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Differential sprouting ability during micropropagation of *Nidularium minutum* Mez (Bromeliaceae)

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ABSTRACT. *Nidularium minutum* is an ornamental bromeliad from the Brazilian Rainforest. The micropropagation of this species is essential for obtaining plants available for conservation programs or commercial use. Our study aimed to establish an efficient plant production method by *in vitro* sprouting. This bromeliad takes a long time to sprout *in vitro,* and 10% of the plants produce shoots in a culture medium without plant growth regulators (PGRs). When subcultured in a PGR-free medium, these individualized shoots can sprout like the mother plant. The Murashige and Skoog basal medium (MS) containing 1.0 mg L^{-1} of 6-benzylaminopurine (BAP) promoted the induction of adventitious shoots in greater than 90% of the plants after 240 days of culture with an average of more than eight shoots per plant. Approximately 100% of the *in vitro*-produced shoots survived after acclimatization, reaching the flowering stage. Therefore, our results showed that *in vitro* regeneration of *N. minutum* depends on the cultivation period and that plants with a higher sprouting capacity can be selected and used as micropropagation matrices, contributing to the production of this endangered bromeliad.

Keywords: bromeliad; endangered species; ornamental plant; shooting; plant regeneration.

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Introduction

Nidularium minutum Mez is a terricolous bromeliad endemic to the tropical rainforest, occurring exclusively in the State of São Paulo (Leme, 2000) and classified as vulnerable in the List of Endangered Species in Brazil (Brazil, 2022). Its ornamental features, such as the green and red primary bracts and the white flowers and fruits, make it a potential target for illegal extraction, which could further aggravate its endangered status. Furthermore, flowers and fruits are highly predated by wild animals, which may compromise the sexual propagation of this species.

Micropropagation is an efficient *ex-situ* conservation strategy for endangered species, which allows for keeping the species in a small space and obtaining high multiplication rates under controlled growth conditions (Engelmann, 2011). No studies on micropropagation of *N. minutum* by side sprouting are available in the literature, although this bromeliad has been cultivated *in vitro* for physiological and biochemical studies (Andrade & Tamaki, 2012; Andrade, Costa, Kurita, Carvalho, & Tamaki, 2012; Carvalho, Hayashi, Braga, & Nievola, 2013; Kurita, Machado, Teixeira, César, & Tamaki, 2014; Andrade & Tamaki, 2016).

Our preliminary results showed that *N. minutum* has low shoot production in medium without PGR and takes a long time to develop shoots even with PGRs added to the medium. Some studies have reported *in vitro* bud production by Bromeliaceae without exogenous inducers (Arrabal, Amancio, Carneiro, Neves, & Mansur, 2002; Santos, Tamaki, & Nievola, 2010), while others have shown that PGRs are essential for micropropagation (Hirimburegama & Wijesinghe, 1992; Mercier & Nievola, 2003; Mendes et al., 2007; Pasqual et al., 2008; Viehmannova, Cepkova, Vitamvas, Streblova, & Kisilova, 2016).

PGRs are involved in cell differentiation during new shoot formation (Faria et al., 2018) and are considered, in many cases, essential for micropropagation. Cytokinins and auxins, such as 6-benzylaminopurine (BAP) and 1-naphthylacetic acid (NAA), respectively, are the PGRs most widely used for producing Bromeliaceae sprouts *in vitro* (Silva et al., 2012; Faria et al., 2018; Dias, Silva, Carneiro, & Sousa, 2020).

Therefore, this study aimed to establish a micropropagation method for producing *N. minutum* plants.

Material and methods

Seeds of *Nidularium minutum* Mez were harvested from fruits of plants growing in the "Alto da Serra de Paranapiacaba" Biological Reserve, São Paulo, São Paulo State, Brazil (23°46'18"-23°47'05" South Latitude and 46°20'24"-46°18'15" West Longitude). Harvesting was authorized by the *Secretaria do Meio Ambiente do Estado de São Paulo* (IBt – 20.733/2005). The seeds were disinfected with 70% ethanol for 2 minutes and commercial bleach (2.5% active chlorine) plus Tween 20 (Labsynth, Brazil) for 15 minutes and rinsed three times with sterile distilled water. Afterward, the seeds were placed in a medium containing distilled water supplemented with sucrose (2%) and solidified with agar (0.6%) at pH 5.8 (Kurita et al., 2014). The seeds were maintained in a growth room at 25°C with a 12 hours (LED lamps) photoperiod for 30 days.

Thirty flasks with 10 seedlings germinated *in vitro* (as described above) were transferred to MS medium (Murashige & Skoog, 1962) supplemented with sucrose (30 mg L^{-1}), glycine (2 mg L^{-1}), myo-Inositol (100 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine-HCl (0.5 mg L^{-1}), thiamine-HCl (0.1 mg L^{-1}), solidified with 0.6% agar, at pH 5.8. The flasks were kept in a growth room at 25°C with a photoperiod of 16 hours (LED lamps, ~100 µmol m^{-2} s^{-1}) for 240 days when the number of shoots per seedling was counted. Shoots were individualized and subcultured in a new basal MS medium without PGRs. Plants that did not produce shoots were also transferred to a new basal MS medium for a shorter time (45 days) to check their shooting ability.

To analyze the PGR efficiency in *N. minutum* shooting, seedlings were transferred to MS medium with sucrose (3%), glycine (2 mg L^{-1}), myo-Inositol (100 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine-HCl (0.5 mg L^{-1}), thiamine-HCl (0.1 mg L^{-1}), and agar (0.6%), at pH 5.8. Five treatments were performed: 0.1 mg L^{-1} of NAA; 1.0 mg L^{-1} of BAP; 0.1 mg L^{-1} of NAA + 0.5 mg L^{-1} of BAP; 0.1 mg L^{-1} of NAA + 1.0 mg L^{-1} of BAP; and control (PGR-free). The flasks were maintained in a growth room at 25°C with a 16 hours photoperiod (~100 µmol m^{-2} s^{-1}). A randomized block design was carried out with four blocks of two flasks containing 10 plants per treatment (N = 80).

Every 30 days, the number of sprouted plants was checked without removing them from the culture flask. After 240 days of cultivation, the plants were removed from the flasks, the number of shoots per plant was counted, and biometric analyses were performed (aerial part length, root length, aerial part fresh and dry masses, root fresh and dry masses).

Plants were oven-dried at 65°C for seven days to determine the dry mass. The fresh and dry masses were determined using three plants per flask from each block (N = 12).

The data obtained were subjected to a one-way analysis of variance (ANOVA). Differences between means were evaluated with the Skott-Knot test at $p < 0.05$, using Sisvar version 5.7. Results were presented as mean ± standard error of the mean (SEM).

Plant acclimatization was evaluated from 50 shoots isolated from five individuals of each treatment (10 shoots per plant). The shoots were transferred to trays with *Pinus* substrate (Maxfertil, Brazil). The same procedure was carried out with unsprouted plants. The trays were kept in a greenhouse and irrigated twice daily for seven minutes. After 60 days, the number of surviving shoots was counted.

Results and discussion

Only 10% of *N. minutum* seedlings grown in medium without PGRs produced shoots in 240 days. The number of shoots per seedling was not constant (Figure 1), indicating a differential sprouting capacity among individuals. Three seedlings had a high sprouting capacity, with 29, 19, and 15 shoots, respectively. However, only one-third of the seedlings sprouted above average (5.9 per plant), taking a long time to sprout (240 days). Some bromeliads grown *in vitro* in a medium without PGRs had higher percentages of shoot-producing plants than *N. minutum*. For example, *Bromelia balansae* had 25% after 249 days (Dias et al., 2020), and *Sincoraea mucugensis* had 77% after 60 days (Lima, Brito, & Santana, 2020).

The sprouting capacity also varied when *N. minutum* shoots (clones) were individualized and subcultured in a PGR-free medium for 45 days. The clones of the three individuals with the highest shoot potential produced 16, 6, and 4 shoots (Figure 1). Our results allowed us to select individuals with greater shooting capacity; however, identifying them took us a long time. We established a 45-day interval for subculturing because this or a similar period has been described in protocols for obtaining bromeliads by lateral sprouting (Martínez et al., 2013; Viehmannova et al., 2016; Rosa et al., 2018).

The method summarized in figure 2 shows the differential multiplication capacity of *N. minutum* individuals. Seedlings of unsprouted plants did not produce new sprouts after subculturing (A). When shoots

(clones) of sprouted seedlings were individualized and subcultured in a PGR-free medium, approximately one-quarter of the clones sprouted in 45 days (B), indicating that sprouted clones can produce shoots like the mother plant. The *in vitro* shooting capacity of *N. minutum* might be related to genes that control the levels of hormones involved in bud branching, as reported by Tantikanjana et al. (2001) for *Arabidopsis*. Furthermore, according to Leyser (2009), specific genes control the increase in the number and sprouting of the initial buds.

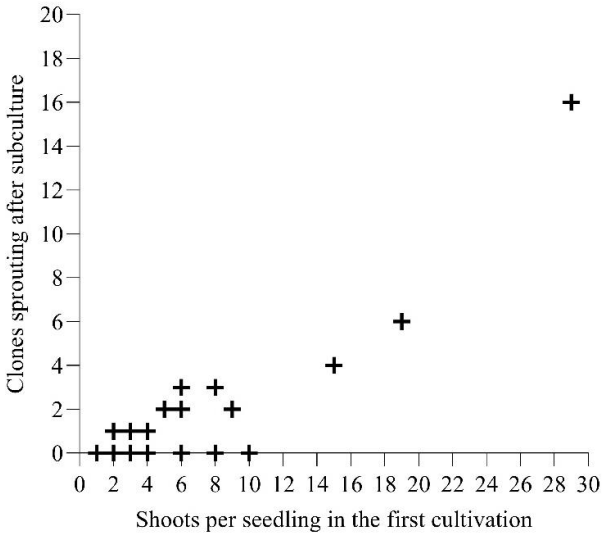

Figure 1. Variation of sprouting capacity in *Nidularium minutum* seedlings during the first 240 days of culture and sprouted clones after subculturing them for 45 days in a PGR-free medium. Each + represents a seedling of the 30 observed (some + overlap, not being visible on the graph).

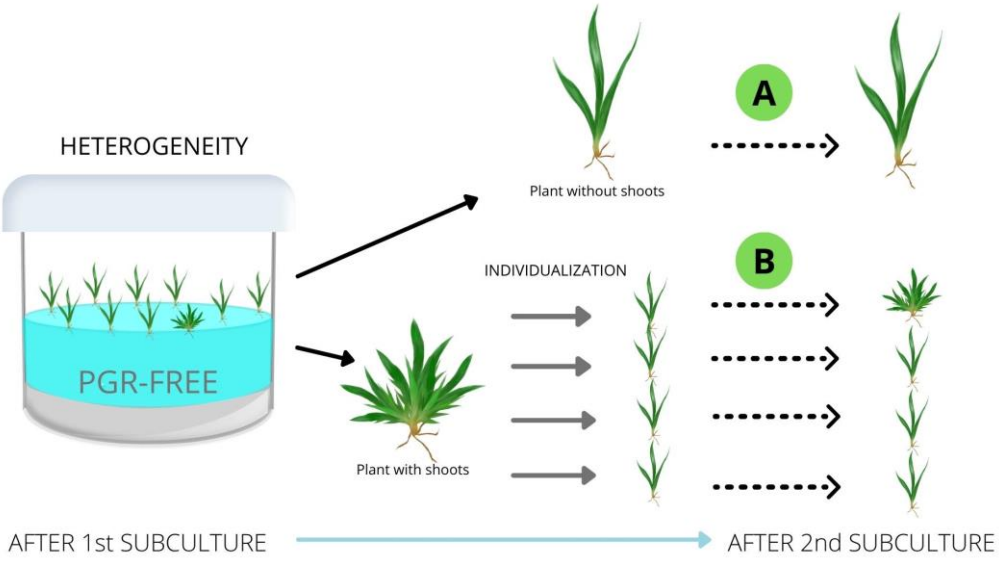

Figure 2. Differential multiplication capacity of *Nidularium minutum* individuals grown in a PGR-free medium.

Regarding the efficiency of PGRs in *N. minutum* sprouting, our results showed that even with the addition of PGRs to the culture medium, a long period is needed to reach a high sprout production rate. The addition of BAP, alone or with NAA, promoted more than 90% of sprouted plants in 240 days. Approximately 10% of the plants produced shoots in the treatment with only 0.1 mg L^{-1} NAA (Figure 3 A). The average number of shoots per plant in the medium containing only auxin did not differ from the PGR-free medium. The treatments with BAP promoted the highest average number of shoots per plant at 240 days (Figure 3B).

Some studies have reported that the synergistic effect between BAP and NAA increased the *in vitro* shoot production of different bromeliad species, such as *Aechmea blanchetiana* and *A. distichantha* (Santa-Rosa, Souza, Vidal, & Ledo, 2013). However, treatments combining NAA and BAP did not increase shoot production in *N. minutum* compared to those with only BAP. This result might be related to cytokinin action, which

decreased apical dominance and induced shoot formation (Pierik, 1997). Mercier, Souza, Kraus, Hamasaki, and Sotta (2003) observed increased shoot formation in *Ananas comosus* leaves cultivated in a medium with BAP and NAA when the apical dominance was broken. These authors suggested that the BAP and NAA joint presence increases the endogenous iP cytokinin content, indicating that iP production is regulated in response to the uptake of these PGRs. In our study, the auxin production by the apical bud was not interrupted since we used the entire *N. minutum* plantlet. Furthermore, using NAA and BAP in the culture medium was not more efficient than BAP alone, indicating that adding auxin does not favor *N. minutum* multiplication, unlike what has been reported for other bromeliad species (Silva et al., 2012).

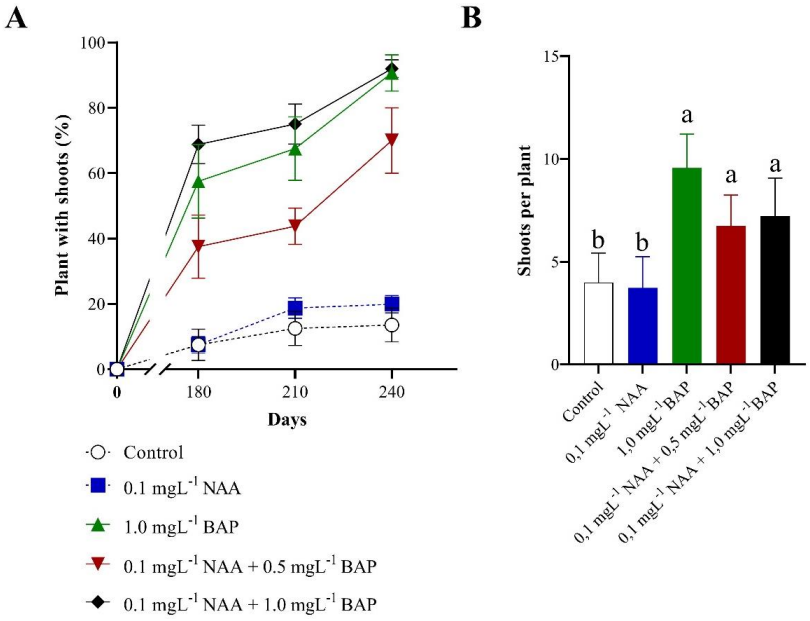

Figure 3. Shooting in *Nidularium minutum* grown in a culture medium with the addition of 6-benzylaminopurine (BAP) and/or 1-naphthylacetic acid (NAA). Control medium = PGR-free. (A) Percentage of *Nidularium minutum* plants with shoots grown for 240 days; (B) Number of shoots per *N. minutum* plant after 240 days of cultivation. Means followed by the same letters do not differ significantly according to the Scott-Knott test ($p \geqslant 0.05$).

BAP is the most used cytokinin for *in vitro* propagation due to its remarkable ability to act in the aerial part, producing more shoots than other PGRs (Coelho, Gonçalves, González-Benito, & Romano, 2012; Bekircan, Yaşar, Yıldırım, Sökmen, & Sökmen, 2018; Komakech et al., 2020; Kornatskiy, 2020). In a medium with 5 µM BAP (~ 1.13 mg L^{-1}), the bromeliad *Aechmea blanchetiana* showed 100% sprouted plants after 60 days (Rosa et al., 2018). *Puya berteroniana* cultivated *in vitro* in a medium with 0.44 µM BAP (~ 0.1 mg L^{-1}) for 28 days had an average of 5.5 shoots per plant (Viehmannova et al., 2016). In this study, using BAP significantly increased shoot production in *N. minutum*. However, *N. minutum* needed a more extended period (240 days) than *Nidularium procerum* and *Nidularium innocentii* to obtain more than 90% sprouting. According to Silva et al. (2012), these other *Nidularium* species require 120 days to reach 100% and 61% shoot production, respectively, using 4 µM (~ 1 mg L^{-1}) of BAP and 2 µM of NAA (0.37 mg L^{-1}). We required PGR concentrations close to those mentioned in previous studies to induce shoot production in *N. minutum*. We preliminary tested higher BAP concentrations (2 and 2.5 mg L^{-1}) but shoot formation did not increase.

Cytokinin added to the medium inhibited the elongation of aerial parts and roots. As shown in Table 1, control plants, or those only with NAA in the medium, had larger aerial parts and roots. Nonetheless, the biomass accumulation in *N. minutum* did not differ significantly between PGR treatments. These results could be associated with cytokinins' positive and negative regulatory roles in cell proliferation, stimulating mitosis in some tissues or differentiation in others (Schaller, Street, & Kieber, 2014). Previous studies have also revealed that there is most likely some resource partition between the mother plant and the new clones in the shoots, possibly due to BAP-mediated coordination of shoot production, which involves nutrient availability signaling (Schmülling, 2002; Tamaki & Mercier, 2007).

Figure 4 illustrates the steps in *N. minutum in vitro* propagation from plantlets (A. Seedling; B-C. Growth and shoot induction; D. Individual shoot production; E-F. Individualization of shoots from the mother plant; and G. Acclimatization).

Table 1. Biometric parameters of *Nidularium minutum* after 240 days of growth in MS medium with and without the addition of 6-Benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA)*.

PGR (mg L^{-1})		Length (cm)				Mass (g)							
						Fresh				Dry			
NAA	BAP	Aerial Part		Root		Aerial Part		Root		Aerial Part		Root	
0	0	6.39	±0.59a	3.01	±0.37a	0.56	±0.08a	0.09	±0.02a	0.04	±0.01a	0.01	±0.004a
0.1	0	7.15	±0.38a	3.33	±0.49a	0.63	±0.07a	0.15	±0.01a	0.06	±0.01a	0.02	±0.003a
0	1.0	2.40	±0.21b	0.46	±0.22b	0.49	±0.13a	0.06	±0.01a	0.05	±0.01a	0.01	±0.002a
0.1	0.5	3.10	±0.53b	0.98	±0.44b	0.38	±0.13a	0.08	±0.03a	0.04	±0.01a	0.01	±0.003a
0.1	1.0	2.13	±0.43b	0.50	±0.15b	0.34	±0.06a	0.11	±0.04a	0.06	±0.02a	0.02	±0.008a

(*) Different letters between columns indicate that the means differ significantly by the Scott-Knott test ($p < 0.05$)

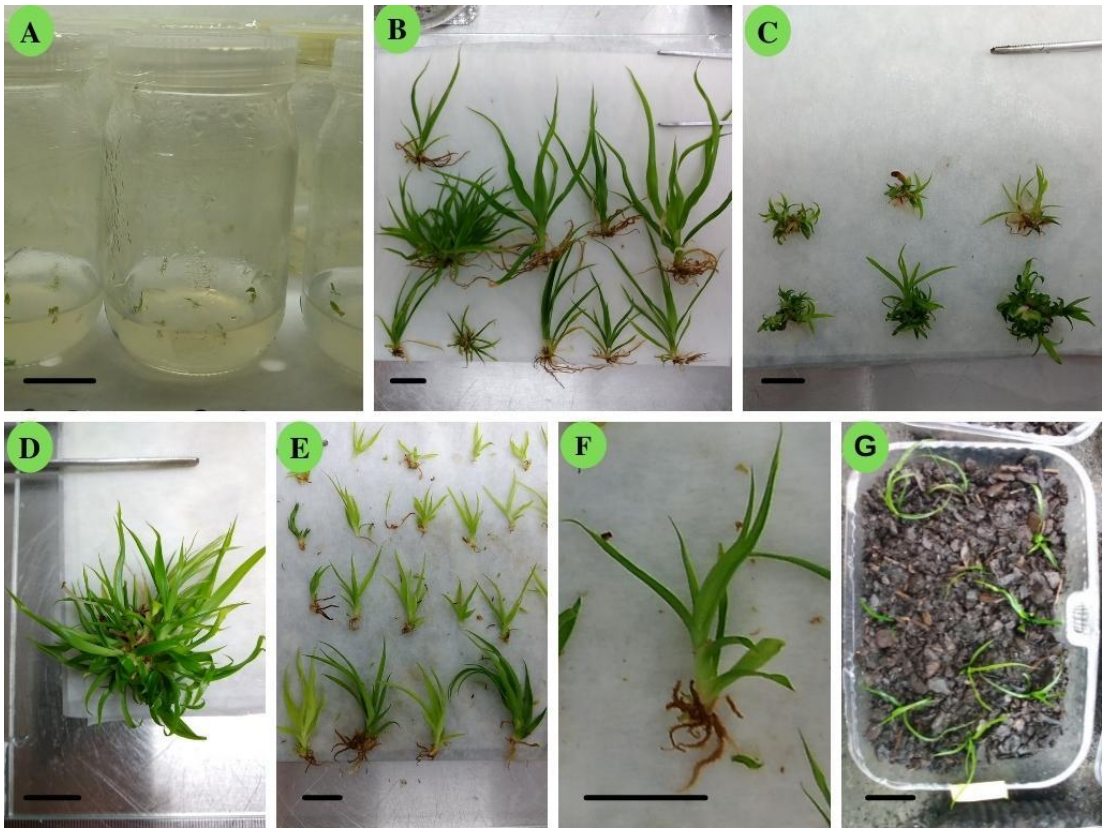

Figure 4. Steps in *Nidularium minutum* propagation. (A) Seedlings approximately 30 days after germination. (B) Plants obtained from the PGR-free medium after 240 days. (C) Treatment plants with 1.0 mg L^{-1} BAP after 240 days. (D) Detail of the shoot proliferation from one seedling. (E) Shoots separated from the mother plant (the mother plant is no longer recognized). (F) Detail of the individualized shoot with roots. (G) Plants with 60 days of acclimatization. (Bars = 2 cm.)

This study showed a method to identify individuals with different shoot production capacities for the first time. Likewise, it evaluated the plant production capacity for micropropagation of *N. minutum* by lateral buds, including acclimatization. Sixty days after acclimatization, the plants showed 100% survival. Individual shoots and unsprouted seedlings were successfully acclimatized. Other bromeliad species have also demonstrated a high percentage of survival after acclimatization (Santa-Rosa et al., 2013; Faria et al., 2018; Lima et al., 2020; Silva, Souza, Souza, Nepomucen, & Costa, 2020).

Conclusion

For the first time, the differential sprouting ability of *N. minutum* plants *in vitro* was demonstrated. Some individuals showed a higher sprouting capacity, even in a PGR-free medium. The clones of these individuals also tended to sprout, which could aid the micropropagation of this species and support genetic improvement studies. Additionally, a high micropropagation performance is achieved using only BAP, despite the long period required to respond to PGR stimuli. The differential sprouting capacity during micropropagation can be used as a production strategy for repopulation programs and/or commercial purposes, reducing the impact of extractivism of this endemic and endangered bromeliad.

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