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# *In Vitro* Rooting and Multiplication of *Myrcianthes pungens* (O. Berg) D. Legrand

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**Abstract**

The propagation of *guabijuzeiro* (*M. pungens*) by seed is originating plants with high genetic variability and a long juvenile period. Therefore, the objective was to study the multiplication and rooting *in vitro* of *guabijuzeiro* in order to get information to enable the clonal propagation. In the multiplication, apical and nodal segments were used, taken from *in vitro* plantlets, combined with 6-Benzylaminopurine (BAP), naphthaleneacetic acid (NAA) and gibberellic acid (GA). In the rooting, NAA and IBA (indolebutyric acid) were used. The results showed that in the multiplication there was superiority in media with BAP, while rooting was not satisfactory in media with NAA or IBA. Thus, it is possible to multiply *M. pungens in vitro* using BAP phytoregulator in the multiplication stage. However, the rooting stage needs more tests to induce the production of roots and facilitate acclimatization of the plants obtained *in vitro*.

**Keywords:** environmental economics, forest genetics and improvement, wood preservation.

## 1. INTRODUCTION AND OBJECTIVES

Brazil holds the largest biodiversity in the world (Forzza et al., 2012). In addition, despite this richness of native plant species, Brazilian agriculture is still supported by the exploration of some exotic domesticated species. This condition generates food insecurity as a result of the growing demand for food, in addition to other resources (clothing, medicine, construction) (Moretto et al., 2014). Native plant resources are sources of food, fiber, wood, pigments, spices, flavorings, energy and the main active ingredients for drug production (Ferro et al., 2006; Sousa et al., 2017). Therefore, the exploration of the potential of use of these native vegetable resources depends on a bigger knowledge of these species. The interest in native fruit species has been growing in recent years, which encourages the development of studies on their agronomic, nutritional and pharmaceutical characteristics (Pereira & Pasqualeto, 2011).

The botanic family Myrtaceae is represented by approximately 132 genera and 5,950 species with diversity centers in Australia, Southeast Asia, and in America; it ranges from the tropical zone to the temperate south (Stevens, 2013). In Brazil, the native fruits of the Myrtaceae family are the most known ones because of the biggest number of species with economic potential (Pereira et al., 2012), among which *guabijuzeiro* (*Myrcianthes pungens* (O. Berg) D. Legrand) stands out. Its natural distribution occurs from the state of São Paulo to Rio Grande do Sul, in the semideciduous forests of altitude and the Uruguay and Paraná river basins (Lorenzi et al., 2006).

*Guabijuzeiro* presents several uses. Its wood is elastic, dense and resistant, suitable for luxury joinery, lathe and civil construction, tool handles and farming implements. Its flowers are white, melliferous and the tree is ornamental and used in urban afforestation (Lopes & Gonçalves, 2006; Lorenzi et al., 2006). The fruits have juicy, sweet and pleasant flavor pulp, and can be consumed fresh or processed (juices, ice creams, yogurts, liqueurs, cereal bars, sweets, jellies) (Lorenzi et al., 2006). In addition to being tasty, the fruits present high antioxidant activity, with high levels of anthocyanins and carotenoids (Nora et al., 2014). Recent studies have demonstrated the

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potential of leaves as a potentially effective new analgesic agent for pain control (Nesello et al., 2016).

Information that support the production of seedlings of this species is scarce and the works found in the literature are on its chemical composition and the physiological quality of the *guabijuzeiro* seeds in order to prolong their viability during storage (Fior et al., 2010; Hossel et al., 2016).

*Guabijuzeiro* is mainly propagated by seeds (Lorenzi et al., 2006), a technique which results in plants with high genetic variability, high vigor and extended juvenile period (Peña et al., 2015), which is not recommended for the production of seedlings for commercial orchards (Franzon et al., 2010). Hence, micropropagation stands out as an alternative for producing seedlings. This technique is used to propagate some native forest species and to establish matrices for seed production (Oliveira et al., 2013), in addition to producing seedlings regardless the time of the year (Dutra et al., 2009). It presents several stages, the phases of multiplication and rooting are those of fundamental importance to achieve micropropagated plants (Dutra et al., 2009; Oliveira et al., 2013).

The micropropagation process is influenced by several factors, such as genetic characteristics and *in vitro* culture conditions. During the multiplication phase, the presence of phytoregulators is a key factor, particularly cytokinins and auxins (Dutra et al., 2009; Oliveira et al., 2013). Cytokinins are used in culture media to promote the multiplication of several species. Concentrations between 0.006 µM and 8.8 µM show greater results in the cultivation of shoot apices of several woody species (Dutra et al., 2009). In the rooting phase, the auxins are the most commonly used, in which indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) stand out. According to Dias et al. (2012), the rooting phase is the most difficult for woody species, as is the case of *guabijuzeiro*. So, the objective of this study was to evaluate the multiplication and *in vitro* rooting of *guabijuzeiro* by testing explants and growth regulators.

## 2. MATERIALS AND METHODS

Seeds of two matrices collected at the Fundação Estadual de Pesquisa Agropecuária (Fepagro), in the municipality of Maquiné, were sent to the Horticulture Biotechnology Laboratory of the Federal University of Rio Grande do Sul (UFRGS), where they were *in vitro* germinated to obtain the seedlings, which served as source of explants for the induction of sprouts. The base culture medium used in the experiments was WPM-Woody Plant Medium (Lloyd & McCown, 1980) added with 30 g $L^{-1}$ of sucrose and 7 g $L^{-1}$ of agar. The growth regulators naphthaleneacetic acid, 6-benzylaminopurine and gibberellic acid (NAA, BAP and GA, respectively) were added according to the micropropagation phase. The pH was adjusted to 5.8 before sterilization, which was performed by autoclaving (15 minutes at 121 °C and 1.2 atm pressure). After incubation, the cultures were kept in a growth room at a temperature of 25 °C ± 3 °C and photoperiod of 16 hours (32-w fluorescent lamps). The experiments were conducted in two stages: multiplication (with two subcultivation) and rooting.

### *2.1. Multiplication*

For the multiplication stage, apical and nodal segments at approximately 0.6 cm and 0.4 cm were used. The apical segments, containing two opposing axillary buds, were obtained from seedlings germinated *in vitro* for 120 days. In addition to the two types of explants, media without phytoregulators were tested, with BAP (0.4 mg $L^{-1}$) (Souza et al., 2011), and with BAP + NAA (0.4 ± 0.04 mg $L^{-1}$, respectively). The experimental design was completely randomized, in a 3 × 2 factorial arrangement (three combinations of phytoregulators × two types of explants). Four replicates per treatment were used, each replicate was composed of five flasks with three explants.

At day 30, the first subculture of sprouts from the apical and nodal segments was performed. Sprouts were incubated in medium without phytoregulators, with a higher concentration of BAP (0.6 mg $L^{-1}$) and BAP + NAA (0.4 + 0.04 mg $L^{-1}$, respectively). The experimental design was completely randomized, in a 2 × 2 factorial arrangement (two combinations of phytoregulators × two types of explants). Seven replicates per treatment were used, each replicate was composed of one flask with five explants.

At day 60 (30 days after the first subcultivation), the second subcultivation was performed with the sprouts originated in the first subculivation. Culture medium with BAP (0.6 mg $L^{-1}$) and BAP + NAA + GA (0.03; 0.2 and 0.3 mg $L^{-1}$, respectively) was used. The experimental design was completely randomized in a 2 × 2 factorial arrangement (two combinations of phytoregulators × two types of explants). Seven replicates were used per treatment, each replicate was composed of one flask containing five explants.

In each of the stages (multiplication, subculture 1 and subculture 2), the following variables were evaluated: sprouting and oxidation percentage, average number of shoots and leaves, number and height of sprouts per explant and percentage of explant survival. Data were submitted to analysis of variance (Anova) and a comparison of means by the test of Duncan at 5% of error probability, from SigmaStat software.

### 2.2. Rooting

For the rooting stage, sprouts (0.6 and 2.5 cm in height) produced in the second subculture of the multiplication stage were used. The treatments consisted of concentrations of 0.2; 0.4; 0.6 mg $L^{-1}$ of NAA and IBA. The explants were incubated in glass flaks containing approximately 30 mL of WPM culture medium, sealed with high density polyethylene caps.

Forty-five days after incubation the following variables were evaluated: percentage of living sprouts, with calluses, oxidized and rooted. The experimental design was completely randomized in a 2 × 3 factorial arrangement (two phytoregulators × three concentrations of the phytoregulators), with 12 replicates per treatment, each replicate was composed of one flask with three explants. Data were submitted to the analysis of generalized linear models and a comparison of means were carried out using the Tukey test at 5% error probability level, with the aid of the SPSS Statistics 23 and SigmaPlot 11.0 software.

## 3. RESULTS AND DISCUSSION

### 3.1. Multiplication

The results demonstrated the development of *guabijuzeiro* shootings in all tested treatments, even in the absence of growth regulator. However, the medium containing BAP was higher in all analyzed variables, regardless of the explant used, differing from the other treatments (Table 1). Similar results were found by Mantovani et al. (2001), in which the multiplication rate and height of shootings of *Cordia trichotoma* were higher in the medium with BAP when compared to medium containing the NAA + BAP combination. In addition, Ali & Lüdders (2001) verified a higher multiplication rate in *Psidium guajava* at the concentration of 0.5 mg $L^{-1}$ of BAP.

The combination BAP + NAA was greater to the control (medium without phytoregulator), promoting the rise in leaf emission, height of the explants and more shoots per explant. Despite this positive result of the combination in relation to the control, it was demonstrated that the use of 0.4 mg $L^{-1}$ of BAP was the most efficient, since all variables analyzed were greater than the other treatments.

Regarding the types of segments tested, a difference was only observed in the oxidation variable (Figure 1). The apical segment presented the best results in the culture medium without phytoregulator, with 16% of oxidation, in the BAP + NAA medium presented only 2% of oxidation. The BAP medium resulted in the lowest oxidation levels between the apical and nodal segments used, 2% and 3%, respectively.

No difference was found between the type of segment used in the initial cultivation for the variables' survival percentage, oxidation and leaf number of shoots (Table 2) in the first subculture. However, there were differences between the combinations of the phytoregulators used, and BAP medium (0.6 mg $L^{-1}$) was higher in all evaluated variables. These results were also observed in the multiplication phase, indicating that, for this species, there is no need to use auxin (NAA) associated with BAP, since in the medium containing only BAP, a high survival (90%), low oxidation (9%) and high number of leaves per explant (18 on average) were achieved in the first subculture.

**Table 1.** Development of apical and nodal explants of *guabijuzeiro* (*Myrcianthes pungens*) in the multiplication phase after 30 days in WPM medium supplemented with BAP (0.4 mg $L^{-1}$), BAP + NAA (0.4 + 0.04 mg $L^{-1}$) and control (without the addition of phytoregulator).

| Treatments | | Shoots (%) | No. leaves | Height (cm) | No. shoots/expl. |
|---|---|---|---|---|---|
| Apical | Control | 18 c | 2.33 c | 0.23 c | 0.5 c |
| | BAP | 73 a | 7.35 a | 0.55 a | 1.2 a |
| | BAP + NAA | 57 b | 5.58 b | 0.46 b | 0.8 b |
| Nodal | Control | 12 c | 2.17 c | 0.13 c | 0.3 c |
| | BAP | 80 a | 11.63 a | 0.82 a | 1.6 a |
| | BAP + NAA | 58 b | 5.45 b | 0.44 b | 0.9 b |
| *p* value | Explant | 0.914 | 0.206 | 0.497 | 0.974 |
| | Phytoregulator | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | Explant × Phytoregulator | 0.597 | 0.070 | 0.050 | 0.177 |
| CV (%) | | 9.13 | 13.6 | 4.88 | 29.3 |

BAP: 6-benzylaminopurine; NAA: naphthaleneacetic acid; No.: number; No. of shoots/expl.: number of shoots per explant; CV: coefficient of variation. Means followed by the same lowercase letter for the phytoregulator combinations do not differ from each other at 5% error probability level by Duncan test. Data were transformed into $\sqrt{x+5}$

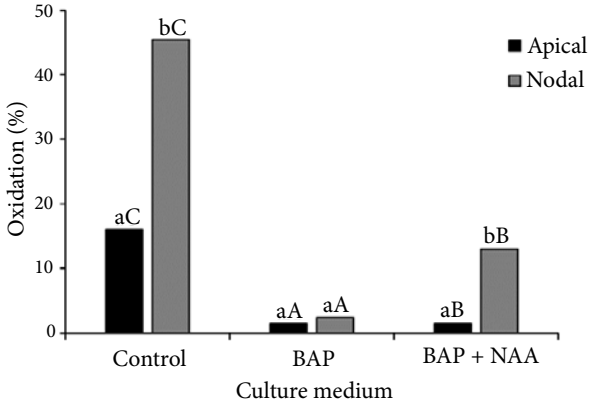


**Figure 1.** Percentage of oxidation of apical and nodal explants of *guabijuzeiro* cultivated in WPM medium supplemented with BAP (0.4 mg $L^{-1}$), BAP + NAA (0.4 + 0.04 mg $L^{-1}$) and control (without the addition of phytoregulator).

Same capital letter for combinations of phytoregulators and lowercase for segments (apical and nodal) do not differ by Duncan test at 5% error probability level.

**Table 2.** Percentage of survival, oxidation and number of leaves of the shoots emitted by the apical and nodal segments of *guabijuzeiro* in the multiplication phase of the first subculture in WPM medium added with BAP (0.6 mg $L^{-1}$), BAP + NAA (0.4 + 0.04 mg $L^{-1}$).

| Treatments | | Survival (%) | Oxidation (%) | No. leaves |
|---|---|---|---|---|
| Apical | BAP + ANA | 67 b | 37 b | 6.8 b |
| | BAP | 90 a | 9 a | 17.6 a |
| Nodal | BAP + ANA | 51 b | 33 b | 6.5 b |
| | BAP | 89 a | 9 a | 18.3 b |
| *p* value | Phytoregulator | < 0.001 | 0.002 | < 0.001 |
| | Explant | 0.269 | 0.965 | 0.895 |
| | Explant × Phytoregulator | 0.312 | 0.563 | 0.716 |
| CV (%) | | 25.8 | 20.6 | 28.9 |

BAP: 6-benzylaminopurine; NAA: naphthaleneacetic acid; No.: number; CV: coefficient of variation. Means followed by the same lowercase letter for the combinations of phytoregulators do not differ statistically by Duncan test at 5% error probability level. Data were transformed into $\log(x+10)$ .

The increase in BAP concentration in the culture medium from 0.4 mg $L^{-1}$ to 0.6 mg $L^{-1}$ was favorable, since it was possible to obtain more shoots per explant, besides improving their visual appearance (explants with greener color and larger leaves). The BAP + NAA medium, although lower than the BAP containing medium, presented a percentage of survival over 50%, causing higher oxidation (37%) and lower number of leaves per explant (6.8), however.

The number of shoots per explant, as well as their height, presented statistical differences in relation to the combinations of phytoregulators and to the type of explant used in the initial cultivation (Figure 2). Superiority was found for the shooting variable in the BAP medium, with no difference between the types of segment used. In the middle with BAP + NAA, the nodal segments had a bigger number of shoots per explants than the apical segments.

The development of the lateral buds with the apical bud is completely related, since the development of the lateral meristems is inhibited by the apical dominance due to the presence of auxin in the meristems of the apical buds; in addition, it acts as a drain of nutrients and cytokinin (Santos et al., 2010; Vieira et al., 2009). Thus, the superiority of shoot emission in non-apical segments may be related to apical dominance, favored by the higher concentration of auxin at the apex of the branches, promoting a negative effect on the proliferation of axillary buds in the plants. After the apical dominance is broken, with the removal of the apical bud, the growth of the lateral buds is initiated, which can be promoted with the use of synthetic cytokinins (Cline, 1997).

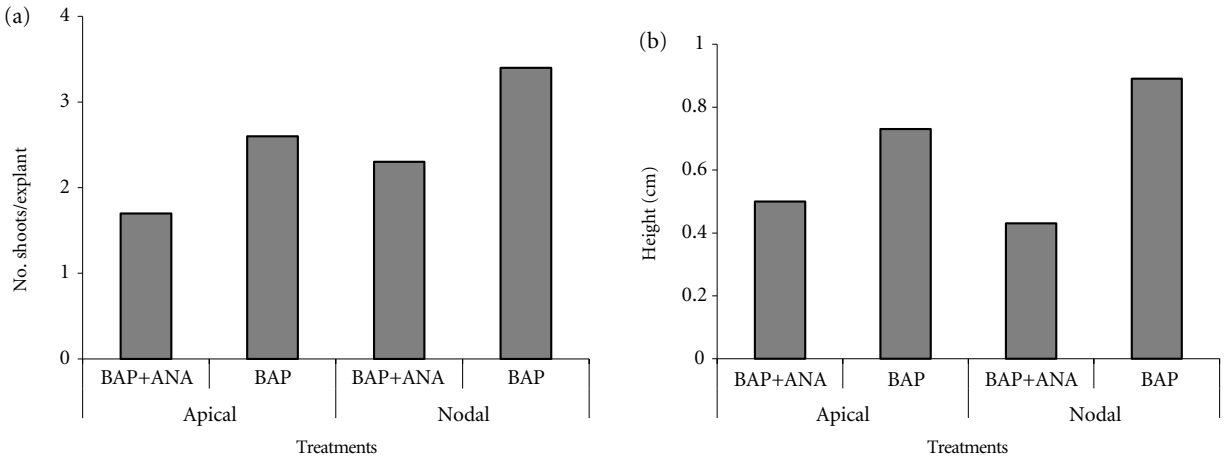


**Figure 2.** Average number of shoots (a) and height of shoots (b) of *guabijuzeiro* in the first subculture, maintaining the identification of the type of explant used in the initial crop (apical and nodal) in the multiplication phase, after 30 days in media WPM plus BAP (0.6 mg $L^{-1}$) or BAP + NAA (0.4 + 0.04 mg $L^{-1}$).

Same capital letter for explants and lowercase for combinations of phytoregulators do not differ by Duncan test at 5% error probability level.

Regarding shoot height, the treatment with explants from nodal segments shoots, cultivated in the medium with BAP, were superior to the other treatments. On the other hand, the explants from shoots of apical segments, cultivated in the medium with BAP, were superior to the explants coming from shootings of apical and nodal segments cultivated in the medium with BAP + NAA. These results suggest that the presence of NAA does not influence the shoot height variable regardless the origin of the segment (apical or nodal). The presence of BAP increased the height and number of shoots. It be explained by the role of cytokinins in plants. Similar results were observed by Araujo et al. (2008) in the *in vitro* multiplication of "smooth cayenne" pineapple.

In the second subculture, medium was maintained with BAP (0.6 mg $L^{-1}$) as it was efficient in the previous culture. At the same time, the cultivation in medium was compared with the presence of the three associated phytoregulators (3 FIT), where the use of GA aimed the generation of more elongated shoots, since in the previous experiments the formation of short shoots was noted as, according to Mantovani et al. (2001), this plant regulator has been frequently used in the micropropagation to promote the stem elongation of the inter-nodes.

The treatments used at this stage allowed high survival rates. In the BAP treatment, the explants from shoots formed by apical segments showed a survival of 94.3%, whereas of nodal segments were 100%. The 3 FIT treatment showed one of 95.7% and 98.6%, respectively.

Callus was present in all treatments, which may negatively affect morphogenesis (Soares et al., 2012). In the two evaluated variables (percentage of survival and callus), for the combinations of phytoregulators and for the types of explants used, no statistical difference was observed. For the number of shoots and number of leaves, the BAP medium remained higher, in which the number of shoots per explant was on average 3.3, and the number of leaves was between 19.7 and 23.9, not differing from the origin of the explants used (Table 3).

**Table 3.** Number of shoots per explant, number of leaves and shoot height (cm) of the second subculture of *guabijuzeiro*, using apical and nodal segments as explants in WPM medium added with BAP (0.6 mg $L^{-1}$), 3 FIT (0.03 BAP + 0.2 NAA + 0.3 GA mg $L^{-1}$).

| Treatments | | No. shoots/ expl. | No. leaves | Height (cm) |
|---|---|---|---|---|
| Apical | BAP | 3.00 a | 23.9 a | 0.75 Ba |
| | 3 FIT | 1.65 b | 10.4 b | 0.56 Ba |
| Nodal | BAP | 3.60 a | 19.7 a | 1.00 Aa |
| | 3 FIT | 1.90 b | 13.3 b | 0.94 Aa |
| *p* value | Phytoregulator | < 0.001 | < 0.001 | 0.429 |
| | Explant | 0.509 | 0.727 | 0.016 |
| | Explant × Phytoregulator | 0.621 | 0.042 | 0.529 |
| CV (%) | | 27.9 | 26.2 | 20.4 |

BAP: 6-benzylaminopurine; 3 FIT: 0.03 BAP + 0.2 NAA + 0.3 AG mg $L^{-1}$; No.: number; No. shoots/expl.: number of shoots per explant; CV: coefficient of variation. Means followed by the same lowercase letter for the combinations of phytoregulators and uppercase letter for explant, do not differ statistically by Duncan test at 5% error probability level. Data on the height data were transformed to $\sqrt{x}$.

In relation to shoot height, the explants cultivated in the medium with BAP did not differ from the explants cultivated in FIT medium 3 but were significantly different in relation to the types of explants tested, being those from shoots of nodal segments (in initial culture) to apical segments. In addition, the shoots were sufficiently elongated in the medium containing BAP, which eliminates an *in vitro* culture step, and consequently, reducing costs.

### 3.2. Rooting

In the rooting phase, a high survival of the shoots occurred regardless of the auxin used in the culture medium (Table 4). In addition, the use of NAA provided superiority in relation to the percentage of calluses and roots. These results agree with those verified by Melo et al. (2001), who observed that NAA allowed greater formation of *Syagrus oleracea* fasciculate roots while IBA was inefficient.

In the NAA treatments, rooting was very low (7%); however, it was superior to the use of IBA, which presented superiority in the percentage of oxidation. The percentage of callus formation was higher with NAA (73%) compared to IBA (15%). A linear trend in the percentage of formed calluses according to the increase in NAA concentration can be observed. Although NAA can induce root formation, being superior to IBA for some species, it may also cause undesirable effects (Martins et al., 2011); for example, when it is used in excessive concentrations, stimulating callus production (Brondani et al., 2009). Thus, the concentrations of NAA in this work can be considered high, since the percentage of callus formation increased as the concentration of this phytoregulator increased as well. In a study with *louro-pardo* (*Cordia trichotoma*), NAA auxin caused an excess in the number of callus by inhibiting the multiplication and development of shoots (Mantovani et al., 2001). Similar results to the calogenic action of the NAA were also observed in guavasteen by Dal Vesco & Guerra (1999).

When working with the establishment and *in vitro* development of *Eugenia involucrate*, Golle et al. (2012) tested the WPM, MS (Murashige & Skoog, 1962) and 1/2 MS media obtaining the best result for rooting the species in 1/2 MS medium. They concluded that this rhizogenic response may be related to the carbon/nitrogen ratio (C/N), in which the reduction of nitrogen (1/2 MS) and the increase of carbohydrate sources (using 30 g $L^{-1}$) favored the rooting processes, which occurs at the expense of a lot of energy. In addition, the same authors found superior response of callus formation in WPM medium, which has nitrogen concentration quite below the MS in its composition. The use of the MS medium, not tested in this work, may be an alternative for *guabijuzeiro in vitro* rooting.

The *in vitro* multiplication of the species *Myrcianthes pungens* is possible by using apical or nodal segments from seedlings obtained by *in vitro* germination in WPM medium, with concentrations of 0.4 and 0.6 mg $L^{-1}$ of BAP. As a result, high sprouting rates (between 70% and 90%) are obtained. For the rooting of these shoots, it is necessary to carry out further studies regarding the concentration and the type of phytoregulator, besides using other means of cultivation for comparison. In this work, due to the formation of callus in the bases of the shoots of the second subculture, which did not occur in the first subculture, it is suggested that the multiplication is interrupted at the end of the first subculture, thus going to the rooting step.

**Table 4.** Percentage of living explants and with callus after 45 days of incubation in media with NAA (0.2, 0.4 and 0.6 mg $L^{-1}$) and IBA (0.2, 0.4 and 0.6 mg $L^{-1}$).

| Treatments (mg $L^{-1}$) | | Survival (%) | Callus (%) |
|---|---|---|---|
| IBA | 0.2 | 100.00 Aa | 16.58 Ba |
| | 0.4 | 91.58 Aab | 16.50 Ba |
| | 0.6 | 77.67 Bb | 11.00 Ba |
| NAA | 0.2 | 91.67 Aa | 44.08 Ab |
| | 0.4 | 100.00 Aa | 86.00 Aa |
| | 0.6 | 100.00 Aa | 88.83 Aa |
| *p* value | Phytoregulator | 0.109 | 0.00 |
| | Concentration | 0.369 | 0.012 |
| | Phytoregulator × Concentration | 0.027 | 0.003 |

IBA: indolebutyric acid; NAA: naphthaleneacetic acid. Averages followed by the same uppercase letter for the phytoregulators and lowercase letter for the concentrations do not differ statistically by the Tukey test at 5% error probability level.

## 4. CONCLUSIONS

The *in vitro* multiplication phase of *guabijuzeiro* showed higher efficiency with the use of apical or non-apical segments in WPM culture medium with BAP at concentrations of 0.4 and 0.6 mg $L^{-1}$.

The *in vitro* rooting phase of *guabijuzeiro* shoots was not satisfactory in WPM medium with NAA and IBA, indicating the need for further studies in order to test concentrations and types of phytoregulators.

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