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In vitro multiplication and rooting of grapevine: Culture media and plant growth regulators

Daniele Camargo Nascimento[1*], Maximiliano Dini[2], Norton Victor Sampaio[1], Márcia Wulff Schuch[2]

ABSTRACT

Micropropagation is a technique that allows to obtain quality plants, making it an important tool in the production of grapevine matrices. Composition of the culture media and plant growth regulators are commonly tested to adapt micropropagation protocols. The objective of this study was to test concentrations of Murashige & Skoog (MS) culture media and BAP (6-benzylaminopurine) and IBA (indolbutyric acid) plant growth regulators in the multiplication and *in vitro* rooting stages as part of the elaboration of a Chardonnay grapevine micropropagation protocol. In both experiments, multiplication and rooting, the experimental design was completely randomized, with a bifactorial scheme, testing culture media (MS and MS/2) and BAP concentrations (0; 5; 10 µM) in the multiplication and of IBA (0; 0.5; 1.0; 1.5 µM) in rooting. For multiplication, the best results for number of buds, leaves and shoots were obtained with 10 µM BAP, and the concentrations of the culture media had no significant effect for most of the variables. For rooting, the MS media provided better results. In relation to IBA, the number and average root length were influenced by the concentration used, with 0.5 and 1.0 µM providing the best results. Grapevine explants may be multiplied in MS/2 media supplemented with 10 µM BAP and subsequently rooted in MS media with 0.5 µM IBA.

Index terms: *Vitis vinifera*; tissue culture; micropropagation; cytokinin; auxin.

INTRODUCTION

Micropropagation for grapevines is an important tool in obtaining healthy and uniform plants, allowing the improvement of vineyard quality, since one of the biggest problems in the wine sector is the lack of plants with warranty phytosanitary quality, offered to growers (Biasi, 2003; Ribeiro et al., 2010). The technique may be applied to initiate the propagation process, using the micropropagated plants as mother plants for the formation of a clonal micro-garden (stock plant) and, from this, carry out the plants production (Yancheva et al., 2018).

To carry out the micropropagation it is necessary to master all process stages, from *in vitro* establishment, multiplication, rooting, to acclimatization of plants. In the multiplication stage, whose purpose is to increase the amount of propagated material, it is important to adapt a methodology so that, in addition, the quality, genetic stability and uniformity of the explants are maintained. In the rooting stage, the goal is root formation in the explants, allowing the formation of complete plants with aerial part and root system developed for later acclimatization to *ex vitro* conditions (Dutra; Wendling; Brondani, 2009; Fachinello; Hoffmann; Nachtigal, 2005).

The main difficulty in these stages is adjusting the concentrations of the elements that constitute the culture media, which must be adequate according to the need of each culture (Rodrigues et al., 2013). However, for the grapevine, there are studies that show the possibility of developing micropropagation protocols, obtaining good results and overcoming these difficulties (Bernd et al., 2007; San Pedro et al., 2017).

In general, several studies indicate the use of the Murashige & Skoog (Murashige; Skoog, 1962) culture media complete or modified with half of salts concentration. In the case of using half the salts concentration, there is also the possibility of cost reduction (Amiri; Mohammadi; Akbari, 2019; Ayub et al., 2010; Mukherjee et al., 2010; Rodrigues et al., 2013; Santos; Rodrigues; Carvalho, 2017; Villa et al., 2006).

During the elaboration of a micropropagation protocol, it is also important to verify the need for the use of plant growth regulators adjusting their concentration. Furthermore, the explants development depends on the interaction between the hormones that occur naturally in the plant and the regulators added to the culture media (Pinhal et al., 2011). Plant growth regulators, such as auxins and cytokinins, have been cited in the bibliography

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[1]Universidade Federal do Pampa/UNIPAMPA, Dom Pedrito, RS, Brasil
[2]Universidade Federal de Pelotas/UFPel, Capão do Leão, RS, Brasil
[*]Corresponding author: dcn.biologia@gmail.com

as essential to promote regeneration and propagation of plants in some species (Camargo et al., 2018; Keshari; Pradhan; Deo, 2016; Oliveira-Cauduro et al., 2016; Santos; Rodrigues; Carvalho, 2017; Silva et al., 2018). Presenting paradoxically antagonistic and complementary actions, these two hormones provide robustness to the development process, enabling the regeneration of a plant from a small fragment (Schaller; Bishopp; Kieber, 2015).

In the multiplication stage, in which cytokinins are recommended, 6benzylaminopurine (BAP) is most commonly used. Although its application to the grapevine has already been reported by several authors, the concentrations for the best multiplication of shoots may vary between different genotypes. Later, in the rooting stage, the auxins are used, since they act in the formation of the root system. Among the auxins, indolebutyric acid (IBA) is one of the most indicated, as it is a photostable and low toxicity substance for most species (Fachinello; Hoffmann; Nachtigal, 2005; Han; Zhang; Sun, 2009; Rodrigues et al., 2013; Silva et al., 2018; Stuepp et al., 2017).

One of the main difficulties encountered in these stages is to suit the concentrations of the elements that compose the culture media, which must be adjusted according to the need of the culture with which it is being worked (Rodrigues et al., 2013). In addition, varieties of the same species of grapevine may present different responses in relation to micropropagation and the use of plant regulators, making it necessary to adapt specific protocols for each one (Carvalho et al., 2011; Leitzke; Damiani; Schuch, 2009; Tronco et al., 2015).

The objective of this study was test concentrations of Murashige & Skoog (MS) culture media and BAP (6-benzylaminopurine) and AIB (indolbutyric acid) plant growth regulators in the multiplication and *in vitro* rooting stages as part of the elaboration of a Chardonnay grapevine micropropagation protocol.

MATERIAL AND METHODS

The plant material used for the experiment was collected from a vineyard located in Dom Pedrito, Rio Grande do Sul state, Brazil (31°01'13" S, 54°36'13" W, 131 m above sea level). The experiments were performed in the Botanical Laboratory of the Federal University of Pampa (Unipampa - Dom Pedrito), the first being made in the multiplication stage and the second later *in vitro* rooting.

Multiplication

In the multiplication experiment, the experimental design was completely randomized, with a factorial scheme, with two levels for the culture media (MS and MS/2) and three levels for the BAP concentration (0; 5; 10 µM), being six treatments with five replicates, each replicate consisting of a glass flask containing five explants.

Were used nodal segments of Chardonnay grapevine, with approximately 1 cm long, containing an axillary bud and a leaf, obtained from the material established *in vitro* by means of meristems in MS culture media plus 2.2 µM BAP and after a subculture, in MS culture media without addition of plant growth regulator.

The culture media used was Murashige & Skoog, with a complete concentration of salts (MS) and half salt concentration (MS/2), plus BAP (0; 5; 10 µM), 100 mg L^{-1} myoinositol, 30 g L^{-1} sucrose and 7 g L^{-1} agar. The pH of the media was adjusted to 5.8 before addition of water and then autoclaved at 121 °C and 1.5 atm for 20 minutes. Glass flasks with 200 mL capacity were used, with 30 mL of culture media per flask.

After inoculation, the flasks containing the explants were kept in a growth chamber, with 25 ± 2 °C temperature and 16 hours of light. After 30 days of cultivation, being recorded bud number, leaf number, number and average length of shoots were evaluated.

Rooting

In the rooting experiment, the experimental design was completely randomized, with a factorial scheme, with two levels for the culture media (MS and MS/2) and four levels for the IBA concentration (0; 0.5; 1.0; 1.5 µM), being eight treatments with ten replicates, each replicate consisting of a glass flask containing five explants.

Nodal segments, approximately 1 cm of long, containing a axillary bud and a leaf, obtained from two subcultures in MS/2 media supplemented with 10 µM BAP, were used as explants.

The preparation of the culture media was as described in the previous experiment, differing only by

the plant growth regulator, in this case, using IBA. After 45 days of cultivation, shoot height, percentage of rooting and of callus formation, number and average length of roots were evaluated.

Statistical analysis

Data were submitted to analysis of variance (ANOVA) and, when significant, the means of the treatments were statistically compared by Tukey´s test at $p \leq 0.05$. For the statistical analyses, the data expressed as a percentage were transformed into √ x / 100, to meet the assumptions of homogeneity of the variance and normality of the residues. In order to verify the behavior of the variables in function of the increase of the IBA concentration, when significant the ANOVA, the regression analysis was used. The data were analyzed using InfoStat 2016 version statistical software (Di Rienzo et al., 2017).

RESULTS AND DISCUSSION

Multiplication

According to the analysis of variance, there was a significant interaction between the factors of culture media and BAP, only for the average shoots length (ASL). Comparing the means of culture media used in each BAP concentration tested, only in the treatment using 5 µM BAP, there were significant differences, where the highest mean was obtained using the MS media (1.20 cm). Comparing the concentrations of BAP, within the MS media showed significant differences, where concentrations 5 and 10 µM obtained the highest averages (1.20 and 1.07 cm, respectively) (Table 1).

The culture media concentration had a significant effect only on the leaf number, where the highest value (3.98) was obtained with the MS media (Table 1). When testing variations of MS media in the *in vitro* multiplication of rootstock VR 04343, Villa et al. (2006) observed that the difference between the use of MS and MS/2was very small, so if the objective of the study is to reduce costs, it is viable to use MS/2. The use of the MS culture media with half of salts concentration aims to reduce costs in the micropropagation process, since this is considered a costly technique. The results of this study show that this change in the culture media did not negatively affect the multiplication rates for most of the variables analyzed.

In relation to BAP, the concentration of 10 µM provided the highest averages for bud number (2.23), leaf number (4.13) and shoot number (1.81), although in the first two variables there was no statistical difference relative to the 5 µM (Table 1). The multiplicity of the explants may increase according to the increased concentration of cytokinins, as they stimulate cell division, acting on morphogenesis. The appropriate concentration may vary for each genotype and, at very high concentrations, may become harmful, causing the formation of abnormal and/or hyperhidric (Ayub et al., 2010).

Table 1 – Average shoot length (ASL), bud number (BN), leaf number (LN) and shoot number (SN) of Chardonnay grapevine explants submitted to concentrations of the MS culture media and BAP.

BAP (µM)	ASL (cm)			BN			LN			SN		
	MS	MS/2	Mean	MS	MS/2	Mean	MS	MS/2	Mean	MS	MS/2	Mean
0	1.00aB[1]	1.10aA	1.05	1.36	1.44	1.40B	2.96	2.72	2.84B	1.08	1.00	1.04B
5	1.20aA	1.02bA	1.11	2.28	1.60	1.94AB	4.68	3.20	3.94A	1.20	1.00	1.10B
10	1.07aAB	1.00aA	1.04	2.10	2.36	2.23A	4.30	3.96	4.13A	1.70	1.92	1.81A
Mean	1.09	1.04		1.91	1.80		3.98a	3.29b		1.33	1.31	
$F_{Media \times BAP}$		4.53*			1.69^{ns}			1.70^{ns}			1.07^{ns}	
F_{Media}		1.76^{ns}			0.26^{ns}			5.06*			0.03^{ns}	
F_{BAP}		1.53^{ns}			4.83*			6.95**			16.83***	
VC (%)		9.66			32.66			22.98			25.07	

[1]Means followed by the same lowercase letter in the row and the same capital letter in the column do not differ by the Tukey test at $p \leq 0.05$. Interaction F-value = $F_{Media \times BAP}$; Main component F-value = F_{Media} and F_{BAP}; ns, *, ** and *** = non-significant, significant at 5, 1 and 0.1% by the F-test, respectively.

In the multiplication of the rootstock Paulsen 1103, Coletto, Martins and Goulart (2008) observed that, increasing BAP levels up to 2.5 µM, there is a continuous increase in explant growth, however, increasing the concentration to 5 µM, toxic effect occurs and, consequently, a reduction in explant growth. Carvalho et al. (2013) obtained the best results for the *in vitro* regeneration of nodal segments of the Bordô and Chardonnay varieties, using the concentration of 5 µM BAP in MS culture media.

Grapevine explants are sensitive to the use of cytokinins in the culture media and concentrations up to 5 µM are generally recommended. However, each genotype may respond differently, so the importance of testing the protocols when performing the propagation of a new cultivar.

Regarding the BAP concentrations in the culture media, these should be suitable for each variety, optimizing multiplication of the explants and aiming for the quality of the material, even if the multiplication rates are not so high (Ayub et al., 2010; Coletto; Martins; Goulart, 2008).

In the present study, the culture media tested did not present significant differences for most of the tested variables, verifying that it is possible to multiply Chardonnay grapevine explants using the MS/2 culture media. Regarding BAP, the concentration of 10 µM provided better multiplication of the explants, without presenting problems with toxicity. Therefore, for the next stage, of rooting, were used explants grown on MS/2 media with 10 µM BAP.

Rooting

In the rooting stage, the ANOVA showed that the interaction between the culture media and AIB factors was not significant ($p > 0.05$), and therefore, the main effects of these factors were studied and the data of these variables are presented graphically (Figure 1).

The average rooting of the explants was very close and satisfactory in all evaluated treatments (between 88 and 100%). It is worth mentioning that the highest percentage of rooting was obtained with the concentration of 0.5 µM IBA (97%) and, in the treatment in which this same concentration was added to the MS media, 100% rooting of the explants was obtained (Figure 1A).

The mean of callus formation was low (<5%) in treatments using the MS media and with IBA concentrations less than 1.0 µM. There was a higher percentage of callus formation (40%) in the treatment with higher IBA concentration, but the second highest mean was in the treatment with the MS/2 media without the use of the regulator (Figure 1B).

The formation of callus in the rooting zone is not always an indication of the adventitious roots formation. In some cases, the presence of callus may affect the quality of the roots, especially, in relation to the vascular connection with the plant (Fachinello; Hoffmann; Nachtigal, 2005). In this experiment, the presence of callus did not impair root formation, however, in the treatment in which the auxin concentration was higher (1.5 µM IBA) and had the highest percentage of callus formation, was

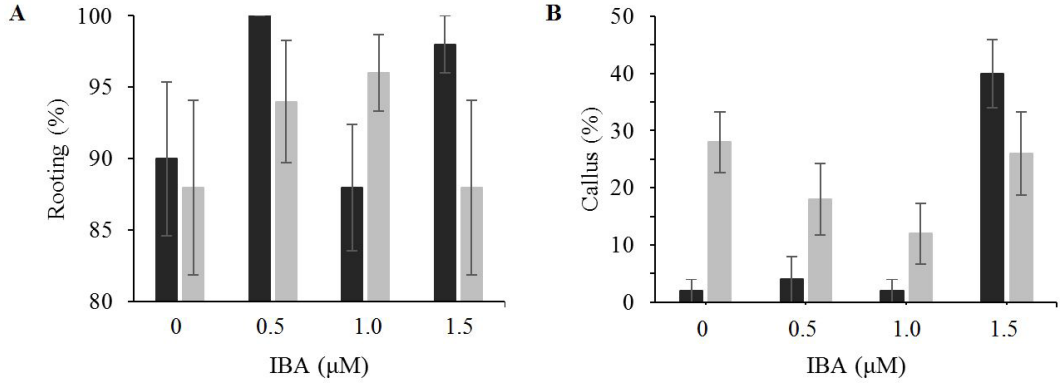

Figure 1 – Rooting (A) and callus (B) percentages of Chardonnay grapevine explants *in vitro* rooting stage. The bars represent the standard error of the mean.

the one with the lowest sprouting height, which may have been impaired due to the presence of callus.

The concentrations of the culture media had a significant effect on the variables shoot height and average length of roots. For shoot height, the MS media had the highest mean (3.68 cm) differing statistically ($p < 0.0001$) from the MS/2 (2.93 cm). Regardless of the IBA concentrations tested, the explants presented homogeneity in relation to height, which was between 2.93 and 3.53 cm. For the average length of roots, the MS culture media also presented better results, the highest mean being 4.57 cm, differing statistically from the MS/2 (3.95 cm) ($p < 0.05$).

In relation to the concentrations of IBA, these, had a significant effect on root number and average length of roots. For the root number, there was a positive linear behavior, in which the increase of IBA concentrations promoted an increase in the root number (Figure 2A). Therefore, the highest mean was obtained with the concentration of 1.5 µM (2.65 cm), and the lowest was in the treatment without the use of IBA (1.87 cm). For the average length of shoots, there was a negative linear behavior, and with the increase of IBA concentrations there was a reduction in average length of roots (Figure 2B). In this case, the highest mean was obtained in the treatment without the use of IBA (4.94 cm) and, the lowest, witch the concentration of 1.5 µM (3.67 cm).

It can be observed that, in the treatments in which the explants presented more root number, these, presented smaller length. Therefore, the root behavior was inversely proportional to the IBA concentrations tested. The higher the IBA concentration, the higher the root number, however, smaller is the length. The reduction in root length can be explained by the fact that auxins are necessary only in the induction phase of the rhizogenesis, and can be detrimental in the elongation phase of the roots. For this reason, some authors recommend the use of two culture media at this stage, being a first containing auxin, favoring the induction of rhizogenesis and, later, the transfer of the material to a media without auxin, stimulating the roots growth (Leitzke; Damiani; Schuch, 2009; Reinhart; Biasi, 2017; Schaller; Bishopp; Kieber, 2015).

There must be a balance between the number and length of roots. A greater number of roots are not sufficient if they are not long enough to sustain the plant when transplanted in the next stage of acclimatization. Also, it cannot be said that a single root of considerable length would be sufficient for the survival of the plant during acclimatization. Even if only a culture media is used for rooting, it is necessary to find the most appropriate concentration to ensure a good roots development (Alizadeh; Singh; Patel, 2010).

When testing IBA concentrations in rooting of four varieties of *Vitis vinifera* (Red Globe, Crimson Seedless, Autumn Royal and Thompson), Ali, Humera and Sabahat (2017), obtained the best results (92% rooting) with the MS/2 media plus 10 µM IBA.

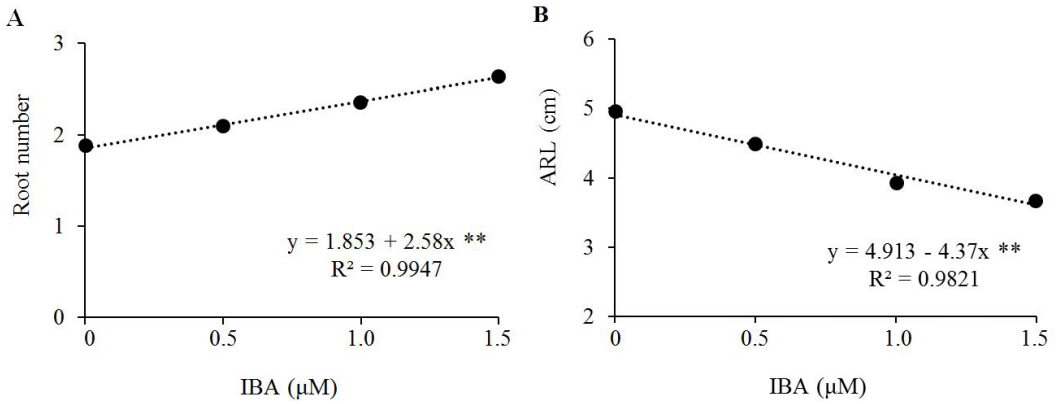

Figure 2 – Root number (A) and average root length (ARL) (B) of Chardonnay grapevine explants, under IBA concentrations, *in vitro* rooting stage.

** Significant at 1% probability of error; R^2 = determination coefficient.

Roubelakis-Angelakis and Zivanovitc (1991) evaluated the *in vitro* rooting of different grapevine genotypes, including grape varieties and rootstocks. They found that the results varied among genotypes, but in general, even without the use of plant regulators in the culture media, the explants rooted, however, the best results were obtained when IBA was added at concentrations between 3 and 5 µM.

Other authors presented similar results, obtaining rooting of grapevine nodal segments even without the addition of plant growth regulators in the culture media, however, the use of auxins, in general, promoted better rooting rates (Bernd et al., 2007; Mukherjee et al., 2010).

CONCLUSION

Chardonnay grapevine explants can be multiplied in MS/2 media supplemented with 10 µM BAP and, subsequently, rooted in MS media with 0.5 µM IBA.

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