

Full Length Research Paper

Cryopreservation and effect of lighting conditions and cytokinins on *in vitro* multiplication of *Miconia ligustroides* (DC.) Naudin

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Miconia ligustroides is a species that is native to Brazil and has medicinal and ecological importance. However, the species shows a lack of uniformity and delay in *ex vitro* germination. Thus, this study aimed to establish *in vitro* propagation for the species and to develop a protocol for the cryopreservation of seeds. For *in vitro* germination, activated charcoal (0.0, 0.5, 1.0, and 2.0 g L⁻¹) was tested in Murashige and Skoog (MS) culture medium. Lateral buds excised from the plants were germinated *in vitro* and were encapsulated in an alginate matrix supplemented with 6-benzylaminopurine (BAP), kinetin, and thidiazuron (TDZ: 0.0, 2.0, 8.0, and 16.0 µM). Shoots derived from encapsulated units were inoculated in MS culture medium supplemented with different concentrations of BAP (0.0, 2.5, 5.0, and 10.0 µM) under white or Gro-Lux fluorescent lamps for multiplication. For cryopreservation, the toxicity of the cryoprotectant solution PVS2 (0, 15, 30, 60, 120, and 180 min) was evaluated before the seeds were immersed in liquid nitrogen. The MS culture medium supplemented with 1.0 g L⁻¹ of activated charcoal yielded the highest percentage of germination (78%). The encapsulated units presented the largest percentages of regeneration (75%) with 8.0 µM BAP, which assisted in the formation of shoots that were 8.03 cm in length. For shoot production, the highest mean number (3.03 shoots) was obtained in the MS medium containing 5.41 µM BAP. When seeds were subjected to cryopreservation, the immersion time in the PVS2 did not affect the survival of the seeds, which was satisfactory (70%). The protocols developed are considered viable alternatives for use in the conservation of the species, production of seedlings for commercialization purposes, and use in programs of reintroduction in degraded environments.

Key words: Melastomataceae, *in vitro* conservation, encapsulated units, 6-benzylaminopurine.

INTRODUCTION

The family Melastomataceae is among the most representative of South America and is the sixth largest

angiosperm family in Brazil (Romero and Martins, 2002). Representing virtually all plant formations, this family is

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composed of a wide variety of species, ranging from trees to vines and epiphytes (Renner, 1993). In Brazil, this family has 68 genera, of which 10 are endemic, with more than 1500 species spread throughout the country. Among the genera, *Miconia* Ruiz & Pav. is distinguished within the Melastomataceae by its numerous species (Romero and Martins, 2002; Michelangeli et al., 2004).

Among the *Miconia* species considered important from a medical viewpoint is *Miconia ligustroides*. The species occurs in the Cerrado (savanna forest and woodlands), Atlantic Forest, and Caatinga (thorn scrub savanna and forest) regions (Goldenberg et al., 2013) and its medicinal importance is related to its analgesic antimicrobial (Cunha et al., 2003) and trypanocidal actions (Cunha et al., 2006). Another interesting characteristic is the listing since 2001 of *M. ligustroides* as a species recommended for the recovery of riparian forests (SMA 21/01). Although the species exhibits the positive features mentioned, it faces problems related to the maturation of fruits and an unequal number of diaspores (Chaves et al., 2013), culminating in unequal and delayed germination. Such characteristics may be associated with a relatively low percentage of seed germination, approximately 55% (Chaves et al., 2011) which can hinder the propagation and even the survival of this species in the natural environment.

The problems related to the difficulties in germination of the species, however, can be minimized or even overcome through maximization and standardization of germination using *in vitro* cultivation techniques (Reis et al., 2015). Through the correct use of these techniques, regeneration of the meristematic regions (Ferreira et al., 2007) can be established to encapsulate the lateral buds of the material regenerated in an alginate matrix (Sandoval-Yugar et al., 2009). When combined with the use of growth regulators (Jona and Webb, 1978; Bhojwani et al., 1984) and adequate light sources (Rocha et al., 2010, 2013) this technique can generate multiple healthy shoots in a short period from a single plant germinated *in vitro*.

In addition to the *in vitro* germination protocols used to obtain healthy seedlings, seed cryopreservation protocols are also available, which are useful for conserving and preserving endangered species as well as those of commercial interest (Cejas et al., 2012; Prudente et al., 2016). Currently, *M. ligustroides* stored in conventional germplasm banks, which require large physical spaces and constant maintenance and are subject to contamination and significant losses (Pritchard and Nadarajan, 2008; Engelmann, 2011).

Given the need to overcome these problems inherent germplasm banks and the scarcity of studies on the cryopreservation of this species, new studies focusing on cryopreservation can contribute more effective alternatives to the current methods employed in the conservation of the species.

Studies have suggested that the longevity of

cryopreserved seeds (-196°C) can be up to 175 times greater than the longevity obtained at the temperature used in seed banks (Pritchard, 1995; Walters et al., 2004; Cejas et al., 2012). In addition, the storage in cryogenic banks requires a smaller financial investment over time compared to other genetic material conservation systems available (Dulloo et al., 2009).

Given the aforementioned, this study aimed to establish an *in vitro* propagation protocol, through the encapsulation of lateral buds, and a seed cryopreservation protocol for the species *M. ligustroides*.

MATERIALS AND METHODS

Plant and *in vitro* germination

The seeds were extracted from mature fruits with uniform size and state of conservation, and stored in a cold chamber (4°C) for 30 days. The seeds were removed manually with tweezers, washed in distilled water and distributed on two sheets of paper towel. Subsequently, they were taken to the laminar flow chamber, immersed in 70% alcohol for 60 s, in sodium hypochlorite solution (NaOCl) with 1% active chlorine for 10 min and, at the end, washed five times with distilled water autoclaved.

For *in vitro* germination step, different concentrations of activated charcoal (AC) (0.0, 0.5, 1.0, 2.0 g L⁻¹) were tested in MS culture medium (Murashige and Skoog, 1962), plus 30 g L⁻¹ sucrose and gelled with 7 g L⁻¹ agar (Sigma®). The seeds were inoculated into 90 × 15 mm crystal polystyrene Petri dishes (J. Prolab®, Brazil) containing 25 ml aliquots of culture medium and sealed with polyvinyl chloride (PVC) film (Rolopac®). The seeds were kept in a growth room under photon irradiance of 86 μmol m⁻² s⁻¹ (40 w white fluorescent lamp, Osram, Brazil) at a temperature of 25 ± 2°C and a photoperiod of 16 h. Each treatment consisted of five Petri dishes, with five seeds per plate. After 30 days of *in vitro* cultivation were evaluated the percentage of seed germination, number of leaves, number of roots and length of the shoots (cm).

Encapsulation of lateral buds

Plants from seeds germinated *in vitro* in MS culture supplemented with 1.0 g L⁻¹ of activated charcoal (AC) were used as a source of explants for encapsulation of lateral buds. Lateral buds approximately 1.0 mm² in length were excised and immersed in MS medium and were supplemented with different concentrations (0.0, 2.0, 8.0, and 16.0 μM) of 6-benzylaminopurine (BAP), kinetin (KIN), and thidiazuron (TDZ) and with sodium alginate (2.5% w/v) added to the medium. Next, with the aid of an automatic pipette, the encapsulated units were individually retrieved and dropped into a calcium chloride solution (CaCl₂·2H₂O) (100 mM), where they remained for 20 min for complexation. The encapsulated units, individually formed by a lateral bud covered with a sodium alginate matrix, were subjected to three washes with autoclaved distilled water to remove the excess CaCl₂·2H₂O. Then, the encapsulated units were immersed in a potassium nitrate solution (KNO₃) (100 mM) for 15 min for decomplexation and, then, were washed again with autoclaved distilled water before inoculation into the basal MS medium. The capsules were placed in a growth chamber with a photoperiod that included 16 h irradiance with 86 μmol m⁻² s⁻¹ (40 W white fluorescent lamp, Osram, Brazil) and at a temperature of 25 ± 2°C. Each treatment comprised five Petri dishes, with five capsules per dish. The variables evaluated were the percentage of regeneration of the encapsulated buds (after 45 days of *in vitro*

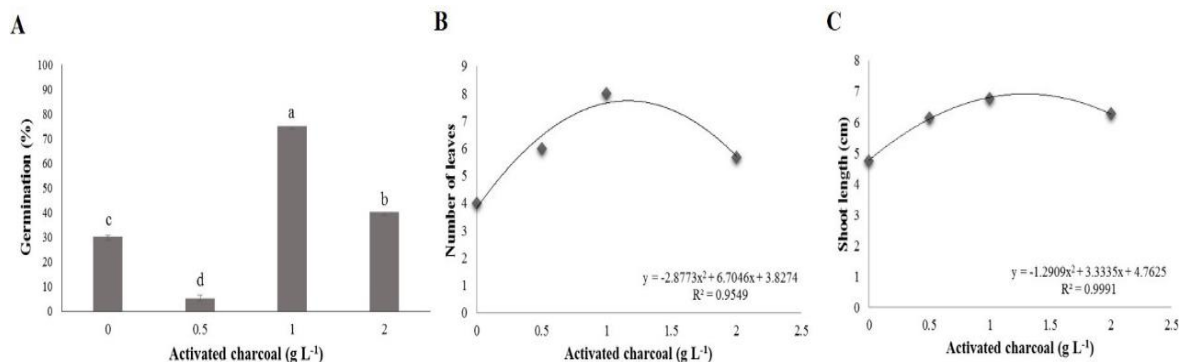


Figure 1. Effect of different concentrations of activated charcoal (0.0, 0.5, 1.0, and 2.0 g L⁻¹) in the MS culture medium on the percentage of seed germination (A), number of leaves (B), and shoot length (D) of *M. ligustroides* seedlings after 30 days of cultivation. Means followed by the same letter in columns in (A) did not differ among themselves, according to the Tukey test at 5% significance.

cultivation), the length of the regenerated shoots (cm), and the number of leaves per shoot (after 60 days of *in vitro* cultivation).

Effect of BAP and light on *in vitro* multiplication of shoots

To evaluate the growth *in vitro*, shoots from encapsulated units germinated *in vitro* after 60 days of cultivation were used, which had been previously inoculated in MS culture medium without the presence of growth regulators for 15 days. After this period, the shoots were standardized at 4.0 cm in length and having three pairs of leaves and were inoculated in MS culture medium supplemented with 30 g L⁻¹ sucrose and 7 g L⁻¹ agar. The treatments consisted of a bifactorial combination of four concentrations of BAP in the medium (0.0, 2.5, 5.0, and 10.0 μM) and two types of light source: (i) 40 W white fluorescent lamp with a white light with irradiance of 86 μmol m⁻² s⁻¹ (Osram, Brazil) and (ii) Gro-Lux 40 W fluorescent lamp with a red light with irradiance of 94 μmol m⁻² s⁻¹ (Sylvania, Brazil). The explants were grown at 25 ± 2°C in a photoperiod with 16 h of light. The treatments followed a completely randomized design with a 4x2 factorial arrangement (concentrations of BAP x light sources) with 30 replicates. The variables evaluated after 60 days of *in vitro* cultivation were the number of shoots per explant and the length of the shoots (cm).

Cryopreservation of seeds

For the cryopreservation, the initial water content of the seeds was determined according to the oven method at 105 ± 2°C for 24 h (Brasil, 2009), using five samples with 0.5 g of seeds each. The seeds were immersed in Plant Vitrification Solution (PVS2) for different times (0, 15, 30, 60, 120 and 180 min) and immersed in liquid nitrogen (NL) (-196°C) for 90 min. Subsequently, the seeds were thawed in a water bath (37°C) for 3 min and inoculated in MS culture medium plus 1.0 g L⁻¹ CA. The seeds were kept in a room of growth under photon irradiance of 86 μmol m⁻² s⁻¹ (white fluorescent lamp 40 w, Osram, Brazil), temperature of 25 ± 2°C and photoperiod of 16 h. Each treatment consisted of five Petri dishes, with five seeds per plate. After 30 days of *in vitro* cultivation, the percentage of germination of the seeds was evaluated.

Statistical analysis

The experimental design was completely randomized for all

experiments. Data were submitted to analysis of variance (ANOVA) using the statistical software SISVAR (Ferreira, 2014). According to the results of the ANOVA, data from the qualitative factors were compared by Tukey's test ($P < 0.05$), and data from quantitative factors were analyzed by polynomial regression ($P < 0.05$).

RESULTS

In vitro germination

The largest percentage of germination (78%) of the seeds was obtained in the MS medium containing 1.0 g L⁻¹ activated charcoal (AC) (Figure 1A). The highest mean number of leaves (7.7 leaves) and the largest mean length of seedlings (6.9 cm) were obtained from the inoculation of seeds with the MS medium containing 1.2 and 1.3 g L⁻¹ AC, respectively, according to the fit of the data to a quadratic curve (Figure 1B and C). The number of seedling roots was the only variable with no significant difference ($P < 0.05$) among the concentrations of AC tested.

Encapsulation of lateral buds

The largest percentages of shoot regeneration from encapsulated lateral buds (75%) were observed at a concentration of 8.0 μM BAP (Figure 2).

A significant interaction ($P \leq 0.05$) was observed between the cytokinin types and their concentrations for both the length of the shoots and the number of leaves per shoot regenerated from the encapsulated buds.

For the length of the shoots, the analyses of the cytokinin types within each concentration demonstrated no significant differences between BAP, KIN, and TDZ only at the concentration of 2.0 μM. For the concentration of 8.0 μM, BAP was shown to be the most suitable cytokinin, leading to the formation of shoots with 8.03 cm

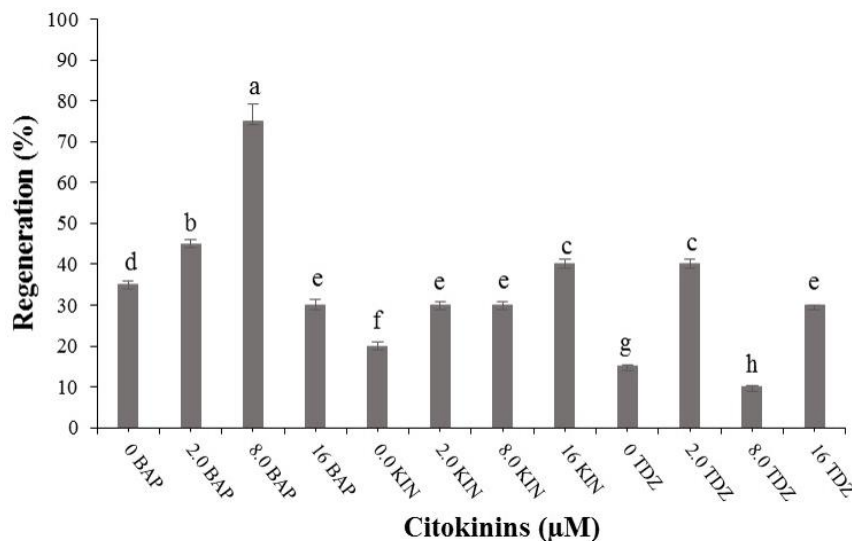


Figure 2. Percentage of shoot regeneration from lateral buds encapsulated in different types and concentrations of cytokinins: BAP, KIN, and TDZ-(0.0, 2.0, 8.0, and 16.0 µM). Means followed by the same lowercase letter within each concentration did not differ among themselves according to the Tukey test ($P \leq 0.05$).

lengths (Figure 3C). Finally, at the concentration of 16.0 µM, both KIN and TDZ were more efficient than BAP, influencing the generation of shoots 6.17 and 6.14 cm in length, respectively (Figure 3E). In the analysis of concentrations within each cytokinin, all presented a significant difference ($P \leq 0.05$). In the MS culture medium containing BAP, the largest length of shoots was 8.0 cm when using 7.9 µM of this cytokinin (Figure 3B). In the media containing TDZ, the largest mean length of the shoots was 6.51 cm in the presence of 11.39 µM of the plant growth regulator (Figure 3F). For both BAP and TDZ, the data fit a negative quadratic trend, unlike the data for the length of the shoots inoculated in medium containing KIN (positive quadratic trend). In the latter case, the smallest length (4.05 cm) was obtained for 7.72 µM KIN (Figure 3D).

For the leaf number of shoots, analysis of the cytokinin types within each concentration showed a significant difference ($P \leq 0.05$) between cytokinins (Figure 4A) only at a concentration of 8.0 µM, with BAP being the most efficient (mean of 4.57 leaves per shoot) (Figure 5). The analysis of the concentrations within each type of cytokinin showed that only BAP showed significant differences among the concentrations used, with the largest mean number of leaves (4.26 leaves) being observed with 8.70 µM, according to the fit of the data to the quadratic curve (Figure 4B).

Effects of BAP and light on *in vitro* shoot multiplication

A significant interaction ($P \leq 0.05$) was observed between

the concentrations of BAP and the light source types only for the leaf number per shoot. For the variables number of shoots and length of shoots, only the concentrations of BAP showed a significant difference at the 5% significance level.

The largest mean number of shoots (3.03 shoots) was obtained in the MS culture medium containing 5.41 µM BAP (Figure 6A). The largest length of shoots was 7.48 cm in the presence of 10.0 µM BAP (Figure 6B).

For the leaf number per shoot, an analysis of the types of light within each concentration of BAP showed that only 5.0 µM BAP generated a significant difference ($P \leq 0.05$) between the two types of light source used, white and Gro-Lux fluorescent lamps, where the Gro-Lux lamps were more efficient in generating shoots with more leaves (mean of 9.2 leaves) than white fluorescent lamps (mean of 6.5 leaves) (Figure 6C). In the analysis of concentrations of BAP within each type of light source, only the Gro-Lux lamp generated a significant difference at the 5% significance level between the concentrations of BAP, with the highest mean leaf number per shoot (8.63 leaves) being obtained in the presence of 5.73 µM cytokinin, according to the data fit to a quadratic curve (Figure 6D).

Cryopreservation of seeds

The water content of the seeds was 11%, and the immersion time in the cryoprotectant solution PVS2 did not affect the survival process. After immersion in liquid nitrogen (LN), the seeds germinated satisfactorily in MS medium containing 1.0 g L⁻¹ AC, reaching a maximum of

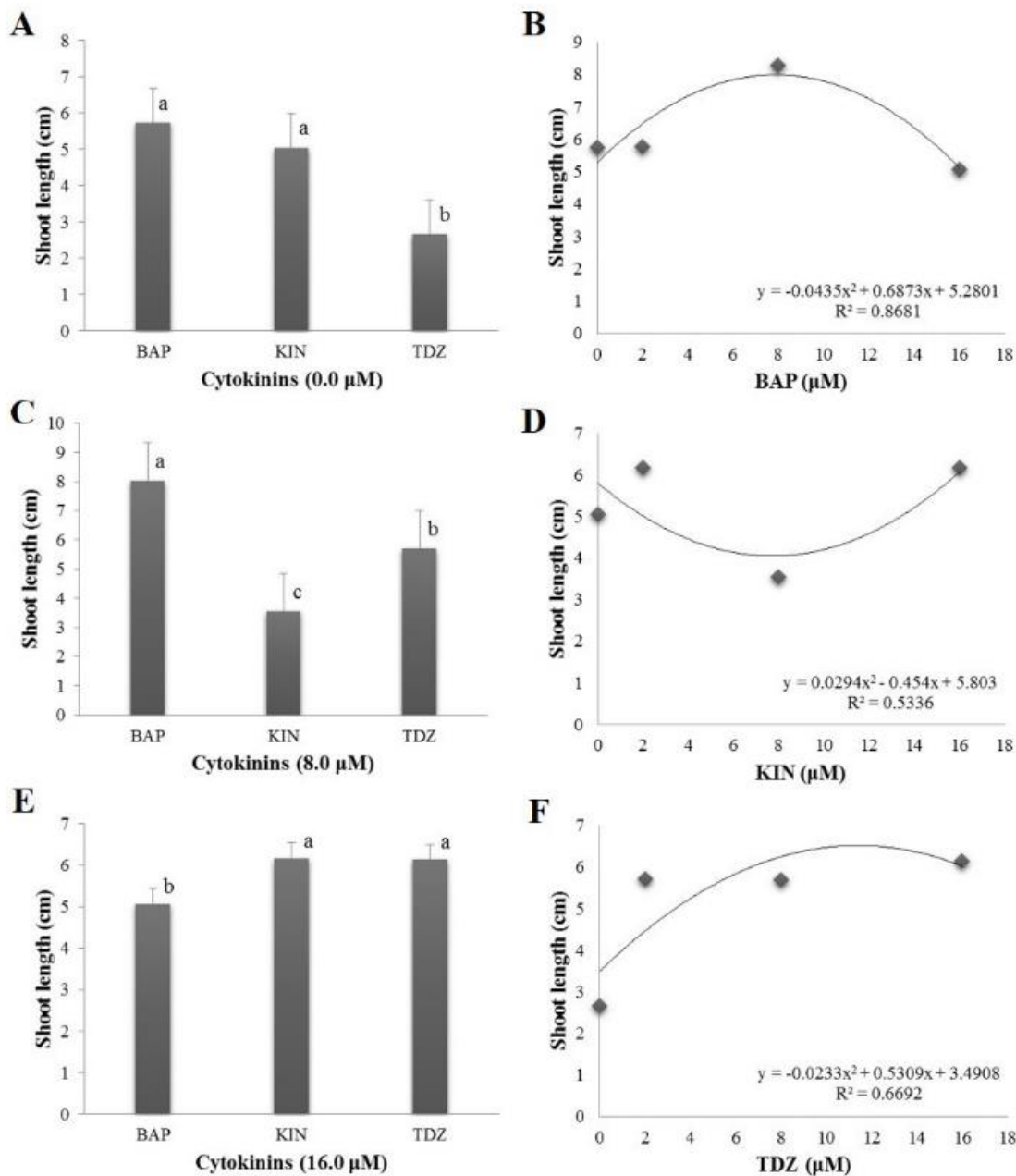


Figure 3. Effect of 0.0 µM (A), 8.0 µM (C), and 16.0 µM (E) cytokinins (BAP, KIN, and TDZ) and different concentrations (0.0, 2.0, 8.0, and 16.0 µM) of BAP (B), KIN (D), and TDZ (F) on the length of the shoots from the encapsulated buds regenerated in MS culture medium after 60 days of cultivation. Means followed by the same letter in columns (A), (C), and (E) do not differ among themselves, according to the Tukey test at 5% significance.

70% germination after 120 min in PVS2 (Figures 7 and 8).

DISCUSSION

Based on the results obtained in this study, we report that AC presented a significant positive effect on the *in vitro*

germination of *M. ligustroides*. This effects related to the physical characteristics of AC, namely its tiny pores that cover a wide area of the culture medium, thus providing a large adsorption capacity (López-Pérez et al., 2015). As a result, a drastic reduction occurs in the oxidation of the phenolic compounds that many species, including *M. ligustroides*, exude (Thomas, 2008). In agreement with the results obtained in this study, *Elaeis guineensis*

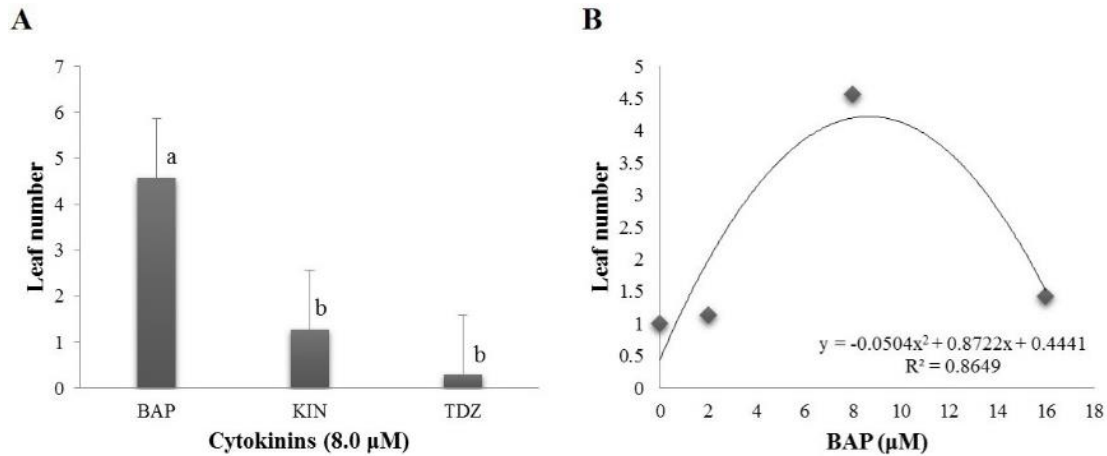


Figure 4. Effect of 8.0 µM BAP, KIN, and TDZ (A) and different concentrations of BAP (0.0, 2.0, 8.0, and 16.0 µM) (B) on the leaf number of shoots from the encapsulated buds regenerated in the MS culture medium after 60 days of cultivation. Means followed by the same letter in columns in (A) do not differ among themselves, according to the Tukey test at 5% significance.

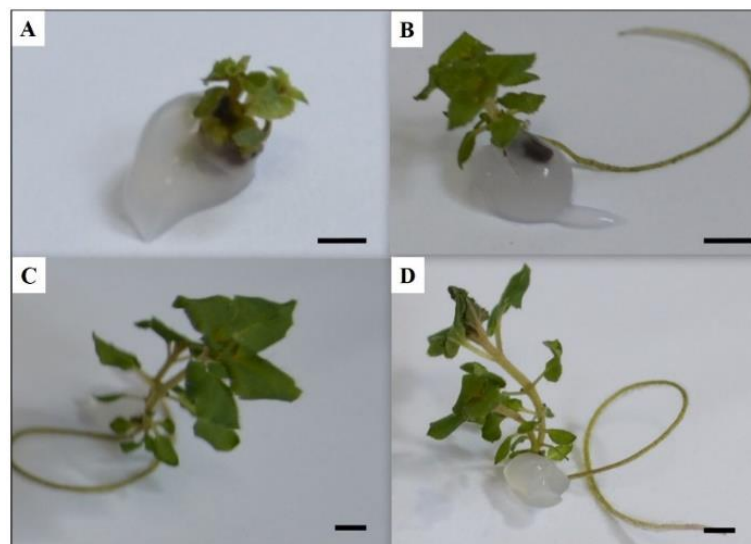


Figure 5. Visual appearance of the lateral buds encapsulated in a sodium alginate matrix in MS culture medium supplemented with 8.0 µM BAP at 30 days (A) bar = 0.5 cm; 45 days, bar = 0.5 cm (B); 60 days, bar = 1.0 cm (C); and 75 days of cultivation *in vitro*, bar = 1.0 cm (D).

embryos grown in MS culture medium supplemented with 2.0 g L⁻¹ AC presented increased development (97%) in comparison to those embryos grown in media without AC (Suranthran et al., 2011).

Once a culture medium that maximizes the germination rate is obtained, the cryopreservation of the plant material in the form of seeds can be tested and widely used (Silva et al., 2013). The effect of the cryoprotectant PVS2 at different immersion times was evaluated after immersion in LN through the vitrification technique; however, no significant difference was observed in the survival of the

seeds. The seeds of *M. ligustroides* have hard coats (Chaves et al., 2013), which may have affected the absorption of the cryoprotectant. The cryoprotectant solution cannot only influence the vitreous state of the internal solutes but can also lead to chemical toxicity (Prada et al., 2015) accelerating the loss of water and causing lethal damage during cryopreservation, mainly for species as *M. ligustroides* which has orthodox seeds and presents low water content (Engelmann and Gonzalez-Arnao, 2013). Therefore, the vitrification technique, without the need for prior immersion in PVS2,

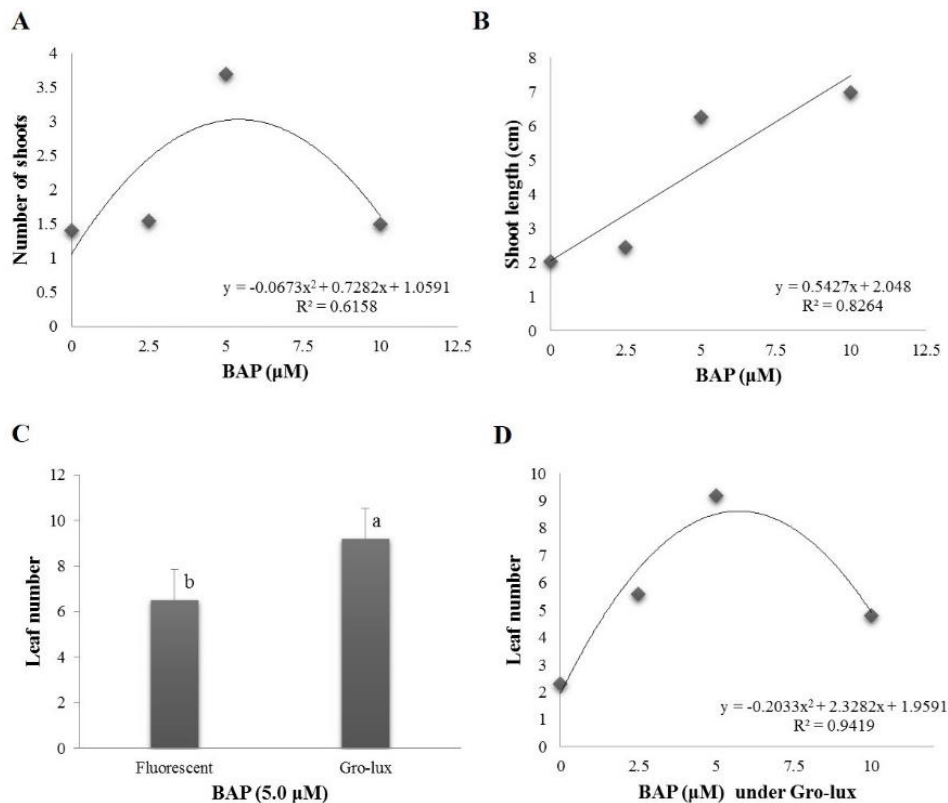


Figure 6. Effect of different concentrations of BAP (0.0, 2.5, 5.0, and 10.0 μM), regardless of the light source type used, on the number of shoots (A) and the length of shoots (B) of *M. ligustroides* after 60 days of *in vitro* cultivation in MS culture medium. Effect of the light source type – white or Gro-Lux fluorescent lamps – in the presence of 5.0 μM BAP on the leaf number per shoot (C) and the influence of different concentrations of BAP (0.0, 2.5, 5.0, and 10.0 μM) in the presence of the Gro-Lux light source on the leaf number (D). Means followed by the same letter in columns in (C) did not differ among themselves, according to the Tukey test at 5% significance.

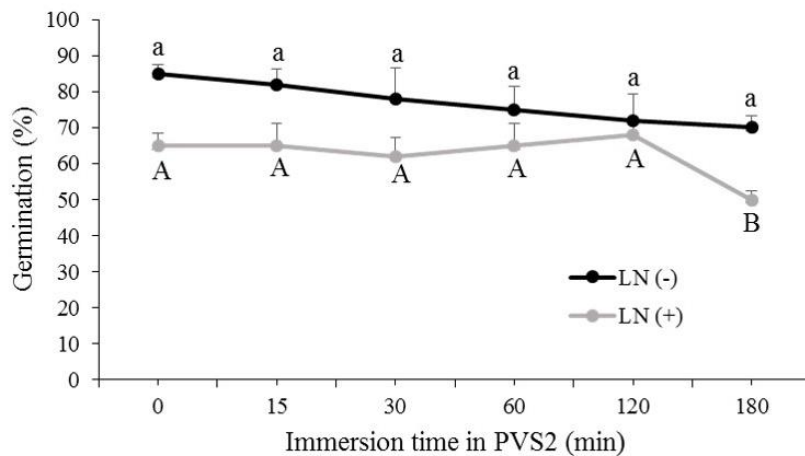


Figure 7. Percentage of germination of cryopreserved (LN +) and non-cryopreserved (LN -) seeds at 30 days of *in vitro* cultivation after exposure to the cryoprotectant solution PVS2 for different times (0, 15, 30, 60, 120, and 180 minutes). Means followed by the same lowercase letter in LN (-) or uppercase in LN (+) do not differ among themselves, according to the Tukey test at 5% significance.

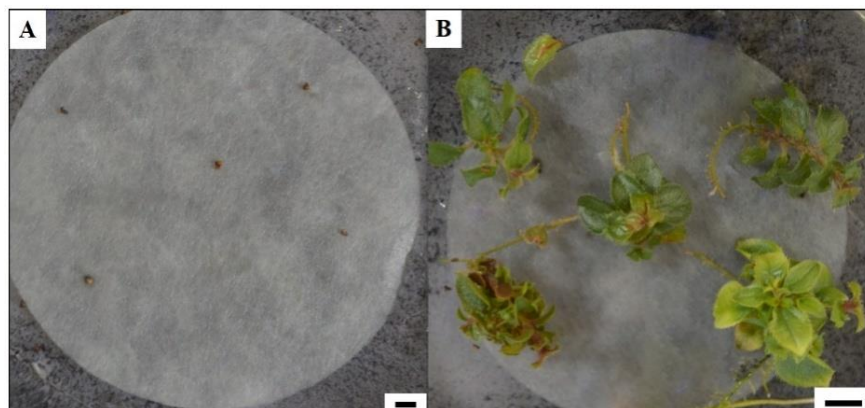


Figure 8. Visual appearance of *M. ligustroides* seeds recently removed from the cryopreservation process (bar= 0.5 cm) (A) and after 60 days of *in vitro* cultivation (bar= 0.5 cm) (B). The seeds were exposed to the cryoprotectant solution PVS2 for 120 min.

presented a high *in vitro* survival rate and can be used in future applications for the species.

Recently, the use of the encapsulation of plant explants in alginate matrix has become a beneficial alternative for *in vitro* multiplication (Fatima et al., 2013). The sodium alginate serves as artificial endosperm and must provide nutrients for the growth of the encapsulated explants (Kumar et al., 2005; Piatczak and Wysokinska, 2013). The addition of growth regulators can improve the development of plant material because the capsules provide a direct contact area with the explant with the small contact surface in test tubes or Petri dishes (Etienne and Berthouly, 2002; Polzin et al., 2014), thus increasing the desired effects.

Among the plant growth regulators used to supplement the culture medium, cytokinins act by inducing the proliferation of auxiliary buds, acting directly on cell division, elongation, and differentiation (Zhang et al., 2011). However, the type and concentration of the cytokinin varied in the *in vitro* multiplication process for each species. In this study, the cytokinin BAP, at a concentration of 8.0 μM , provided the best condition for the growth of encapsulated lateral buds, enabling rupture of the capsules and shoot development at a higher frequency.

The cytokinin TDZ, used in positive regenerative responses in a wide variety of species (Hosseini-Nasr and Rashid, 2002; Yancheva et al., 2003; Matand and Prakash, 2007), acts systematically in responses linked to somatic embryogenesis (Guo et al., 2011; Faisal et al., 2014). The cytokinin KIN presents various biological properties, such as promotion of transcription, cell cycle control, calcium flux, and antioxidant activity (Barciszewski et al., 1999; Verbeke et al., 2000), however, kinetin showed no positive effects for the variables evaluated during the development of *M. ligustroides* lateral buds.

For the shoot multiplication, an interaction was observed between BAP and the different types of fluorescent light (white or Gro-Lux), with the Gro-Lux red light bulbs generating larger values for the aforementioned characteristics. Such results could be related to a higher carbon gain through increased net photosynthetic rate and/or indirectly caused by plant hormonal changes resulting from changes in the light spectrum, which is important for the accumulation of starch and consequently greater investment in young sink organs (Folta, 2004; Rocha et al., 2010). Thus, several authors have studied this wavelength in order to ensure the maximization of the growth and development of plants cultivated *in vitro* (Tanaka et al., 1998; Galdiano Júnior et al., 2012).

Conclusions

The protocols developed here are considered suitable alternatives for the conservation of *M. ligustroides* and production of seedlings for commercialization purposes and use in programs of reintroduction in degraded environments.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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