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In vitro multiplication of apuleia (*Apuleia leiocarpa* (Vogel) J. F. Macbride)

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ABSTRACT

The aim of this study was to evaluate the influence of explant position in the seedling and the composition of culture medium on the *in vitro* multiplication of apuleia [*Apuleia leiocarpa* (Vogel) J. F. Macbride]. In order to evaluate the effect of explant position and 6-benzylaminopurine (BAP) on the multiplication, nodal segments of 0.8 to 1.2 cm in length were excised at the basal, middle and apical parts of aseptic seedlings with 30 days of cultivation, which were grown in WPM culture medium with 0; 2.2; 4.4; 6.6 and 8.8 µM of BAP. Nodal segments were cultivated in WPM culture medium supplemented with 0; 2.2; 4.4; 6.6 and 8.8 µM of BAP, with or without 1.5 g L^{-1} of activated charcoal. Concentrations of 0; 2.3; 4.6; 6.9 and 9.2 µM of kinetin (KIN) were also tested in WPM culture medium, with or without 1.5 g L^{-1} of activated charcoal. In all experiments, the percentage of shoot, callus and root formation, the total number and length of shoots (cm) and the number of leaves were evaluated at 30 days of cultivation. Explants from the basal portion of apuleia seedlings had higher shooting capacity than those from the middle and apical ones. Adding KIN to the culture medium did not affect the *in vitro* multiplication of apuleia. Culture medium supplemented with 6.6 µM of BAP and 1.5 g L^{-1} of activated charcoal favors shooting and rooting of apuleia explants. The absence of activated charcoal in the culture medium, despite stimulating adventitious shoots through greater availability of cytokinin, resulted in the formation of less vigorous shoots. Culture medium without activated charcoal favored callus formation in the explants, which were responsive to indirect organogenesis. Thus, basal segments cultivated in WPM medium supplemented with BAP is feasible for the *in vitro* multiplication of apuleia.

INTRODUCTION

Apuleia [*Apuleia leiocarpa* (Vogel) J. F. Macbride], native tree species from Rio Grande do Sul, Brazil, is considered noble for its wood characteristics and priority in the actions regarding the *in situ* and *in vitro* conservation, as it is of economic importance and a vulnerable species to extinction (Sema, 2006). The production of quality seedlings has significant impact on the conservation of the species and most of the existing reports regarding apuleia propagation are the production of seminal seedlings. However, the production of seedlings is hindered as of seed picking, taking into consideration that apuleia shows irregular fruiting. Furthermore, seeds present coat dormancy, which requires the use of specific treatments to achieve uniform germination (Carvalho, 2003).

Vegetative propagation might be an alternative to maximize seedling production for those species that present seedling production limited by germination difficulties or low seed production. In addition, vegetative propagation is essential when it is intended to multiply genotypes with desirable traits, since the individuals produced are genetically identical to the stock plant (Hartmann *et al*., 2011). Among the vegetative propagation

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techniques, micro-propagation has excelled in forestry, especially for serving as an auxiliary tool in breeding programs and for being the foundation of other biotechnological techniques (Penchel *et al*., 2007).

Micro-propagation is one of the widely-used tissue culture applications, and it can be an appropriate economical alternative to traditional propagation methods of native forest species, especially when it comes to those of difficult rooting and high commercial value (Teixeira, 2001). The main objective of *in vitro* multiplication is to maximize the production of shoots with competency for adventitious rooting, either *in vitro* or *ex vitro* (Oliveira *et al*., 2013). The multiplication rate depends on the interaction between explant potential and phytohormone content (Wendling *et al*., 2006), being typically utilized nodal and apical segments aiming for proliferation of preexisting axillary buds (Xavier *et al*., 2013; Oliveira *et al*., 2013).

Phytohormone can be added to culture medium to direct the morphogenetic responses in the explants (Hartmann *et al*., 2011), considering that the choice of type, concentration and combinations constitute one of the main challenges to achieve success on micro-propagation of woody species (Oliveira *et al*., 2013). Cytokines are commonly employed to induce adventitious shoot formation, among which the 6-benzylaminopurine (BAP), kinetin (KIN), zeatin (ZEA) and2-isopenteniladenine (2-iP). BAP is the most used for the micro-propagation of forest species, which might be employed alone or in combination with other cytokines (Oliveira *et al*, 2013.) in the *in vitro* multiplication of *Amburana Acre* (Ducke) A C Smith (Fermino Junior; Scherwinski-Pereira, 2012), *Aspidosperma ramiflorum* Müll. Arg. (HUBNER *et al*., 2007), *Luehea divaricata* Mart. & Zucc. (Flores *et al*., 2011) and *Ocotea porosa* (Pelegrini *et al*., 2011). Adjustments in the protocol of multiplication can also be gotten with the addition of other compounds, such as activated charcoal, which acts on the adsorption of phenolic compounds and tannin exudates by the explant (Andrade *et al*., 2000).

It is known the potential utilization of micro-propagation in forestry production and forest-based industry (Penchel *et al*., 2007). However, we did not find any report on *in vitro* multiplication of apuleia, which has curbed the establishment of protocols for this species. Thus, the identification of optimal growing conditions, even for one of the *in vitro* culture steps, is an important advance regarding micro-propagation of native forest species. The objective of this study was to evaluate the influence of explant position in the seedling and the composition of culture medium on the *in vitro* multiplication of apuleia.

MATERIAL AND METHODS

The trials were carried out from March to October, 2012, at the Plant Breeding and Asexual Propagation Center, Department of Plant Science of the Federal University of Santa Maria (UFSM), Santa Maria, RS.

In one experiment, nodal segments of apuleia with 0.8 to 1.2 cm long were excised at the basal, middle and apical portions of aseptic seedlings cultivated *in vitro* for 30 days. Five explants of each position had their diameters recorded in the central portion. The explants were cultivated in WPM medium (Lloyd and McCown, 1980) added with 0; 2.2; 4.4; 6.6 and 8.8 µM of 6-benzylaminopurine (BAP). The trial was carried out in a factorial (explant positions x BAP concentrations) in a complete random design, with five replications of three segments.

In other experiment, nodal segments of apuleia with 0.8 to 1.2 cm long were excised from aseptic seedlings cultivated *in vitro* for 15 days. The explants were cultivated in WPM medium supplemented with 0; 2.2; 4.4; 6.6 and 8.8 µM of BAP, with or without the addition of 1.5 g L^{-1} of activated charcoal. The trial was carried out in a factorial (BAP concentrations x presence of activated charcoal) in a complete random design, with five replications of four segments.

In another experiment, nodal segments of apuleia with 0.8 to 1.2 cm long were excised from aseptic seedlings cultivated *in vitro* for 15 days. The explants were cultivated in WPM medium supplemented with 0; 2.3; 4.6; 6.9 and 9.2 µM of Kinetin (KIN), with or without the addition of 1.5 g L^{-1} of activated charcoal. The trial was carried out in a factorial (KIN concentrations x presence of activated charcoal) in a complete random design, with five replications of four segments.

In all experiments, the pH of the WPM culture medium was adjusted to 5.8 before autoclaving. About 10 mL of the medium were poured into test tubes (20.5 mm wide and 150 mm high). The test tubes were sealed with aluminum foil and autoclaved for 20 minutes at 121 °C and 1 atm. Each test tube was inoculated with one explant. The inoculated tubes were maintained in a growth room at 25 ± 2 °C and 16 h photoperiod under light intensity of 14.3 µE m^{-2} S^{-1}supplied by fluorescent lamps. In all experiments, the percentage of shoot, callus and root formation, the total number and length of shoots (cm) and the number of leaves were evaluated at 30 days of cultivation.

In order to meet normality, the percentage data were transformed to arc sine$\sqrt{x/100}$ and that of counting to$\sqrt{x+0.5}$.Treatment means with significant differences ($p \leq 0.05$) were compared by Tukey´s test or polynomial regression, with the ESTAT program (UNESP - Jaboticabal).

Results:

There was no significant interaction between explant positions in the seedling and BAP concentrations in the culture media for any variables. There was 100% of explant sprouting for all treatments. The number and length of shoots were only affected by the explant position, being the basal segments better than middle and apical ones (Table 1). There was no influence of the explant position and BAP concentration on the number of leaves and the percentages of callus and rooting, with an average of 2.5 leaves per shoot, 82.5% of callus formation and 1.6% of rooted explants.

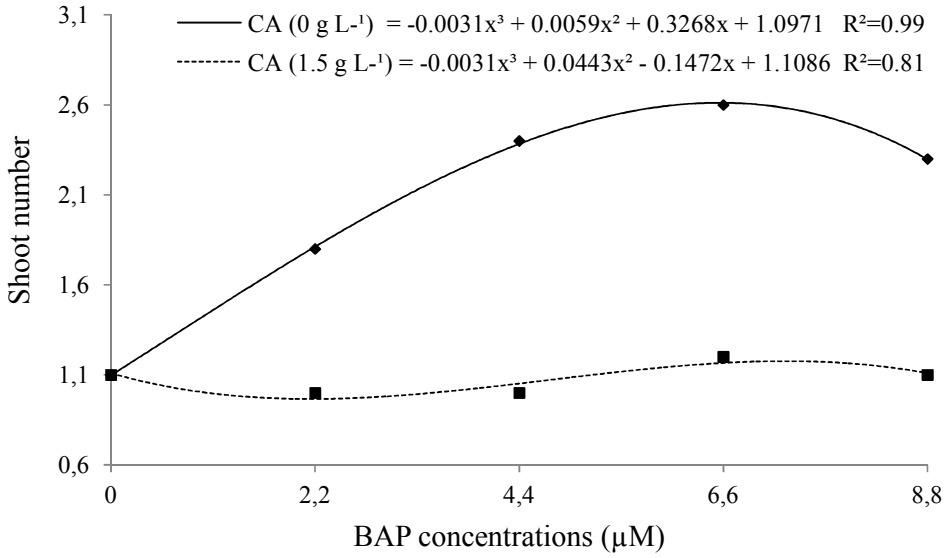
There was a significant interaction between BAP concentration and the presence of activated charcoal in the culture media for the number of shoots and the percentage of callus formation in the explants. A cubic linear regression was the most suitable model to explain these variables (Figure 1). The highest number of shoots occurred when explants were grown in WPM medium with the addition of 6.6 µM of BAP without activated charcoal. All explants cultivated in WPM medium only with the addition of BAP showed callus formation. The WPM culture medium without BAP and activated charcoal had 55% of the explants with callus formation, but when activated charcoal was added to the medium only 25% of the explants formed callus. The addition of 1.5 g L^{-1} of activated charcoal to the WPM medium increase the percentage of rooting and the number of leaves (Table 2), but the addition of BAP had no effect at all. The sprouting percentage and shoot length were not affected by both the presence of BAP and activated charcoal in the culture media, with an average of 99% of explants with shoots of 1.2 cm long.

There was no interaction between KIN and activated charcoal in the multiplication of apuleia. The addition of KIN to the culture media did not affect the *in vitro* multiplication of apuleia. Neither KIN nor activated charcoal addition to the culture media affected the percentage of shooting and rooting, and the number of shoots, with an average of 95.8%, 7.0% and 1.1 shoot per explant, respectively. The presence of activated charcoal in the culture medium increased shoot length and leaf number, but reduced the percentage of callus formation in the explants (Table 3).

Table 1: Number and length of shoots in nodal segments from different position of apuleia aseptic seedlings grown in WPM culture medium at 30 days of cultivation.

Explant position	Shoot number	Shoot length (cm)
Basal	1.9 a*	1.4 a
Middle	1.4 b	0.7 b
Apical	1.3 b	0.5 c
Mean	1.5	0.8
CV (%)	14.6	15.8

* Values followed by the same letter did not differ by the Tukey´s test at 5% probability of error.



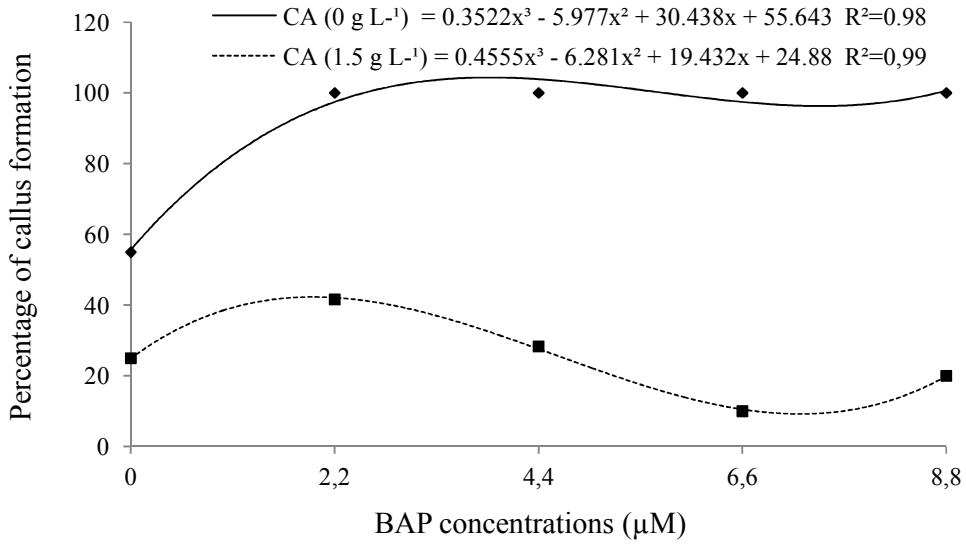


Fig. 1: Number of shoots (top) and percentage of callus (below) from nodal segments of apuleia grown in WPM medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and/or 1.5gL^{-1} of activated charcoal(AC) at 30 days.

Table 2: Rooting percentage and number of leaves in nodal segments of apuleia grown in WPM medium supplemented with 6-benzylaminopurine (BAP) with or without 1.5 g L^{-1} of activated charcoal at 30 days of cultivation.

Activated charcoal	Rooting percentage	Leaf number
1.5 g L^{-1}	8.0 a*	2.4 a
0 g L^{-1}	1.0 b	1.7 b
Mean	4.5	2.1
CV (%)	118.6	15.3

* Values followed by the same letter did not differ by the Tukey´s test at 5% probability of error.

Table 3: Shoot length, number of leaves and percentage of callus formation in nodal segments of apuleia grown in WPM medium supplemented with kinetin (KIN) with or without 1.5 g L^{-1} of activated charcoal at 30 days of cultivation.

Activated charcoal	*Shoot length*(cm)	*Number of leaves*	*Percentage of callus*
1.5 g L^{-1}	1.1 a*	2.5 a	20.0 b
0 g L^{-1}	0.9 b	1.9 b	74.0 a
Média	1.0	2.2	47.0
CV (%)	10.4	12.6	48.7

* Values followed by the same letter did not differ by the Tukey´s test at 5% probability of error.

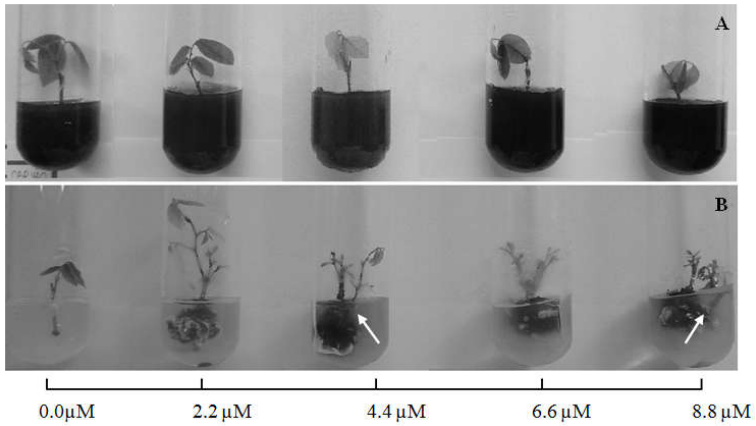


Fig. 2: Nodal segments of apuleia grown in WPM medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and with 1.5 g L^{-1} of activated charcoal (A) or without activated charcoal (B) at 30 days of cultivation. Arrows indicate indirect organogenesis of shoots.

Discussion:

Previous micro-propagation studies showed that the morphogenetic response depends upon the balance between auxin and cytokine concentrations in the explant. Root formation occurs when auxin concentration is higher than cytokine and when the opposite takes place results in shoot formation (Skoog and Miller, 1957). In this work, it is possible that nodal and apical segments of apuleia had endogenous concentrations of cytokinin sufficient for shoot induction, since shoot formation occurred even when the culture medium had no cytokine, averaging 97.9% of segments that formed shoots. Explants from basal position of the seedling produced a higher number and length of shoots than middle and apical ones (Table 1). A similar result was gotten with *Hancornia speciosa* var speciosa Gomes, in which basal and middle segments showed higher sprouting responses compared to apical segments (Ledo *et al*., 2011; Sa *et al*., 2012.). The apuleia explants were obtained from *in vitro*-grown seedlings, consisting of shoot and root with phytohormone concentrations sufficient to sustain their growth and development. The basal segments are the first ones to receive the cytokinin flow coming from roots. As the endogenous hormone levels are often responsible for the success of *in vitro* morphogenesis, the best response should be associated with the position in the seedling of the nodal segment (George *et al*., 2008). Furthermore, the basal segment has a larger diameter and volume, which may also have affected the shooting response, since the thicker the diameter the higher the contents of carbohydrates, starch and other nutritive substances in cuttings (Hartmann *et al*., 2011).

The addition of BAP concentrations up to 6.6 µM in the absence of activated charcoal in the culture medium increased shoot formation, but the addition of activated charcoal eliminated the BAP effect in the *in vitro* multiplication of apuleia (Figure 1). This result might be related to the capacity of the activated charcoal to adsorb, among other substances, phytohormones present in the culture medium, but little is known in terms of the adsorbed amount (Leitzke *et al*., 2009). Furthermore, activated charcoal can promote or even inhibit *in vitro* growth, depending on the species and type of explant (George and Sherrington, 1984). In this case, the presence of activated charcoal in the culture medium should have reduced the concentration of exogenous cytokines and their effect on apical dominance breaking, resulting in a smaller number of shoots with greater number of leaves, as observed in these experiments with BAP and KIN (Figure 1 and Tables 2 and 3). Cytokinins also play a role in cell division and proliferation of the meristem of the apical shoot (Taiz and Zeiger, 2013). Therefore, only the endogenous cytokinin was responsible for increasing shoot elongation and number of leaves and, in the case of KIN, it was also associated withlonger shoots (Table 3). A similar result was obtained for prunus rootstocks (*Prunu spersica* (L.) Batsch. x *P. Amygdalus* Batsch.), in which there was a greater number of leaves on shoots formed in culture medium-grown explants with activated charcoal in the presence of BAP and GA^3 (Sotiropoulos and Fotopoulos, 2005).

The addition of BAP to the medium culture resulted in vigorous shoots and intense green color and it was not affected by the addition of activated charcoal (Figure 2A). Shoot formation in culture medium without activated charcoal had less developed leaves and yellow coloration (Figure 2B). The presence of activated charcoal in *in vitro* culture of *Pterodon pubescens* (Benth.) Benth., favored vigor of shoots, which showed leaflets with greener color than those cultivated in its absence (Coelho *et al*., 2001),which is in agreement with the results of this study. In culture medium without activated charcoal, reduction in shoot vigor may have been caused by the presence of larger amounts of phenolic substances released by the plant tissue in the culture medium, since there was not activated charcoal to absorb them. It is well known the negative effect of the presence of phenolic compounds in the culture medium on shoot vigor (Cid and Teixeira, 2010), since the accumulation of polyphenol compounds and oxidation products, such as melanin, suberin, lignin and cutin, leads to modifications of the culture medium composition and, consequently, the absorption of metabolites (Andrade *et al*., 2000).

In addition to reducing the vigor of apuleia shoots, there was also the formation of callus in 100% of the explants grown in culture medium with BAP without activated charcoal (Figure 1). The activated charcoal added to the culture medium reduced callus formation in the explants, even in the absence of BAP (Figures 1 and 2). KIN also resulted in higher callus formation (74%) in apuleia explants grown in culture medium without activated charcoal. Actually, callus formation was reduced in the culture medium with activated charcoal (Table 3). In leaf discs of *Caesalpinia echinata* Lam, species of the same botanical family as apuleia, adding 2 g $L^{-1}$of activated charcoal in MS medium supplemented with 10 mg $L^{-1}$of 2,4-D and 2.0 mg $L^{-1}$of BAP reduced callus formation in 40% of the explants (Werner *et al*., 2009).

Apuleia callus showed nodular characteristic, spongy aspect and it was not friable, with white color, becoming brown with time (Figure 2B). Moreover, emission of multiple shoots coming out of the callus was observed (Figure 2), suggesting that this type of explant also has morphogenic capacity and can be employed for the multiplication of this species through indirect organogenesis. Indirect organogenesis takes place by cell dedifferentiation and redifferentiation, leading to the formation of a complete plant from meristematic activity in differentiated mature cells (Xavier *et al*., 2013). In the process, differentiation and cell specialization are reversed and the explant gives rise to a new tissue composed of unspecialized meristematic cells. Although the callus continues disorganized during cell multiplication, some specialized cell types may be randomly formed

through morphogenesis centers. These specialized cells are capable of initiating the formation of organs, such as roots, shoots and somatic embryos (George *et al*., 2008).

The development of callus can be independent of auxins and cytokinins or even be dependent on both, since high levels of them favor the development of callus (Jain *et al*., 1995; Hartmann *et al*., 2011.). The combination of cytokinins and auxins has not been tested in this study; however, it suggests that high callus formation in apuleia explants could possibly be related to the endogenous levels of auxins, which were sufficient, in the absence of activated charcoal, to equalize the levels of exogenous cytokinin leading to the formation of callus, as suggested by the theory of hormonal balance formulated by Skoog and Miller (1957). The addition of activated charcoal to the culture medium has possibly reduced the level of exogenous cytokinin, because of its adsorptive characteristics. It probably resulted in hormonal balance favoring endogenous auxin of the explant, reducing the formation of callus and promoting root formation. The activated charcoal has residual charges that promote adsorption of substances from the culture medium, one of them are phytohormones (Teixeira *et al*., 1993). However, it is believed that only the cytokinin added to the culture medium has been adsorbed by the activated charcoal, which did not happen to endogenous auxin. By taking part of the plant tissue, its elimination from the explant would require the establishment of water potential gradient between explant and culture medium, so that favoring the output of exudates. Plant cells have water potential ranging from -0.2 to - 10.0 MPa depending on the growth conditions and the type of plant (Taiz and Zeiger, 2013) and MS medium supplemented with 30 g $L^{-1}$of sucrose has a water potential of -0.2170 MPa (Lima-Brito *et al*., 2011), which would prevent the flow of auxin and other explant substances to the culture medium. Moreover, the high percentage of rooting in the experiments with activated charcoal can be ascribed to greater aeration and reduced light intensity in regions of root formation (Assis and Teixeira, 1998). Taking into consideration that the starting point for micro-propagation is based on the induction of axillary buds, enabling the formation of vigorous shoots capable of *in vitro* or *ex vitro* rooting, culture medium supplementation with BAP and activated charcoal favors shoot and root vigor of apuleia explants. The absence of activated charcoal in the culture medium, despite stimulating adventitious shoots through greater availability of cytokinin, resulted in the formation of less vigorous shoots. *In vitro* cultivation in medium without activated charcoal resulted in callus formation in apuleia explants, which have proved responsive to indirect organogenesis. In general, the apuleia multiplication with preexisting buds demonstrated to be feasible, with values greater than 95% of shoot formation, suggesting that further studies should be done with a sight on the multiplication of apuleia using other types of explants, as well as callus culture.

Conclusion:

Basal segments of apuleia are more responsive for shoot formation than middle and apical ones. BAP-enriched culture medium in the absence of activated charcoal favors shoot production and in its presence increases vigor and percentage of rooting.

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