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Micropropagation enables the mass propagation and conservation of *Dyckia distachya* Hassler

Marcelo Francisco Pompelli1, and Miguel Pedro Guerra1

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ABSTRACT - *The Atlantic Forest has a rich biological diversity and elevated levels of endemism in which bromeliads are frequent, but endangered. Tissue culture techniques raise high hopes for the rescue, mass propagation for commercial aims or the conservation of natural populations threatened with extinction. The present study evaluated aspects of seed-based* D. distachya *micropropagation. A high rate of* in vitro *regeneration (78.93 shoot gram-1) was obtained in response to liquid MS culture medium supplemented with Benzylaminopurine 2 µM after 120 days in culture, or supplemented with Naphthaleneacetic acid 2 µM, BA 4 µM and Paclobutrazol 6 µM (133.58 shoots explant-1) after 142 days in culture. Not less than 1.5 cm long shoots were successfully acclimatized to* ex vitro *conditions.* D. distachya *is responsive to the application of tissue culture techniques for mass propagation and conservation purposes. The use of seeds as initial explants allows the maintenance of the genetic diversity observed in the natural populations and mass regeneration is a promising technique for the conservation of this endangered bromeliad.*

Key words: *Dyckia distachya* Hassler, micropropagation, organogenesis, tissue culture, bromeliads.

INTRODUCTION

The Atlantic Forest is a biome of high biological diversity showing elevated levels of endemism (Myers et al. 2000). The destruction of this biome and its associated ecosystems caused an impressive reduction of its biodiversity. This impact also cut back the epiphytic flora rich in bromeliad species. The devastation of the Atlantic Forest, hydroelectric plant constructions and expressive endemism have restricted the *Dyckia distachya* bromeliads to a few subsisting along the rocky banks of the Uruguay River in the west of Santa Catarina and the south of Paraná state and in restricted areas of Paraguay and Argentina. This species is similar to *D. brevifolia* from which it differs mainly in the number of leaves, the more appealing rosette and the robust and ramified inflorescence (Strehl 1994).

The conventional propagation methods of bromeliads are not appropriate for a large-scale plant production as required for ornamental or endangered species (Carneiro et al. 1999). Tissue culture-based techniques are up-and-coming to overcome these constraints.

1Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-000, Viçosa, MG, Brasil. *E-mail: mpompelli@yahoo.com.br
2Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, 88.040-900, Florianópolis, SC, Brasil

The consistency of the culture medium influences the *in vitro* rate. Jellified and liquid culture media are used in different *in vitro* protocols. Liquid culture medium allows the best contact of the culture with the medium (Teisson et al. 1996). Liquid culture medium enhanced the induction and development of adventitious shoots in the bromeliads *Quesnelia quesneliana, Vriesea poelmanii* and *Aechmea fasciata* (Hosoki and Asahira 1980).

The present study investigated inductive conditions for the *in vitro* morphogenesis of *Dyckia distachya* aiming at the conservation and mass propagation of this endangered Brazilian bromeliad.

MATERIAL AND METHODS

Direct shoot induction from seeds

Mature seeds were collected from plants of natural populations dwelling in the Uruguay River basin, western Santa Catarina State. Seed-containing capsules were sealed in polyethylene bags and mailed to the Laboratory of Developmental Physiology and Genetics of the Federal University of Santa Catarina, Florianópolis, Santa Catarina, south of Brazil.

The seeds were immersed in ethanol (70%) for 3 min under aseptic conditions, and then in a 5% NaOCl solution for 30 min and rinsed five times with sterilized water. Ten seeds each were inoculated in Petri dishes (100 mm x 20 mm) containing 30 mL MS culture medium (Murashige and Skoog 1962), agar (Vetec) 6 g L^{-1}, and supplemented with 6-Benzylaminopurine (BA) or 6-Furfurylaminopurine (Kinetin - Kin) (0, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 and 30.0 μM). The concentration zero represents a treatment without plant growth regulators.

Each treatment consisted of three replications in four Petri dishes arranged in a completely randomized design. After 60 days of culture the fresh weight and multiplication rates were recorded. The cultures were subsequently reinoculated in 250 mL flasks containing 25 mL liquid culture medium. After 60 more days the cultures were evaluated for the fresh weight values and multiplication rates.

The data of fresh weight were transformed by $\sqrt{x+0.5}$ and the number of shoots by log (x+1) and then submitted to ANOVA and SNK test of mean separation (SNK $\leq$ P 0.05).

Shoot induction

Seeds were collected and disinfected as described above and inoculated in Petri dishes containing 30mL KC culture medium (Knudson 1946) jellified with agar 10 g L^{-1}. Each Petri dish was inoculated with ten seeds. After germination the plantlets were individually transferred to test tubes (150 mm x 25 mm) containing 10 mL of the same culture medium as described above. Plantlet roots of at least 1.5cm length were excised and inoculated in 250 mL flasks containing 30 mL of culture medium as described below.

Thirteen treatments with a combination of BA, α-Naphthaleneacetic acid (NAA) and Paclobutrazol (PBZ) were selected in two liquid and semi-solid consistencies (see Table 1). The basal medium composition was based on liquid or jellified (agar 6 g L^{-1}) MS culture medium, supplemented with sucrose (30 g L^{-1}).

Each treatment consisted of three replications in four flasks or Petri dishes arranged in a completely randomized design. Subcultures were established in fresh medium every 30 days. The data of fresh weight were transformed by $\sqrt{x+0.5}$ and the number of shoots by log (x+2), then submitted to ANOVA and the mean separation test (SNK P $\leq$ 0.05).

Acclimatization

Shoots of at least 1.5cm length were acclimatized in trays containing a substrate mixture of peat, vermiculite and sand (2:2:1), and maintained in a greenhouse under 50% light intensity reduction.

Table 1 – Treatment used in this work

Medium consistency	NAA (μM)	BA (μM)	PBZ (μM)	Abbreviation
liquid	0	0	0	PGR Free liquid
jellified	0	0	0	PGR Free jellified
liquid	2	4	0	N2B4 liquid
jellified	2	4	0	N2B4 jellified
liquid	2	4	3	N2B4P3 liquid
jellified	2	4	3	N2B4P3 jellified
liquid	2	4	6	N2B4P6 liquid
jellified	2	4	6	N2B4P6 jellified
liquid	0	0	5	PBZ 5 liquid
jellified	0	0	5	PBZ 5 jellified
liquid	1	1	0	N1B1 liquid
liquid	1	2	0	N1B2 liquid
liquid	1	2	3	N1B2P3 liquid

RESULTS AND DISCUSSION

Direct organogenesis from seeds

After 30 days in culture all viable seeds germinated, most of them presenting the formation of adventitious shoots induced by the PGR (plant growth regulator) in the culture medium. Similarly, Fischer and Zimmer (1988) reported that bromeliad seeds germinated in cytokinin-supplemented culture medium originated more than oneshoot.

BA and Kin levels of 2 μM attained the highest values of regeneration of adventitious shoots (Figure 1). However, at a level of 3 μM of BA or Kin the regeneration rate dropped, expressing toxicity. No statistically significant differences were observed in the regeneration rate in response to BA at 15, 20 or 30 μM while highest values were obtained with 20 μM (78.93 shoots per gram of initial culture). The regeneration rate in *Centella asiatica* micropropagation was enhanced in response to 22 μM BA (Tiwari et al. 2000).

No significant differences were observed in response to Kin; the concentration of 20 μM induced 26.23 shoots per gram of inoculum (Figure 1).

In comparison, BA induced 64.30 and Kin only 17.14 shoots per gram of inoculum (Figure 1). In the micropropagation of apricot, TDZ attained best results (Tornero et al. 2000). Cytokinin was however less effective han BA in the *in vitro* multiplication of *Cryptanthus sinuosus*, a Brazilian bromeliad threatened with extinction (Carneiro et al. 1998).

Shoot multiplication

The highest *in vitro* regeneration rate was observed in the liquid culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (6 μM). This treatment resulted in the induction of 21.31, 80.96, 121.93, and 133.58 shoots per explant after 45, 75, 112, and 142 days in culture, respectively (Figure 2). The best multiplication rate (72.94) was observed in response to the jellified MS culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (6 μM) after 112 days in culture (Figure 3).

Compared to the multiplication rates commonly observed in response to different micropropagation protocols the regenerative rates observed in the present study were high. For example, the best multiplication rate in *Aechmea fasciata* was 4.53 shoots per explant in response to the culture medium supplemented with 2.0 mg L^{-1} Kin and IAA (Vinterhalter and Vinterhalter 1994). Similar results were reported by Hosoki and Asahira (1980) in the micropropagation of *Vriesea poelmanii, Aechmea fasciata* and *Quesnelia quesneliana* where 4.4 and 8.0

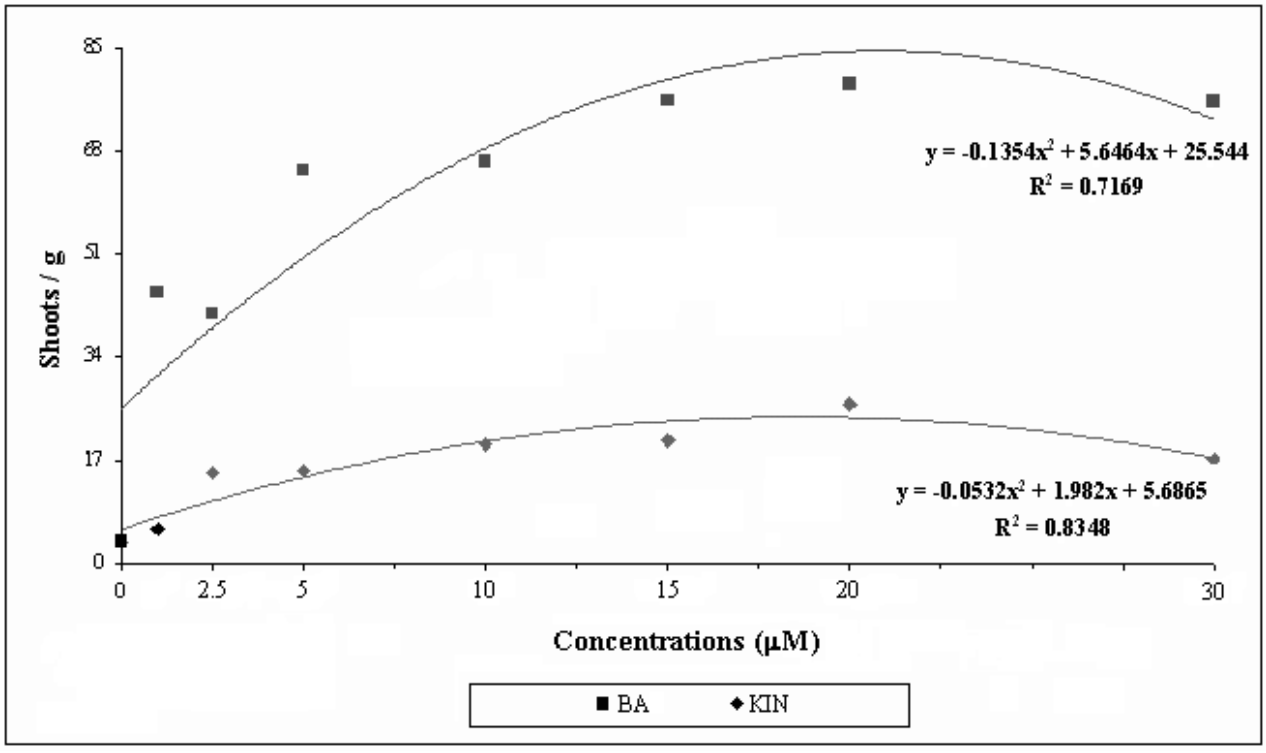

Figure 1. *In vitro* regeneration rate of *Dyckia distachya* in response to BA and Kin, after 120 days in culture

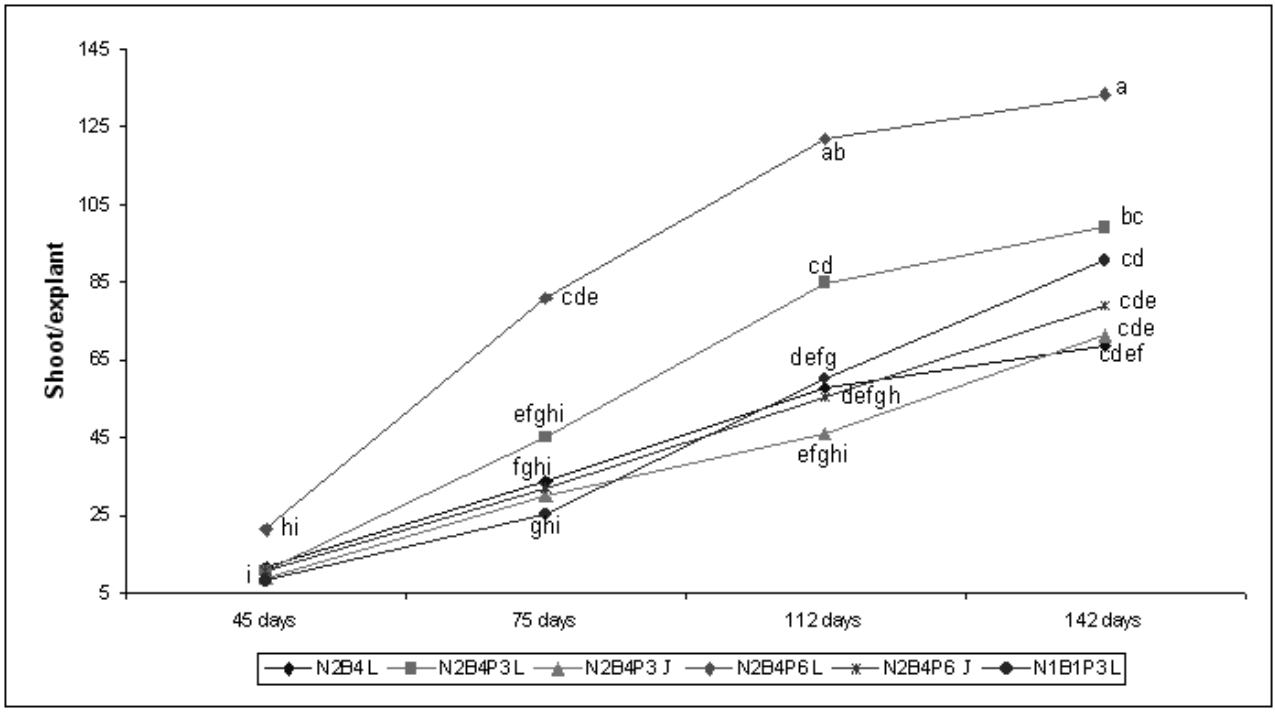

Figure 2. Evolution of number of shoots per explant of *D. distachya* cultivated in jellified (J) or liquid (L) MS culture medium supplemented with 0, 1 and 2 μM NAA (N), 0, 1, 2 and 4 μM BA (B) and 0, 3, 5 and 6 μM PBZ (P). Means followed by the same letter are not statistically different (SNK P $\leq$ 0.05)

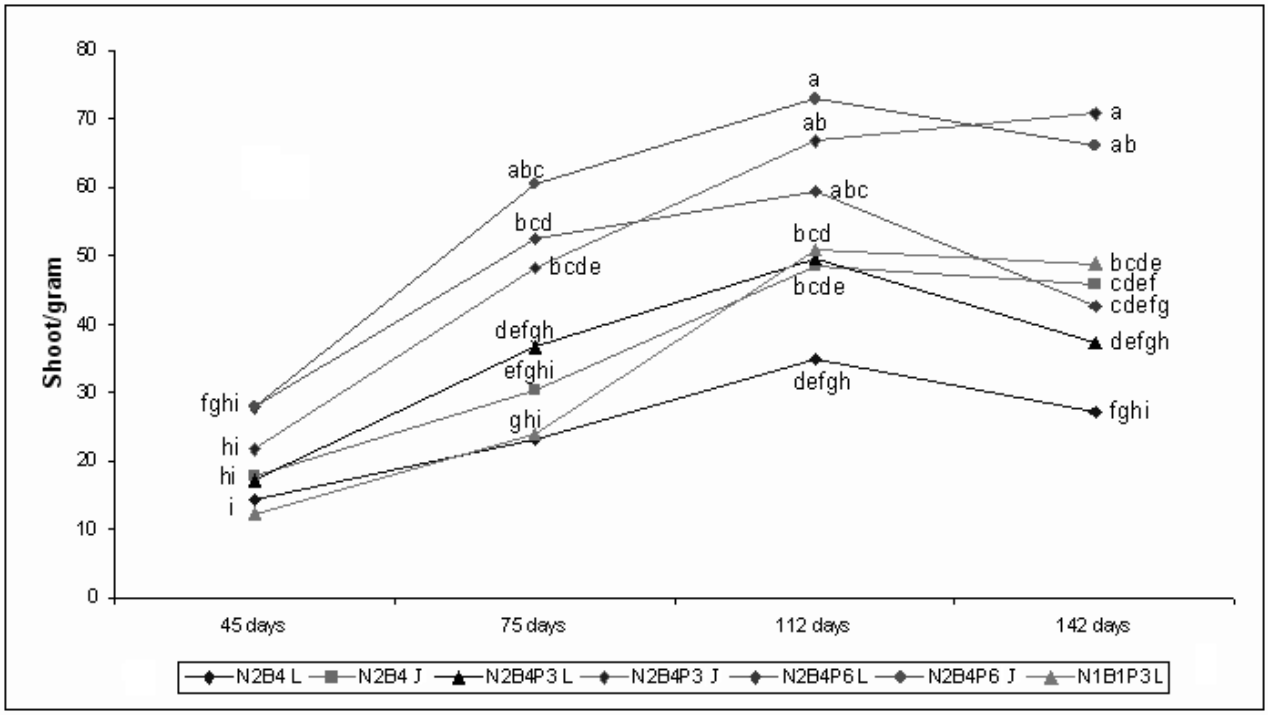

Figure 3. Evolution of number of shoots per gram of inoculum of *D. distachya* cultivated in jellified (J) or liquid (L) MS culture medium supplemented with 0, 1 and 2 µM NAA (N), 0, 1, 2 and 4 µM BA (B) and 0, 3, 5 and 6 µM PBZ (P). Means followed by the same letter are not statistically different (SNK P $\leq$ 0.05)

adventitious shoots were induced, respectively, in response to 1.0 mg L^{-1} NAA and BA.

In pineapple micropropagation the highest multiplication rate was 13.5 shoots per explant in response to the culture medium supplemented with 2 μM NAA and 4 μM BA (Dal Vesco et al. 2001). For *Tillandsia cyanea*, 14.6 shoots per explant were obtained in response to the culture medium supplemented with 0.2 mg L^{-1} BA (Pierik and Sprenkels, 1991). The highest regeneration rate (22.5 shoots per explant) in *Vriesea fosteriana* was observed in response to the KC culture medium supplemented with 2.7 μM NAA and 8.9 μM BA (Mercier and Kerbauy 1992). The micropropagation of *Vriesea friburgensis* achieved 231.86 shoots starting from 0.10 g microbuds after 60 days in culture medium supplemented with 2 μM NAA and 4 μM BA (Alves and Guerra 2001). Only one study on *Dyckia* was found in the revised literature where *Dyckia macedoi* showed a multiplication rate of 9 shoots per explant in response to the culture medium supplemented with 0.1 mg L^{-1} NAA and 5.0 mg L^{-1} BA (Mercier and Kerbauy 1993).

In the present study the MS liquid culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (6 μM) resulted in the regeneration of 89.44 shoots per explant. However jellified MS culture media supplemented with NAA (2 μM), BA (4 μM) and PBZ (3 and 6 μM) resulted in mean values of 51.86 and 56.88 shoots per gram of inoculum, respectively. These values did not show differences from those observed in response to the MS liquid medium supplemented with 2 μM NAA, 4 μM BA and 6 μM PBZ but were statistically different from those observed in the other treatments.

The treatments with a 1/3 higher regeneration rate than the regeneration rate in response to the culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (6 μM) were selected for a more detailed study into the subculture effects (dotted line in Figures 4 and 5 shows the limit to be used). Six treatments were selected for the parameter shoots per explants: liquid MS culture medium supplemented with NAA (2 μM) and BA (4 μM); jellified and liquid MS culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (3 and 6 μM), and; liquid MS culture medium supplemented with NAA (1 μM), BA (1 μM) and PBZ (3 μM). Considering the parameter shoots/gof inoculum seven treatments were selected: jellified and liquid MS culture medium supplemented with NAA (2 μM) and BA (4 μM); jellified and liquid MS culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (3 and 6 μM), and; liquid MS culture medium supplemented with NAA (1 μM), BA (1 μM) and PBZ (3 μM). Among these it is possible to show the efficiency of the liquid MS culture medium supplemented with NAA (2 μM), BA (4 μM) and PBZ (6 μM) (Figure 2).

The highest multiplication rate for most of the treatments was generally observed in the third subculture, with a reduction in the fourth subculture. The highest value in the third subculture (72.94 shoots per gram of inoculum) was observed in the jellified MS culture medium supplemented with 2 μM NAA, 4 μM BA and 6 μM PBZ (Figure 3).

The presence of hyperhydric shoots was observed in most of the treatments in the fourth subculture (Figure 6-H and I). Hyperhydric tissue cells show alterations in the cytoplasm with consequent loosening of the cell walls and a low photosynthetic rate responsible for the translucent aspect of hyperhydric plants (Kevers et al. 1988). Hyperhydric shoots present less apical dominance and damage in the cell wall (Kevers et al. 1988). In tissue culture, cytokinins can lead to a hyperhydricity of shoots with restrictive effects in the micropropagation (Nobre et al. 1996). This occurrence was also reported for *Fragaria* x *ananassa* (Lopez-Aranda et al. 1994) and *Vriesea friburgensis* (Alves and Guerra 2001) *in vitro* cultures.

Liquid medium enhanced the multiplication rate and was effective at reducing the explant oxidation and improving the *in vitro* regeneration of *Neoregelia cruenta*, a Brazilian bromeliad (Carneiro et al. 1999). It was also observed that lateral buds grew faster in liquid than in solid medium (Hosoki and Asahira 1980). It is well documented that the nutrient uptake in bromeliads occurs not only through the roots but the leaves as well (Benzing 2000). This fact could explain the efficiency of liquid medium we observed in the present study. It should be stressed that agar is one of the most expensive components of the culture medium and consequently increases the production costs of micropropagation (George 1993).

In the present study it was also observed that cultures maintained in the presence of PBZ formed thick and short roots. Conversely, the induction of long and thin roots was observed in PGR-free culture medium.

Cultures maintained in PBZ-supplemented culture medium frequently showed toxic effects ranging from a yellow coloring to the culture's death (Figure 6-G).

PBZ affects the gibberellin synthesis resulting in a reduced growth of *in vitro* cultures. These effects were also observed in sugarcane (Lorenzo et al. 2001) and pineapple (Feuser et al. 2001).

In the treatments with low PBZ levels an enhancement in the development of shoots with normal features was observed (Figure 6-J). When cultured in the absence of PBZ the shoots were often etiolated (Figure 6-K).

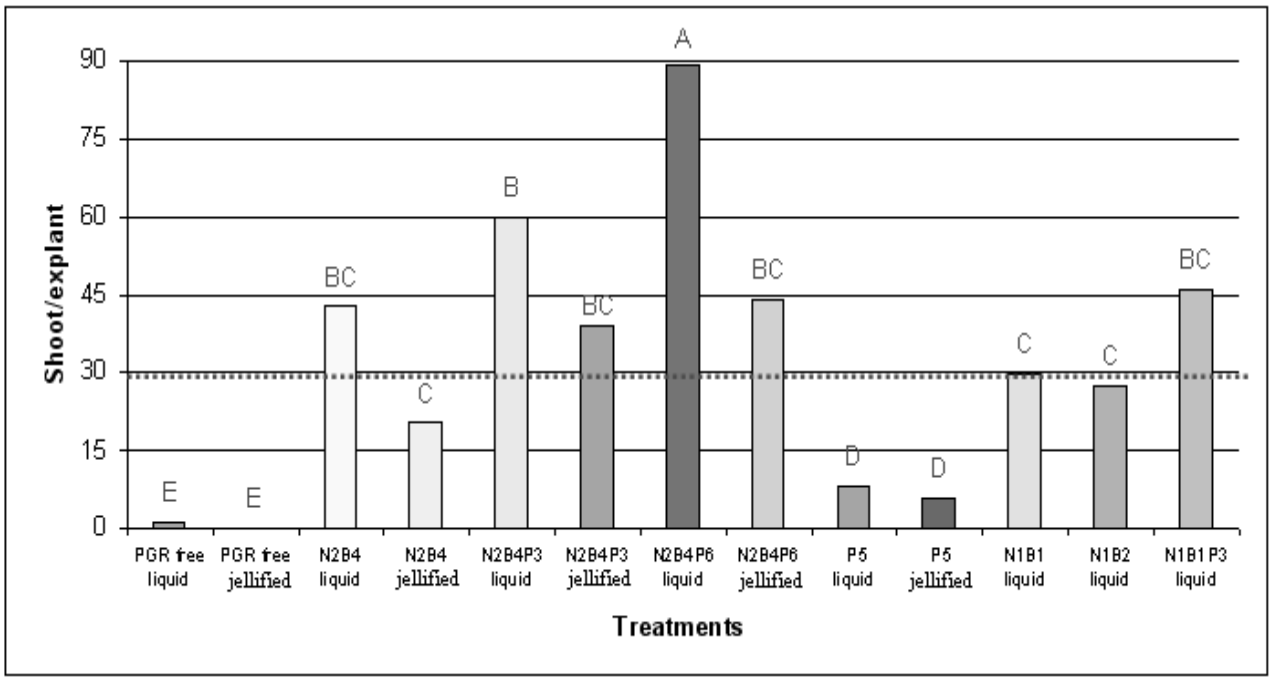

Figure 4. Average shoot number per explant of *Dyckia distachya* cultivated in jellified or liquid MS culture medium supplemented with NAA (N), BA (B) and PBZ (P) after 142 days in culture. Means followed by the same letter are not statistically different (SNK ≤ 0.05)
*PGR-free = culture medium without plant growth regulator

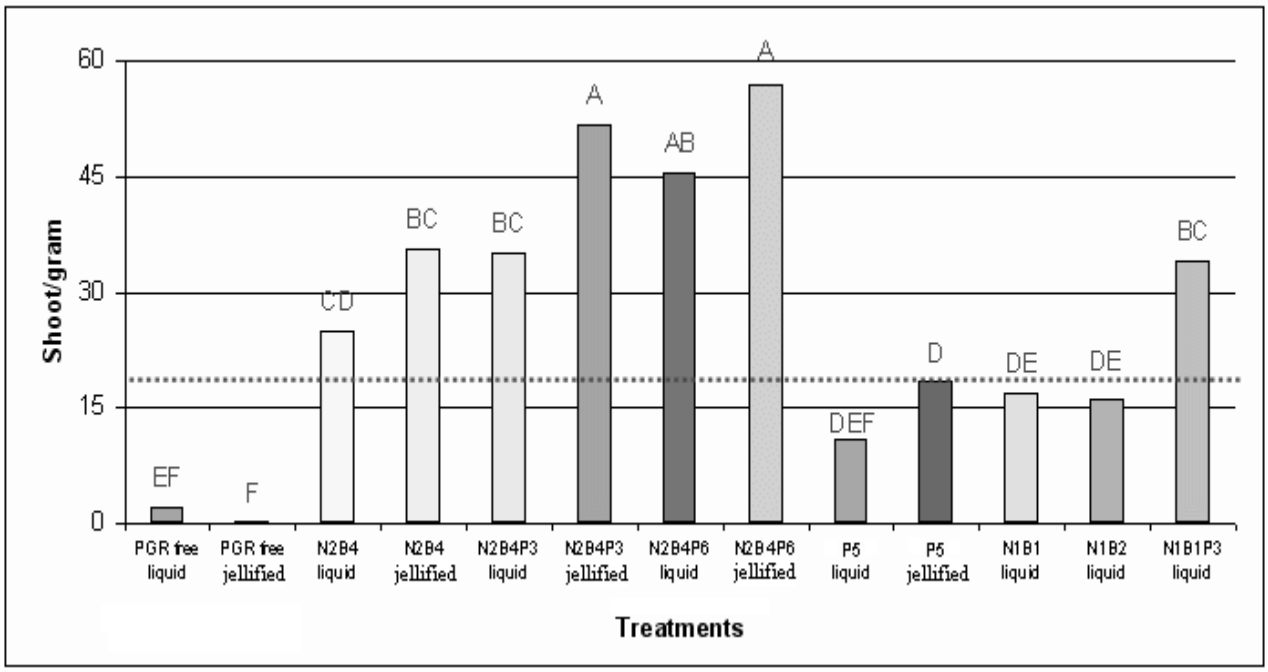

Figure 5. Mean shoot number per gram of inoculum of *Dyckia distachya* cultivated in jellified or liquid MS culture medium supplemented with NAA (N), BA (B) and PBZ (P) after 142 days in culture. Means followed by the same letter are not statistically different (SNK ≤ 0.05)
*PGR-free = culture medium without plant growth regulator

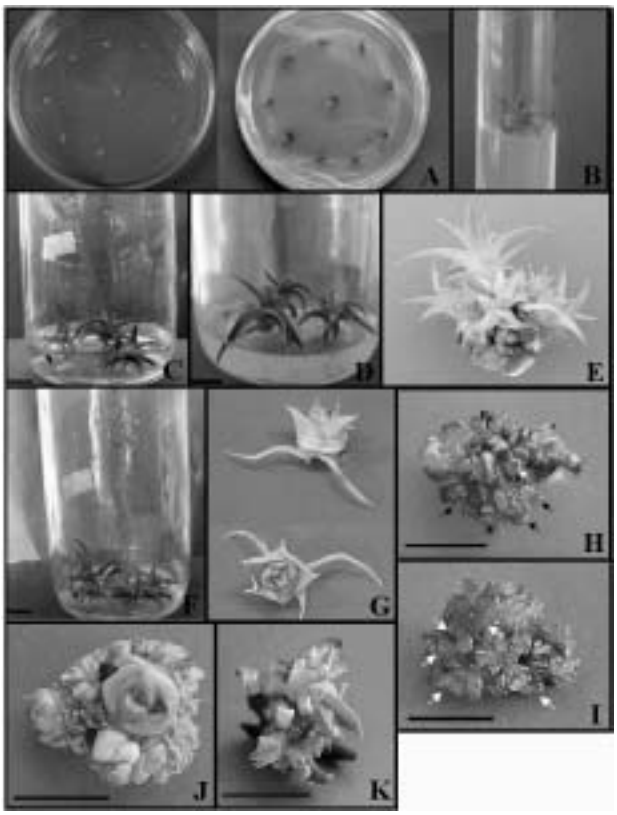

Figure 6. Micropropagation of *Dyckia distachya*. **A.** Seed inoculation (left) and germination after 30 days (right). **B.** Plantlets elongated individually in test tubes. **C.** Subculture in liquid medium, and **D.** jellified. Bar - 1 cm. **E.** Cluster of shoots (arrows). **F.** Elongation of shoots in liquid MS culture medium free of PGR. Bar - 1 cm. **G.** Phytotoxicity caused by PBZ. **H, I.** Hyperhydricity (see arrows) of shoots in liquid culture medium. Bar 1 cm and 5mm. **J.** Normal shoots formed in PBZ-supplemented culture medium. Bar 1 cm. **K**. Etiolated shoots. Bar - 1 cm.

Elongated shoots (at least 1.5 cm) in MS culture medium free of plant growth regulators were successfully transferred and acclimatized to *ex vitro* conditions.

Shoots shorter than 1.5 cm were not able to survive *ex vitro*. Consequently, the shoots need to be maintained *in vitro* until they reach this minimum size. A survival rate of 92.6% was observed after 120 days of *ex vitro* conditions. The acclimatized plants showed normal morphological features after two years in a greenhouse.

In conclusion, the results of the present study demonstrated that tissue culture techniques are useful tools for *D. distachya* mass propagation. High rates of *in vitro* regeneration were obtained in response to liquid MS culture medium supplemented with BA 2 μM after 120 days in culture, or supplemented with NAA 2 μM, BA 4 μM and PBZ 6 μM after 142 days in culture. The use of seeds as initial explants allows the maintenance of the genetic diversity observed in the natural populations. This is an important approach to the conservation of this endangered bromeliad.

Micropropagação massal clonal e conservação of *Dyckia distachya* Hassler

RESUMO - *A Mata Atlântica possui alta diversidade biológica, elevado índice de endemismo, onde as bromélias são muito freqüentes, porém ameaçadas. As técnicas de cultura de tecidos têm auxiliado e muito no resgate de populações ameaçadas de extinção, e na produção de um grande número de plantas, como requerido para os fins comerciais. Esse trabalho avaliou os pontos de controle da micropropagação de* D. distachya*, utilizando sementes como material inicial. Esta espécie apresentou um alto potencial de regeneração* in vitro*, com uma taxa de multiplicação de 78,93 brotos grama^{-1} em resposta ao meio de cultura MS suplementado com BAP 2 μM, após 120 dias, ou 133,58 brotos explante^{-1} na presença de ANA (2 μM), BAP (4 μM) e PBZ (6 μM) após 142 dias de cultivo. Tendo em vista que sementes foram utilizadas como material inicial, a estrutura genética da população original pode ser mantida e o protocolo regenerativo desenvolvido pode ser empregado com sucesso para a regeneração massal dessa bromélia ameaçada de extinção.*

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