

**MICROPROPAGATION OF *Aspidosperma polyneuron* Müll. Arg. FROM *IN VITRO* GERMINATED SEEDLINGS**

MICROPROPAGAÇÃO DE *Aspidosperma polyneuron*. Müll. Arg. A PARTIR DE PLÂNTULAS GERMINADAS *IN VITRO*

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**ABSTRACT**

In this study, an efficient method for regenerating plants from nodal cultures of seedlings was developed for *Aspidosperma polyneuron*. Mature seeds were surface-sterilized and embryos were germinated in Woody Plant Medium (WPM). Epicotyl and hypocotyl nodal segments, excised from 3-week-old *in vitro*-grown seedlings, were cultured in WPM medium supplemented with 6-benzyladenine (BA) (2.5, 5.0, and 10  $\mu$ M), alone or combined with indole-3-butyric acid (IBA) or  $\alpha$ -naphthaleneacetic acid (NAA) (0.5  $\mu$ M) for culture initiation and three subcultures. For root induction, IBA (2.5, 5.0, and 10 mM) pulse treatments (15 minutes) were initially applied, followed by transfer to growth regulator-free WPM for five weeks. Regenerated plants were first transplanted to trays containing soil and Plantmax® substrate (3:1) and later to polyethylene bags for acclimatization in a greenhouse. Hypocotyl explants exhibited superior shoot regeneration rate and rooting percentages compared with those of epicotyl explants. The highest numbers of shoots per explant (7–8) were obtained at the third subculture when using the culture medium supplemented with 10  $\mu$ M BA. When combined with BA, the addition of 0.5  $\mu$ M IBA or NAA did not affect the regeneration of shoots. An IBA pulse treatment of 5–10 mM for 15 minutes induced 60% of rooting. The regenerated plants were acclimatized and successfully established in a greenhouse, with a 90% survival rate observed after three months. Based on the results of this study, the micropropagation of *Aspidosperma polyneuron* from *in vitro* seedlings is feasible and could be a useful tool for the conservation and propagation of this important endangered species.

**Keywords:** forestry species; juvenile explant; multiplication; rooting.

**RESUMO**

Foi desenvolvido um método eficiente de regeneração de plantas para *Aspidosperma polyneuron* a partir de culturas nodais de plântulas. Sementes maduras foram desinfestadas e os embriões germinaram em meio de cultura *Woody Plant Medium* (WPM). Segmentos nodais do epicótilo e hipocótilo retirados de plântulas de três semanas crescendo *in vitro* foram cultivados em meio WPM, suplementado com 6-benziladenina (BA) (2,5; 5,0 e 10  $\mu$ M), isolado ou combinado com ácido indol-3-butírico (AIB) ou ácido  $\alpha$ -naftalenoacético (ANA) (0,5  $\mu$ M) durante o cultivo inicial e três subcultivos. Para indução de raízes, inicialmente, foram testados tratamentos-pulso de AIB (2,5; 5,0 ou 10 mM) por 15 minutos, seguidos de transferência para meio WPM, sem regulador de crescimento por cinco semanas. Plantas regeneradas foram transplantadas

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para bandejas contendo solo e substrato Plantmax® (3:1) e depois transferidas para sacos plásticos para aclimatização em casa de vegetação. Os explantes do hipocótilo apresentaram resultados superiores de regeneração de brotações e enraizamento, quando comparados com os do epicótilo. Os maiores números de brotações por explante (7-8) foram obtidos em meios de cultura, suplementados com 10  $\mu\text{M}$  de BA, no terceiro subcultivo. A adição de 0,5  $\mu\text{M}$  de AIB ou ANA combinada com BA não influenciou a regeneração de brotações. O tratamento-pulso de AIB (5-10 mM) por 15 minutos induziu 60% de enraizamento. As plantas regeneradas foram aclimatizadas e bem estabelecidas em casa de vegetação com 90% de sobrevivência, após três meses. Baseado nos resultados desse estudo, a micropropagação de *Aspidosperma polyneuron* a partir de plântulas cultivadas *in vitro* é viável e pode ser uma boa opção para conservação e propagação dessa espécie importante que está em extinção.

**Palavras-chave:** espécie florestal; explante juvenil; multiplicação; enraizamento.

## INTRODUCTION

*Aspidosperma polyneuron* Müll. Arg. (peroba-rosa) is a native species of the Atlantic Forest, and ranges from the southwest of Mato Grosso do Sul state to Argentina (CARVALHO, 1994). In the past, it was overexploited because of the high quality and value of its wood; however, in the beginning of 1990s, it was declared an endangered species (HATSCHBACH; ZILLER, 1995). The natural genetic populations of *Aspidosperma polyneuron* have been become smaller because of the selective exploitation of superior genotypes and the expansion of the agriculture (BRUNE; MELCHIOR, 1976). The propagation of this tree species is limited, because of its seasonal seed production, irregular seed germination, and the difficulty of collecting seeds (CARVALHO, 1994). The seeds of *Aspidosperma polyneuron* display polyembryony, i.e., they commonly show more than one developed embryo, and smaller ones exhibit different sizes and stages of development. Although the occurrence of polyembryony in *Aspidosperma polyneuron* has not been completely investigated yet (SOUZA; MOSCHETTA, 1992), it is possible that the additional embryos could be identical to the mother plant; thus, the mass multiplication of an elite tree could be feasible using mature seeds.

Slow growth and poor adventitious rooting of cuttings are two of the problems that prevent the mass propagation of *Aspidosperma polyneuron*. Most commercial forestry species are preferably propagated using seeds of superior genotypes obtained from seed orchards (when such seeds are available). However, for mostly forestry improvement programs, the lengthy growth cycle, size of trees and seasonal seed production are major obstacles for success (GROSSNICKLES; CYR; POLONENKO, 1996). For these reasons, a protocol for the *in vitro* propagation of *Aspidosperma polyneuron* would provide an alternative method of propagation for trees with superior phenotypic characteristics. Ribas et al. (2003) obtained aseptic cultures using apical shoots from two-year-old *Aspidosperma polyneuron* seedlings collected in a greenhouse in each of the seasons during one year. Afterwards, a micropropagation protocol using these explants was established for the species (RIBAS et al., 2005). The objective of the present study was to determine a protocol for the *in vitro* propagation of *Aspidosperma polyneuron* using epicotyl and hypocotyl explants obtained from *in vitro* germinated seeds.

## MATERIAL AND METHOD

### Plant material and culture conditions

Mature seeds were collected in July from open pollinated trees in natural stands of the São Paulo Forestry Institute headquarters, Brazil. The seeds were surface sterilized in 1% (v/v) sodium hypochlorite plus 0.1% Tween 20 for 20 minutes and then rinsed six times with sterile distilled water. The seed coats were removed aseptically, and embryos were cultured on Woody Plant Medium (WPM) as the basal medium (LLOYD; MCCOWN, 1980) for *in vitro* germination. The WPM was supplemented with sucrose 3% (w/v) and agar Micromed® (0.65%) (w/v). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Cultures were incubated at 25  $\pm$  2°C in a 16-h photoperiod, which was provided by cool-white fluorescent light that gave a photon flux density of 40  $\mu\text{mol.m}^{-2} \cdot \text{s}^{-1}$ . After three weeks, the seedlings were

excised and separated into two portions: hypocotyl segment including the cotyledonary nodes and epicotyl segment with the apex.

### Shoot induction

Hypocotyl and epicotyl explants (2 cm in length) were transferred to WPM supplemented with 6-benzyladenine (BA) (0, 2.5, 5.0, and 10.0  $\mu\text{M}$ ) alone or combined with indole-3-butyric acid (IBA) or  $\alpha$ -naphthaleneacetic acid (NAA) (0.5  $\mu\text{M}$ ). The number of shoots per explant was evaluated at monthly intervals. Each monthly subculture of shoots was designated as the first-subculture ( $S_1$ ), second-subculture ( $S_2$ ), and third-subculture ( $S_3$ ). The initial stage of induction of shoots on hypocotyl and epicotyl explants was designated as culture initiation ( $S_0$ ).

Each treatment had six replications for epicotyls and eight for hypocotyls, with five explants per replication. ANOVA was used to compare the mean number of shoots per epicotyl and hypocotyl explants, and the F test was applied for orthogonal contrasts.

### *In vitro* rooting

Adventitious roots were induced on individual shoots (2 cm in height) with a double bevel cut at the base. These shoots had previously been subcultured in WPM, with 0.1% activated charcoal but without growth regulators, for six weeks. Subsequently, the shoot base (0.5 cm) was subjected to a pulse-treatment in the aqueous solution of 0, 2.5, 5, or 10 mM IBA for 15 minutes. The IBA solutions were prepared using 20% ethanol and were filter-sterilized. Shoots were cultivated on WPM in the dark for 8 days and then transferred to light (40  $\mu\text{mol. m}^{-2}\text{s}^{-1}$  and a 16 hr-photoperiod) for five weeks. The percentage of rooted shoots was evaluated after six weeks.

Four replications were conducted, with five flasks per replication. Each flask had four shoots and the experiment was repeated twice. The experimental design was completely randomized and the mean rooting percentages were compared using ANOVA and Tukey's test. The effect of IBA concentrations on rooting was analyzed with linear regression.

### Transplanting and acclimatization

Plants (2-3 cm in height) were transplanted to trays where they were planted singly in plastic cell-packs that held mixtures of previously sifted soil and Plantmax substrate (3:1). Plants were cultivated in a commercial greenhouse model Van der Hoeven. The percentage of plant survival was evaluated after one month. After three months, the plants were transplanted into polyethylene bags containing the same substrate used for transplanting.

## RESULTS AND DISCUSSION

### Plant material, culture conditions and *in vitro* germination

The seed disinfection procedure was efficient (90% survival) when seeds were treated with 1% sodium hypochlorite for 20 minutes. Seed germination was initiated after five days, and after three weeks of *in vitro* growth in the WPM medium, the seedlings reached a height of approximately 8 cm (Figure 1 A). In previous studies, WPM was also efficient for *in vitro* germination of other forest species, including *Quercus semecarpifolia* (TAMTA et al., 2008) and *Amburana cearensis* (CAMPOS et al., 2013), which were produced as explants for shoot multiplication.

### Shoot induction

The mean number of shoots from epicotyl explants was not significantly affected by the presence

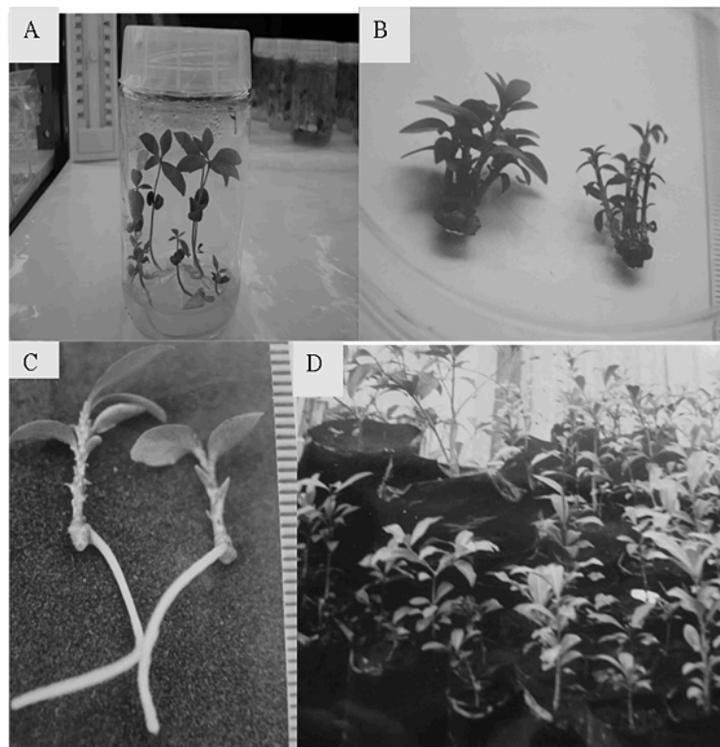


FIGURE 1: Micropropagation of *Aspidosperma polyneuron*. (A) Seedlings germinated in WPM medium for three weeks. (B) Shoots from the third subculture developing in WPM medium supplemented with 10  $\mu\text{M}$  BA. (C) Rooted shoots treated with 5 mM IBA for 15 minutes and then transferred to WPM medium without plant growth regulator for six weeks. (D) Acclimatized plants grown in a greenhouse for three months.

FIGURA 1: Micropropagação de *Aspidosperma polyneuron*. (A) Plântulas germinadas em meio WPM, após três semanas. (B) Brotações do terceiro subcultivo se desenvolvendo em meio WPM, suplementado com 10  $\mu\text{M}$  BA. (C) Brotações enraizadas tratadas com 5 mM de AIB por 15 minutos e transferidas para meio WPM, sem regulador de crescimento. (D) Plantas aclimatizadas crescendo em casa de vegetação após três meses.

of BA, IBA or NAA, nor there was a synergistic interaction between these growth regulators during culture initiation and first subculture; however, there were significant differences between the treatments in the second and third subcultures (F Test,  $P \leq 0.01$ ). In addition, supplementing the BA-containing medium with IBA or NAA did not affect the mean number of shoots (F Test, orthogonal contrasts,  $P \geq 0.05$ ). However, the number of shoots was enhanced by increasing the concentration of BA in the culture medium independent of the presence of IBA or NAA (Table 1).

In culture medium supplemented with 10  $\mu\text{M}$  BA, the average number of shoots in the third subculture was higher than that in previous subcultures (i.e.,  $S_0$ ,  $S_1$  and  $S_2$ ). Epicotyl explants cultivated in the presence of NAA exhibited a reduced number of shoots when compared with explants grown in other treatments (F Test, orthogonal contrasts,  $P \leq 0.05$ ). For explants grown in medium supplemented with concentrations of 2.5 and 5.0  $\mu\text{M}$  of BA, the presence of NAA and IBA had no influence on the average number of shoots (Table 1, F Test, orthogonal contrasts,  $P \leq 0.05$ ).

The average number of shoots formed on the epicotyl explants was low during the culture initiation and first subculture (Figures 2A and 2B). Regression analysis indicated a correlation between concentrations of BA (0–10  $\mu\text{M}$ ) and the average number of shoots produced on the epicotyl explants (Figures 2C and 2D), with most shoots produced at the third subculture (Figure 1B). Tang, Ishii and Ohba (1996) observed similar results; during the initiation culture on a basal medium WPM, containing 2–8  $\mu\text{M}$  of BA, it was

TABLE 1: Mean number of shoots from epicotyl explants of *Aspidosperma polyneuron* cultivated in WPM<sup>1</sup> medium supplemented with BA alone or in combination with IBA or NAA.TABELA 1: Número médio de brotações de explantes do epicótilo de *Aspidosperma polyneuron* cultivadas em meio WPM, suplementado com BA, isolada ou combinada com AIB ou ANA.

BA ( $\mu\text{M}$ )	0				IBA (0.5 $\mu\text{M}$ )				NAA (0.5 $\mu\text{M}$ )			
	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>
0	1.00	1.00	1.00	1.03	1.00	1.03	1.03	1.03	1.00	1.00	1.00	1.00
2.5	1.00	1.03	1.13	1.67	1.00	1.03	1.20	1.83	1.00	1.00	1.07	1.40
5.0	1.03	1.17	1.70	3.50	1.00	1.00	1.67	3.20	1.00	1.03	1.50	3.13
10.0	1.00	1.00	2.90	7.50	1.00	1.10	2.63	7.80	1.00	1.13	2.87	6.97

Where in: <sup>1</sup>Lloyd and Mc Cown (1980). Monthly transfer or subculture of shoots was denominated as follows: culture initiation (S<sub>0</sub>), first-subculture (S<sub>1</sub>), second-subculture (S<sub>2</sub>), and third-subculture (S<sub>3</sub>).

observed that epicotyl explants of *Alnus cremastogyne* did not elongate, and adventitious buds and shoots were not formed.

In contrast to the results obtained from epicotyl explants, the presence of cytokinin in the medium significantly affected the number of shoots formed from hypocotyl segments treated with BA at culture initiation and first subculture (F Test,  $P \leq 0.05$ , Figures 3A and 3B). Addition of IBA or NAA to the culture medium did not influence the average number of the produced shoots; however, the average number of regenerated shoots did increase with increasing concentrations of BA (F Test, orthogonal contrasts,  $P \geq 0.05$ ) (Table 2). Hypocotyl explants treated with 10  $\mu\text{M}$  of BA plus 0.5  $\mu\text{M}$  of IBA or NAA at the first subculture exhibited a reduced average number of shoots (F Test, orthogonal contrasts,  $P \geq 0.05$ ; Table 2; Figure 3B).

ANOVA showed that concentrations of BA and the presence of IBA or NAA significantly affected hypocotyl segments of *Aspidosperma polyneuron* (F test,  $P \leq 0.05$ ). Specifically, the addition of 0.5  $\mu\text{M}$  of IBA or NAA to the BA-supplemented culture medium reduced the average number of shoots (F test, orthogonal contrasts,  $P \leq 0.05$ ). Table 2 and Figure 3C indicate that an increase in the average number of shoots occurred with higher concentrations of BA (F test, orthogonal contrasts,  $P \leq 0.05$ ).

The presence of BA, IBA, or NAA in the culture medium, as well as the interaction between these factors, significantly affected the average number of shoots produced in hypocotyl explants of *Aspidosperma polyneuron* during the third subculture (F Test,  $P \leq 0.05$ ). In particular, culture media supplemented with IBA or NAA in combination with 5.0 or 10.0  $\mu\text{M}$  BA significantly increased the average number of regenerated shoots (F Test, orthogonal contrasts,  $P \leq 0.05$ ).

Higher rates of shoot regeneration were obtained by increasing the concentration of BA at the third subculture, and this effect was also observed in earlier subcultures of hypocotyl explants (F Test, orthogonal contrasts,  $P \leq 0.05$ ; Table 2; Figure 3D). In a previous study, similar responses were observed for *Cedrela fissilis* grown in MS medium supplemented with 1.25–5.0  $\mu\text{M}$  BA, where the multiplication rate per single cotyledonary node cutting increased to 6–7-fold after 60–90 days in the second subculture cycle (NUNES et al., 2002).

Hypocotyl explants presented a higher average number of regenerated shoots compared with epicotyl explants, and this trend was observed from the culture initiation to the second subculture in all treatments where growth regulators were present in the media. However, during the third subculture, concentrations of 10  $\mu\text{M}$  BA produced the highest average number of shoots in epicotyl explants rather than hypocotyls (Figures 2D and 3D). The different performances of epicotyl and hypocotyl explants could be attributed to the effects of apical dominance in apical shoots; the multiplication ratio of shoots could be increased by the removal of the apical region, as well as by the presence of more developed axillary buds located at basal segments (SANCHEZ et al., 1997). Pattnaik, Sahoo and Chand (1996) have suggested that these morphogenetic responses could be related to differences in physiological conditions between shoots distributed through different portions of the stem. Mongomaké et al. (2009) reported that

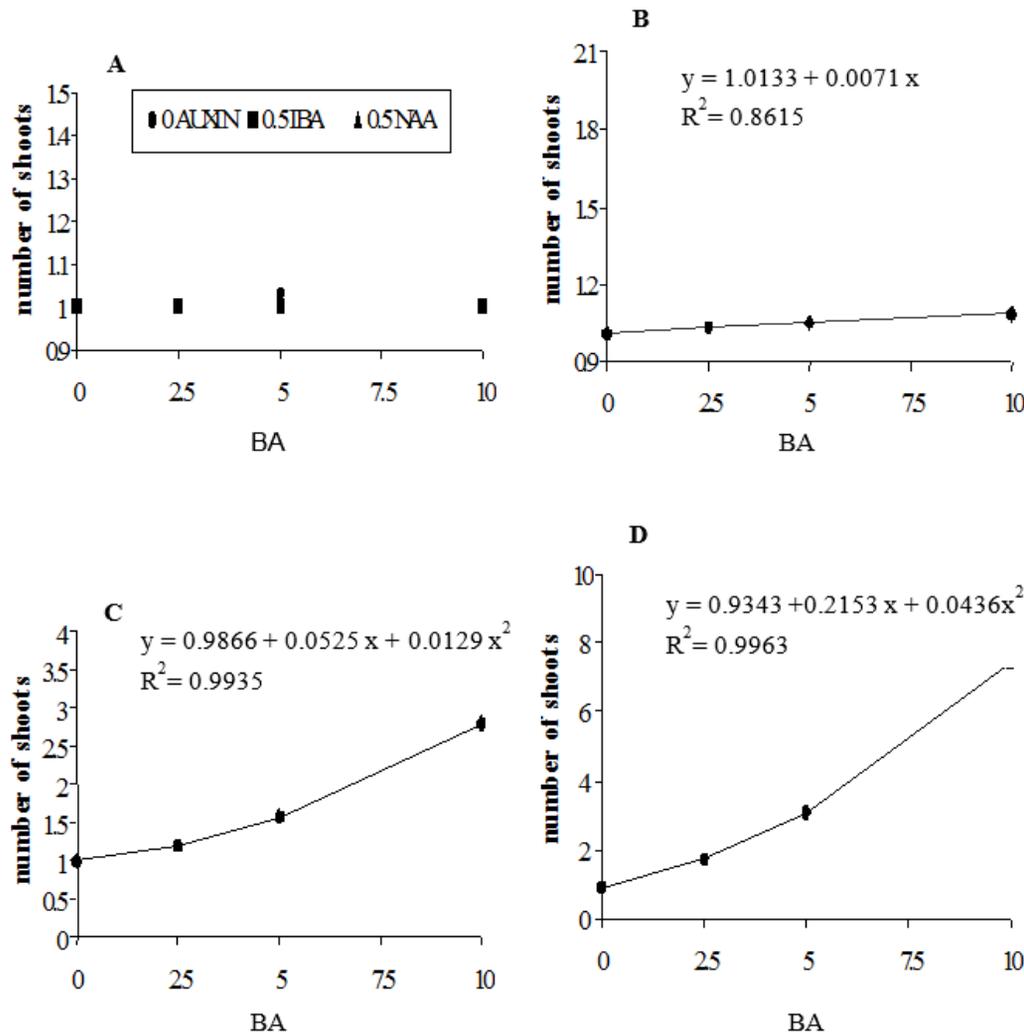


FIGURE 2: Effect of BA, with and without IBA or NAA, on the shoot regeneration rate in explants from epicotyls of *Aspidosperma polyneuron*. Results were obtained during: (A) the culture initiation ( $S_0$ ), (B) first subculture ( $S_1$ ), (C) second subculture ( $S_2$ ), and (D) third subculture ( $S_3$ ).

FIGURA 2: Efeito de BA, com ou sem AIB ou ANA, em explantes do epicótilo de *Aspidosperma polyneuron*. Os resultados foram obtidos no: (A) cultivo inicial ( $S_0$ ), (B) primeiro subcultivo ( $S_1$ ), (C) segundo subcultivo ( $S_2$ ) e (D) terceiro subcultivo.

the ability of hypocotyl and epicotyl explants of *Vigna subterranean* to produce shoots was dependent of BA concentration, and 8.8  $\mu$ M BA provided the highest rate of shoot production (3.7 shoots per explant from hypocotyls and 5.8 shoots per explant from epicotyls). In addition, they found that multiple shoots were induced for both explants, but that regeneration efficiency was higher when epicotyl explants were used. This result is similar to the one observed in this study after two subcultures for *A. polyneuron*.

Considering all treatments tested, the average number of regenerated shoots per explant was low at the end of the culture initiation and first subculture, and this result was independent of the origin of the explants tested. Feyissa, Welander and Negash (2005) observed that the rate of multiplication in explants of *Hagenia abyssinica* originating from juvenile material was low at the first subculture and increased at the second and later subcultures, especially for explants originating from seedlings.

The presence of BA in the culture medium was necessary for production of shoots in hypocotyl and epicotyl explants of *Aspidosperma polyneuron*. When used alone, the cytokinin has also been effective for the cultivation of other forest species such as *Cedrela fissilis* (NUNES et al., 2002), *Albizia odorissima*

TABLE 2: Mean number of shoots formed from hypocotyl explants of *Aspidosperma polyneuron* when cultivated in WPM<sup>1</sup> medium supplemented with BA alone or in combination with IBA or NAA.

Tabela 2: Número médio de brotações de *Aspidosperma polyneuron* formadas a partir de explantes do hipocótilo, quando cultivadas em meio WPM, suplementado com BA, isolada ou combinada com AIB ou ANA.

BA ( $\mu\text{M}$ )	0				IBA (0.5 $\mu\text{M}$ )				NAA (0.5 $\mu\text{M}$ )			
	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>0</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>
0	1.07	1.15	1.15	1.15	1.00	1.07	1.07	1.07	1.00	1.07	1.00	1.00
2.5	1.05	1.17	2.32	2.52	1.07	1.32	2.00	2.32	1.17	1.35	1.82	2.30
5.0	1.15	1.70	3.10	3.90	1.02	1.70	2.82	4.05	1.12	1.70	3.02	5.07
10.0	1.42	2.20	3.72	6.92	1.32	1.97	3.37	5.87	1.35	1.87	3.24	6.95

Where in: <sup>1</sup>Lloyd and McCown (1980). Monthly transference or subcultures of shoots were denoted as culture initiation (S<sub>0</sub>), first-subculture (S<sub>1</sub>), second-subculture (S<sub>2</sub>), and third-subculture (S<sub>3</sub>).

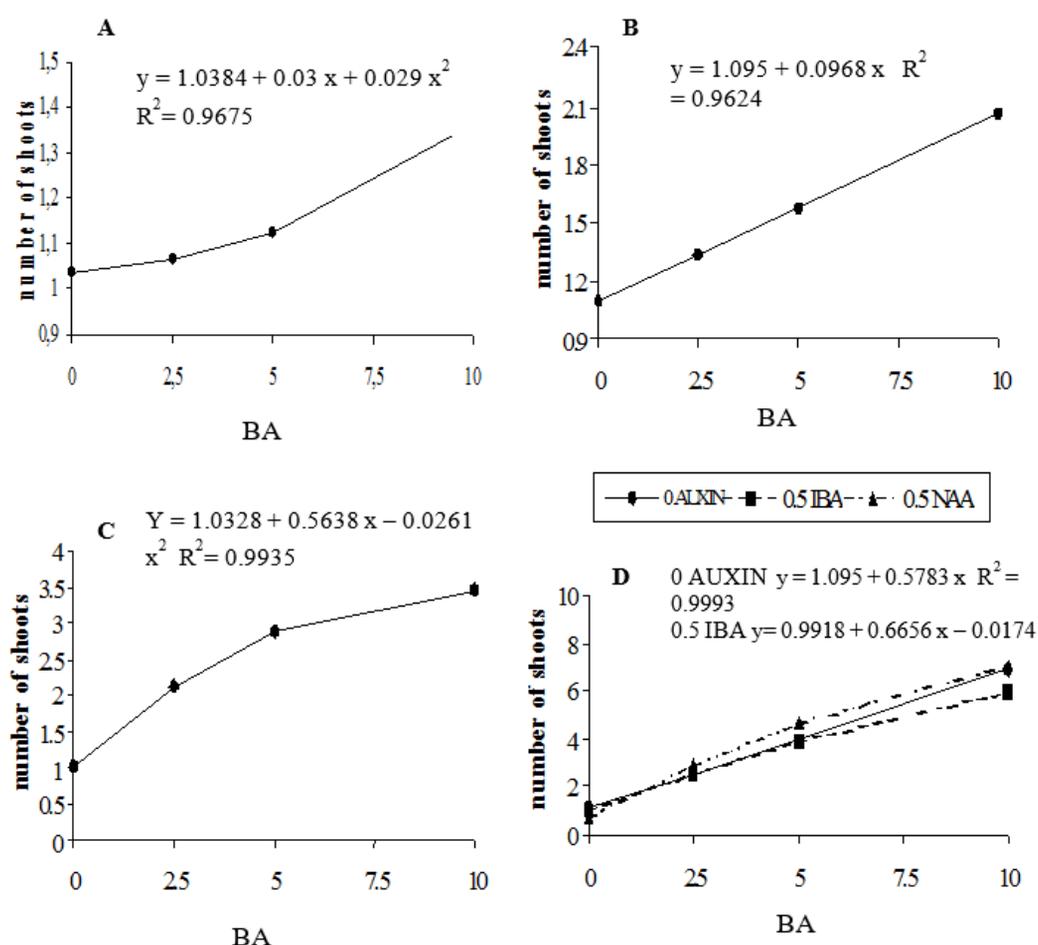


FIGURE 3: Effect of BA treatment, with or without IBA or NAA, on shoot regeneration rate in nodal segments from hypocotyls of *Aspidosperma polyneuron*. Results were obtained during: (A) the culture initiation (S<sub>0</sub>), (B) first subculture (S<sub>1</sub>), (C) second subculture (S<sub>2</sub>), and (D) third subculture (S<sub>3</sub>).

FIGURA 3: Efeito do tratamento com BA, com ou sem AIB ou ANA, na taxa de regeneração de brotações de segmentos nodais do hipocótilo de *Aspidosperma polyneuron*. Os resultados foram obtidos durante: (A) cultivo inicial (S<sub>0</sub>), (B) primeiro subcultivo (S<sub>1</sub>), (C) segundo subcultivo (S<sub>2</sub>) e (D) terceiro subcultivo.

(RAJESWARI; PALIWAL, 2008), *Quercus semecarpifolia* (TAMTA et al., 2008), *Caesalpinia echinata* (ARAGÃO; ALOUFA; COSTA, 2011), and *Amburana cearensis* (CAMPOS et al., 2013). In the present study, the shoot induction rate was higher at the third subculture (7 – 8 shoots) when the explants of *Aspidosperma polyneuron* were cultivated in the basal medium supplemented with the highest concentration of cytokinin, i.e., 10  $\mu\text{M}$  of BA. In other studies, the most effective BA concentration for inducing shoots varied with species, e.g. 2.5  $\mu\text{M}$  for *Caesalpinia echinata* (ARAGÃO; ALOUFA; COSTA, 2011) and *Crataeva nurvala* (WALIA; SINHA; BABBAR, 2003), 4.4  $\mu\text{M}$  for *Amburana cearensis* (CAMPOS et al., 2013), and 20  $\mu\text{M}$  for *Quercus semecarpifolia* (TAMTA et al., 2008). Each of these species exhibited lower shoot production than *Aspidosperma polyneuron*.

For *Aspidosperma polyneuron*, combining BA with auxins was ineffective for induction and development of shoots in both types of explants. Although the initial multiplication ratios were quite low as previously mentioned, they increased with further subcultures. There are three phases through which a shoot must progress to be successfully propagated *in vitro*: isolation, stabilization, and production. Isolation involves decontamination; once this stage is accomplished, *in vitro* morphogenesis can be achieved following standard patterns. For *Aspidosperma polyneuron*, at the isolation phase, tissue grew relatively quickly, and few axillary shoots were formed. These shoots originated from pre-formed quiescent buds already present in the epicotyl and hypocotyl explants, which were the tissues most responsive to the presence of growth regulators. The stabilization phase involves a change in growth characteristics from unpredictable, often abnormal shoot development at first, to a uniform predictable growth pattern (HARTMANN et al., 2002). During this phase, further morphogenesis events depend on the new meristematic tissues previously generated *in vitro*. The difficulty in stabilization appears to be associated with growth phase: the more juvenile explant, the more easily it is stabilized (HARTMANN et al., 2002). Thorpe, Harry and Kumar (1991) suggested that growth is rapid during the isolation period, and that the few axillary shoots that are produced are usually an expression of buds already present on the original explant. Frequent subculture of shoot apices in cytokinin-containing medium has resulted in reactivated meristems in many species, including *Prunus* spp., *Eucalyptus* spp., *Pinus pinaster* and *Sequoia* spp. The sequence of subculture itself appears to be a rejuvenating process that ultimately leads to increased competence and promotion of morphogenetic events (THORPE; HARRY; KUMAR, 1991) this tendency was also observed for *Aspidosperma polyneuron* in the present study. The most effective shoot regeneration was achieved using hypocotyl or nodal cotyledonary segments. These results resemble those observed for *Melissa officinalis* (TAVARES; PIMENTA; GONÇALVES, 1996), *Sterculia urens* (PUROHIT; DAVE, 1996) and *Castanea sativa* (SANCHEZ et al., 1997) cultured on media supplemented with 8.8 – 22.12  $\mu\text{M}$  BA.

In general, when BA-containing culture medium was supplemented with 0.5  $\mu\text{M}$  IBA or NAA, the average number of regenerated shoots on *Aspidosperma polyneuron* did not improve. However, IBA was more effective than NAA for the promotion of shoot production by axillary budding, and similar observations have been made for other species such as *Eucalyptus globulus* (TRINDADE et al., 1990) and *Fraxinus angustifolia* (PEREZ-PARRON; GONZALEZ-BENITO; PEREZ, 1994). Similar results were also reported in previous work, where combinations of auxins and BA were tested as promoters of shoot multiplication and development in *Betula celtiberica* (PEREZ; POSTIGO, 1989), *Cornus florida* (DECLERCK; KORBAN, 1994) and *Vigna subterranea* (MONGOMAKÉ et al., 2009).

When different types of explants of *Aspidosperma polyneuron* were tested, apical shoots originating from two-year-old seedlings were able to produce and regenerate axillary shoots from the beginning of culture initiation, whereas shoots from epicotyl explants exhibited increased shoot production after the second subculture only. Distinct physiological and internal settings of growth regulators for both explant types cultivated *in vitro* could account for the differences found under experimental conditions (RIBAS et al., 2005). Letham and Palmi (1983) suggested that several factors could influence the results of cytokinin treatment. Examples of these factors include the ratio of absorption in culture medium, the speed and transport efficiency within the plant tissue, catabolism or degree of absorption of growth regulators and the overall genetic capacity of the explant to metabolize cytokinins (AUER et al., 1992). Besides these factors, exogenous application of cytokinins could interact with endogenous growth regulators and thereby interfere with development of plant morphogenesis *in vitro*. The application of cytokinins is also known to interfere with the metabolism of the indole-3-acetic acid (BOUZA et al., 1993).

***In vitro* rooting**

*In vitro* rooting of *Aspidosperma polyneuron* was significantly affected by IBA concentration and explant origin (F Test,  $P \leq 0.05$ ). When the percentage of rooting was compared between shoots derived from hypocotyl and epicotyl explants, the former was superior when cultivated in the presence of IBA at 5 mM. (Tukey's Test,  $P \leq 0.05$ ; Table 3). Regression analyses showed an increase in rooting percentage for both explant types when concentrations of 2.5 mM and 5 mM of IBA were used. No further increase in rooting was observed above 5 mM (i.e., at 10 mM IBA) (Figure 4).

TABLE 3: Percentage of rooting from shoots of *Aspidosperma polyneuron* formed from epicotyl and hypocotyl explants following pulse treatments with various concentrations of IBA for 15 minutes.

TABELA 3: Percentagem de enraizamento de brotações de *Aspidosperma polyneuron* formadas a partir de explantes de epicótilo e hipocótilo, após tratamentos-pulso com várias concentrações de AIB por 15 minutos.

IBA (mM)	Epicotyl	Hypocotyl
0	0.00 a	0.00 a
2.5	18.27 a	21.62 a
5.0	53.33 b	63.34 a
10.0	60.00 a	56.67 a

Where in: Means followed by different letters on lines are significantly different at  $P \leq 0.05$  according to Tukey's Test.

The maximum rooting percentage (63.34%) for *Aspidosperma polyneuron* was achieved by using hypocotyl explants subjected to a pulse treatment with 5 mM of IBA for 15 minutes, and cultivated on growth regulator-free WPM for five weeks. Similar results were reported for *Ocotea porosa*, which exhibited 62.6% root formation with a pulse treatment of 10 mM IBA for 10 min (PELEGRINI et al., 2011). A pulse treatment of 2.5 mM IBA for 10 minutes also improved rooting and reduced callus formation at the base of the shoots of *S. urens* (PUROHIT; DAVE, 1996).

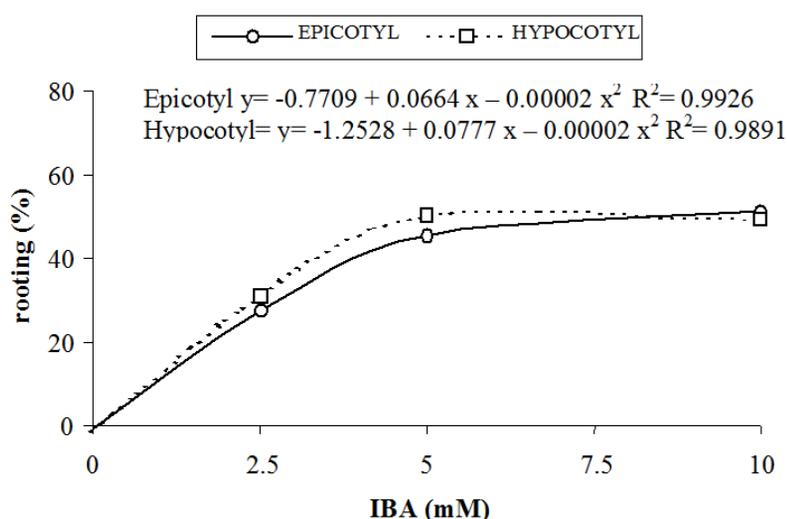


FIGURE 4: Effect of pulse treatments with solutions of IBA on the rooting rate (%) of *Aspidosperma polyneuron* explants cultivated from hypocotyls and epicotyls, after six weeks.

FIGURA 4: Efeito do tratamento pulso com soluções de AIB na porcentagem de enraizamento de explantes do hipocótilo e epicótilo de *Aspidosperma polyneuron*, após seis semanas.

## Transplanting and acclimatization

Although *Aspidosperma polyneuron* cultivated using *in vitro* root-inducing treatments initially presented only one or two roots (Figure 1 C), when they were acclimatized, they achieved survival rates > 90%. After transplanting, the plants produced new leaves and began the growing process once again. Later, the plants were transplanted to trays and planted singly in plastic cell-packs that held mixtures of previously sifted soil and Plantmax<sup>®</sup> substrate (3:1, v/v). They remained in this condition for a period of 8–12 weeks and developed new roots. The plants were then transplanted to polyethylene bags containing the same substrate composition and placed in a van der Hoeven<sup>®</sup> greenhouse, where they were subjected to controlled aeration, temperature, and a mist irrigation system (Figure 1 D). The use of Plantmax<sup>®</sup> as a substrate was also efficient for other forestry species, including *Tectona grandis* (FERMINO JUNIOR; RAPOSO; SCHERWINSKI-PEREIRA, 2011), and *Pinus taeda* (OLIVEIRA et al., 2011), which achieved 100% and 90% of survival, respectively.

In conclusion, the results of the present work show that the micropropagation of *Aspidosperma polyneuron* by organogenesis using epicotyl and hypocotyl explants is a feasible method for mass propagation and conservation. The results on shoot multiplication show that BA was indispensable, and the number of shoots produced increased with the increasing of cytokinin concentration. This species demonstrated the good rooting capacity achieved with IBA pulse treatment and plantlets were successfully acclimatized using a mixture of soil:Plantmax<sup>®</sup> (3:1). The fact that the starting plant material used as explant was derived from juvenile plants could be analyzed in the light of its polyembryogenic behavior. These embryos could produce seedlings for use as explants in clonal propagation. The embryo resulting from the zygote could also be used to obtain explants for conservation purposes, since it would be possible to preserve the genetic variability of the remnant populations.

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