

Introduction of nodal segments and *in vitro* rooting of *Apuleia leiocarpa*

Introdução de segmentos nodais e enraizamento *in vitro* de *Apuleia leiocarpa*

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Abstract

Apuleia leiocarpa Vogel J. F. Macbr. is a tree species native to Brazil that has high wood quality, and is currently threatened with extinction. Seminal seedlings of grápia are rarely commercialized, and vegetative propagation techniques, such as micropropagation, are considered as alternative methods for the production of grápia trees. However, there is a lack of available information about the *in vitro* introduction of nodal segments, and little information is available regarding *in vitro* rooting of grápia trees. As such, there are knowledge gaps that need to be addressed in order to define a micropropagation protocol for this species. Thus, the objective of this study was to evaluate different disinfection solutions in the introduction of nodal segments and of different types and concentrations of auxins in the *in vitro* rooting of grápia explants. For the introduction of nodal segments, different types of disinfection solutions and immersion times were evaluated. For rooting, different concentrations and types of auxins were evaluated. The best disinfection treatment was found to be immersion in sodium hypochlorite (NaOCl) solution with 2.5% active chlorine for 5 minutes. For rooting, treatment with 1.0 mg L⁻¹ of naphthalene acetic acid (NAA, C₁₀H₇CH₂CO₂H) resulted in the highest rates of adventitious rooting. Grápia nodal segments can be established *in vitro*, and provide a good source of propagules for micropropagation, and rooting can be favored through the application of NAA.

Keywords: Asepsis; Auxin; Micropropagation; Grápia

Resumo

Apuleia leiocarpa Vogel J. F. Macbr. é uma espécie arbórea nativa do Brasil que possui madeira de alta qualidade e, atualmente, se encontra ameaçada de extinção. Mudanças seminais de grápia dificilmente são comercializadas, sendo técnicas de propagação vegetativa, a exemplo da micropropagação, uma alternativa a ser considerada para produção de mudas. No entanto, há uma falta de informação disponível sobre a introdução *in vitro* de segmentos nodais e pouca informação está disponível sobre o enraizamento adventício de grápia. Como tal, existem lacunas de conhecimento que precisam ser abordadas para definir um protocolo de micropropagação para essa espécie. Assim, o objetivo deste estudo foi avaliar diferentes soluções de desinfestação na introdução de segmentos nodais e de diferentes tipos e concentrações de auxinas no enraizamento *in vitro* de explantes de grápia. Para a introdução, foram testados diferentes tipos de solução de desinfestação e tempos de imersão. Para o enraizamento, foram avaliadas diferentes concentrações e tipo de auxina. O melhor tratamento de desinfestação foi a imersão em solução de hipoclorito de sódio (NaOCl) a 2,5% de cloro ativo por 5 minutos. Para o enraizamento, o tratamento com 1,0 mg L⁻¹ de ácido naftalenoacético (ANA, C₁₀H₇CH₂CO₂H) resultou nas maiores taxas de enraizamento adventício. Os segmentos nodais de grápia podem ser estabelecidos *in vitro*, consistindo em boa fonte de propágulos para a micropropagação, e o enraizamento pode ser favorecido pela aplicação de ANA.

Palavras-chave: Assepsia; Auxina; Micropropagação; Grápia

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Introduction

Grápia (*Apuleia leiocarpa* Vogel J. F. Macbr.) is a tree species in the Fabaceae family that is native to the Atlantic Forest and widely distributed in Brazil, where it is found from the southern to the northern and northeastern parts of the country. In addition to providing excellent quality wood, grápia is a pioneer species in secondary vegetation that efficiently promotes the development of the forest structure (BACKES; IRGANG, 2004). As a result of the large-scale exploitation of grápia in the search for wood, this species is classified as 'vulnerable' in the Red List of the National Center for Conservation of Flora (CENTRO NACIONAL DE CONSERVAÇÃO DA FLORA, 2012), and is classified as 'critically endangered' in the State of Rio Grande do Sul by Decree n° 52,109/2014 (BRASIL, 2014), as amended by Decree n° 54,171/2018 (BRASIL, 2018).

Although grápia is considered a priority for conservation, forest restoration plantations rarely include endangered species, such as grápia, due to irregular fruiting over the years, and because species such as grápia produce seeds with water-impervious teguments, which makes germination and obtaining seedlings difficult (CARVALHO, 2003). As such, studies that address the identification of production technologies for grápia seedlings are relevant, and micropropagation may be an option for propagation of this species. Studies related to the vegetative propagation of grápia have been carried out in recent years, and such studies have led to the establishment of aseptic plants to supply explants (LENCINA *et al.*, 2014), *in vitro* multiplication (LENCINA *et al.*, 2016; 2017a), *in vitro* and *ex vitro* rooting, and plant acclimatization (LENCINA *et al.*, 2017b). However, no previous studies have addressed the introduction of propagules *in vitro*, and there is little available data related to adventitious rooting in grápia. In many cases, the introduction of propagules *in vitro* is an essential step for the rescue of selected plants, as well as for the definition of micropropagation protocols. The introduction of propagules *in vitro* is one of the most critical phases, due to the high losses of propagating material caused by fungal and bacterial contamination, and requires superficial disinfestation procedures (OLIVEIRA; DIAS; BRONDANI, 2013).

Chemical substances can be used for the superficial disinfestation of nodal segments, and the application of such substances aims to eliminate the microorganisms present on the plant surface, as these may limit the establishment of cultures *in vitro* (XAVIER; WENDLING; SILVA, 2013). Contaminating microorganisms compete with explants for nutrients from the culture medium and emit toxic metabolites, which can cause the explant to die (PEREIRA; CORREA; BOLIANI, 2011). Therefore, it is necessary to define an efficient disinfestation methodology, which includes identifying the appropriate chemicals, concentrations, and time of exposure that result in greater effectiveness in controlling contamination, whilst limiting the damage to explants as much as possible (SALLES *et al.*, 2017).

As is the case for rooting, grápia presents recalcitrance to vegetative propagation. For example, in one study only a low percentage ($\leq 26.4\%$) of explants rooted *in vitro* when treated with 2.0 mg L^{-1} indolebutyric acid (IBA, $\text{C}_{12}\text{H}_{13}\text{NO}_2$) (LENCINA *et al.*, 2017b). It should be noted that adventitious rooting is essential for the success of vegetative propagation, and is a process of great complexity which depends on both endogenous and exogenous factors that have not yet been fully elucidated (SOUZA; PEREIRA, 2007). Among the exogenous factors evaluated, the application of auxin is a strategy commonly used to obtain adventitious rooting. Auxins are plant phytohormones that act on the signaling of cells responsive to rooting, and are frequently used to induce adventitious roots in several species (HARTMANN; DAVIES; GENEVE, 2011).

It is evident that there are gaps in the protocol of micropropagation of grápia, which justifies the continuation of specific studies related to the introduction of propagules *in vitro*, as well as to obtain new methodologies and strategies for rhizogenesis of the species, aiming at the production of plantlets *in vitro*. Thus, the objective of this study was to evaluate different disinfestation solutions in the introduction of nodal segments and of different types and concentrations of auxins in the *in vitro* rooting of grápia explants.

Material and methods

This study was carried out at the Plant Breeding and Vegetative Propagation Center of the Federal University of Santa Maria, Santa Maria, RS, in 2017 and 2018. The culture media were sterilized by autoclaving for 20 min. at a temperature of 121 °C and a pressure of 1 atm. The cultures were kept in a growth room at a temperature of 25 ± 2 °C and a photoperiod of 16 h, and under a light intensity of 14.3 μE m⁻² S⁻¹ provided by fluorescent lamps.

For *in vitro* introduction, shoots from plants kept in a clonal mini-garden were sectioned into nodal segments of a bud of approximately 2.0 cm length, washed with neutral detergent, and washed three times with distilled water. The explants were then subjected to superficial disinfestation with the following solutions: sodium hypochlorite (NaOCl) at 2.5%, 5%, or 10% active chlorine; and 3% glutaraldehyde (C₅H₈O₂) for different immersion times (5, 10, or 15 minutes) in a laminar flow chamber. The control treatment consisted only of distilled water. The solutions were prepared in distilled and autoclaved water. Posteriorly, the explants were kept immersed in 70% ethanol for 1 min., followed by a triple wash with distilled water. The nodal segments were grown in 50 mL glass flasks containing approximately 10 mL of wood plant medium (WPM) culture medium (LLOYD; MCCOWN, 1980) composed of 30 g L⁻¹ sucrose and 6 g L⁻¹ agar.

The experiment included a 5 × 3 factorial design (types of solution and immersion times) in a completely randomized design, with 10 repetitions of three nodal segments each. After 30 days of cultivation in a growth room, the nodal segments were evaluated for the percentage of survival, shooting, and fungal contamination. The means of treatments with significant differences ($p < 0.05$) were compared using the Tukey test at a 5% probability of error.

The established nodal segments were multiplied *in vitro* in WPM culture medium (LLOYD; MCCOWN, 1980) plus 2.0 mg L⁻¹ of benzylaminopurine (BAP) according to the methodology of Lencina *et al.* (2016) for the production of shoots, which in turn, were used to assess adventitious rooting. Thus, shoots of 1.0 to 1.5 cm in length were grown in WPM culture medium plus 30 g L⁻¹ sucrose, 6 g L⁻¹ agar, and 0.1 g L⁻¹ inositol (LENCINA *et al.*, 2016) and the following concentrations of synthetic auxins: indolebutyric acid (IBA, C₁₂H₁₃NO₂), naphthalene acetic acid (NAA, C₁₂H₁₀O₂), and dichlorophenoxyacetic acid (2,4-D, C₈H₆Cl₂O₃): 0.0, 1.0, 2.0, 3.0 or 4.0 mg L⁻¹.

The experiment included a 3 × 5 factorial design (types of auxins and concentrations) in a completely randomized design, with five repetitions of five shoots each. The evaluations were carried out at 30 and 60 days of cultivation, and included recording the percentages of survival, callus formation and rooting, the number of roots, and the number of leaves. To meet the assumption of normality, the percentage data were transformed to arcsine $\sqrt{x/100}$ and count and length were transformed as $\sqrt{x + 0.5}$ and submitted to analysis of variance (ANOVA). The treatment means with significant differences ($p < 0.05$) were compared using the Scott-Knott Test, with a 5% probability of error. Analyses were conducted using Rbio Software (BHERING, 2017).

Results and discussion

The *in vitro* introduction of nodal segments from the clonal mini-garden of grápia showed a high rate of survival and shooting, and median rates of fungal contamination, which indicates that the disinfestation treatments used were adequate. In addition, disinfestation and introduction of nodal segments proved to be a good alternative for use in tissue culture in order to rescue genotypes of interest. This is a promising result, since the introduction of propagating material *in vitro* is one of the most critical stages of the micropropagation process, due to the high losses caused by microbial contamination. Even though no previous treatment was used in the clonal mini-garden, no bacterial contamination was observed in the explants.

There was no significant interaction between the types of disinfection solution and the immersion times for the *in vitro* establishment of grápia nodal segments. A significant effect of the type of disinfection solution used was observed for all variables (Table 1), with the best survival and shooting responses observed in the control treatment, and in the treatment consisting of sodium hypochlorite with 2.5% active chlorine. In contrast, the control treatment resulted in the highest percentage of fungal contamination, which differed significantly from the other treatments. This result was expected, since this treatment consisted only of distilled water.

Table 1 – Percentage of survival, shooting, and fungal contamination in nodule segments of *Apuleia leiocarpa* subjected to different types of disinfection solutions after 30 days of *in vitro* cultivation

Tabela 1 – Porcentagem de sobrevivência, de brotação e de contaminação fúngica em segmentos nodais de *Apuleia leiocarpa* submetidos a diferentes tipos de soluções de desinfestação aos 30 dias de cultivo *in vitro*

Treatments	Survival (%)	Shooting (%)	Fungal contamination (%)
Disinfection solution			
Control (distilled water)	100.0 a*	60.0 a	83.3 a
Sodium hypochlorite 2.5%	94.4 a	60.0 a	48.9 b
Sodium hypochlorite 5%	94.5 a	36.7 b	51.1 b
Sodium hypochlorite 10%	75.6 b	25.6 b	47.8 b
Glutaraldehyde 3%	81.1 b	41.1 ab	35.6 b
Immersion time (min.)			
5	92.7 a*	44.7 a	56.7 a
10	89.3 ab	43.3 a	56.7 a
15	85.3 b	46.0 a	46.7 a
Average	89.1	44.7	53.4
CV (%)	17.2	62.2	53.0

Source: Authors (2020)

Where: *Values followed by the same letter are not significantly different from each other (Tukey's test, $p < 0.05$).

It is important to note that there were no significant differences found with regards to the type of disinfection substance used. Chlorine-based solutions, such as the sodium hypochlorite used in the present study, are frequently used, and are effective for using in the superficial cleaning of explants of several forest species (OLIVEIRA; DIAS; BRONDANI, 2013). Glutaraldehyde, on the other hand, although it has a biocidal function with a wide spectrum of action against bacteria, fungi, and viruses (TIPPLE *et al.*, 2004), is rarely used in disinfection treatments of explants for introduction *in vitro*, and is commonly used to fix cellular structures in vegetal anatomical analysis (LIMA *et al.*, 2014). Although little used in surface disinfection of explants, our objective was to evaluate its biocidal potential in the search for efficient treatments for the elimination of fungi and bacteria for use in the culture of plant tissues. However, sodium hypochlorite is less toxic than glutaraldehyde, since the latter is a volatile chemical substance.

Regarding the immersion time, this only affected the percentage of survival, in which the highest survival rates were observed in the explants treated for 5 min., without differing from treatment with 10 min. (Table 1). Thus, treating nodules with a solution containing a 2.5%

concentration of active chlorine for 5 min. can be used to obtain aseptic *in vitro* cultures of grápia, and a responsive material for multiplication, since this treatment combines contamination control and high explant survival and shooting rates. In a similar study, the best responses for *in vitro* disinfestation of nodal segments from adult teak plants (*Tectona grandis* Linn. F.) were observed after treatment with 2.5% sodium hypochlorite for 30 min. (FERMINO JÚNIOR; NAGAO; SCHERWINSKI-PEREIRA, 2009). The determination of the disinfestation time is an important variable, since at its maximum efficiency, it will result in the elimination of microorganisms without causing plant tissue damage or death.

For *in vitro* rooting, survival, and number of roots, a significant interaction was observed between the treatments evaluated at 60 days of cultivation (Table 2). Survival exceeded 90% in all treatments evaluated, with the exception of treatment with 3.0 mg L⁻¹ NAA. Rooting, on the other hand, showed greater variation in response, depending on the evaluated treatment. The highest percentage of rooted explants was observed in the treatment with 1.0 mg L⁻¹ of NAA (61.1%), but it was not statistically different from the other concentrations. However, at concentrations of 1.0 mg L⁻¹, there were statistical differences in rooting between the type of auxin used, with higher rooting percentages in the NAA treatment than in the 2,4-D and IBA treatments, which is corroborated by the general rooting averages. In addition, treatment with 2,4-D at a concentration of 4.0 mg L⁻¹ resulted in the lowest rooting percentage (5.5%, Table 2). The low rooting percentage (5.5%) when 2,4-D was applied at a concentration of 4.0 mg L⁻¹ of 2,4-D may indicate an inhibitory effect of this auxin in the induction of adventitious roots when applied at higher concentrations. For number of roots, the best responses were when treated with ANA at a concentration of 1.0 mg L⁻¹, but without differing from other concentrations. According to Geiss, Gutierrez and Bellini (2009) the application of auxin plays a central role in the formation of adventitious roots, however the pattern of action of auxins is still little known, the control of the development of adventitious roots is a complex characteristic, as well as of high phenotypic plasticity (GEISS, GUIERREZ; BELLINI, 2009; BELLINI; PACURAR; PERRONE, 2014).

Table 2 – Percentage of survival and rooting, and number of roots in *Apuleia leiocarpa* nodal segments maintained in different types and concentrations of auxins after 60 days of *in vitro* cultivation

Tabela 2 – Porcentagem de sobrevivência e de enraizamento, e número de raízes em segmentos nodais de *Apuleia leiocarpa* mantidas em diferentes tipos de auxinas aos 60 dias de cultivo *in vitro*

[] mg L ⁻¹	Survival (%)			Rooting (%)			Number of roots		
	NAA ¹	2,4-D ²	IBA ³	NAA ¹	2,4-D ²	IBA ³	NAA ¹	2,4-D ²	IBA ³
0.0	100.0 aA*	100.0 aA	100.0 aA	27.7 aA	50.0 aA	27.7 aA	0.7 aA	1.2 aA	0.4 aA
1.0	100.0 aA	100.0 aA	97.2 aA	61.1 aA	30.5 aB	12.0 bB	2.1 aA	1.2 aB	0.2 aB
2.0	100.0 aA	100.0 aA	97.2 aA	50.0 aA	50.0 aA	42.9 aA	1.2 aA	1.9 aA	0.5 aA
3.0	75.0 bB	100.0 aA	97.8 aA	50.0 aA	30.5 aA	31.5 aA	1.4 aA	0.8 aA	0.5 aA
4.0	94.4 aA	94.4 aA	95.5 aA	38.8 aA	5.5 bA	30.5 aA	1.7 aA	0.1 bB	0.5 aB
Average	93.9	98.9	97.5	45.5	33.3	28.9	1.4	1.0	0.4
CV(%)		11.0			82.4			37.6	

Source: Authors (2020)

Where: ¹NAA = naphthalene acetic acid; ²2,4-D = dichlorophenoxyacetic acid; ³IBA = indolebutyric acid.

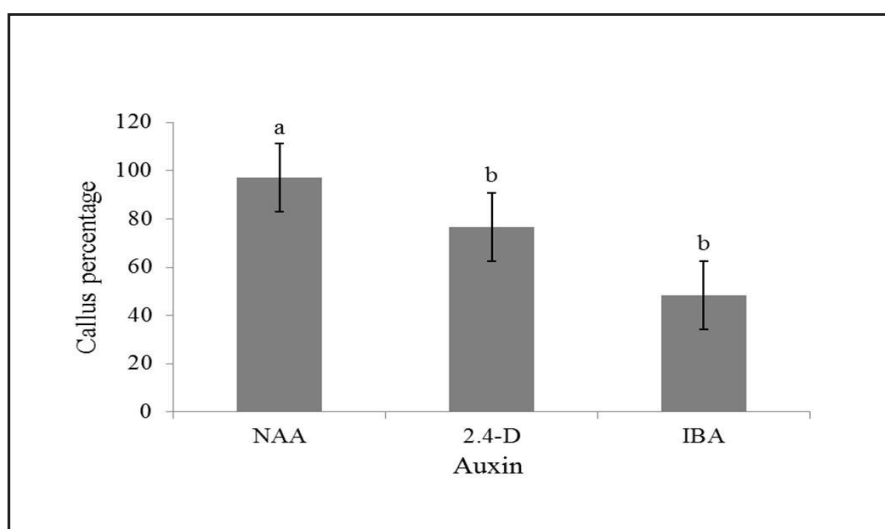
*Values followed by the same lowercase letter in each column and uppercase letter in each row are not statistically significantly different from each other (Scott-Knott test, p < 0.05).

Our results indicate that the grápia nodal segments are more responsive to NAA, regardless of concentration. Studies in other species, such as mahogany (*Swietenia macrophylla* King), found that concentrations of 2.0 and 5.0 mg L⁻¹ of NAA were more efficient in inducing rooting, with rooting percentages above 70% recorded after 30 days (LOPES *et al.*, 2001). For rosewood (*Aniba rosaeodora* Ducke), Murashige and Skoog medium (MS) containing 3.0 mg L⁻¹ of NAA was the most efficient for inducing rooting after 90 days (JARDIM *et al.*, 2010). The results of the current study are relevant because the rooting percentages found here are higher than those found in the literature (maximum of 26.4% *in vitro* when treated with 2.0 mg L⁻¹ of IBA and 14.6% *ex vitro* regardless of IBA concentration, LENCINA *et al.*, 2017b). These previously reported percentages are low enough to consider the species as recalcitrant to vegetative propagation (LENCINA *et al.*, 2017b), and make the *in vitro* rooting technique unfeasible for seedling production. Sarmast, Salehi and Khosh-Khui (2012) obtained a maximum of 33% *in vitro* rooting for Norfolk Island pine (*Araucaria excelsa* R. Br. var. *glauca*), and concluded that this technique was not an efficient way to produce seedlings of this species.

For the callus percentage, there was an isolated effect of the type of auxin tested, with the highest induction observed in the NAA treatment (Figure 1 and Figure 2B) with 97.2% of callogenesis. Although significantly lower than in the NAA treatment, the callus formation values in the treatments with 2.4-D and IBA were also high (76.7% and 48.4%, respectively, Figure 1). This is in agreement with the results obtained by Lencina *et al.* (2016), which showed that grápia presents high callus formation at the base of explants when grown *in vitro*, both in multiplication and rooting. Callus induction often occurs in tissue culture and does not necessarily involve cell de-differentiation to create a mass of cells, but rather represents a form of organogenesis (XU; HUANG, 2014). In addition, Sugimoto, Jiao and Meyerowitz (2011) report that the callus is composed of cells with characteristics similar to the cells that form the root meristem. Evidently, several genes and processes involved in the formation of lateral roots also occur in the formation of callus. For grápia, we found that, in treatments with greater callus formation, greater root induction occurred, suggesting a beneficial effect of callogenesis on *in vitro* rooting of this species.

Figure 1 – Percentage of callus in *Apuleia leiocarpa* nodal segments treated with different types of auxins after 60 days of cultivation

Figura 1 – Porcentagem de calo em segmentos nodais de *Apuleia leiocarpa* cultivadas em diferentes tipos de auxinas aos 60 dias de cultivo



Source: Authors (2020)

Where: Different letters indicated significant differences between treatments as tested by the Scott-Knott test at 5% probability.

In this study, the formation of secondary roots in the root system of plants was verified (Figure 2). This can positively influence acclimatization, as these structures play an important role in increasing the root surface and allowing the roots to explore a greater volume of substrate.

Figure 2 – *Apuleia leiocarpa* plants produced by *in vitro* rooting in wood plant medium (WPM) culture medium without auxin (A), and supplemented with 2.5 mg L⁻¹ of naphthalene acetic acid (NAA) (B) at 60 days of cultivation. Bar = 1 cm

Figura 2 – Plantas de *Apuleia leiocarpa* produzidas pelo enraizamento *in vitro* em meio de cultura WPM sem auxina (A) e suplementado com 2,5 mg L⁻¹ de ANA (B) aos 60 dias de cultivo. Barra = 1 cm



Source: Authors (2020)

The results of the present study show that it is possible to perform disinfection and *in vitro* introduction of nodal segments of grápia, through treatment with hypochlorite solution with 2.5% active chlorine for 5 minutes. This is an important step for micropropagation of plants, and is essential for the rescue of selected plants, or those with a lack of seeds for *in vitro* establishment. Once established, explants can be multiplied following the methodology described by Lencina et al. (2016; 2017a), rooted *in vitro* in WPM culture medium supplemented with 1.0 mg L⁻¹ of NAA, and acclimated according to Lencina et al. (2017b). Therefore, the results of this study, combined with those of Lencina et al. (2014; 2016; 2017a; 2017b), provide a complete micropropagation methodology and, as a result, an *in vitro* conservation strategy for grápia, a native species that is of ecological interest and an important timber species that is threatened with extinction.

Conclusions

Nodal segments of the clonal mini-garden of grápia can be introduced *in vitro* after disinfection treatment with active chlorine at a concentration of 2.5% for 5 minutes, and this result in a good source of propagules for micropropagation. The *in vitro* rooting of grápia explants can be performed in culture medium supplemented with 1.0 mg L⁻¹ of NAA.

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