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ARTIGOS

FREE CULTURE MEDIA OF GROWTH REGULATORS ON MICROPROPAGATION OF GRAPEVINE (*VITIS LABRUSCA* L.) 'BORDÔ' CULTIVAR THROUGH NODAL SEGMENTS

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Abstract

The micropropagation is an important biotechnological tool for obtaining and maintaining mother grapevine plants with high quality plant health. The objective was to evaluate the establishment and multiplication *in vitro* and *ex vitro* acclimatization of grapevine cultivate Bordô in different culture media without adding growth regulators. Grapevine nodal segments were cultured in five culture media formulations without adding growth regulators. It was evaluated the number of leaves and roots, length of roots and shoots, replication rate, relative chlorophyll index, percentage of regenerated and rooted plants, dry biomass of shoot, root and total plants grown *in vitro* and after acclimatization. *In vitro* propagation of grapevine Bordô through nodal segments provided high rates of regeneration and rooting. High survival rates were obtained in the acclimatization of Bordô. Considering all the varia-

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bles, culture media Roubelakis and Zlenko showed the best growth rates and development for shoots and roots, and *in vitro* multiplication rate of Bordô grapevine cultivar.
Keywords: *In vitro* propagation. Vegetative propagation. *Vitis*.

Meios de cultura livres de reguladores de crescimento na micropropagação da videira (Vitis labrusca* L.*) cultivar 'Bordô' através de segmentos nodais

Resumo

A micropropagação é uma importante ferramenta biotecnológica para a obtenção e manutenção de plantas matrizes de videira de alta qualidade fitossanitária. O objetivo do trabalho foi avaliar o estabelecimento e a multiplicação in vitro e a aclimatização ex vitro da videira cv. Bordô em diferentes meios de cultura sem a adição de reguladores de crescimento. Segmentos nodais foram cultivados em cinco formulações de meios de cultura sem adição de reguladores de crescimento. Foram avaliados o número de folhas e de raízes, comprimento da maior raiz e da parte aérea, taxa de replicação, índice relativo de clorofila, biomassa seca da parte aérea, radicular e total, porcentagem de plantas regeneradas e enraizadas, de plantas cultivadas in vitro e após aclimatização. A propagação in vitro da cv. Bordô através de segmentos nodais proporcionou elevadas taxas de regeneração e enraizamento. Elevadas taxas de sobrevivência foram obtidas na aclimatização da cv. Bordô. Considerando todas as variáveis analisadas, as formulações salinas Roubelakis e Zlenko proporcionaram o melhor crescimento e desenvolvimento da parte aérea e radicular e taxa de multiplicação in vitro da cultivar de videira Bordô.
Palavras-chave: Propagação in vitro. Propagação vegetativa. Vitis.

1 **INTRODUCTION**

In the last decade, following global trends, the grape and wine national market is undergoing a processing resulting from the Brazilian consumer habits change. In the season 2014/2015 domestic production was 1.436.074 tons; that amount 46.89 % of the grapes were aimed at production of wine, juices and derivatives and 53.11 % for fresh consumption.[1] The search for a healthy diet associated with the attractive flavor of grape juice makes this market an interesting income option for Brazilian grape growers.

In southern Brazil, viticulture consolidated with the cultivation of American origin grapes. The Bordô grape (*Vitis labrusca* L.), introduced in Rio Grande do Sul in 1839, is one of the cultivars, which is due to several factors, such as high productivity, use of diversity (wine, juice, table grape, jelly and vinegar) and rusticity.[2] So, consumption is given in different ways, the must of grapes Bordô is considered the dye base of juice and Brazilian table wine. Also creates a typical wine with aroma and fruity and foxy flavor, appreciated by a particular group of consumers.

To ensure the longevity of the orchard and the quality of the grapes produced, the acquisition of propagative materials with good phytosanitary conditions is required. However, the availability of

free of pathogens materials is a limiting factor for the sustainability of agricultural production and the permanence in the activity.[3]

In this sense, micropropagation techniques are important tools for obtaining and maintaining stock plants of good quality phytosanitary.[4] The process enables the mass multiplication of plants and the formation of free virus matrices; besides preservation materials of interest in an aseptic environment.[5,6,7]

The culture media, responsible for supplying the basic nutritional needs for growth and development of explants is a relevant factor in the micropropagation process.[8] Thus, the choice of a suitable salt formulation has a direct relationship with the successful development of in vitro cultures.[3,8] Published protocol with *Vitis* suggest the addition of plant growth regulators in the culture media, particularly from the group of the cytokinins, to promote multibrotation.[9] However, inadequate concentrations of cytokinin can promote the formation of anomalous shoots and multiplication in the form callus,[8] which is undesirable for vegetative propagation protocols, especially the risk of induced somaclonal mutation. Recent study described by Bettoni et al.,[3] with *Vitis*, demonstrate that free salt formulations of growth regulators provided high *in vitro* multiplication rates and grapevines had root formation in various free culture media growth regulators and or specific phase for rooting. Thus, the aim of this study was to evaluate the establishment and multiplication in vitro in different culture media without adding growth regulators. and ex vitro acclimatization of grape cv. Bordô.

2 **MATERIALS AND METHODS**

Mother plants from Bordô grape (*Vitis labrusca* L.) were kept in greenhouse for providing explants. Experiments were performed in completely randomized design, with five treatments (saline formula) and thirty repetitions for each treatment in the evaluations during *in vitro* phase, and ten repetitions during acclimatization phase.

It was tested saline formulation proposed by Galzy, Haffner and Compan,[6] Roubelakis-Angelakis and Zivanovitc,[10] C_2D,[11] DSD1[12] and Zlenko, Troshin and Kotikov[13] for *in vitro* grapevine cultivation, supplemented with 20 g L^{-1} of sucrose and 7 g L^{-1} of agar, without growth regulators.

2-cm-nodal segments, with a single bud were used as explants source for *in vitro* cultivation. Under aseptic conditions, nodal segments were superficially sterilized, embedded in 7 0% alcohol (v/v) for 15 seconds and rinsed two times in sterile water; after, they were immersed in 80 % sodium hypochlorite solution (v/v) and 0.1 % Tween 20 (v/v) during 15 minutes and rinsed three times in sterile water. Explants were transferred into the test tubes (110 mm X 23 mm) with 10 mL of different culture media.

Cultures were maintained in the test room at 25 °C ± 2 °C during the fourth days in the dark, and after for a photoperiod of 16 hours of light per day^{-1} and luminous intensity of 50 μmol m^{-2} s^{-1}. Sub-crops were done each 45 days, segmenting sprouts in nodal segments of 1 cm length with a single bud. After the second sub-crop, *in vitro* cultures were maintained in growth for 60 days and were

evaluated for number of leaves and roots, length of the bigger root and the aerial part, replication rate, chlorophyll index, dry matter from aerial parts and roots after drying in the oven at 60 ºC for 48 hours, percentage of regenerated and rooted plants.

Replication rate was obtained by counting explants number which are originated when the material is replicated. For indirect measures of chlorophyll (chlorophyll index) in SPAD value, readings from adaxial face of the leaf located on the medium part of each bud, selecting completely expanded leaves through Handheld Chlorophyll Gauge SPAD-502 (Soil Plant Analysis Development, Konica Minolta®, Japan). Readings values performed with the gauge were calculated based on the quantity of transmitted light by the leaf on two wavelengths; the light that has passed through the leaf reaches a receptor which converts light on analog signals, and those ones on digital signals that are used to calculate SPAD values.[14]

Ex vitro acclimatization was performed with the third sub- cultures after 60 days in *in vitro* growing. Roots were washed in water and pruned (2 cm length); aerial part was maintained with 2 or 3 basal leaves. Buds were transferred to honey-combed trays with 72 cells (100 mL) containing sterilized substrate at 121 ºC for 1 hour with Dystroferric Red Nitosol, sand and commercial substrate Tecnomax® (1:1:1, v/v/v). Trays were packed into plastic boxes, covered with glass and put in an acclimatization room during 60 days. Analyzed variables were the same as the experiments performed *in vitro*, exception to replication rate.

Statistical models were considered according to the nature of the variable response. For the variables Roots Number, Leaves Number and Replication Rate, it was used Poisson's distribution. Model prepositions were verified using Kolmogorov-Smirnov's Tests for normality of residuals, and Bartlett's test for variance homogeneity.

In order to verify the model adjustment, it was used normal plot with simulated envelopes for *deviance* residual.[15] Data were submitted to variance analysis and, if significant, averages were compared by Tukey's test at 5% significance level in R environment.[16]

3 **RESULTS AND DISCUSSION**

Morphological studies related with anatomical characteristics of grapevine plants in vitro were described by several authors,[3,17-19,6] and variations of evaluation results usually are related to the formulations of culture media and genotype used.

Independently of the saline formulation of each nutritive media, *in vitro* cultures of Bordô cultivar showed high potential for regeneration and rooting, so that all explants that were placed *in vitro* regenerated and virtually all had root formation, except C2D formulation, which occurred the rooting of 85 % of inoculated explants (Table 1). These results suggest that there is no need of use of growth regulators in culture media to promote multiplication, rooting or specific phase for rooting. Similar results were described by Biasi, Passos and Pommer[19] and other authors[8] in *in vitro* propagation of Jales and VR 043-43 grapevine rootstock, respectively, in saline formulations without growth regulators.

For the variable number of leaves, no significant differences were observed among formulations. At the end of the third subculture was obtained an overall average of 6.1 leaves per explant (Table 1). These results are similar to those found by other research[18] in *in vitro* propagation of VR 043-43 and SO4 grapevine rootstock, and lower than reported by researches[20] with SO4, Riparia, Grav, 140Ru and Fercal rootstocks, that after 60 days of cultivation in DSD1 formulation obtained around 10 leaves per explant.

Roubelakis' formulation promoted greater number of roots, with average formation of 2.7 main roots per plant, not differing significantly from the ZL, DSD1and C2D formulations, which formed on average 2.1 roots per plant respectively (Table 1). Roubelakis-Angelakis and Zivanovitc,[10] by comparing the Murashigee and Skoog (MS)[21] and Roubelakis formulations in micropropagation of 15 grapevine cultivars, found better development and increased formation of primary roots of in vitro plants in Roubelakis media.

Similarly to the previous variable, the greater length of roots occurred when cv. Bordô was cultivated in Roubelakis formulation (12.5 cm), which was higher than Galzy formulation and not different from the formulations ZL, DSD1 and C2D, that obtained on average a length of 10.3 cm (Table 1). Variations in genotype responses to different culture media associated substantially with the composition of the nutrient formulations. The same statement was reported in a study[22] with evaluation of eight culture media in vitro propagation of cv. Red Globe, where different nutrient sources made up the formulations and exercised influence on the development of cultivar.

The aerial parts length of Bordô cultivar ranges from 3.8 and 4.7 cm, no significant differences between the formulations of culture media (Table 1). These values are higher than those found in a study[9] with the same cultivar, in different culture media formulations with and without growth regulators and lower than those found by other authors[8,20] in the multiplication of grapevine rootstock in without growth regulators formulations.

Table 1 – Number of leaves and roots, length (cm) of the longest root and aerial part and replication rate (TR), relativachlorophyll index (IRC), dry matter (mg) of roots and aerial part, total biomass (mg), regeneration (R) and rooting (E) of Bordô grapevine nodal segments cultivated in different culture media†

Culture Media	Number		Length (cm)		TR	IRC
	Leaves	Roots	Longer Root	Aerial Part		
Roubelakis	6.2 a	2.7 a	12.5 a	4.4 a	5.2 a	26.9 ab
ZL	6.1 a	2.2 ab	10.5 ab	4.2 a	5.2 a	26.2 ab
DSD1	6.3 a	1.8 ab	11.2 ab	4.7 a	5.4 a	23.9 b
Galzy	5.8 a	1.5 b	8.7 b	4.4 a	5.0 a	24.5 b
C2D	6.2 a	2.2 ab	9.1 b	3.8 a	4.5 a	29.2 a
Mean	6.1	2.1	10.4	4.3	5.1	26.1
C.V. (%)	24.96	23.12	42.93	17.04	34.43	15.56
Culture Media	Dry Matter (mg)				R (%)	E (%)
	Roots	Aerial Part	Total Biomass			
Roubelakis	14.9 a	27.9 a	42.8 a		100.0	100.0
ZL	12.7 ab	26.9 a	39.7 ab		100.0	100.0
DSD1	14.0 a	32.2 a	46.2 a		100.0	100.0
Galzy	11.7 ab	30.6 a	42.4 a		100.0	100.0
C2D	8.6 b	30.9 a	36.3 b		100.0	85.0
Mean	12.4	29.7	41.5		100.0	97.0
C.V. (%)	40.15	9.05	8.37		-------	-------

Replication rate reflects the quantity of propagules derived from a single explant. As a reflection of 'number of leaves' and 'stem length' variables, no significant differences among formulations in relation to the rate of replication. When cultures of cv. Bordô are pricked generate an average of 5.1 new plants (Table 1). On the contrary, some authors[23] showed the influence of the composition of the culture media on the in vitro development deGrasset grapevine rootstock (*Vitis champinii* Planch.), and related formulations express effects on aerial growing rates which in turn are linked to the replication capacity of the inoculated explant. The results for the replication rate found in this study are higher than those evidenced by Krizan, Ondrusiková and Moudrá[24] when multiplied Kober 5BB, 125AA Kober and Teleki 5C grapevine rootstocks in different culture media.

In relation to chlorophyll index, values of SPAD-502 reading showed culture media formulations influenced chlorophyll rate (Table 1). The highest chlorophyll indexes (IRC) were found when the Bordô cv. was cultivated on C2D, Roubelakis and ZL formulations, with IRC in the range of 26.2 to 29.2; saline formulation ZL and Roubelakis did not differ from culture media DSD1 and Galzy. Likewise, authors[25] related the influence of culture media on Criolla Grande and Pedro Giménez grapevine culti-

† Averages followed by the same letter in the column do not differ among them by Tukey test (p<0,005).

vars, and the biggest values were found to Criolla Grande cultivated on ½MS formula. According to the authors, IRC of Cereza, Criolla Chica and Torrontés Riojano cultivars in different culture medium have varied from 23.3 to 31.2, similar to those described for Bordô cv. in investigated formulations. Results in this study can be explained by the difference on mineral constitution of saline formulations; nitrogen and magnesium contents are found in the investigated formulations and those which show higher IRC have higher concentration of these nutrients in the formula. Nitrogen and magnesium are nutrients that have participation on chlorophyll molecule synthesis and structure, therefore when there is an increment of those nutrient sources, higher chlorophyll contents are observed.[26]

In relation to the allocation of *in vitro* reserves, Bordô cv. showed around 90 % of dry matter accumulation in the aerial part, without significant difference among the culture mediua (Table 1). Results are in accordance with those by Ribeiro[27] and other authors,[20] who observed higher accumulation of leaves and stem dry matter from Paulsen 1103 and VR 043-43 grapevine rootstocks and from Cabernet Sauvignon cv. in relation to roots dry matter.

The higher root dry matter accumulation of Bordô cv. was obtained when cultivating in Roubelakis and DSD1 formulations, with 14.9 mg of dry roots, more than C2D formulation (8.6 mg) and without differing significantly from ZL and Galzy formulations, with an accumulation around 12 mg of dry roots (Table 1). According to Silva and Doazan[12] dry biomass, stem length and leaf area are the more reliable parameters evaluate *in vitro* development and multiplication of grapevine genotypes.

In the production of total biomass, the higher accumulation of dry matter were achieved when the cv. Bordô was cultivated in DSD1, Roubelakis and Galzy formulations, with average formation of 43.8 mg of dry matter, differing from C2D formulation; ZL does not differ from the other formulations (Table 1). These values were higher than those related by Ribeiro,[27] in evaluating culture media for in vitro propagation VR043-43 and Paulsen 1103 grapevine rootstocks and the Cabernet Sauvignon cv. and those reported by other researches[18] (2003) at *in vitro* propagation of SO4 and Paulsen 1103 grapevine rootstocks, which after 60 days of *in vitro* culture in DSD1 formulation showed 34.8 and 35.6 mg dry matter accumulation.

The acclimatization process of Bordô cultivar showed, after 60 days, survival above 85% in all saline formulations studied. Losses were found only for the C2D formulation in which only two plants did not survive the acclimation (Table 2). These results indicate the efficiency of the method that was used for the *in vitro* transfer of plants to *ex vitro* conditions. Working with the same cultivar, other authors[28] compared different substrates for the acclimatization of plants cv. Bordô, they not found differences among different substrate formulations and survival ranged from 96% to 100%.

Table 2 – Number of leaves and roots, length (cm) of the longest root and aerial part, relative chlorophyll index (IRC), dry mass (mg) of roots and aerial part, total biomass (mg) and survival index (TS) of acclimatized Bordô grapevine seedlings cultivated in different culture media‡

Culture Media	Number		Length (cm)		IRC
	Leaves	Roots	Longer Root	Aerial Part	
Roubelakis	6.9 a	7.7 a	15.9 a	11.8 a	21.2 a
ZL	6.6 a	7.5 a	14.3 a	11.3 ab	21.1 a
DSD1	6.8 a	7.2 a	14.4 a	10.4 bc	21.1 a
C2D	6.3 a	7.7 a	13.6 a	9.8 c	21.3 a
Galzy	6.6 a	4.4 b	14.4 a	10.2 bc	21.0 a
Mean	6.6	6.9	14.5	10.7	21.1
C.V. (%)	13.70	38.73	20.36	19.95	6.96

Culture Media	Dry Matter (mg)			TS (%)
	Roots	Aerial Part	Total Biomass	
Roubelakis	51.6 a	167.9 a	219.5 a	100.0
ZL	41.5 ab	141.4 ab	182.9 ab	100.0
DSD1	36.4 b	125.7 ab	162.2 ab	100.0
C2D	34.8 b	111.5 b	146.4 b	85.7
Galzy	36.0 b	129.9 ab	165.9 ab	100.0
Mean	40.1	135.3	175.4	97.1
C.V. (%)	12.78	38.00	32.52	-------

In relation to the effects of acclimatization environment on survival rate of Jales' grapevine rootstock, Biasi, Passos and Pommer[19] observed plant survival from 92.5 to 100.0% which were acclimatized in an environment under misting, in opened and closed containers, respectively. Moreover, Dzazio, Biasi and Zanette[29] comparing different substracts for '420- grapevine rootstock acclimatization, observed high survival rates regardless of the type of particulated substract. These researches and others relating[3,30] high survival on grapevine acclimatization have showed ease grapevine adaptability on transferring in vitro to ex vitro conditions.

After acclimatization, it was possible to detect effects of formulations have on acclimatized plants (Table 2). For the variable number of leaves, no significant differences were observed between the formulations. Formation occurred on average of 6.6 leaves per plant, these results are superior to those presented by other authors researched[28] with the same cultivar.

Greater amounts of roots are formed when plants Bordô cv. were cultivated previously with Roubelakis, ZL, DSD1 and C2D formulations, on average 7.6 roots per plant, differing significantly from Galzy formulation which forms only 4.4 roots per plant (Table 2). In contrast to the variable length of roots is not verified significant difference among formulations which after 60 days of acclimatization, they have an average length of 14.5 cm. These results are similar to those found by another

‡ Averages followed by the same letter in the column do not differ among them by Tukey test (p<0,005).

research[28] evaluating different substrates for acclimatization Bordô cultivar, obtained the best average roots lengths around 14.9 cm in Plantmax® substract; however, the evaluation was performed at 36 days of *ex vitro* cultivation.

Longer aerial part of Bordô cv. are found when being previously cultivated in Roubelakis formulation, with 11.8 cm, significantly differing from DSD, C2D and Galzy formulations, and did not differ from ZL formulation; ZL did not differ from DSD1 and Galzy and, finally, C2D not differ from DSD1 and Galzy (Table 2).

The IRC of acclimatized plants was lower than the values of the readings *in vitro* and contains no effects of culture media at this stage, possibly because the plants were subjected to the same nutrient conditions, unlike the phase in vitro (Table 2). Likewise, authors[18] observed that acclimatized plants from VR043-43, VR039-16 and Paulsen 1103 grapevine rootstocks showed lower chlorophyll content in relation *in vitro* plants, the authors showed that these plants potentially are in the process of adaptation to new environmental conditions. Thus it is evident that according to the values of the estimates of chlorophyll in plants *in vitro*, are not limiting the photosynthetic operation.

After 60 days of acclimatization, the higher accumulations of total biomass were observed when Bordô cv. was previously cultivated in Roubelakis formulation, with accumulation of 219.5 mg of dry matter, significantly differing from the C2D formulation (146.4 mg) and did not differ from ZL formulations DSD1 and Galzy, which accumulated an average of 170.4 mg of dry matter (Table 2). This result demonstrates that the conditions of availability of nutrients from the culture media, *in vitro* micropropagation phase, has an influence on plant growth in the later stage of acclimatization periods, during adaptation of heterotrophic conditions. Regarding of biomass allocation in different acclimatized plant parts, the greatest accumulation occurred on the aerial part and the best results for the dry matter variable followed the same order of total biomass (Table 2).

Regarding the dry mass of the roots, the greater accumulations were found when. Bordô cv. was previously cultivated in Roubelakis formulation, forming 51.6 mg of dry roots, differing significantly from DSD1, C2D and Galzy formulations, with average of 35.8 mg of dry roots and did not differ from ZL formulation, which in turn is the same as the others (Table 2). The values found are in the range of 24 to 57 mg of the dry roots accumulation defined by an important study[28] in the acclimatization of. Bordô cv in different substrates.

Formulation of culture media showed effects on morphological and physiological parameters of in vitro propagation and acclimatization of grapevine Bordô cultivar. Results in this research are in agreement with those found by Roubelakis-Angelakis and Zivanovitc[9] who demonstrated the influence of culture medium composition on the development of in vitro grapevine cultivars. Protocols of in vitro introduction and multiplication and acclimatization were applied successfully and showed higher indexes of survival, regeneration and rooting.

4 **CONCLUSIONS**

In vitro propagation of Bordô cv. through nodal segments obtained from mother plants maintained in greenhouse has promoted high indexes of regeneration and rooting.

This study demonstrate that in vitro cultivation of the 'Bordô' is an efficient propagation technique due to the elevated rates of multiplication and acclimatization achieved, in additional important information was discussed there is no need of use of growth regulators in culture media to promote multiplication, rooting or specific phase for rooting of the 'Bordô'.

Considering all the analyzed variables, saline formulations Roubelakis and Zlenko has promoted better growing and development of aerial parts and roots as well the best in vitro multiplication of 'Bordô'.

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