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In vitro introduction of healthy and virus-infected genotypes of native Croatian grapevine cultivars

Research Article

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Abstract: We evaluated the response of eight economically important Croatian grapevine cultivars and studied the impact of their sanitary status on *in vitro* introduction, by comparing the response of healthy and virus-infected genotypes of one cultivar. Nodal explant survival on three media, M1 (half-strength MS), M2 (full-strength MS) or M3 (full-strength MS with 4.4 μM L^{-1} benzylaminopurine) was measured after 2 weeks and regrowth after 8 weeks. After 8 weeks, average shoot length and node number were significantly higher on M2 compared to M1 and M3. M3 induced significantly shorter average internode length, compared to M1 and M2. Survival of one healthy and of five cultivar Plavac mali genotypes infected with GFLV, GLRaV-1, GLRaV-3, GLRaV-3+GVA and GLRaV-1+GLRaV-3 was 97.5 and 82.8-87.5%, respectively. Regrowth of the healthy genotype reached 95.5%, but dropped to 5.5-31.4% in infected ones. The healthy genotype showed significantly higher shoot length (6.3 cm) and node number (7.3) compared to infected genotypes, with shoot length between 1.2-2.6 cm and node number between 1.2-3.0. By contrast, internode length was not significantly different between the healthy and the infected genotypes. The present work represents the first successful *in vitro* introduction for three of the eight native Croatian cultivars studied.

Keywords: *Grapevine • Croatian autochthonous cultivars • In vitro inoculation • Growth parameters • Infected genotypes*

1. Introduction

Croatian viticulture has a very long history (the first written evidence of its existence dates back to 300 B.C. [1]), which has been affected by many natural and historical factors. Beside this long history and the favourable climatic conditions for grapevine culture, the geographical isolation of the Dalmatia region from the rest of Croatia had a strong influence on the development of a large genetic diversity and speciation of local cultivars. At the beginning of the 20th century, more than 400 varieties were grown in Croatia [2], with over 200 described in Dalmatia alone [3]. Since that time, vineyard destruction caused by new fungal diseases and pests (*e.g. Plasmopara*, *Uncinula* and *Phylloxera*) brought about the loss of many cultivars. Genetic diversity was also reduced by modern production demands for high yields and the consequent introduction of popular international cultivars.

Native cultivar conservation in Croatia has greatly improved over the last decades through steady collecting, genotyping and characterisation of native varieties [4-7]. Nowadays, approximately 130 different varieties have been identified as native to Croatia. About 2/3 of these - among which 14 are considered to be economically important - are underutilized and highly endangered because of their very small populations and the non-availability of grafted plant material on the market [8].

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Besides conserving native varieties in *ex situ* collections, a lot of efforts have been put into revitalizing the commercial growing of those with valuable economic characteristics. One of the main prerequisites is the production of healthy plant material. As there is no certified planting material of native cultivars on the market yet, clonal selection has been initiated with positive mass selection of Croatian autochthonous cultivars including cv. Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina and Vugava, together with screening for the most important grapevine viruses. The results of the surveys [9,10] revealed a high incidence of virus infection in almost all Croatian native cultivars. In all these studies, Grapevine leafroll-associated virus 3 (GLRaV-3) was the dominant virus, with incidence rates varying from 72 to 100%, followed by Grapevine leafroll-associated virus 1 (GLRaV-1) and Grapevine virus A (GVA). It was not rare to find vines infected with two, three or even more viruses. In Dalmatia, the most common virus combinations were found to be GLRaV-1 + GLRaV-3 and GLRaV-3 + GVA.

According to these studies, no healthy vines have been found so far in several cultivars characterized by small populations grown in limited areas. Even in cultivars with great economic importance such as Plavac mali (the most important red variety in Croatia), only a very small number of non-infected vines could be detected [10,11]. Therefore, it is necessary to undertake the sanitation of Croatian varieties in order to avoid losing some rare and endangered native cultivars or losing intravarietal variability in economically important ones.

In vitro culture has been developed as an alternative and a supplementary measure in response to problems related to the conservation, management, sanitation and propagation of plant germplasm [12]. In grapevine, these methods have been successfully used for rapid clonal multiplication and virus elimination [13,14]. Efforts have also been put into the development of efficient protocols for long-term maintenance of grapevine biodiversity *in vitro* as a safer and more cost-efficient alternative to field collections [15].

A prerequisite to developing such approaches for grapevine is the development of an efficient *in vitro* regeneration protocol. However, such protocols are usually highly genotype-specific and need to be adjusted to certain cultivars [16,17]. Some authors have indicated that the optimal composition of grapevine culture medium depends on the species and cultivar under consideration, so that the results obtained with one genotype on a given medium may differ from those obtained with other genotypes [18,19].

There have been very few reports on *in vitro* propagation of Croatian native grapevine cultivars [20-22]; moreover the cultivars tested were of unknown sanitary status and information about their specific culture requirements is still scant.

Establishing an efficient *in vitro* culture protocol for Croatian native grapevine cultivars is a prerequisite for their micropropagation and virus sanitation as well as for research work on cryopreservation and cryotherapy protocol development, which is in progress [23]. The objectives of this work were to evaluate the response to *in vitro* introduction of eight economically important Croatian grapevine cultivars, and to study the impact of their sanitary status, by comparing the response to *in vitro* introduction of healthy and virus-infected genotypes of one cultivar.

2. Experimental procedures

2.1 Plant material

For this study, eight Croatian grapevine varieties were selected, including Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina and Vugava. All of them are commercially grown in Dalmatia and are thus of great economic importance. In order to ensure production of certified plant material, virus screening and mass positive selection were conducted during three consecutive production years. Healthy genotypes showing good production characteristics were propagated and planted in the pre-base plantation “Baštica” near Zadar.

Plant material of healthy genotypes was taken from this plantation, while material of infected genotypes of cultivar Plavac mali originated from the grapevine germplasm repository of the Institute for Adriatic Crops and Carst Reclamation near Split. Comprehensive studies have been performed recently on Croatian autochthonous cultivars, allowing their classification in several groups (A, B, C, D) according to their genetic distance [7]. Cultivars were compared accordingly in our study. Hardwood cuttings were taken at the beginning of December 2010 and were further exposed to low temperature (-10°C) for 2 weeks in order to break dormancy and ensure uniform shoot grow.

3. Methods

3.1 Virus testing

Although selected genotypes had been tested for the presence of viruses during the process of mass positive selection, according to the European Union Council Directive 92/34/EEC, they were retested for the purposes of our research. Virological tests were carried out at the Department of Phytopathology of

the Faculty of Agriculture, University of Zagreb. A total of 176 samples of six genotypes of cv. Plavac mali, (PMC 201, PMC 008/1, PMC 010/1, PMC 064/3, PMC 225/2 and PMC 201/6) were tested for presence of GLRaV-1, GLRaV-3 and for GFLV using enzyme-linked immunosorbent assay (ELISA). An additional test using RT-PCR was conducted for GVA. For the ELISA test, phloem tissues from mature canes, collected throughout the dormant season, were used as potential sources of antigen. They were ground in a mortar after addition of liquid nitrogen and diluted in extraction buffer (pH 8.2) with a 1/15 ratio. The extraction buffer was composed of 37.2 g L^{-1} TRIS-HCl, 32 g L^{-1} TRIS-base, 8 g L^{-1} NaCl, 20 g L^{-1} PVP MW 24000, 10 g L^{-1} PEG MW 6000 and 0.5 g L^{-1} Tween 20. All other ELISA steps were conducted according to manufacturer's instructions (Agritest, Valenzano, Italy). For GVA, total RNA was extracted from cortical scrapings using the RNeasy plant mini kit (QIAGEN, Germany) according to manufacturer's instructions and RT-PCR was conducted as described by MacKenzie [24].

3.2 Establishment of *in vitro* cultures

Hardwood cuttings were cultured in a growth chamber to obtain shoot growth from winter buds. Shoots developed from these hardwood cuttings with leaves removed were first washed with tap water for 15 min, immersed in 70 or 96% ethanol for 30 s, then in 5% sodium hypochlorite (NaOCl) for 30 min and rinsed three times for 5 min each in sterile distilled water. Shoots were then cut into 1.5 cm long nodal segments which were planted in individual test tubes (100 mm x15 mm) containing 10 ml culture medium.

For healthy genotypes, three media based on MS [25] basal salts as the most commonly used in tissue culture of different grapevine varieties [26] were compared: 1) M1: half-strength MS; 2) M2: full-strength MS; and 3) M3: full-strength MS + 4.4 µM L^{-1} benzylaminopurine (BAP), each containing 30 g L^{-1} sucrose and 8 g L^{-1} agar. Experiments with virus-infected Plavac mali genotypes were conducted using M1. Cultures were incubated under a 16 h light/28°C - 8 h dark/24°C dark photo/thermoperiod, with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

3.3 Observations performed

For each cultivar, three repetitions of 15 test tubes per medium were performed. Survival was evaluated 2 weeks after inoculation by counting the number of shoots which showed any type of growth, while regrowth was defined as the development of plants with at least four leaves, 8 weeks after inoculation. Contamination, recorded 2 weeks after inoculation, was consistently below 5% (data not shown). Contaminated test tubes were not included in survival evaluation. After 8 weeks, two growth variables, shoot length (cm) and number of nodes, were recorded on grown plants for all tested cultivars. For infected genotypes of cv. Plavac mali, survival was evaluated after 4 weeks and regrowth after 12 weeks, due to their low shoot growth. Shoot length and number of nodes were recorded 12 weeks after inoculation. Survival and regrowth data are presented in % of inoculated explants.

3.4 Statistical analyses of results

The influence of genotype and culture medium and their interaction on survival and recovery were analyzed using logistic regression. Success of survival/recovery was coded as binary variable (1 for success and 0 for lack of survival/recovery).

Factorial ANOVA was performed for shoot length and number of nodes based on cultivar, medium and their interaction. One-way ANOVA and means comparison was performed using Duncan's multiple range test for shoot length and number of nodes between eight cultivars and three media.

A canonical discriminant analysis was performed using PROC CANDISC in the SAS software [27], based on the following variables: shoot length, number of nodes and average length of nodes of the eight cultivars studied grown on the three different media tested and the first two canonical variables were plotted.

The Ryan-Einot-Gabriel-Welsh test (REGW, 28) was performed using the SAS software [27], for comparison of non-parametric values for survival and regrowth percentages between healthy and infected genotypes of cultivar Plavac mali ($P \leq 0.05$).

4. Results

4.1 Effect of cultivar and medium on survival and regrowth

For the evaluation of survival and regrowth, the cultivars studied were divided in three groups according to their genetic characterization [7]. In group A, high survival (84.5-100.0%) was reached on M1 for all cultivars (Table 1). Survival of cultivar Plavac mali was above 90% whatever the culture medium used. Differences in survival were observed between the other cultivars on M2 and M3 (containing BAP). On M2, survival was lower for cultivars Debit, Grk and Lasina (57.1-61.0%). On M3, survival of cultivars Grk and Plavina was only 36.4 % and 64.3%, respectively. Survival of cultivars of groups B and C was highest on M3 and lowest on M1.

Cultivar	Genetic group*	Survival (%)			Regrowth (%)		
		M1	M2	M3	M1	M2	M3
Debit	A	100.0	61.0	87.5	42.1	61.0	83.3
Grk	A	90.9	60.0	36.4	59.0	20.0	22.7
Lasina	A	93.3	57.1	100.0	83.3	19.0	100.0
Plavac mali	A	97.5	96.0	91.7	95.0	80.0	91.7
Plavina	A	84.6	89.3	64.3	46.2	39.3	46.4
Pošip	B	82.2	96.7	100.0	80.0	80.6	100.0
Vugava	B	65.6	70.0	93.3	37.5	36.6	93.3
Maraština	C	66.6	84.3	100.0	66.6	62.5	100.0

		Survival			Regrowth	
Effect	DF	Chi-Square	Pr > Chi-Square	DF	Chi-Square	Pr > Chi-Square
Cultivar	7	22.4272	0.0021	7	22.4272	0.0021
Medium	2	2.5546	0.2788	2	2.5546	0.2788
Cultivar*Medium	13	25.1122	0.0223	13	25.1122	0.0223

Table 1. Survival (%) and regrowth (%) of nodal segments of eight native Croatian grapevine cultivars from three genetic groups after *in vitro* inoculation on three different media (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 μM L^{-1} BAP) and effect of cultivar and medium composition on the survival and regrowth of nodal segments of eight native Croatian grapevine cultivars, as revealed by logistic regression analysis.

After 8 weeks, Plavac mali was the most reactive cultivar of group A, with regrowth between 80.0 and 95.0% on the three media tested (Table 1). The lowest regrowth, between 20.0% on M2 and 59.0% on M1, was observed for Grk, followed by Plavina, with 39.3% regrowth on M2, 46.2% on M1 and 46.4% on M3. Regrowth of cultivar Debit was high on M2 and M3 and low on M1 (42.1%), while cultivar Lasina showed very high regrowth on M1 and M3 but only 19.0% on M2. In groups B and C, cultivar Pošip showed very high regrowth on all three media tested, while regrowth of cultivars Vugava and Maraština was lower on M1 and M2 compared to M3.

When studying the effect of cultivar, medium and cultivar*medium interaction on survival and regrowth of the eight cultivars studied, logistic regression analysis showed that only the cultivar and the cultivar*medium interaction had a significant effect on survival and regrowth (Table 1).

4.2 Effect of cultivar and medium on shoot growth

After 8 weeks in culture, the length of shoots, the number of nodes and the length of nodes produced from inoculated nodal segments were measured. Variance analysis indicated a high level of significance both for the factors considered (cultivar and culture medium) and their interaction (medium*cultivar) (Supplemental data).

On average for the eight cultivars studied, significant differences in shoot length were observed between the three media tested (Table 2). M2 induced the highest average shoot length; M1 gave intermediate results while M3 gave the lowest results. On M2, differences in shoot length between cultivars were not significant, except with Maraština, which displayed the highest shoot length (7.4 cm), and Pošip, which displayed the lowest shoot length (4.4 cm). On M1, shoot length was highest for Plavac mali, intermediate for Lasina and Plavina, and lower for the other five cultivars. On M3, shoot length was highest for Plavina, Grk and Pošip, and lower for the other five cultivars.

The average number of nodes for the eight cultivars studied was significantly higher on M2, compared to M1 and M3 (Table 3). The number of nodes produced on M1 and M2 varied broadly between cultivars, from 2.6 (Vugava) to 8.3 (Plavina) on M1 and from 4.6 (Pošip) to 10.0 (Grk) on M2. By contrast, on medium M3, the difference between cultivars was less marked, with number of nodes between 4.1 (Debit) and 7.0 (Lasina).

Group B and C cultivars and cultivar Debit (group A) produced longer internodes compared to other group A cultivars (Table 4). Internode length was also influenced by the culture medium, with M3 (containing BAP) producing shorter ones compared to M1 and M2. On M3 hyperhydricity was observed with all cultivars (data not shown).

Based on the three parameters studied, shoot length, number of nodes and length of internodes produced by nodal explants, differences between cultivars of the three groups identified were observed (Tables 2, 3 and 4). Group B cultivars (Pošip and Vugava) were the least reactive to *in vitro* culture conditions, while group A cultivars (except Debit) were the most reactive.

Cultivar	Genetic group	Stem length (cm)[a]			F value	p	Average[b]
		M1	M2	M3			
Debit	A	2.8 ± 1.2 b/C	5.6 ± 2.1 a/AB	3.3 ± 1.1 b/C	11.4	0.0002	3.9 CD
Grk	A	3.4 ± 1.3 b/C	6.2 ± 1.0 a/AB	4.4 ± 2.8 ab/AB	3.2	0.063	4.1 BCD
Lasina	A	4.7 ± 0.9 b/B	5.7 ± 2.3 a/AB	3.4 ± 0.9 c/BC	13.5	<0.0001	4.2 BCD
Plavac mali	A	6.3 ± 2.1 a/A	5.9 ± 3.3 a/AB	2.3 ± 0.7 b/D	23.0	<0.0001	5.2 A
Plavina	A	4.8 ± 3.2 a/B	5.3 ± 1.8 a/AB	4.6 ± 2.6 a/A	0.2	0.791	4.9 AB
Pošip	B	2.8 ± 1.0 b/C	4.4 ± 2.0 a/B	3.9 ± 0.9 a/ABC	14.2	<0.0001	3.7 D
Vugava	B	2.6 ± 1.0 b/C	5.2 ± 1.9 a/AB	3.0 ± 1.6 b/CD	9.2	0.0004	3.5 D
Maraština	C	3.6 ± 1.9 b/BC	7.4 ± 2.9 a/A	3.1 ± 0.6 b/CD	47.8	<0.0001	4.7 ABC
	F value	17.8	3.3	5.8			
	p	<0.0001	0.0035	<0.0001			
	Average[c]	4.1b	5.8 a	3.5 c			

Table 2. Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 μM L^{-1} BAP) on shoot length produced from nodal segments of eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition.

[a]Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium. [b]Average of cultivars on three media. [c]Average of medium for eight cultivars

Cultivar	Genetic group	Number of nodes[a]			F value	p	Average[b]
		M1	M2	M3			
Debit	A	3.2 ± 0.4 b/D	5.3 ± 2.0 a/CD	4.1 ± 1.5 ab/D	4.3	0.021	4.3 D
Grk	A	4.9 ± 1.0 b/C	10.0 ± 2.6 a/A	6.0 ± 4.4 b/ABC	5.3	0.146	5.9 ABC
Lasina	A	6.0 ± 1.5 a/BC	5.2 ± 2.8 a/CD	7.0 ± 2.1 a/A	4.7	0.0121	6.6 AB
Plavac mali	A	7.3 ± 2.3 a/AB	6.3 ± 3.1 ab/BCD	5.3 ± 2.5 b/BCD	4.2	0.0178	6.6 AB
Plavina	A	8.3 ± 4.1 a/A	7.0 ± 2.6 a/BCD	6.5 ± 3.9 a/AB	0.5	0.589	7.1 A
Pošip	B	2.9 ± 1.2 c/D	4.6 ± 2.6 b /D	5.8 ± 1.4 a/ABCD	29.4	<0.0001	4.5 D
Vugava	B	2.6 ± 1.1 c/D	7.9 ± 3.0 a/ABC	4.7 ± 2.9 b/CD	11.0	0.0001	5.0 CD
Maraština	C	4.5 ± 2.0 b/C	8.1 ± 2.9 a /AB	4.5 ± 1.3 b/CD	31.7	<0.0001	5.8 BC
	F value	25.3	4.9	5.3			
	P	<0.0001	<0.0001	<0.0001			
	Average[c]	5.0 b	6.5 a	5.5 b			

Table 3. Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 μM L^{-1} BAP) on number of nodes produced from nodal segments of the eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition.

[a]Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium. [b] Average of cultivars on three media [c]Average of medium for eight cultivars

Maraština, the only representative of group C, displayed intermediate reactivity.

A canonical discriminant analysis of shoot length, number of nodes and average length of nodes of the eight cultivars studied produced on the three media tested was performed. The first two canonical variables (CAN1 and CAN2) explained 92.7% of the total variability between cultivars grown on different media. Based on CAN1 (which explained 54.5% of total variability) and CAN2 (which explained 38.2% of total variability) a scatter plot was designed (Figure 1). Shoot length was highly positively correlated (0.93) with CAN1, while CAN2 was highly positively correlated with number of nodes (0.88) and highly negatively correlated (0.81)

Cultivar	Genetic group	Internode length (cm)[a]			F value	p	Average[b]
		M1	M2	M3			
Debit	A	0.8 ± 0.2 b/BC	1.0 ± 0.2 a/AB	0.8 ± 0.2 b/A	4.0	0.0255	0.9 A
Grk	A	0.6 ± 0.1 a/DC	0.6 ± 0.0 a/C	0.8 ± 0.1 a/A	1.9	0.1686	0.7 C
Lasina	A	0.8 ± 0.2 b/BC	1.1 ± 0.1 a/A	0.5 ± 0.1 c/B	24.5	<.0001	0.6 C
Plavac mali	A	0.8 ± 0.1 a/BC	0.9 ± 0.2 a/ABC	0.5 ± 0.2 b/B	21.8	<.0001	0.7 BC
Plavina	A	0.5 ± 0.1 b/D	0.7 ± 0.1 a/BC	0.7 ± 0.1 a/A	5.9	0.0076	0.7 C
Pošip	B	1.0 ± 0.3 a/AB	1.0 ± 0.4 a/AB	0.6 ± 0.1 b/A	18.3	<.0001	0.9 A
Vugava	B	1.1 ± 0.5 a/A	0.7 ± 0.3 b/C	0.7 ± 0.4 b/A	3.3	0.0452	0.8 AB
Maraština	C	0.7 ± 0.2 ab/BC	0.9 ± 0.1 a/ABC	0.7 ± 0.1 b/A	3.8	0.0271	0.8 ABC
	F value	5.5	3.8	6.4			
	p	<0.001	<0.001	<0.001			
	Average[c]	0.8 a	0.9 a	0.6 b			

Table 4. Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 μM L^{-1} BAP) on average length (cm) of internodes of shoots produced from nodal segments of the eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition.

[a]Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium. [b]Average of cultivars on three media [c]Average of medium for eight cultivars

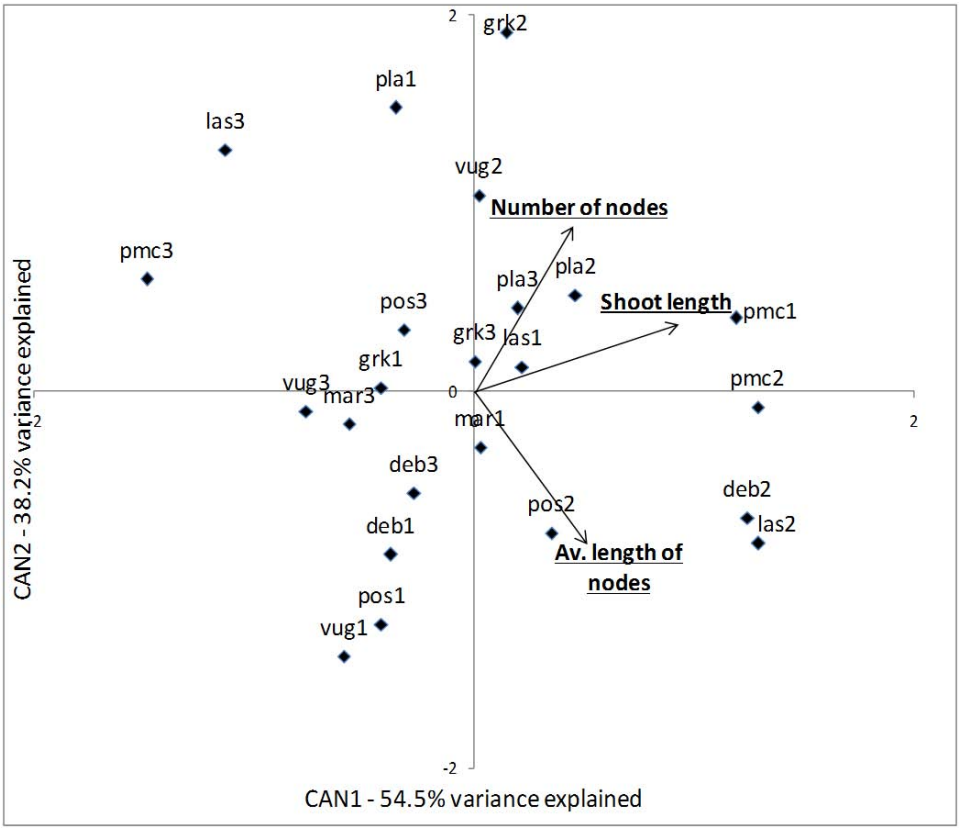

Figure 1. Position of the eight Croatian grapevine cultivars studied grown on three different media in the space defined by the first two canonical variables of the canonical discriminant analysis performed using three growth parameters (shoot length, number of nodes and average internodes length) and vector diagram of correlations between the three growth parameters studied (shoot length, number of nodes and average.

with average length of nodes. Based on the position on the scatter plot of centroids of the eight cultivars grown on three different media and on the correlation of the variables studied with CAN1 and CAN2, the growth differences of the eight cultivars studied on the three media tested could be explained.

4.3 Effect of virus infection on *in vitro* growth parameters

In order to test the influence of sanitary status on *in vitro* growth parameters, nodal segments of virus-infected genotypes of cultivar Plavac mali were introduced *in vitro*. Genotypes with single and double virus infection were selected. The virus infection status of selected genotypes, as revealed by ELISA and RT-PCR, is presented in Table 4.

No significant difference in survival was observed between the healthy control genotype (PMC201) and infected ones, with values ranging between 82.8 and 97.5% (Table 5). By contrast, significant differences in regrowth were noted between the healthy control and infected genotypes. Almost all surviving explants of the healthy genotype displayed regrowth, while regrowth of infected ones dropped to 5.5-31.4%.

Statistically significant differences in shoot length and number of nodes were observed between healthy and infected genotypes (Table 6). Shoots of the healthy genotype reached an average length of 6.3 cm, while PMC 201/6, the most reactive infected genotype, reached only 2.6 cm. Shoots of the other infected genotypes measured between 1.2 and 2.6 cm. There was no significant effect of the phytosanitary status of Plavac mali genotypes on internode length.

Genotype	Virus
PMC 201	none detected
PMC 008/1	GLRaV-3 + GVA
PMC 010/1	GLRaV-1 + GLRaV-3
PMC 064/3	GLRaV - 1
PMC 225/2	GLRaV - 3
PMC 201/6	GFLV

Table 5. Virus infection status determined by ELISA and RT-PCR (GVA) testing of selected genotypes of grapevine cultivar Plavac mali. All samples were tested for the presence of GFLV, GLRaV-1, GLRaV-3 and GVA viruses.

5. Discussion

In this paper, we studied the response of eight autochthonous cultivars (Debit, Grk, Plavina, Lasina, Plavac mali, Pošip, Vugava and Maraština) essential to Croatian viticulture following their introduction *in vitro*. The results revealed that cultivar and interaction between cultivar and medium had a significant effect on survival and regrowth, whereas the effect of medium was not significant. Differences were observed between the three groups of cultivars selected according to their genetic distance [7]. Group A cultivars showed good survival and regrowth on M1 (half strength MS medium devoid of cytokinin), while group B and C cultivars developed better on M3 (full strength MS medium with BAP). Plavac mali yielded the best overall results while Grk had the worst performance for *in vitro* inoculation.

After 8 weeks in culture, variance analysis for shoot length and number of nodes showed a high degree of significance both for the principal factors considered (cultivar and culture medium) and their interaction (medium*cultivar). For all cultivars, the most suitable medium regarding the obtained shoot length and number of nodes appeared to be M2 (full-strength MS medium) and the least suitable M3. The canonical discriminant analysis performed showed a positive correlation between node number and shoot length, while average internode length was not correlated with stem length. Moreover, only a low negative correlation existed

Genotype	Virus infection	Survival (%)	Regrowth (%)
PMC 201	healthy	97.5a	95.0a
PMC 201/6	GFLV	85.1a	18.5bc
PMC 010/1	GLRaV-1 + GLRaV-3	82.8a	28.5bc
PMC 064/3	GLRaV-1	84.3a	15.6bc
PMC 008/1	GLRaV-3 + GVA	88.5a	31.4b
PMC 225/2	GLRaV-3	86.1a	5.5c

Table 6. Effect of virus infection status on survival (%) and regrowth (%) of nodal segments of six genotypes of grapevine cultivar Plavac mali.

In columns, values followed by the same letter are not significantly different according to Ryan's multiple range test.

Genotype	Viral status	Number of tested plants	Shoot length (cm)	Number of nodes (n)	Average internode length (cm)
PMC 201	healthy	39	6.3 ± 2.1a	7.3 ± 2.3a	0.85a
PMC 201/6	GFLV	27	2.6 ± 1.8b	3.0 ± 1.9b	0.87a
PMC 010/1	GLRaV-1 + GLRaV-3	29	2.4 ± 1.7b	2.6 ± 1.6bc	0.91a
PMC 064/3	GLRaV-1	27	1.6 ± 0.7cb	1.7 ± 0.6cd	0.97a
PMC 008/1	GLRaV-3+GVA	23	1.5 ± 0.7cb	1.8 ± 1.3cd	0.86a
PMC 225/2	GLRaV-3	31	1.2 ± 0.4c	1.2 ± 0.5d	0.99a

Table 7. Effect of virus infection status on shoot length (cm), number of nodes and average internodes length (cm) produced from nodal segments of six genotypes of grapevine cultivar Plavac mali.

In columns, mean values followed by the same letter are not significantly different according to Duncan's multiple range test (P ≤ 0.05).

between internode length and the number of nodes. Nevertheless, according to our results some cultivars such as Grk, Plavina, Lasina and Plavac mali showed a tendency towards shorter internodes and Debit towards longer internodes. Internode elongation was influenced by the medium as well, and cultivars grown on M3 (containing BAP) usually produced shorter internodes compared with the other two media. Decrease of internode length in presence of BAP has been observed in many species [29] including *Vitis* [30,31].

The variability observed in *in vitro* performance of the eight cultivars studied confirmed that the efficiency of *in vitro* multiplication is strongly genotype-dependent, as previously reported in *Vitis* [32,33]. Some studies [34,35] suggested that the variable efficiency of *in vitro* techniques between grapevine varieties could be linked to differences in endogenous plant regulator content and/or to different responses to exogenous growth regulators. This hypothesis is supported by our results, which showed that growth initiation for some cultivars was enhanced by the addition of BAP to the culture medium, while this was not the case for other cultivars.

Previous studies have shown that the cytokinin BAP is effective in inducing shoot development in *Vitis* [36]. At relatively low concentrations (2.5-5.0 μM L^{-1}), BAP enhanced bud multiplication, whereas a higher concentration (10 μM L^{-1}) induced hyperhydricity [16]. The addition of cytokinin inhibited shoot elongation in some cultivars such as Refošk [37]. In our study, M3 (full strength MS medium supplemented with 4.4 μM L^{-1} BAP) enhanced survival and regrowth of cultivars, especially those of groups B and C, as well as Lasina and Plavac mali (group A). However, in most cultivars, it induced a decrease in growth parameters, notably internode length shortening and favoured hyperhydricity.

Micropropagation of cultivars Debit, Plavac mali, Plavina, Pošip and Vugava was first performed by Hartl *et al*. [20] using a MS medium supplemented with 2.85 μM L^{-1} indole acetic acid (IAA), 0.44 μM L^{-1} BAP and 3% sucrose. Hartl and Maleš [20] reported that *in vitro* culture of cultivars Plavac mali and Vugava on MS medium with 4.4 μM L^{-1} BAP was suitable for inducing bud growth, but that increasing BAP concentration to 8.9 μM L^{-1} caused multiple shoot proliferation with compact nodal segments and abnormal leaf shape. However, these authors emphasized that growth of Plavac mali shoots was erratic and *in vitro* propagation of this cultivar was strongly limited. By contrast, in our conditions, Plavac mali was the most reactive cultivar based on survival, regrowth (regardless of medium) and shoot elongation (on medium M1 and M2); however, decrease in shoot growth and nodal segment compaction were observed on the medium even with lower concentrations of BAP [21]. The differences observed in the reactivity of the eight cultivars studied imply that, even though *in vitro* introduction was achieved with all of them, the specific requirements of each cultivar should be thoroughly tested. In accordance with our results, Péros *et al*. [38] observed a high variability in the response to *in vitro* culture of 23 grapevine cultivars. Some cultivars showed very good growth, whereas others exhibited relatively poor growth. As also observed in our study, these authors indicated that shoot length was highly positively correlated with the number of nodes.

The surveys of Croatian native cultivars performed in recent years [7,9,10] highlighted the serious problem of virus infestation in their populations. For cultivars showing a high percentage of vines infected with viruses having high economic impact (GFLV, GLRV-1, GLRV-3 and GVA), the initiation of a virus eradication program is urgently needed. *In vitro* techniques have been successfully used for virus eradication in *Vitis* [13,14,39,40] but a prerequisite to their utilisation is the establishment of *in vitro* cultures of infected genotypes. This provided the

scientific basis for our study on the influence of the most common single and double virus infections on *in vitro* introduction of infected Plavac mali genotypes. Studies by Barba *et al*. [41] and Monis and Bestwick [42] revealed that the virus titre of *in vitro* propagated grapevine tissues was higher (up to 30 times higher in some samples) than in the original actively growing vines. Correspondingly, the growth rate of infected genotypes was significantly lower than that of healthy ones [41,42]. It has been reported that virus infection strongly influences regeneration potential [43]. However, the results published on the influence of specific virus strains and virus combinations on *in vitro* growth of grapevine are not consistent. Indeed, Buciumeanu *et al*. [44] observed a higher multiplication rate of GFLV-infected plants compared to healthy plants, while very poor growth of plants infected with GLRaV-1+3 was noted. By contrast, Walter [45] and Abracheva *et al*. [46] reported that GFLV caused growth reduction of *in vitro* grapevine plantlets. In our experiments comparing the *in vitro* growth of one healthy and several infected Plavac mali genotypes, all growth parameters values measured were significantly higher in the healthy genotype. Differences in growth parameters between genotypes infected with different viruses or virus combinations have been reported [47]. In our conditions however, no such differences were observed between the five infected Plavac mali genotypes studied, regardless of their virus contamination status.

In conclusion, the present study reported the successful *in vitro* introduction of eight native Croatian cultivars, despite differences between genotypes in survival, regrowth and growth parameters. This was the first report of *in vitro* introduction for three (Grk, Lasina, and Maraština) of the eight cultivars studied. Although infected genotypes were less reactive compared to the healthy control, plant material could be obtained in sufficient quantity to implement virus eradication procedures. Future research will focus on the optimization of the micropropagation protocol for these Croatian cultivars.

References

[1] Maletić, E., Karoglan Kontić J., Pejić, I., 2008: Grapevine: Ampelography, Ecology, Breeding. Školska Knjiga, Zagreb.

[2] Jelaska, M., 1960: Ampelography of Dalmatian cultivars. Library of Institut for Adriatic Crops and Karst Reclamation, Split, Croatia.

[3] Bulić, S., 1949: Ampelography of Dalmatia. Poljoprivredni Nakladni Zavod, Zagreb.

[4] Šimon, S., Malenica, N., Žulj, M., Gasi, F., Zdunić, G., Preiner, D., Pejić, I., 2010: REMAP as a tool for preliminary grapevine accession screening. Acta Hortic. 859, 155-159.

[5] Piljac, L., Maletić, E., Kontic, J.K., Dangl, G.S., Pejić, I., Mirosević, N., Meredith, C.P., 2002: The parentage of Pošip bijeli, a major white wine cultivar of Croatia. Vitis 41, 83-87.

[6] Preiner, D., Karoglan Kontić, J., Šimon, S., Marković, Z., Stupić, D., Maletić, E., 2012: Intravarietal agronomic variability in croatian native Vitis vinifera l. cultivar Grk with female flower and seedless berries. Am. J. Enol. Vitic. 63, 291-295.

[7] Zdunić, G., Preece, J.E., Dangl, D.S., Koehmstedt, A., Mucalo, A., Maletić, E., Pejić, I., 2013: Genetic characterization of grapevine cultivars collected throughout the Dalmatian region. Am. J. Enol. Vitic. doi: 10.5344/ajev.2012.12085.

[8] Maletić, E., Pejić, I., Kontić, J.K., Piljac, J., Dangl, G.S., Vokurka, A., Lacombe, T., Mirosević, N., Meredith, C.P., 2004: Zinfandel, Dobricic, and Plavac mali: the genetic relationship among three cultivars of the Dalmatian Coast of Croatia. Am. J. Enol. Vitic. 55, 174–180.

[9] Poljuha, D., Sladonja, B., Peršurić, Đ., 2004: Survey of five indigenous istrian cultivars for the presence of six grape viruses. Am. J. Enol. Vitic. 55, 286-287.

[10] Karoglan Kontić, J., Preiner, D., Šimon, S., Zdunić, G., Poljuha, D., Maletić, E., 2009: Sanitary Status of Croatian Native Grapevine Varieties. Agric. Conspec. Sci. 74, 99-103.

[11] Zdunić, G., Maletić, E., Vokurka, A., Karoglan Kontić, J., Pezo, I., Pejić, I., 2007: Phenotypical, sanitary and ampelometric variability within the population of cv. Plavac Mali (Vitis vinifera L.). Agric. Conspec. Sci. 72, 117-128.

[12] Silva, R.C., Luis, G.Z., Pereira, J.E.S., 2012: Short term storage in vitro and large scale propagation of grapevine genotypes. Pesqui. Agropecu. Bras. 47, 344-350.

[13] Skiada, F.G., Grigoriadou, K., Maliogka, V.K., Katis, N.I., Eleftheriou, E.P., 2009: Elimination of grapevine leafroll-associated virus 1 and grapevine rupestris stem pitting-associated virus from grapevine cv. Agiorgitiko and a micropropagation protocol for mass production of virus-free plantlets. J. Plant Pathol. 91, 177-184.

[14] Panattoni, A., Luvisi, A., Triolo, E., 2013: Review. Elimination of viruses in plants: twenty years of progress. Span J Agric Res 11(1), 173-188.
[15] Roubelakis-Angelakis, K.A., 2009: Grapevine Molecular Physiology and Biotechnology. Springer, New York.
[16] Banilas, G., Korkas, E., 2007: Rapid micro-propagation of grapevine (cv. Agiorgitiko) through lateral bud development. e-J. Sci. Tech. 2, 31-38.
[17] Mederos-Molina, S., 2007: Culture medium requirements for micropropagation of Vitis vinifera L. cv. Listan Blanco. Acta Hort. 754, 265-271.
[18] Reisch, B.I., 1986: Influence of genotype and cytokinins on in vitro shoot proliferation of grapes. J. Am. Soc. Hortic. Sci. 111, 138-141.
[19] Botti, C., Garay, L., Reginato, G., 1993: The influence of culture dates, genotype and size and type of shoot apices on in vitro shoot proliferation of Vitis vinifera cvs. Thompson Seedless, Ribier and Black Seedless. Vitis 32, 125-126.
[20] Hartl, D., Maleš, P., Jelaska, S., 1989: Vegetative multiplication of five Dalmatian grapevine cultivars in vitro. In: Abstracts of the Contributions presented at the 5th International Symposium on Grape Breeding. Bundesforschungsanstalt fur Rebenzuchtung Geilweilerhof, D-6741-Siebeldingen.
[21] Hartl, D., Maleš, P., 2000: Factors affecting of grapevine cultivars, Vugava and Plavac mali micropropagation from in vitro cultured minicuttings. Biologia (Bratislava) 55, 121-129.
[22] Hartl, D., Hančević, K., Bućan, L., 2005: Rejuvenation of cv. Vugava grapevine (Vitis vinifera L.) in vitro. Biologia (Bratislava) 60, 437-442.
[23] Marković, Z., Chatelet, P., Sylvestre, I., Karoglan Kontić, J., Engelmann, F., 2012: Duration of culture of grapevine (Vitis vinifera) microcuttings on medium with zeatin riboside affects shoot tip recovery after cryopreservation, in: GRAPIN, A., KELLER, E.R.J., LYNCH, P.T., PANIS, B., REVILLA BAHILLO, A., ENGELMANN F., (Eds.), Cryopreservation of Crop Species in Europe Proceedings of the final meeting, Agrocampus Ouest INPH, Angers, France, 8-11 Feb. 2011, COST Office, Brussels, pp. 145-147.
[24] Mackenzie, D.J.: 1997: A standard protocol for the detection of viruses and viroids using a reverse transcription-polymerase chain reaction technique. Document CPHBTRTPCR1.00. Canadian Food Inspection Agency.
[25] Murashige, T., Skoog, F., 1962: A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. 15, 473-497.
[26] Torregrosa, L., Bouquet, A., Goussard, P.G., 2001: In vitro culture and propagation of grapevine, in: Molecular Biology and Biotechnology of the Grapevine Roubelakis-Angelakis K.A. (Ed.), Springer, New York, pp 281-326.
[27] S.A.S., 2004: System for Windows Version 8.02. SAS Institute, Cary, NC, USA.
[28] Ryan, T.A., 1960: Significance tests for multiple comparison of proportions, variances, and other statistics. Psychol. Bull. 57, 318-328.
[29] George, E.F., 1993: Plant Production by Tissue Culture. Exegetics Ltd., U.K.
[30] Goussard, P.G., 1981: Effects of cytokinins on elongation, proliferation and total mass of shoots derived from shoot apices of grapevine cultured in vitro. Vitis 20, 228-234.
[31] Goussard, P.G., 1982: Morphological responses of shoot apices of grapevine cultured in vitro. Effects of cytokinins in routine subculturing. Vitis 21, 295-298.
[32] Mayerson, M.E., Benton C.M., Gray, D.J., 1994: A comparison of shoot micropropagation among bunch and muscadine grape species and cultivars. Proc. Florida State Soc. 107, 311-312.
[33] Torregrosa, L., Bouquet, A., 1996: Adventitious bud formation and shoot development from in vitro leaves of Vitis × Muscadinia hybrids. Plant Cell Tiss. Org. Cult. 45, 245-252.
[34] Grönroos, L., Von Arnold, S., Ericsson, T., 1989: Somatic embryos from callus of Salix viminalis L. Ann. Sci. Forest. 46, 108-109.
[35] Roubelakis-Angelakis, K.A., Zivanovitc, S.B., 1991: A new culture medium for in vitro rhizogenesis of grapevine (Vitis spp.) genotypes. HortSci. 26, 1551-1553.
[36] Alizadeh, M., Singh, S.K., Patel, V.B., 2010: Comparative performance of in vitro multiplication in four grape (Vitis spp.) rootstock genotypes. Int. J. Plant Produc. 4, 41-50.
[37] Koruza, B., Jelaska, S., 1993: Influence of meristem culture and virus elimination on phenotypical modifications of grapevine (Vitis vinifera L. cv. Refošk). Vitis 32, 59-60.
[38] Péros, J.P., Torregrosa, L., Berger, G., 1998: Variability among Vitis vinifera cultivars in micropropagation, organogenesis and antibiotic sensitivity. J. Exp. Bot. 49, 171-179.
[39] Gambino, G., Bondaz, J., Gribaudo, I., 2006: Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. Eur. J. Plant Pathol. 114, 397-404.
[40] Youssef, S.A., Al-Dhaher, M.M.A., Shalaby, A.A., 2009: Elimination of Grapevine fanleaf virus (GFLV) and grapevine leaf roll-associated virus-1

(GLRaV-1) from infected grapevine plants using meristem tip culture. Int. J. Virol. 5, 89-99.

[41] Barba, M., Cupidi, A., Casorri, L., 1993: Influence of virus and virus-like diseases of grapevine in shoot cultures. Extended Abstracts of the 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, Montreux, Switzerland. September 6-9, 1993. Federal Agricultural Research Station of Changins, CH-1260 Nyon, Switzerland, p 2.

[42] Monis, J., Bestwick, R.K., 1996: Detection and localization of grapevine associated clostero viruses in greenhouse and tissue culture grown plants. Am. J. Enol. Vitic. 47, 199-205.

[43] Smerea, S., Andronic, L., Grigorov, T., Bujoreanu, V., 2010: In vitro regenerative genotypic specifity of meristems from virus infected grapevine cultivars. Rom. Biotech. Lett. 15, 19-25.

[44] Buciumeanu, E.C., Vişoiu, E., Guţă, I.C., Popescu, C.F., 2010: Continuity of grapevine virology at NRDIBH Ştefăneşti-Argeş in the service of a viable viticulture in Romania. Rom. Biotech. Lett. 15, 11-18.

[45] Walter, B., 1988: Examples of physiological reaction of the vine in the presence of virus [Quelques exemples de la réaction physiologique de la vigne en présence de virus]. Bull. O.I.V. 687-688, 383-390.

[46] Abracheva, P., Rozenova, L., Todorova, M., 1994: The influence of grapevine fanleaf virus and stem pitting on in vitro grapevine cultures. Vitis 33, 181-182

[47] Walter, B., Martelli, G.P., 1997: Clonal and sanitary selection of the grapevine, in: Walter, B. (Ed.), Sanitary Selection of the Grapevine. Protocols for Detection of Viruses and Virus- like Diseases, Les Colloques No. 86 INRA Editions, Paris, 43-95.

Supplemental data

Factorial ANOVA for the effect of cultivar, medium and their interaction on shoot length, number of nodes, and average internode length from shoots produced from nodal segments of eight native Croatian grapevine cultivars studied.

Variable	Source of variance	Degree of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Stem length (cm)	Medium	2	224.8	112.4	38.94	<0.0001
	Cultivar	7	123.1	17.6	6.09	<0.0001
	Medium*Cultivar	14	368.9	26.3	9.13	<0.0001
Number of nodes	Medium	2	140.0	69.8	14.52	<0.0001
	Cultivar	7	361.3	51.6	10.73	<0.0001
	Medium*Cultivar	14	547.5	39.1	8.13	<0.0001
Average internode length	Medium	2	2.01	1.01	13.73	<0.0001
	Cultivar	7	2.73	0.39	5.33	<0.0001
	Medium*Cultivar	14	4.61	0.33	4.49	<0.0001