirin (R) and on ribavirin-free medium (Rf) for GLRaV-1+GVA infected vine cv. Servant (1-3= subcultures)

Explant type	**Multiplication rate and elongated shoots ± sd / explant (no)**			
	Subcultures			
One subculture with ribavirin (1R)				
	1R	**1R+1Rf**	**1R+2Rf**	**1R+3Rf**
Apex	1 ± 0.288	2 ± 0.500	5.30 ± 0.763	0 16 ± 0.360 shoots
Axillary bud	2 ± 0.230	4 ± 0.186	4.90 ± 0.661	0 49 ± 0.513$^{\#}$ shoots
Two subcultures with ribavirin (2R)				
	1R	**2R**	**2R+1Rf**	**2R+2Rf**
Apex	1 ± 0.866	2 ± 0.577	3 ± 0.818	2 ± 0.360 2 ± 0.500 shoots
Axillary bud	1.5 ± 0.366	4 ± 0.186	6.25 ± 0.763	1 ± 0.288^{0} 21 ± 0.763 shoots
Three subcultures with ribavirin (3R)				
	1R	**2R**	**3R**	**3R+1Rf**
Apex	1 ± 0.763	2.5 ± 0.577	5.50 ± 0.233*	1.80 ± 0.763* 10 ± 0.763 shoots
Axillary bud	2 ± 0.381	3 ± 0.230	6.40 ± 0.266^{0}	0 14 ± 0.416 shoots
Controls (4Rf)				
	1Rf	**2Rf**	**3Rf**	**4Rf**
Apex	2 ± 0.950	4 ± 0.288	4 ± 0.577	0 15 ± 0.378 shoots
Axillary bud	3 ± 0.404	5 ± 0.381	4 ± 0.700	0 18 ± 0.661 shoots

*, ^{0}, $^{\#}$ significance compared to the control at $P < 0,05$ (apices, axillary buds and respectively shoots number)

The most used ribavirin concentration of 80 μmol L^{-1} has not induced in this experiment the explants mortality during the treatment and the plants regeneration was possible.

Higher concentration of ribavirin (120 μmol L^{-1}) determined visible phytotoxic effects to GLRaV 1+3 - infected Ranâi Magaraci cv., but with no results on virus elimination [14].

The experiments confirmed the grapevine virus elimination difficulty by *in vitro* chemotherapy.

Acclimatization, as a complex process of adapting to *ex vitro* conditions, led to 18% acclimated plants / microshoot rooted after one subculture of axillary bud with viricide, and also, 15% and 85% in the case of cultivation on two subcultures with ribavirin of apex and axillary bud, respectively.

Virus elimination. In the first stage of analysis of acclimatized grapevines using mature leaf tissue as sample material, GVA and / or GLRaV-1 ELISA negative plants were found in all experimental variants. Analysis during the dormant period led to the identification of GVA –free plants in percentage of 33% at 60 days cultivation of apices on medium with viricide and 100% after 30 days with ribavirin of axillary buds. GLRaV-1 was removed at a rate of 11% only when axillary buds have been cultured 30 days with viricide. So, the sanitation rate did not increase with increasing the treatment period. The final percentage of healthy grapevines recovery was 11%. These unlike response are due to the concentration of viruses in woody material and also, to their uneven spread in the plant (Table 2).

Table 2. Percentage of ELISA-negative grapevines cv. Servant

Ribavirin sub-cultures	Explant type	Regenerated plants			
		Leaf sample		Cane sample	
		GVA- free (%)	GLRaV-1 -free (%)	GVA-free (%)	GLRaV- 1-free (%)
1R	Apex	21	26	0	0
	Axillary bud	100	44	100	11
2R	Apex	33	66	33	0
	Axillary bud	35	12	0	0
3R	Apex	15	10	0	0
	Axillary bud	42	14	0	0

Observation on virus-free vine. GVA + GLRaV-1- free plants were normally developed, with a growth rate of 3-6 cm in the active growth of shoots, and the length of mature wood in the end of vegetative cycle was between 22 and 30 cm (Fig 1 a, b)

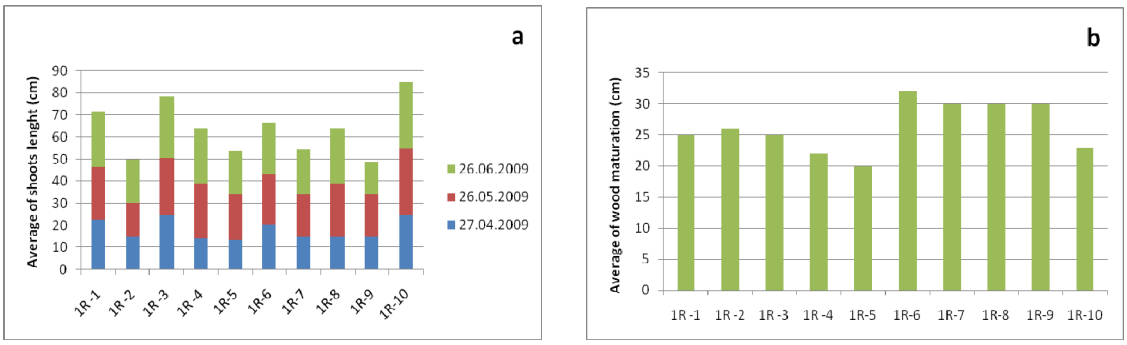

Figure 1. Dynamics of shoots growing (a) of healthy grapevine and mature wood length (b) / ribavirin treated axillary buds for 30 days. 1R 1-10 virus-free vine cv. Servant

Despite the advantages of *in vitro* propagation, factors such as growth regulators, time of culture and media composition appear to be capable of inducing *in vitro* genetic variability in the regenerated plants. As a result, to test the genetic stability and fidelity of recovered plants, random amplified polymorphic DNA method (RAPD) was used elsewhere [15].

Conclusions

Concentration of 80 µmol L^{-1} of ribavirin, the exposure period and the type of explant did not negatively affect the evolution of the culture, so that the grapevine plants could be regenerated in all experimental variants.

Elimination of GVA + GLRaV-1 from grapevine Servant was successfully obtained with *in vitro* 30 days chemotherapy of axillary buds with 80 μmol L^{-1} ribavirin, for 11% of recovered plants; any healthy grapevine derived from apex culture in the presence of ribavirin was identified.

Low percentage of virus -free regenerated grapevines is due to synergistic activity of both viruses and, perhaps, to adaptation of plant to multiple viral infection.

Due to the possibility of viruses concentration and, also, to their uneven distribution in the plant tissues, for a better selection of virus-free regenerated vines, their repeated analysis is necessary.

The treatment with ribavirin did not influence the growth and development processes of healthy regenerated vines by chemotherapy.

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