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Induction of *in vitro* shoots of *Billbergia euphemiae* E. Morren (Bromeliaceae) from leaf explants

Mariela Justiniano Simão[1*], Daniele Vidal Faria[2], Elias Terra Werner[3], Taís Cristina Bastos Soares[3] and Andreia Barcelos Passos Lima Gontijo[4]

*[1]Núcleo de Biotecnologia Vegetal, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier, 524 PHLC, 20550-013, Rio de Janeiro, Rio de Janeiro, Brazil. [2]Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. [3]Universidade Federal do Espírito Santo, Centro de Ciências Agrárias, Alegre, Espírito Santo, Brazil. [4]Universidade Federal do Espírito Santo, Centro Universitário Norte do Espírito Santo, Departamento de Ciências Agrárias e Biológicas, São Mateus, Espírito Santo, Brazil. *Author for correspondence. E-mail: marielajsimao@gmail.com*

ABSTRACT. Bromeliads are an important group for the maintenance of the Atlantic Forest, with many threatened species due to exacerbated extraction and destruction of their natural habitats. Considering the need of developing protocols for the conservation of these species, the aim of this work was to evaluate the effect of different growth regulators in the *in vitro* induction of shoots of *Billbergia euphemiae*. Leaf explants were excised from seedlings derived from *in vitro* germination and grown on MS medium supplemented with NAA (0, 1 or 2 µM) and BA (0, 2, 4 or 6 µM) combinations. The evaluation of the number of shoots per explant, shoot length, number of leaves per shoot and longest leaf length average was carried out after 30 and 60 days of culture. The best *in vitro* responses were observed in the presence of 1 µM NAA after 60 days of culture, which induced the best production of shoots per explant (16.39), as well as the highest rates of shoot length (1.08 cm), number of leaves per shoot (5.00) and the longest leaf length (0.56 cm). This work determined the best conditions for shoot production from leaf explants of *B. euphemiae*, being the first report on micropropagation of this species.

Keywords: BA, bromeliad, micropropagation, NAA, organogenesis.

Indução *in vitro* de brotos de *Billbergia euphemiae* E. Morren (Bromeliaceae) a partir de explantes foliares

RESUMO. As bromélias constituem um importante grupo para a manutenção da Floresta Atlântica, com várias espécies ameaçadas de extinção pelo extrativismo exacerbado e a destruição dos habitats naturais. Considerando a necessidade do desenvolvimento de protocolos para a conservação destas espécies, este trabalho teve como objetivo avaliar o efeito de diferentes reguladores de crescimento na indução de brotos de *Billbergia euphemiae*. Explantes foliares foram excisados de plântulas derivadas da germinação *in vitro* e inoculados em meio MS suplementado com combinações de ANA (0, 1 ou 2 µM) e BA (0, 2, 4 ou 6 µM). O número de brotos por explantes, comprimento dos brotos, número médio de folhas por broto e comprimento médio da maior folha foram avaliados após 30 e 60 dias de cultura. As melhores respostas foram observadas na presença de ANA a 1 µM, após 60 dias de cultura, que induziu a maior produção de brotos por explante (16,39), assim como as maiores taxas de comprimento dos brotos (1,08 cm), número médio de folhas por broto (5,00) e comprimento da maior folha (0,56 cm). Este trabalho determinou as melhores condições para produção de brotos de *B. euphemiae* a partir de explantes foliares, representando o primeiro relato de micropropagação desta espécie.

Palavras-chave: BAP, bromélia, micropropagação, ANA, organogênese.

Introduction

The Atlantic Forest is one of the most important tropical biomes in the world for having a high genetic diversity, presenting around 20,000 vegetal species, in which 40% are endemic (Tabarelli, Pinto, Silva, Hirota, & Bedê, 2005). The remaining fragments make approximately 7.91% of the original Atlantic Forest, including the majority of the officially endangered species, forming the most threatened ecosystem in Brazil (Martinelli, 2000).

Within these species, there are the bromeliads, which belong to the Bromeliaceae family and are largely used as ornamental plants. The combination of the fragmentation of the Atlantic Forest and the uncontrolled extractivism of these species for domestic use drove to a decline in the number of natural populations and, consequently, the loss of a big part of its genetic diversity (Vieira et al., 2013).

The species *Billbergia ephemiae* E. Morren is found at the Brazilian states of Bahia, Espírito Santo, Minas Gerais and Rio de Janeiro, and can be found in vegetations of dense rainforests, semideciduous seasonal forests and restingas (Barros & Costa, 2008; Martinelli, Vieira, Leitman, Costa, & Forzza, 2009).

The *in vitro* culture techniques represent important biotechnogical tools that have helped the use and conservation of the plant genetic resources in the last decades (George et al., 2008). Works with *in vitro* propagation of bromeliads have been developed using explants as seeds (Galvanese et al., 2007; Bencke & Droste, 2008), radicular meristems (Pompelli & Guerra, 2005) and leaves (Koh & Davies, 1997; Carneiro et al., 1999; Silva, Franco, Dornelles, Bortoli, & Quoirin, 2009; Fermino, Lando, Santos, & Pescador, 2014). However, to our knowledge, there aren't any previous reports of *in vitro* propagation of the species *B. euphemiae* or of the utilization of leaf explants for the micropropagation of this genus, what makes this work unprecedented, enabling subsidies to the development of new *in vitro* studies with this bromeliad species.

This work intended to establish a protocol to the induction of shoots and *in vitro* propagation of *B. euphemiae*, aiming the *in vitro* conservation of the species, due to the ornamental and commercial potential of bromeliads, the intense degradation of its natural habitats, which leads to the extinction of many species, and the success of *in vitro* propagation protocols of important bromeliad species.

Material and methods

Fruits of *B. euphemiae* were collected from individuals located in the District of Bururama, city of Cachoeiro de Itapemirim, ES, in the geographic coordinates 20°41'S 41°21'W and dried outdoors in the shade for seven days, in order to complete their dehiscence and facilitate the extraction of the seeds. Subsequently, the fruits were stored in paper packagings at 4ºC until its utilization. The voucher of specimens *B. euphemiae* E. Morren was stored in the herbarium Leopoldo Krieger in *Universidade Federal de Juiz de Fora*, Minas Gerais, Brazil, under the number CESJ 55660.

For the experiment, a bulk of seeds was created, in which seeds from one or two fruits of each individual were extracted manually and mixed to the seeds of the other individuals in order to obtain a representative sample of the population diversity of *B. euphemiae* present in the area. Thereafter, the seeds were washed in distilled water and dried in a B.O.D. at 37ºC for 24h. After this procedure, the seeds were stored in filter paper packaging at 4ºC.

In aseptic conditions, the seeds were decontaminated for one minute in alcohol 70% and in commercial sodium hypochlorite (2.5% of active chlorine) for five minutes and rinsed in sterile distilled water three times. This process was repeated twice and then the seeds were placed in filter paper until the moment of inoculation.

The seeds were placed in MS medium (Murashige & Skoog, 1962) supplemented with sucrose (30 g L^{-1}), solidified with agar (7 g L^{-1}) and kept in culture room at temperature of 25 ± 2ºC, under artificial white light (fluorescent) with fluency of 1.6 W m^{-2} and photoperiod of 16 hours.

Leaves of approximately 1 cm in length were removed from seedlings derived from *in vitro* germination after 42 days of culture and inoculated with the abaxial surface in contact with the medium. The MS medium used was supplemented with different combinations of the plant growth regulators (PGR) naphthaleneacetic acid (NAA) (0, 1 or 2 µM) and 6-benzylaminopurine (BA) (0, 2, 4 or 6 µM). The conditions of the culture room were the same as previously mentioned.

The experiment was conducted in a 3x4 factorial scheme (concentrations of NAA x concentrations of BA) with six repetitions, in a completely randomized experimental design (CRD). The experimental unit was composed by a petri dish containing five explants.

The cultures were evaluated 30 and 60 days after the inoculation of the leaf explants to measure the following variables: number of shoots per explant (NSE), shoot length (SL), average number of leaves per shoot (ANL) and longest leaf length average (LLL). A stereoscopic microscope was used to measure the variables NSE and ANL, and for the variables SL and LLL, a caliper.

To the statistical analysis, we used Shapiro-Wilk tests for normality of residues of the analysis of variance (ANOVA) and Bartlett's for homogeneity between the variances. Having covered these two assumptions of the parametric statistics, the analysis of variance was performed, followed by the Tukey test for comparisons among the averages, both in 1% and 5% of probability using the Assistat software 7.6 beta version.

Results and discussion

We observed a rate of 100% of germination of *B. euphemiae* seeds in MS medium without the addition of growth regulators and free of contaminants such as bacteria and fungi, confirming the efficiency of the protocol of decontamination used.

Leaf explants excised from seedlings derived from *in vitro* germination originated shoots from the basal region in response to all tested combinations of NAA and BA (Figure 1). The same response pattern was described by Hosoki and Asahira (1980), who suggested that the basal region of leafs of bromeliads presents vascular elements that can contain competent cells for dedifferentiation when activated by growth regulators. Posterior works also showed this kind of response for different species of bromeliads (Mercier & Kerbauy, 1997; Carneiro et al. 1999; Pompelli & Guerra, 2005; Alves, Dal Vesco, & Guerra, 2006; Corredor-Prado et al., 2015).

The results of the analysis of variance showed that the NAA and BA factors only had presented significant interaction in the LLL variable after 60 days of culture (Table 1). For the other parameters (NSE, SL and ANL, after 30 and 60 days, and LLL, 30 days) we did not observe significant interaction between the two tested factors.

The NAA effect on the NSE, SL and ANL variables is represented on Table 2. The best results for NSE were observed after 60 days in the concentrations of 1 µM (16.39 shoots) and 2 µM (10.69 shoots) respectively, although they did not show statistical differences between them and the 2 µM concentration did not differ from the control.

The treatment with 1 µM NAA had presented the highest average for SL, 1.08 cm, after 60 days of culture (Table 2 and Figure 2). This same NAA concentration also favored the highest average for the ANL variable after 60 days, with approximately five leaves per shoot (Table 2).

Similar results were also described by Paiva, Naves, Dutra, Paiva and Pasqual (2009) with the bromeliad *Nidularium fulgens*, in which a longer shoot length (1.85 cm) was observed after 120 days of culture in MS medium supplemented with NAA at 2.6 µM.

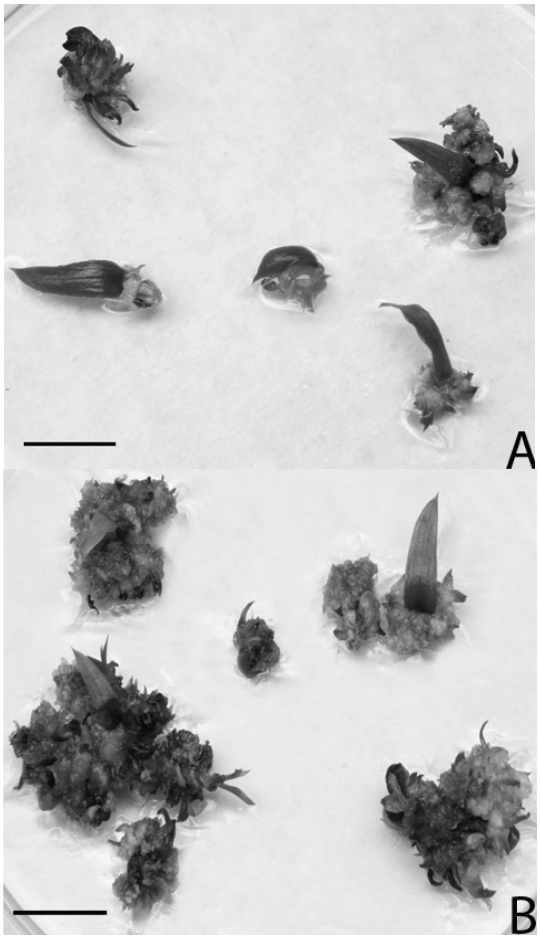

Figure 1. Leaf explants of *Billbergia euphemiae* E. Morren inoculated in MS medium + 2 µM NAA + 6 µM BA. The shoot formation can be seen in the leaf base. A - Explants after 30 days of culture. B - Explants after 60 days of culture. (Bar = 1 cm).
Source: the authors.

Table 1. Analysis of variance for the variables: number of shoots per explant (NSE), shoot length (SL), average number of leaves (ANL) and longest leaf length (LLL) of *Billbergia euphemiae*, after 30 and 60 days of culture in MS medium supplemented with NAA (F1) and BA (F2).

PGR	NSE		SL		ANL		LLL	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
NAA (F1)	8.1168 **	6.4062 **	5.9270 **	12.8430 **	2.9532 ns	14.9510 **	2.9532 ns	14.9510 **
BA (F2)	6.8746 **	6.7893 **	7.5360 **	5.4912 **	1.8296 ns	6.5353 **	1.8296 ns	6.5353 **
Int. F1 x F2	0.5230 ns	0.4569 ns	1.5008 ns	2.2346 ns	0.4500 ns	3.4583 ns	0.9250 ns	4.4687 **

**significatif at 1% ($p < .01$), *significatif at 5% ($.01 \leq p < .05$), ns non-significatif ($p \geq .05$) (*F*-test).

Table 2. NAA effect on the number of shoots per explant (NSE), shoot length (SL), average number of leaves (ANL) of *Billbergia euphemiae* after 30 and 60 days of culture.

Treatments	NSE		SL		ANL	
	30 days	60 days	30 days	60 days	30 days	60 days
0µM NAA	2.35833 b	6.23833 b	0.22292 b	0.44958 b	0.15208 a	1.33333 b
1µM NAA	6.98333 a	16.39333 a	0.45792 a	1.08542 a	0.39167 a	5.53833 a
2µM NAA	4.82500 ab	10.69167 ab	0.33250 ab	0.66458 b	0.05000 a	1.16042 b

Means followed by same column letters do not differ at 1% of probability ($p < 0.01$) (Tukey test).

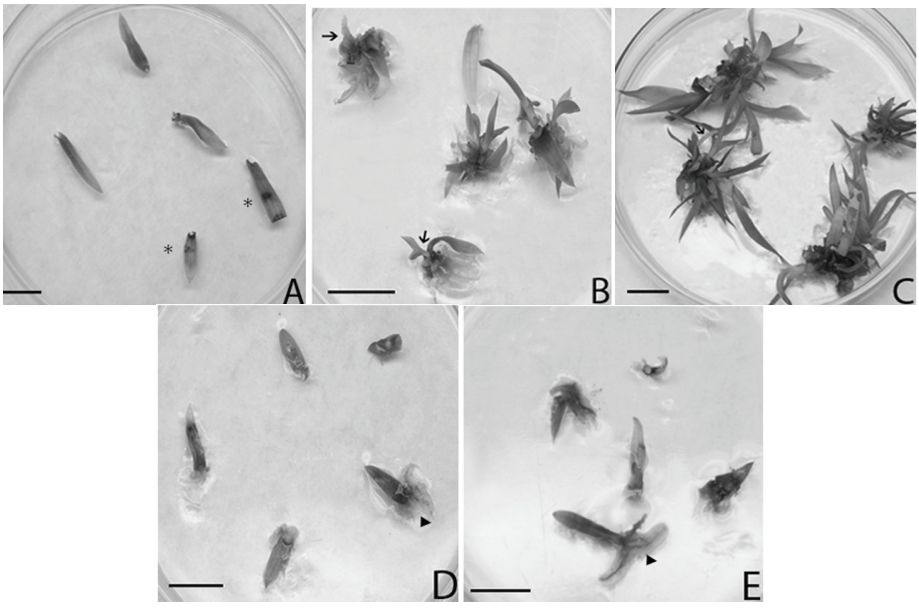

Figure 2. NAA effect on the induction of shoots of *Billbergia euphemiae* E. Morren. **A -** Ageing explants (*) in MS medium after 30 days. MS medium + 1 µM NAA after 30 days (**B)** and after 60 days **(C)**. MS Medium + 2 µM NAA after 30 days (**D)** and after 60 days **(E)**. Arrows indicate shoots and arrow tips point the root. (Bar = 1 cm).
Source: the authors.

Moreover, Pierik, Steegmans and Hendriks (1984) reported that the addition of NAA in the MS culture medium, in concentrations among 2.6 and 4.2 µM, efficiently promoted the growth and rooting of the shoots of three different bromeliad species (*Guzmania minor, G. lingulata* and *Vriesea splendens*). In this work, the endogenous concentration of cytokinins in the *B. euphemiae* leaf explants probably was sufficient to promote a hormonal balance with the auxin present in the culture medium and to stimulate the observed morphogenic responses. This can be justified by the fact that the highest number and longest length of the shoots were observed using only 1 µM of NAA.

Table 3 shows the results of the BA effect on the NSE, SL and ANL variables. All the treatments with BA demonstrated to be efficient for the NSE and SL variables, without significant difference among the tested concentrations and only differentiating significantly from the control (Figure 3). Mendes et al. (2007) and Pardo, Michelangeli, Mogollón and Alvarado (2008) showed that the presence of BA in MS medium promoted a significant increase in the number of shoots in *Billbergia distachia* and *B. rosea*, respectively, indicating that this cytokinin can contribute to the *in vitro* multiplication of plants of this sort. However, to *B. euphemiae* we can observe that the presence of BA in the culture medium does not seem to be necessary, once the best results to the analyzed variables were detected in the treatment with 1 µM of NAA.

In relation to the ANL analysis, there was no statistical difference among the treatments after 30 days, whereas after 60 days, the concentration of 2 µM of BA was superior to the other treatments (5.35 leaves), not differing significantly from the control (Table 3). This indicates that the endogenous level of cytokinins in the leaf explant is sufficient to promote the growth of the leaves. There is no need for any addition of this growth regulator to the culture medium.

The variable longest leaf length (LLL) was not influenced by NAA after 30 days of culture, once the highest averages observed in the concentration of 1 µM did not differ significantly in relation to the control (Table 4).

Table 3. BA effect on the number of shoots per explant (NSE), shoot length (SL), average number of leaves (ANL) of *Billbergia euphemiae* after 30 and 60 days of culture.

Treatments	NSE		SL		ANL	
	30 days	60 days	30 days	60 days	30 days	60 days
0µM BA	1.17778 b	2.48889 b	0.12278 b	0.37056 b	0.13333 a	2.60000 ab
2µM BA	5.38889 a	11.31778 a	0.42000 a	0.83611 a	0.43611 a	5.35611 a
4µM BA	5.45556 a	15.03111 a	0.33889 a	0.82389 a	0.10000 a	1.67778 b
6µM BA	6.86667 a	15.59333 a	0.46944 a	0.90222 a	0.12222 a	1.07556 b

Means followed by same column letters do not differ at 1% of probability ($p < 0.01$) (Tukey test).

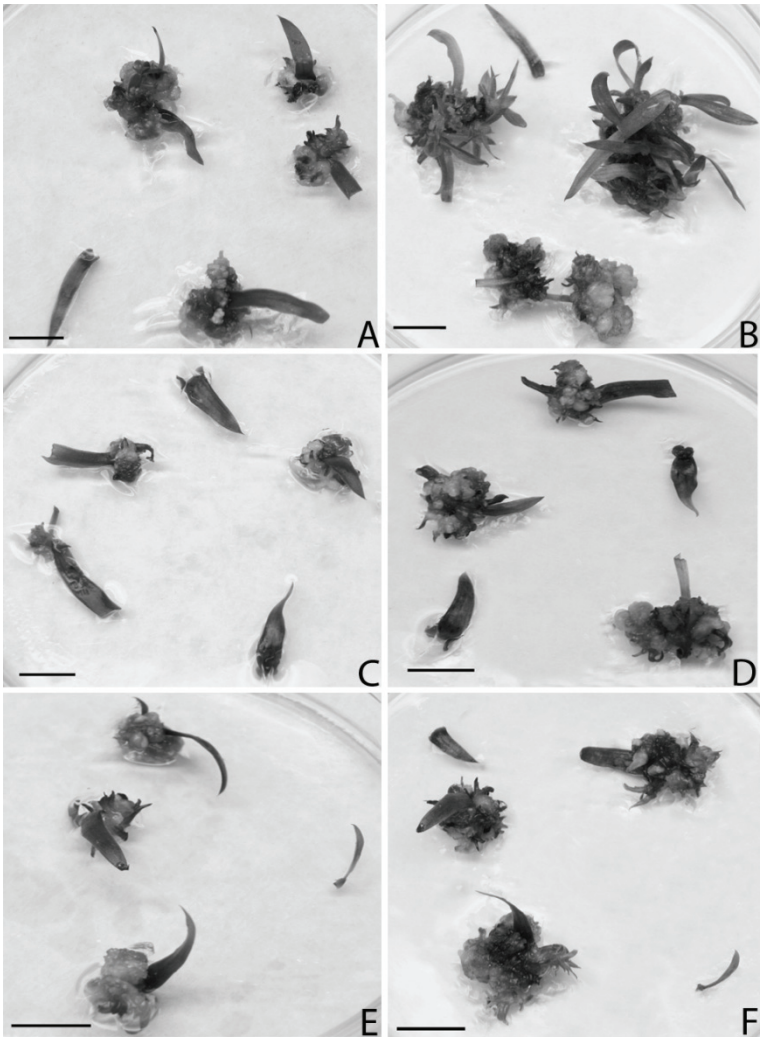

Figure 3. BA effect in the induction of shoots of *Billbergia euphemiae* E. Morren. Explants inoculated in MS medium + 2 µM BA after 30 days of culture **(A)** and 60 days of culture (**B**). MS medium + 4 µM BA after 30 days **(C)** and 60 days **(D)**. MS Medium + 6 µM BA after 30 days **(E)** and 60 days **(F)**. (Bar = 1 cm).

Source: the authors.

Table 4. NAA and BA effect on the longest leaf length (LLL) of *Billbergia euphemiae* after 30 days of culture.

NAA			BA			
0µM	1µM	2µM	0µM	2µM	4µM	6µM
0.02958 ab	0.07917 a	0.02167 b	0.02944 a	0.08889 a	0.03389 a	0.02167 a

Means followed by same letters do not differ at 1% of probability ($p < 0.01$) (Tukey test).

For BA, there was no significant difference among the treatments. These results indicate that the addition of NAA or BA to the culture medium did not influence the leaves length after 30 days of culture, probably due to a satisfactory endogenous level of these regulators in the explants.

When analyzing the variable LLL after 60 days, however, a significant interaction between NAA and BA was remarked (Table 5). The highest value was detected in the interaction of 1 µM NAA with absence of BA (0.560 cm) in MS medium, in which inside from level 0 µM of BA, the concentration of 1 µM NAA differed statistically from 0 and 2 µM NAA. Inside of level 1 µM of NAA, the concentrations of 0 and 2 µM of BA (0.560 and 0.458, respectively) were statistically superior to the concentrations of 4 and 6 µM of BA (0.180 and 0.085, respectively) (Figure 4).

As previously mentioned, some works have reported the role of NAA in the development of the aerial parts in bromeliads (Pierik et al., 1984; Paiva et al., 2009). The presence of 1 µM of NAA was sufficient to promote a biggest growth of the leaves, since responses were not induced in the concentration of 2 µM.

Table 5. NAA and BA effect on the longest leaf length (LLL) of *Billbergia euphemiae* after 60 days of culture.

NAA	BA			
	0 µM	2 µM	4 µM	6 µM
0 µM	0.0000 bB	0.3067abA	0.0000 aB	0.1067aAB
1 µM	0.5600 aA	0.4583 aA	0.1800 aB	0.0850 aB
2 µM	0.0000 bA	0.2033 bA	0.1283 aA	0.1250 aA

Means followed by same column letters and capital letters on lines, do not differ at 1% of probability ($p < 0.01$) (Tukey test).

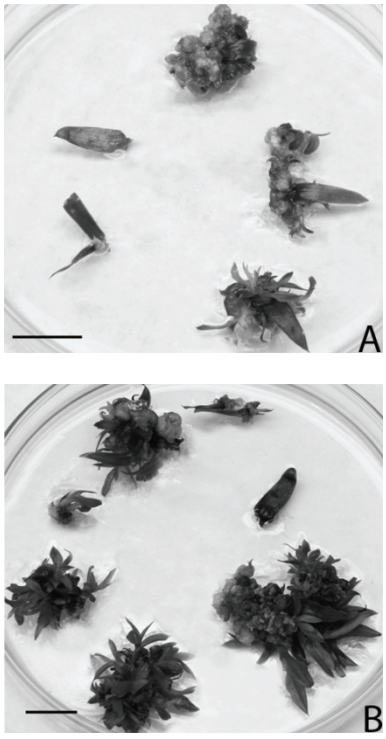

Figure 4. NAA and BA effect in the induction of shoots of *Billbergia euphemiae* E. Morren. Explants inoculated in MS medium + 1 µM NAA + 2 µM BA after 30 days of culture (A) and after 60 days of culture (B) (Bar + 1 cm).
Source: the authors.

Conclusion

In this work, it was possible to establish, for the first time, a protocol to the induction of shoots from *B. euphemiae* leaf explants. The organogenesis was only observed in the base of the leaf explants, in response to NAA and BA, probably because of the activation of competent cells to the dedifferentiation, which highlights the morphogenic potential of this region. Moreover, as the production of shoots occurred by direct organogenesis, the usage of leaf explants to the micropropagation of *B. euphemiae* can also be considered as a complementary strategy to the *in vitro* multiplication and conservation of this species.

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