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SOMATIC EMBRYOGENESIS IN *DYCKIA DISTACHYA* HASSLER (BROMELIACEAE) – AN ENDANGERED BROMELIAD FROM SOUTH BRAZIL

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Abstract

Dyckia distachya Hassler is an endemic bromeliad from the western part of the Santa Catarina state, southern Brazil. This species is currently threatened by extinction, since its original habitat has been severely affected by the construction of hydroelectric plants. Micropropagation techniques are efficient tools for the mass propagation and conservation of elite genotypes. In the present work, we studied the factors associated with the induction of somatic embryogenesis in *D. distachya*. Seeds were employed as an explant source and showed distinct morphogenic responses. Somatic embryogenesis and the regeneration of complete plants were obtained when seeds were inoculated in MS culture medium supplemented with Morel vitamins, Picloran (5-10 μM) and in the presence or absence of 1 μM N^6-Benzyladenine (BA) or Kinetin (Kin).

Key words: Atlantic Forest, Bromeliaceae, conservation, somatic embryogenesis

INTRODUCTION

Tropical forests cover 7% of the Earth's surface and provide a habitat for 50% of the biodiversity of the planet. The Brazilian Atlantic Forest, reduced to 7% of its original area, is one of the most important biomes in the world. This biome contains 20,000 different species, 8,000 from which are endemic (Schäffer and Prochnow 2002). About 45% of all known plant species grow in Brazil, and the Atlantic Forest is one of the most important plant diversity centers in the world (Heringer and Montenegro 2000).

Dyckia distachya Hassler is a bromeliad native to the western part of the Santa Catarina state, southern Brazil. The environment in this region has recently been affected by the establishment of several hydroelectric power plants. As a result, *D. distachya* is now threatened by extinction (Pompelli and Guerra 2004, Lopes 2005).

Crassulaceaen acid metabolism (CAM), which occurs in *D. distachya*, reduces plant reproductive capacity (Herrera 1999). A promising alternative to natural reproduction may be the propagation by tissue culture-based techniques, which allow the fast and continuous production of plants. Among these techniques, somatic embryogenesis receives the most attention because it potentially allows the large-scale propagation of elite or endangered genotypes (Schultheis et al. 1990).

Somatic (asexual) embryogenesis is a process by which somatic cells develop characteristic morphological stages that are similar to those observed in the ontogenesis of the zygote embryo (Yeung 1995). Unlike zygotic embryos, somatic embryos do not have a vascular connection with the mother tissue and, in theory, can be derived from any tissue (Dodeman et al. 1997).

In an explant, some specific cells are pre-conditioned to morphogenic events that lead to somatic embryogenesis (Thorpe 1980). For this reason, the presence of plant growth regulators (PGR) in the culture medium (e.g., 2,4-Dichlorophenoxyacetic acid (2,4-D)), not only initiates the development of the embryos, but also stimulates the clonal multiplication of pre-determined cells (Fehér et al. 2003, Litz and Jarret 1991). The mechanism of auxinic action is associated with the presence of receptors located in the cell membrane, cytoplasm, and , nucleus (Fehér et al. 2002, 2003, Nato et al. 2000). When the nucleus receives a signal, RNA-polymerase II, an enzyme specific to the transcription of genes involved in the regulation of cell division, gets activated (Van der Linde 1990).

Few protocols for micropropagation via somatic

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embryogenesis have been developed for bromeliads and ornamental plants (Sage et al. 2000, Malda et al. 1999, Rout et al. 1999, Castillo and Smith 1997, Torné et al. 1997). Most protocols for micropropagation of bromeliads are based on direct organogenesis. This is because it is relatively easy to induce a shoot from an initial explant (typically a seed). In a previous study, *D. distachya* showed a high regeneration rate – up to 130 plantlets were obtained from a single seed (Pompelli and Guerra 2005).

The aim of the present work was to elucidate and describe the factors associated with the induction of somatic embryogenesis in *D. distachya*, and develop a protocol for the large-scale micropropagation of this threatened species.

MATERIALS AND METHODS

D. distachya seeds were disinfested according to the protocol developed by Pompelli and Guerra (2004, 2005). Ten seeds were inoculated in each Petri dish, which contained 30 ml of MS culture medium (Murashige and Skoog 1962) supplemented with sucrose (30 g l^{-1}) and MW vitamins (Morel and Wetmore 1951), and gelled using agar (6 g l^{-1}). Two independent experiments were carried out in order to evaluate the effect of the type and concentration of auxins.

Experiment I

A total of nine treatments were tested: four were based on the use of 2,4-D (1.25, 2.5, 5 and 10 μM), another four were based on the use of Picloran (1.25, 2.5, 5 and 10 μM), and one was a control treatment. Each treatment consisted of three replicates. The experiment was laid out in a completely randomized design.

Experiment II

Based on the results obtained in the previous experiment**,** seven treatments were tested, including 2,4-D (5 μM), Picloran (5 μM), 2,4-D (5 μM) + BA (1 μM), 2,4-D (5 μM) + Kin (1 μM), Picloran (5 μM) + BA (1 μM), and Picloran (5 μM) + Kin (1 μM), and a control treatment. Each treatment consisted of three replicates. This experiment was also laid out in a completely randomized design. The percentage data of embryogenic induction were scored after 120 days in culture and transformed, if necessary, using the *ArcSin* $\sqrt{x/100}$ transformation. The data on the number of somatic embryos was, if necessary, transformed using the $\sqrt{x+0.5}$ transformation. The data were then analyzed using ANOVA and Tukey's separation test, through the *Statgraphics Plus* program (version 5.1).

Somatic embryo conversion

The embryogenic cultures were transferred to Petri dishes with 30 ml MS culture medium supplemented with sucrose (30 g l^{-1}), MW vitamins, charcoal (15 g l^{-1}) and gelled using agar (6 g l^{-1}). Three treatments were tested for the conversion of somatic embryos: N^6-(2-isopentenil)adenine (2iP) (12 μM) + α-naphthalene acetic acid (NAA) (0.5 μM), gibberellic acid (GA_3) (10 μM), and sucrose (60 g l^{-1}). After 60 days, the percentage of conversion was recorded. Data were then transformed, if necessary, using the *ArcSin* $\sqrt{x/100}$ transformation and analyzed using ANOVA and Tukey's separation test, through the *Statgraphics Plus* program (version 5.1).

The pH of all culture media were adjusted to 5.8 by adding either NaOH or HCl, before sterilization at 121ºC. The cultures were kept in a growth chamber, with a temperature adjusted to 25 ± 2ºC and relative humidity of 60 ± 5%, in the absence of light. For the conversion of somatic embryos to plantlets, the cultures were maintained in a growth room with irradiance of 60 μmol m^{-2} s^{-1} of photons, 16-hour light period, and 25 ± 2ºC.

Histological Evaluations

In order to perform a histological study of the cultures, samples were removed every two days and immersed in Formaldehyde: Acetic Acid: Water (90:5:5) for 48 hours. The material was then dehydrated in ethanol series and impregnated in paraffin/xylol (Sass 1951). Sections were made using a microtome (section thickness = 5 μm), stained with saphranine and fast green, and visualized under a microscope (Olympus BX 40).

RESULTS

Supplementing the culture medium with 5 μM 2,4-D or Picloran resulted in mean embryogenic induction rates of 61% and 34% (110 and 58 somatic embryos per dish), respectively (Table 1). These two values were not statistically different. Therefore, a second experiment was carried out keeping the concentration of auxins fixed at 5 μM and changing the source of cytokinins.

After two days in culture, the germination of the inoculated seeds was initiated (Fig. 1D). However, in the presence of 5 μM 2,4-D or Picloran, an intense process of cellular division occurred after 30 days of culture (Fig. 1B). These cultures showed an intense cell proliferation, which result in the induction and development of somatic embryos in different developmental stages (Fig. 1F).

In the second experiment, distinct morphogenetic responses were observed. When seeds were inoculated in PGR-free culture medium, no formation of embryogenic cultures was observed (Fig. 1C). In contrast, in seeds inoculated in culture medium supplemented with Picloran, high rates of embryogenic induction were observed, regardless of the cytokinin used. The highest

Table 1. Rate of induction and number of somatic embryos (SE) of *Dyckia distachya*, in response to different levels of 2,4-D and Picloran in gelled MS basal culture medium, 120 days after inoculation. Averages followed by the same letter in each column are not significantly different (Tukey, α = 0.01). Overall means (last line) followed by the same capital letter are not significantly different (Tukey, α = 0.01).

Concentrations	Embryogenic Induction (%)		SE per Petri dish	
(μM)	Picloran	2,4-D	Picloran	2,4-D
0.00	0.0 c	0.0 b	0.0 e	0.0 c
1.25	5.5 b	33.6 a	10.2 d	33.2 b
2.50	7.2 b	37.3 a	23.7 c	36.7 b
5.00	60.9 a	34.3 a	109.7 a	58.3 a
10.00	56.5 a	36.7 a	77.8 b	25.8 b
CV (%)	4.9	8.0	8.4	7.6
Mean	26.0 A	28.4 A	40.3 A	30.8 A

rate of embryogenic induction (59.2%) was observed in response to the treatment with Picloran (5 μM) + Kin (1 μM). In this case, the mean number of somatic embryos developed per Petri dish was 125.4 (Table 2). In this experiment, 2,4-D proved to be less efficient than Picloran for the induction of embryogenic cultures. In the presence of 2,4-D (5 μM) + Kin (1 μM), 36.7% of the explants became embryogenic after 120 days in culture (Table 2). The development of 49.8 embryos per Petri dish was observed in this treatment.

When the interaction between the concentration of auxins and cytokinins was analyzed, an advantage of Picloran over 2,4-D was observed. Fifty-eight percent of the explants in the culture medium supplemented with Picloran became embryogenic, whereas in the presence of 2,4-D, this response was observed in less than 34% of the cases. Similar responses were observed regarding the number of somatic embryos – 120 and 36 somatic embryos per dish were developed in response to Picloran and 2,4-D, respectively (Table 2).

Table 2. Rate of induction and number of somatic embryos (SE) of *Dyckia distachya* in response to different combinations of auxins (Picloran or 2,4-D – 5 μM) in the presence or absence of BA or Kin (1 μM each), supplemented to MS basal culture medium, 120 days after inoculation. Averages followed by the same letter in each column are not significantly different (Tukey, α = 0.01). Overall means (last line) followed by the same capital letter are not significantly different (Tukey, α = 0.01).

BA	Kin	Embryogenic Induction (%)		SE per Petri dish	
		Picloran	2,4-D	Picloran	2,4-D
0	0	0.0 b	0.0 c	0.0 b	0.0 c
0	0	56.7 a	39.5 a	115.5 a	40.3 a
1	0	58.1 a	25.8 b	118.2 a	18.1 b
0	1	59.2 a	36.7 a	125.4 a	49.8 a
CV (%)		6.2	9.2	2.1	12.4
Mean		58.0 A	34.0 B	119.7 A	36.1 B

Different stages of somatic embryogenesis, with globular, torpedo, and cotyledonal embryos could be observed in this experiment (Fig. 1G).

In general, rates of conversion were low, although the treatment with 2iP (12 μM) + NAA (0.5 μM) promoted a 16% conversion rate (Figs. 1, 2).

DISCUSSION

The rate of embryogenic induction obtained in this study was high compared to the values reported by Mohamed et al. (2004) – 33.2% using 7.9 μM 2,4-D in *Macrotyloma uniflorum* (Lam.), Torné et al. (1997) – 25% in zygotic embryos of *Araujia sericifera* Brot. cultivated with 9.1 μM 2,4-D, and Chen and Chang (2000) – 29.1% in *Oncidium* 'Gower Ramsey' in the presence of different combinations of NAA and TDZ.

Picloran is used in tissue culture in order to induce and/or maintain calluses or cellular suspensions, or induce the formation of embryogenic calluses. In the present study, the addition of Picloran (5 and 10 μM), regardless of whether it was used in combination with Kin (1 μM), resulted in the highest rates of embryogenic induction (Tables 1 and 2). Similar results were described by Stefanello et al. (2005) based on embryogenic induction in *Feijoa sellowiana* Berg. Other reports of the efficiency of Picloran are also present in the literature (Little et al. 2000, Dineshkumar et al. 1995). In *Freesia hybrida* L.H. Bailey, Picloran induced 3-12 times more somatic embryos than 2,4-D (Bach 1992). In addition to this, Dineshkumar et al. (1995) concluded that Picloran is a potent auxin, which is able to induce embryogenic calluses. However, in *Narcissus pseudonarcissus* L., NAA and 2,4-D promoted the formation of somatic embryos, whereas Picloran inhibited the process (Sage et al. 2000).

In cell suspension of carrots, 2,4-D led to the accumulation of indole-3-acetic acid (IAA) (Michalczuk et al. 1992). Based on this, the authors postulated that the embryogenic competence of the carrot cells is closely related to the increase in IAA concentration caused by the presence of 2,4-D. They also postulated that 2,4-D can act in two different ways: (1) indirectly, by deviating the metabolism of the endogenous IAA, or (2) directly, on the embryogenic induction.

In the present study, kinetin proved to be more efficient as a complement to auxin for the induction of somatic embryogenesis. Wang et al. (2004) observed that the calluses of *Scirpus robustus* Pursh, which formed only in the presence of BA, had a yellowish appearance, but were not embryogenic.

These pro-embryos are stimulated to follow the

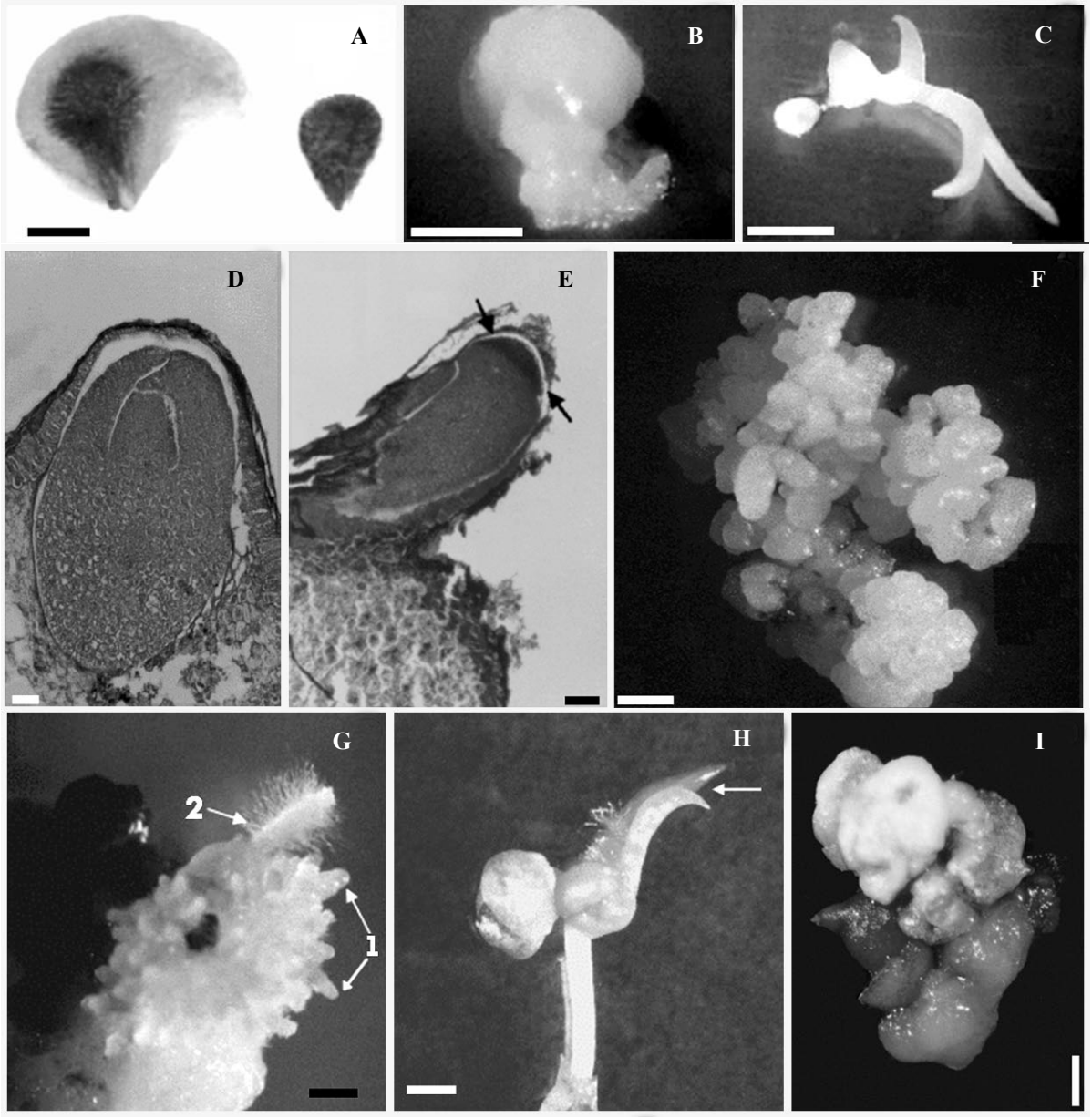


Figure 1. Somatic embryogenesis in *Dyckia distachya*. A) Inoculated seed – Bar 5 mm; B) Calluses after 30 days in the presence of 2,4-D (5 μM) and C) in MS/0 – control – Bars 5mm; D) Seeds in germination in culture medium with 2,4-D (5 μM) – Bar 66.7 μm; E) Seeds after 2 days in culture medium with 2,4-D (5 μM). Note that the embryo first germinates and then gives origin to calluses demonstrated in B. Note also the high cell proliferation (arrows) – Bar 88 μm; F) Embryogenic cultures after 120 days of inoculation– Bar 1.764 mm; G) Somatic embryos in torpedo stage (1) and beginning of rhyzogenesis (2) – Bar 0.704 mm; H) Somatic embryo in germination stage; see the opening of the cotyledon (arrow) – Bar 0.88 mm; I) Initial stage of germination of a somatic embryo in the presence of 2iP (12 μM) + NAA (0.5 μM) 30 days after its transference to light – Bar 0.88 mm;

developmental stages by the removal of auxins from the culture medium and by the addition of abscisic acid (ABA), cytokinins, and other agents (Guerra et al. 1999). The germination of somatic embryos represents a complete bipolar development, with the distinct development of shoot and root axes (Féher et al. 2003).

The utilization of 2iP in the conversion of somatic embryos has already been well documented. In experiments with *Euterpe edulis* Mart., conversion of somatic embryos was obtained when they were subcultivated in a medium supplemented with 2iP (12 μM) + NAA (0.5 μM) (Guerra and Handro 1988).

CONCLUSION

In the present work, it was possible to establish a complete *in vitro* protocol for somatic embryogenesis

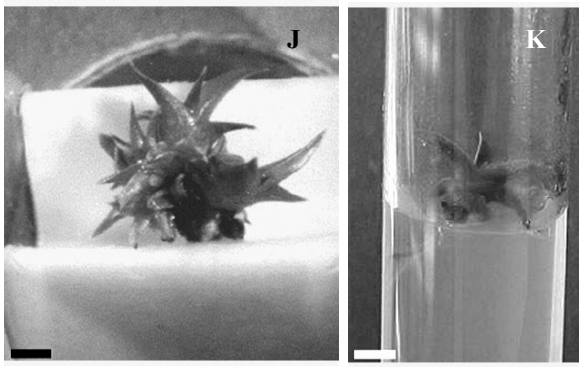


Figure 1. Somatic embryogenesis in *Dyckia distachya*.J) plants in conversion still onto a paper bridge – Bar 1.764 mm and K) individualized in jellified culture medium – Bar 2.514 mm.

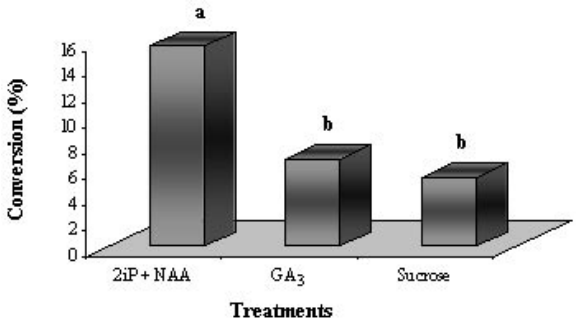
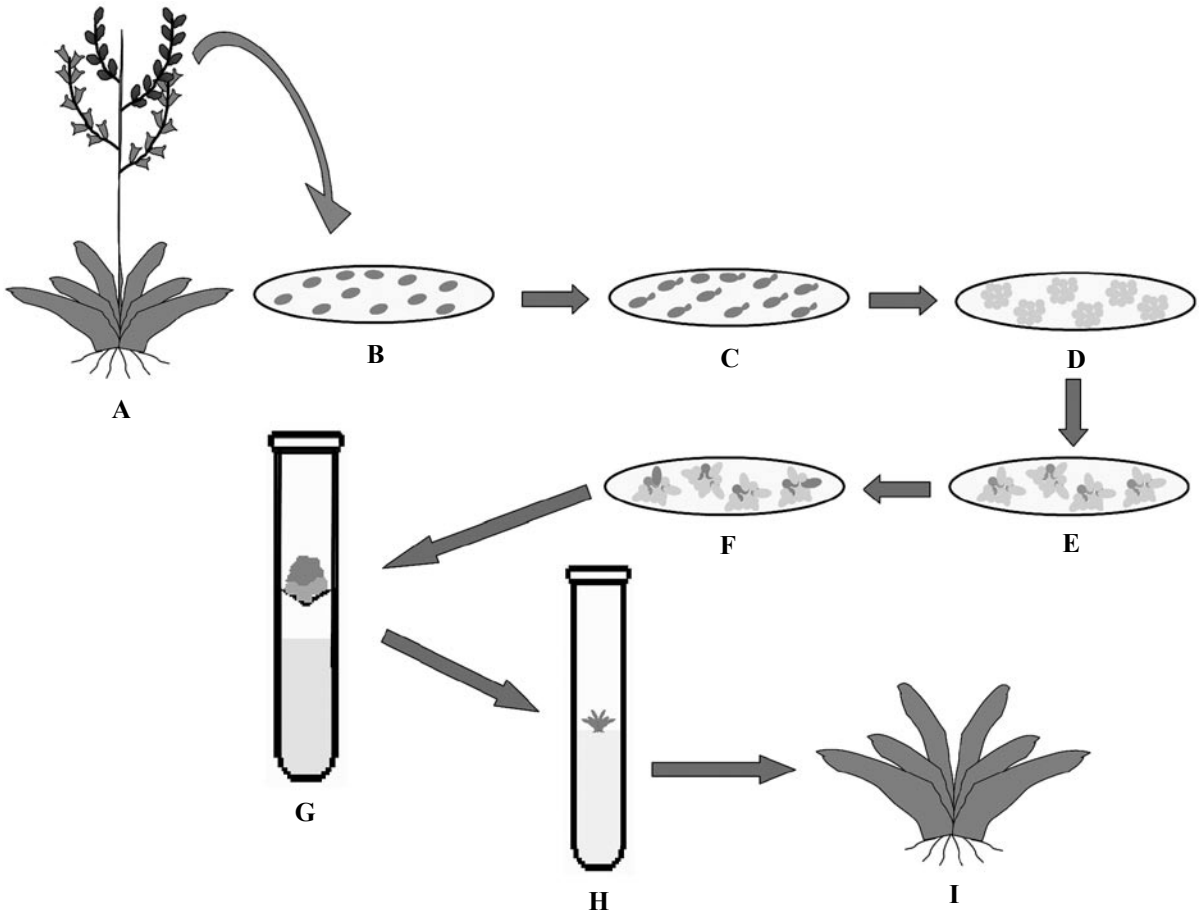


Figure 2. Rate of conversion from somatic embryos to plantlets from seeds of *Dyckia distachya* in response to different plant growth regulators and sucrose supplemented to MS culture medium, 180 days after inoculation. Means followed by the same letter are not significantly different (Tukey, $\alpha = 0.01$).



A) Mother plant in flowering stage; B) Seeds are collected and inoculated in the MS culture medium supplemented with Picloran (5 μM); C) Two days after inoculation the seeds begin to germinate and, at the same time start to dedifferentiate to form a callus; D) After 30 days in culture, the formation of proembryogenic cellular masses is observed. After 120 days, these masses form embryogenic cultures; E) Aliquots of these embryogenic cultures are transferred to a MS culture medium supplemented with 2iP (12 μM) + NAA (0.5 μM) and F) then transferred to light conditions, where, after a period of 30 days in culture, the somatic embryos initiate a process of conversion to plantlets; G) Embryogenic cultures are transferred to test tubes with liquid culture medium over paper filter bridges; H) As somatic embryos develop to mature stage, they are transferred to Petri dishes with gelled MS culture medium; I) Regeneration of complete plantlets to be acclimatized and transplanted to the field.

in *Dyckia distachya,* an endangered bromeliad, which is endemic to the south of Brazil. Seeds inoculated in MS culture medium supplemented with Picloran (5 μM) + Kin (1 μM) exhibited a series of morphogenic events, including induction, development, maturation, conversion, and germination of somatic embryos (Fig. 3). The importance of these findings concerns: (1) the need for conservation of this endangered species in its center of diversity, (2) the ornamental potential of *D. distachya*, and (3) the potential application of (bio) technologies based on large-scale micropropagation for achieving the objectives stated above.

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