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Micropropagation of *Cariniana legalis* (Martius) O. Kuntze, an endangered hardwood tree from the Brazilian Atlantic Forest

Micropropagação de *Cariniana legalis* (Martius) O. Kuntze, uma arbórea ameaçada de extinção da Floresta Atlântica Brasileira

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ABSTRACT

The aim of this study was to establish the best conditions for *in vitro* germination, organogenesis and rooting of micropropagated shoots of *Cariniana legalis*. For *in vitro* germination, the effects of Murashige and Skoog (MS) and woody plant medium (WPM) were tested. The effect of different combinations and concentrations of N6-benzyladenine, α-naphthaleneacetic acid, gibberellic acid and polyamines on direct organogenesis were investigated. The indole-3-butyric acid (IBA) was tested for the *ex vitro* rooting of shoots. The WPM culture medium resulted in higher *in vitro* seed germination (66%) and seedling growth compared to MS culture medium (25%). The development of shoots was achieved in WPM culture medium without addition of plant growth regulators (control treatment). The explants from cotyledonary nodal segments provided a greater number and length of shoots compared to apical nodal in the control treatment. *Ex vitro* rooting of micropropagated shoots was achieved in the absence of IBA. A higher root induction was obtained in shoots arising from cotyledonary nodal explants (75%) compared to those from apical nodal explants (50.3%). This work is one of the first describing the establishment of micropropagation in *C. legalis*, an important wood species, and the results are relevant for establishing further plant conservation strategies for threatened tree species.

Keywords: *In vitro* seed germination; organogenesis; cytokinins; polyamines; *ex vitro* rooting.

RESUMO

O objetivo deste estudo foi estabelecer as melhores condições para a germinação *in vitro*, organogênese e enraizamento de brotos micropropagados de *Cariniana legalis*. Para a germinação *in vitro*, os efeitos dos meios de cultura Murashige e Skoog (MS) e Wood Plant Medium (WPM) foram testados. O efeito de diferentes combinações e concentrações de N6-benziladenina, ácido α-naftalenoacético, ácido giberélico e poliaminas sobre a organogênese direta foram investigados. O ácido indolbutírico (AIB) foi testado para o enraizamento *ex vitro* de brotos micropropagados. O meio de cultura WPM resultou em maior germinação de sementes (66%) e crescimento de plântulas comparado ao meio de cultura MS (25%). O desenvolvimento de brotos foi obtido em meio de cultura WPM sem adição dos reguladores de crescimento vegetal (tratamento controle). Os explantes oriundos de segmentos nodais cotiledonares possibilitaram maior número e comprimento dos brotos comparado aos nodais apicais no tratamento controle. O enraizamento de brotos micropropagados foi obtido através de enraizamento *ex vitro* na ausência de AIB. A maior indução de raízes foi obtida em brotos oriundos de explantes nodais cotiledonares (75%) comparado com os explantes nodais apicais (50.3%). Este trabalho é um dos primeiros a descrever o estabelecimento da micropropagação em *C. legalis*, uma importante espécie arbórea, e os resultados são relevantes para o estabelecimento de novas estratégias de conservação para espécies de árvores que estão ameaçadas de extinção.

Palavras chave: Germinação *in vitro*; organogênese; citocininas; poliaminas; enraizamento *ex vitro*.

INTRODUCTION

Cariniana legalis (Martius) O. Kuntze (Lecythidaceae) is a native tree species of the Brazilian Atlantic Forest, and it currently occurs at a population density of less than 1 tree/ha in the forest (Tambarussi et al., 2015). Because of intensive wood exploitation, *C. legalis* is currently included on the red list of endangered species by the International Union for Conservation of Nature (IUCN), and it is classified as vulnerable (Iucn, 2016). Methods of vegetative propagation, such as traditional mini-cuttings or the use of micropropagation as a biotechnological tool, have not been used for this species.

Micropropagation is a viable method for mass propagation, which is the aim of the research that is being performed for many tree species (Pijut et al., 2012). Therefore, an efficient plant regeneration protocol is a prerequisite for the biotechnological breeding

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of economically and ecologically important species. Micropropagation offers a rapid means to produce clonal plants for forestation programs, woody biomass production and the conservation of germplasm (Giri; Shyamkumar; Anjaneyulu, 2004). This technique has many advantages over conventional propagation, such as the rapid multiplication of elite genotypes, the production of disease-free plants, and season-independent propagation, requiring less space in comparison with seed-grown saplings (Shukla; Shukla; Mishra, 2009). In this sense, biotechnological tools, such as *in vitro* propagation, can be applied to propagate endangered tree species such as *C. legalis*.

Signalling agents such as plant growth regulators (PGRs) are used for micropropagation, and cytokinins and auxins are the most important PGRs (Lee et al., 2011). For tree species, N6-benzyladenine (BA) is the cytokinin most often used to promote the development of axillary buds, the breaking of apical dominance and the stimulation of shoot proliferation (Mohebalipour et al., 2012; Nas et al., 2012). Moreover, the auxin α-naphthaleneacetic acid (NAA), combined with cytokinins such as BA and/or thidiazuron, can promote shoot development *in vitro* (Brijwal; Pandey; Tamta, 2015; Sivanesan; Saini; Kim, 2016) by stimulating cell division, cell elongation and shoot elongation (Basuchaudhuri, 2016). In addition, gibberellic acid (GA_3) has long been known to promote vegetative growth, including the initiation of lateral organs and elongation, furthering the development of more robust micropropagated shoots (Fleet; Sun, 2005; Shani; Yanai; Ori, 2006). Furthermore, polyamines (PAs) are multifunctional aliphatic nitrogen polycationic compounds that interact with macromolecules such as DNA, RNA, phospholipids, cell wall components and proteins (Baron; Stasolla, 2008) . PAs have been implicated in several physiological processes in plants, including organogenesis in tree species (Parimalan; Giridhar; Ravishankar, 2011; Aragão et al., 2016).

Auxins also have a direct effect on adventitious root induction, and indole-3-butyric acid (IBA) is most often used to promote *in vitro* or *ex vitro* rooting in different tree species (Pijut et al., 2012) because of its higher root-inducing capacity and greater stability in response to light (Pacurar; Perrone; Bellini, 2014). In relation to *in vitro* rooting, *ex vitro* rooting is an important step in the establishment of propagation methodologies, and it reduces the costs and the time needed for micropropagation (Ranaweera; Gunasekara; Eeswara, 2013). Another relevant advantage of *ex vitro* rooting is that the plantlets produced do not require any additional acclimatization steps; they exhibit lateral roots similar to the natural root system and achieve better survival in comparison with those that are developed from *in vitro* rooting (Yan et al., 2010; Phulwaria et al., 2012).

Thus, the aim of this study was to establish the best conditions for *in vitro* seed germination, organogenesis development from apical and cotyledonary nodal explants and rooting induction in micropropagated shoots of *C. legalis*.

MATERIAL AND METHODS

Plant material

Mature *C. legalis* seeds were obtained from the Sementes Caiçara nursery, being collected from trees of a natural area located in Brejo Alegre, SP, Brazil (21º10'S and 50º10'W). Seeds were germinated *in vitro* (Figure 1A), and 90-day-old seedlings (Figure 1B) were used as the source of apical and cotyledonary nodal explants in the organogenesis experiments (Figure 1C). Sixty-day-old micropropagated shoots (Figure 1D) were used for the rooting experiments (Figure 1E-F).

Seed disinfestation and germination

Prior to inoculation, the seeds were surface-disinfected according to Aragão et al. (2016). Following, the seeds were cultured on culture medium of Murashige and Skoog (MS) (Murashige; Skoog, 1962) (Phytotechnology Lab, Overland Park, KS, USA) and Woody Plant Medium (WPM) (Lloyd; Mccown, 1980) (Phytotechnology Lab), both supplemented with 20 g L^{-1} sucrose (Merck), 1.5 g L^{-1} activated charcoal (Sigma-Aldrich, St. Louis, MO, USA) and 2 g L^{-1} Phytagel® (Sigma-Aldrich). The pH of each culture medium was adjusted to 5.7; the medium was distributed into culture tubes (150 x 25 mm; 10 mL per tube), and then it was autoclaved at 121 °C for 15 min. The seeds were inoculated in the culture tubes and incubated in a growth room with a 16-h photoperiod at a light intensity of 22 µmol

$m^2 s^{-1}$, at 25 ± 2 °C. Twenty replicates with five seeds per replicate were performed. The germination percentage was evaluated throughout the culture period over 10 weeks.

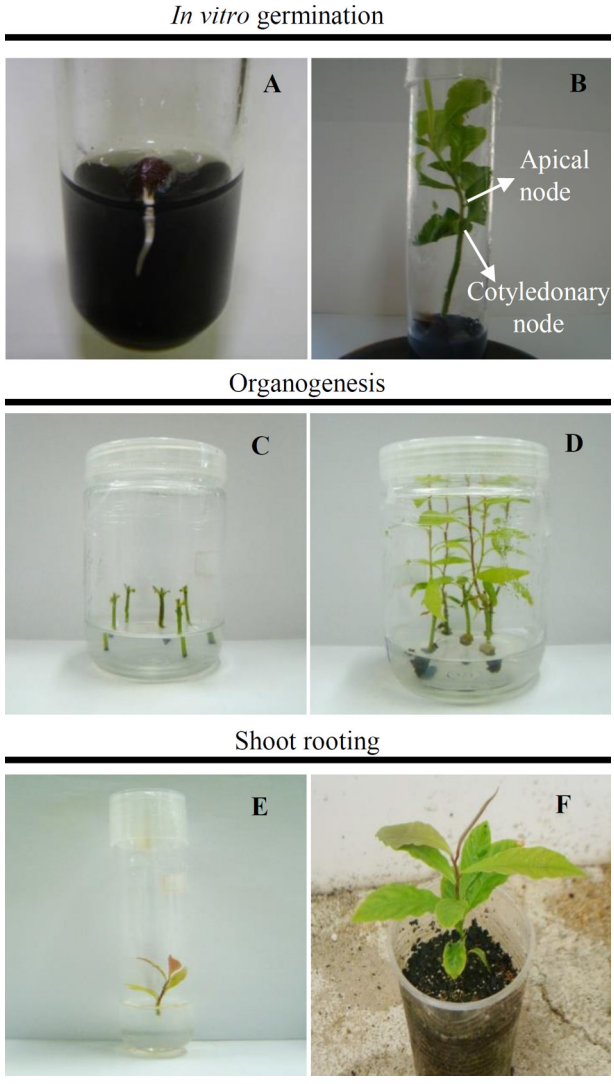

Figure 1 – Morphological aspects during the micropropagation of *C. legalis* with *in vitro* seed germination after 3 weeks of culture in WPM culture medium (A), ninety-day-old seedlings used as a source of apical and cotyledonary nodal explants (arrows) (B), nodal explants obtained from seedlings germinated *in vitro* (C), sixty-day-old shoots obtained *in vitro* arising from apical and cotyledonary nodal explants (D), shoots transferred to WPM culture medium supplemented with IBA (0, 250, 500 and 1000 µM) under *in vitro* conditions, during 7 days of incubation (E) and the *ex vitro* rooting of micropropagated shoots, which produced somatic plantlets after 60 days (F).

The effects of cytokinins, auxins and gibberellin on shoot development

Ninety-day-old *C. legalis* seedlings that were germinated *in vitro* were used as a source of explants. Apical and cotyledonary nodal explants (± 2 cm) were isolated and inoculated on WPM supplemented with 20 g L^{-1} sucrose, 1.5 g L^{-1} activated charcoal (Sigma-Aldrich) and 2 g L^{-1} Phytagel and different concentrations of the PGRs (all from Sigma-Aldrich) as follows: BA (0, 5 and 10 µM), NAA (0 and 5 µM) and GA_3 (0 and 10 µM), which were combined or not. The experiment consisted of nine treatments: control (without any PGRs), 5 µM BA, 10 µM BA, 5 µM BA + 10 µM GA_3, 10 µM BA + 10 µM GA_3, 10 µM GA_3, 5 µM BA + 5 µM NAA, 10 µM BA + 5 µM NAA, and 5 µM NAA. The pH of the culture medium was adjusted to 5.7 and distributed into culture tubes (10 mL per tube), and the tubes were autoclaved for 15 min at 121 °C. After inoculation, the explants were incubated in a growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25±2 °C. Each treatment consisted of six replicates, and each replicate consisted of five explants. The shoot induction (%), number of shoots per explant and shoot length (cm) were analysed after 60 days of culture.

Effect of PAs on shoot development

The effects of the PAs putrescine (Put), spermidine (Spd) and spermine (Spm) on shoot development were investigated using apical and cotyledonary nodal explants (± 2 cm) isolated from 90-day-old *C. legalis* seedlings germinated *in vitro*. The explants were inoculated on WPM, supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel and different concentrations (0, 0.5, 1, 2.5 and 5 mM) of each PA (Put, Spd or Spm) (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.7 and autoclaved at 121 °C for 15 min. The PAs were incorporated into the medium by filter sterilization using a 0.22-µm filter (JetBio-Filtration®, Guangzhou, China) under laminar flow. The culture medium was distributed into culture pots (150 mL) (Aapace, São Paulo, Brazil) containing 30 mL of culture medium per pot.

After the inoculation, the explants were incubated in the growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25±2 °C. Each treatment consisted of eight replicates, and each replicate consisted

of one culture pot containing five explants. The shoot induction (%), number of shoots per explant and shoot length (cm) were recorded after 60 days of culture.

Effect of IBA on shoot rooting

Sixty-day-old shoots (0.5-1.0 cm length) arising from apical and cotyledonary nodal explants that were cultured on WPM in the absence of PGRs were inoculated into WPM culture medium and supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel and different concentrations (0, 250, 500 and 1000 µM) of IBA (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.7 and distributed into culture tubes (10 mL per tube), followed by autoclaving for 15 min at 121 °C. Following inoculation, the explants were incubated in a growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25 ± 2 °C; these conditions were maintained over 7 days for root induction.

After this time, the shoots were transferred to *ex vitro* environment conditions, where they were placed in plastic pots (20 mL) containing a 1:1 mixture (v/v) of PlantMax® (DDL Agroindústria, Paulínia, Brazil) and vermiculite for root development. These shoots were maintained in plastic trays covered with a plastic film to maintain the high humidity needed for root development. The shoots were kept in the grow room under the same conditions described above. After 15 days of incubation, the humidity was gradually reduced until 25 days, when the explants were exposed to the grow room atmosphere, and they were considered acclimatized. Each treatment consisted of eight replicates, and each replicate comprised four shoots. The root induction (%), number of roots per shoot and root length were analysed after 60 days of culture.

Statistical analysis

All experiments were performed using a completely randomised design. The data were analysed with analysis of variance (ANOVA) ($p < 0.05$) followed by a Student-Newman-Keuls (SNK) test (Sokal; Rohlf, 1995) in the R program (R Foundation for Statistical Computing, version 3.1.1, 2014, Vienna, Austria).

RESULTS AND DISCUSSION

The percentage of *C. legalis* seed germination was higher in WPM (66%) than MS (25%) culture medium after 9 weeks of culture (Figure 2).

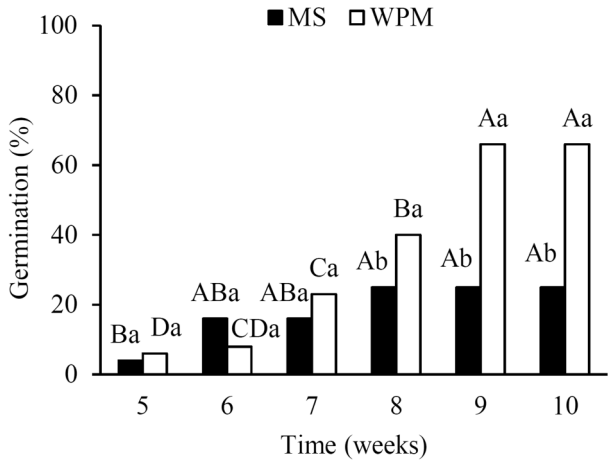

Figure 2 – *In vitro* seed germination (%) of *C. legalis* in MS and WPM culture media after 5, 6, 7, 8, 9 and 10 weeks of culture. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters indicate significant differences among different weeks in the same culture medium. Lowercase letters indicate significant differences between MS and WPM culture media in the same week of culture.

Radicle protrusion, which corresponds to germination, started after 5 weeks of culture in both culture media and stabilised after 9 weeks. *In vitro* seed germination is an important step for obtaining aseptic explants needed for micropropagation studies. Similar results have been reported for other tree species, such as *Ilex paraguariensis* and *Hancornia speciosa* (Zaniolo; Zanette, 2001; Soares et al., 2009), in WPM. These results may be related to the formulation of WPM (Lloyd; Mccown, 1980) , which contains only 25% of the nitrate and ammonium ion concentrations of MS culture medium and consequently has a low concentration of total nitrogen (Rocha et al., 2007). However, WPM has higher levels of potassium and sulphate ions (Bertozzo; Machado, 2010). The combinations of nutrients in WPM may be important for the seed germination of select species, including *C. legalis*.

In vitro shoot development in *C. legalis* can occur without the use of PGRs (BA, NAA or GA_3), and the different types and concentrations of PGRs in use did not induce significant differences in the induction, number and length of shoots compared with the control treatment (Figure 3). Moreover, the cotyledonary nodal explants were more responsive than the apical nodal

ones, showing a greater number of shoots per explant (Figure 3B) and greater shoot lengths (Figure 3C) in all the tested treatments. Therefore, the GA_3 concentrations used, which were combined or not combined with BA, affected the shoot induction, number and length of shoots in a negative manner (Figure 3).

The effects of cytokinins and auxins, whether combined or not, have been investigated in several tree species (Park et al., 2008; Singh et al., 2014; Almeida et al., 2015; Brijwal; Pandey; Tamta, 2015; Aragão et al., 2016; Patil et al., 2016). The greatest number and length of shoots in *Berberis aristata* were recorded in WPM supplemented with 8.88 µM BA in combination with 2.68 µM NAA (Brijwal et al., 2015). Similarly, the maximum induction, number and length of shoots for *Shorea robusta* were obtained in WPM supplemented with 1.0 $mg \cdot L^{-1}$ (4.44 µM) BA combined with 0.5 $mg \cdot L^{-1}$ (2.68 µM) NAA (Singh et al., 2014). Additionally, the 7.5

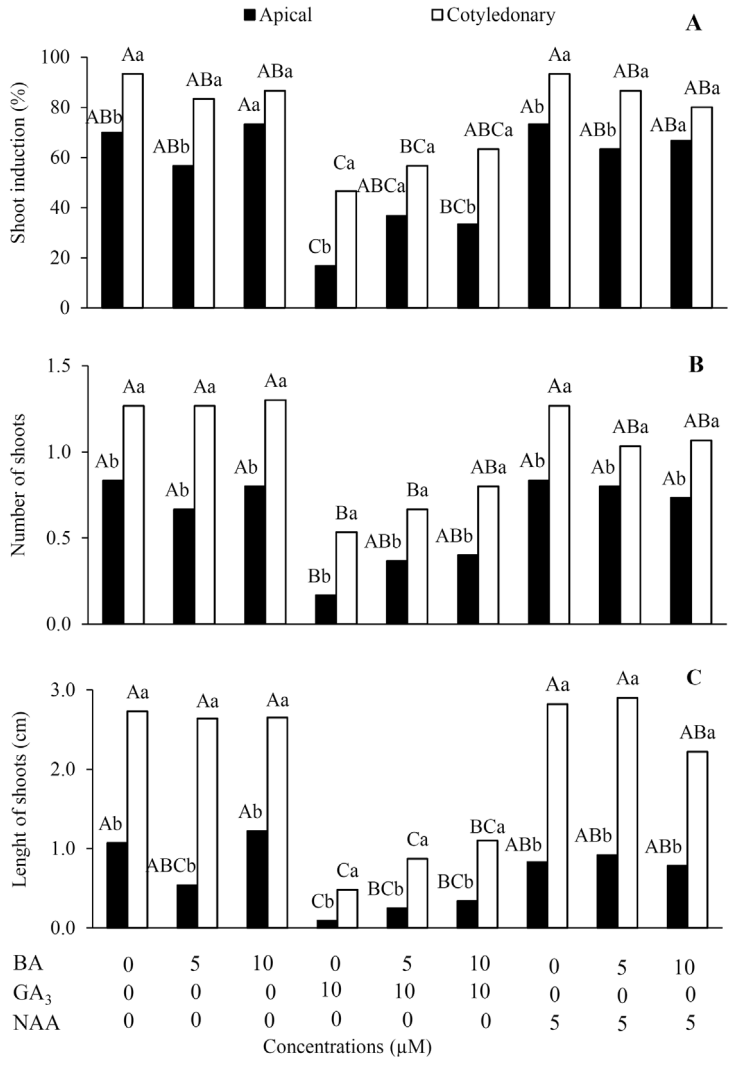

Figure 3 – Induction (A), number (B) and length (C) of shoots developed from apical and cotyledonary nodal explants of *C. legalis* under the influence of the plant growth regulators N6-benzyladenine (BA), α-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3) after 60 days of culture. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences between the different types of explants in the same treatment.

µM BA treatment combined with 0.4 µM NAA in MS culture medium promoted a greater number of shoots in *Aegiphila verticillata* (Almeida et al., 2015). The 0.1 µM NAA concentration combined with 10 µM BA was sufficient for promoting a greater number of shoots in *Acacia ehrenbergiana*, and any increase in the NAA concentrations beyond this optimum concentration will induce a detrimental effect on the number of shoots (Javed et al., 2013). Otherwise, the use of BA alone in WPM promoted the best results in some tree species, such as greater shoot induction in *Salix pseudolasiogyne* with the addition of 2.2 µM BA (Park et al., 2008) and a higher shoot induction and maximum number of shoots per explant in *Garcinia xanthochymus* when using 20 µM BA (Patil et al., 2016). According to Javed et al. (2013), the low concentration of auxin required to promote the greater number of shoots in *Acacia ehrenbergiana* indicates the higher endogenous contents of auxin in the explant. Thus, it is necessary to have a higher concentration of exogenous cytokinin to promote an adequate balance between auxin and cytokinin for the development of shoots. In addition, the endogenous and exogenous cytokinin/auxin balance is necessary for the development and elongation of shoots in *Tectona grandis*, a tree species (Shirin; Rana; Mandal, 2005). In this sense, it is possible that the endogenous contents of auxins and cytokinins in *C. legalis* explants can be equilibrated at a rate that can promote shoot development without PGR addition.

In this study, the supplementation of the culture medium with 0.5 mM Spd increased shoot induction significantly (95%) compared to the control (85%) in apical nodal explants (Figure 4A).

PAs are important for seed germination, zygotic and somatic embryogenesis (Santa-Catarina et al., 2006; Santa-Catarina et al., 2007; Dias et al., 2009; Pieruzzi et al., 2011; Dutra et al., 2013; Vondráková et al., 2015) and shoot regeneration (Parimalan; Giridhar; Ravishankar, 2011; Arun et al., 2014; Aragão et al., 2016). Effects of Spd on shoot development have been observed in other species. In *Cucumis sativus,* greater shoot induction was obtained from apical nodal explants incubated with BA in combination with leucine and Spd (Vasudevan et al., 2008). Exogenous Spd combined with BA increased shoot induction from cotyledonary nodal explants in *Glycine max* (Arun et al., 2014). In addition, Spd provides a nitrogen source to enhance shoot differentiation, as suggested for *C. sativus* (Vasudevan et al., 2008) and *G. max* (Arun et al., 2014). Thus, exogenous Spd can contribute to the increase in the shoot induction, possibly acting directly on the metabolism of nitrogen, and in the cell cycle, increasing the mitotic divisions necessary for the development of morphogenetic processes. Although the shoot induction increased with exogenous 0.5 mM Spd,

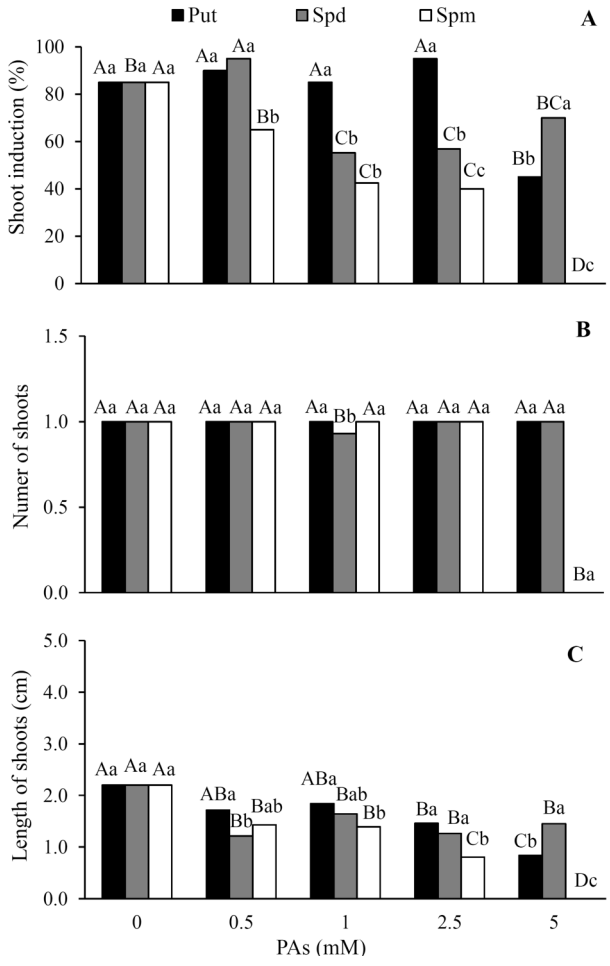

Figure 4 – Induction (A), number (B) and length (C) of shoots developed from apical nodal explants of *C. legalis* under the influence of the polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) after 60 days of culture. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences among the different PAs in the same treatment.

the number and length of shoots were not affected by the addition of different PAs from apical nodal explants (Figure 4 and 5) in *C. legalis*, leading to shoot development without exogenous PA supplementation. In addition, in cotyledonary nodal explants, the induction of shoots (Figure 5A) and the number (Figure 5B) and length (Figure 5C) of shoots were also not affected significantly by adding different PAs.

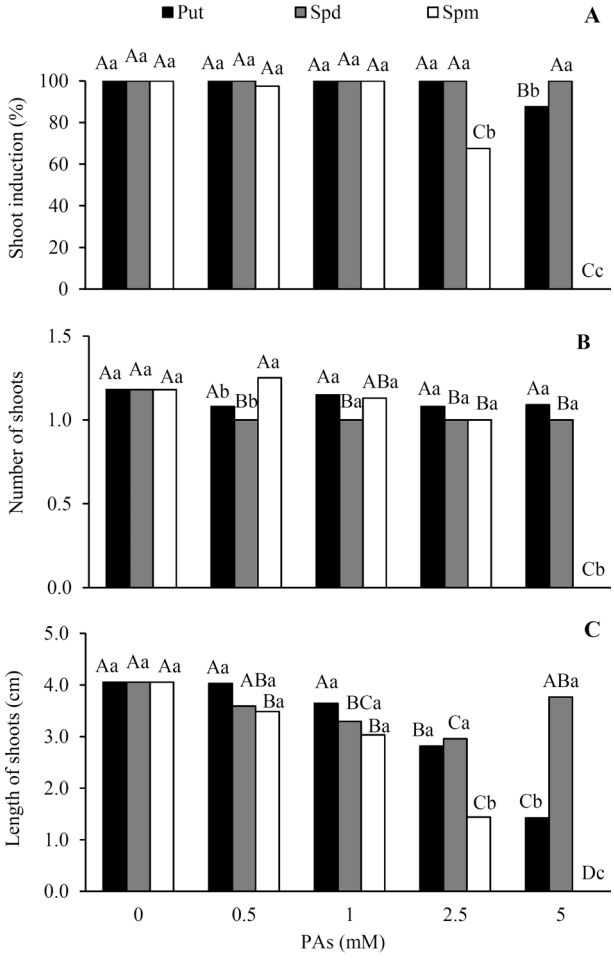

Figure 5 – Induction (A), number (B) and length (C) of shoots developed from cotyledonary nodal explants of *C. legalis* under influence of the the polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) after 60 days of culture. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences among the different PAs in the same treatment.

In this sense, the morphogenetic response of the explant to the exogenous application of different PGRs depends on the endogenous balance of the hormones in the plant tissue (explant), which, in turn, varies with the organ or plant species (Chand; Singh, 2004). Thus, our results showed that direct organogenesis in *C. legalis* can be achieved without PGRs using apical and cotyledonary nodal tissue as a source of explants, with cotyledonary nodal explants being more responsive in terms of the induction, number and length of shoots than the apical tissues (Figure 3).

In preliminary experiments, *in vitro* rooting was performed in *C. legalis* using different concentrations (0, 5, 10 and 20 µM) of IBA, with only 7.7% rooting in the 10 µM IBA treatment (data not shown). Thus, we have developed a rooting system for *C. legalis* using an *in vitro* approach for root induction and an *ex vitro* approach for root development. After 7 days of *in vitro* incubation with different IBA concentrations following 60 days of incubation under *ex vitro* conditions, greater root induction was obtained in shoots arising from cotyledonary (75%) than apical (50.3%) nodal explants in the control treatment (Figure 6A). In addition, a higher number of roots per shoot was observed in the control treatment for both explant types (Figure 6B). Moreover, a higher root length was recorded in shoots arising from cotyledonary nodal explants, and no significant differences were observed between the control (1.84 cm) and 250 µM IBA (1.93 cm) treatments (Figure 6C). Thus, shoots from cotyledonary nodal explants may have had sufficient endogenous auxin content to promote the induction and development of adventitious roots, and the auxin-regulated responses depend on the different levels of this hormone in plants (Pacurar; Perrone; Bellini, 2014).

Ex vitro rooting is more effective than *in vitro* rooting because rooted *ex vitro* plantlets do not require any additional acclimatization step prior to transplantation to regular greenhouse conditions (Yan et al., 2010). Additionally, *ex vitro* rooting has been successfully applied to tree species, such as *Embelia ribes* (Dhavala; Rathore, 2010), *Tecomella undulata* (Varshney; Anis, 2012), *Albizia lebbeck* (Perveen; Anis; Aref, 2013) and *Leptadenia reticulata* (Patel et al., 2014). This system is promising because it can reduce the labour and time needed for somatic plantlet establishment from the laboratory to the soil, which is helpful for reducing manipulation costs (Martin,

2003). It is estimated that *ex vitro* rooting reduces the costs of a micropropagation system by up to 71% (Ranaweera; Gunasekara; Eeswara, 2013). Therefore, our results showed that *ex vitro* rooting without IBA can be a viable alternative for the rooting of micropropagated *C. legalis* shoots.

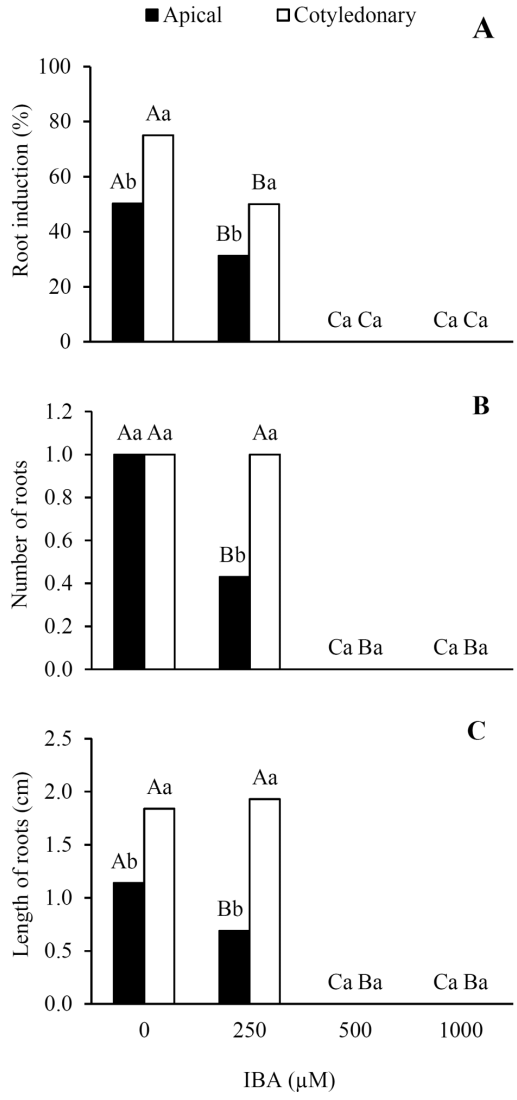

Figure 6 – Induction (A), number (B) and length (C) of roots in micropropagated shoots arising from apical and cotyledonary nodal explants of *C. legalis* after 60 days of *ex vitro* incubation. Different IBA concentrations were applied over 7 days of *in vitro* incubation. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences between the different types of explants in the same treatment.

CONCLUSIONS

This work is one of the first describing the micropropagation research in *C. legalis*, an endangered native hardwood tree species from the Brazilian Atlantic Forest. The best seed germination rate was obtained with WPM culture medium compared to MS. Shoot development was achieved in WPM without adding PGRs, and cotyledonary nodal explants provided greater responses than apical nodal explants. Micropropagated shoots can be rooted *ex vitro* in the absence of IBA. These results are important for establishing further plant conservation strategies for endangered woody species.

ACKNOWLEDGEMENTS

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