



Acta Scientiarum. Biological Sciences

ISSN: 1679-9283

ISSN: 1807-863X

actabiol@uem.br

Universidade Estadual de Maringá

Brasil

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Acta Scientiarum. Biological Sciences, vol. 42, 2020

Universidade Estadual de Maringá

Maringá, Brasil

DOI: <https://doi.org/10.4025/actascibiols.v42i1.52940>

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Micropropagation and *in vitro* conservation of *Alcantarea nahoumii* (Bromeliaceae), an endemic and endangered species of the Brazilian Atlantic Forest

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ABSTRACT. *Alcantarea nahoumii* (Leme) J. R. Grant is a species native to the Atlantic Forest that stands out for ornamental purposes. The objective of this work was to evaluate the *in vitro* germination of *A. nahoumii* seeds and establish a micropropagation protocol for production of seedlings so as to minimize the effects of predatory extractivism and develop an *in vitro* conservation system. Mature seeds were disinfested, established in three culture media (MS, MS½ and MS¼) and incubated at four temperatures (20, 25, 30 and 35°C) in a germination chamber. In the micropropagation experiment, stem segments were introduced in MS medium supplemented with 0.5 µM of 1-naphthaleneacetic acid (NAA) and 0.0, 2.2, 4.4 and 6.6 µM of 6-benzylaminopurine (BAP). For the *in vitro* conservation, plantlets were established in MS or MS½ medium supplemented with 15 g L⁻¹ or 30 g L⁻¹ of sucrose. The plants were acclimated with commercial substrate. The highest seed germination percentages were promoted by temperature conditions of 20 and 25°C, with MS culture medium. The highest multiplication rate of shoots was obtained from the treatment without addition of the growth regulator or when combined with 2.2 µM of BAP + 0.5 µM of NAA. The acclimation of the plants occurred with high survival rate. The species can be conserved *in vitro* under slow growth condition for 24 months when incubated in MS medium supplemented with 30 g L⁻¹ of sucrose.

Keywords: bromeliad; *in vitro* propagation; *in vitro* culture; Tillandsioideae.

Received on April 2, 2020.

Accepted on May 5, 2020.

Introduction

The family Bromeliaceae encompasses 77 genera and 3,629 species (Gouda, Butcher, & Gouda, 2020), of which 40% are found in Brazil, where 70% of these are endemic to the Atlantic Forest (Instituto Brasileiro de Florestas [IBF], 2015). It is considered the second-largest family of vascular epiphytes in this biome (Kersten, 2010), and many species are considered to be vulnerable due to environmental degradation, caused mainly by extractive activities and other anthropic actions. Among these species, *Alcantarea nahoumii* (Leme) J. R. Grant is classified as endangered (Forzza et al., 2013), due to frequent burning of habitat and widespread extraction for use as an ornamental plant.

Alcantarea nahoumii belongs to the subfamily Tillandsioideae and mainly occurs in the Brazilian state of Bahia, where its largest population is found in the Serra da Jibóia (Jibóia Range), located in the municipality of Santa Teresinha (Forzza et al., 2013; Versieux & Wanderley, 2010). Biotechnological techniques have been used as an alternative for preservation of the Bromeliaceae family, which can range from *in vitro* seed germination to slow growth techniques to maintain the plants in ideal conditions for conservation (Simão, Faria, Werner, Soares, & Gontijo, 2016). The germination of seeds *in vitro* can be enhanced to increase the germination percentages and reduce the time when compared to natural conditions, besides maintaining the genetic variability of the population (Pinto, Freitas, & Praxedes, 2010).

The conservation of germplasm of bromeliads should always consider the genetic diversity that exists in the wild, so *in vitro* germination and *in vitro* conservation should be applied together to allow maintenance of this diversity. The maintenance of multiple plants of a single population in natural conditions, as in *in situ* conservation, requires large spaces, which is not always possible.

On the other hand, *in vitro* multiplication has been shown to have good potential to produce bromeliad seedlings compared to conventional methods, assuring rapid and genetically safe multiplication that can meet the strong market demand for these plants, thus significantly minimizing extractive activities.

There are no protocols for either *in vitro* multiplication or conservation of *A. nahoumii*. Therefore, since this species is classified as endangered, research is necessary to develop methods for *ex situ* conservation under *in vitro* conditions, to enable production of seedlings in sufficient number to meet market demand and thus minimize predatory extraction.

The objective of this study was to evaluate the influence of temperature and the concentration of salts in MS culture medium on the *in vitro* germination of *A. nahoumii* seeds and to establish a protocol for micropropagation of this bromeliad, aiming to produce seedlings on a large scale and for *in vitro* conservation.

Material and methods

Seeds from ripe *A. nahoumii* fruits (Figure 1 A-C) were collected at the start of dehiscence in March 2016, in a natural population in Serra da Jibóia, Santa Terezinha, Bahia, Brazil (coordinates 12° 51' 08.19" S and 39° 28' 34.32" W).



Figure 1. A-B) Plants and inflorescences of *Alcantarea nahoumii* under field conditions. C) Ripe fruit. D) Recently removed seeds. E)

Seeds distributed on MS culture medium. F) Germinated seeds after 15 days on MS medium. G) Plants conserved *in vitro* with 30% sucrose (MS and MS $\frac{1}{2}$) 12 months after seeding. H) Plants conserved *in vitro* with 30% sucrose (MS and MS $\frac{1}{2}$) 24 months after seeding. I) Micropropagated plants of the fifth subculture with 6.6 μ M of BAP. J) Micropropagated plants of the fifth subculture with 2.2 μ M of BAP. K) Acclimated plant with 90 days of cultivation.

Biometry of the seeds

The biometric measurement of the seeds (length, width and thickness) was performed on 100 seeds chosen at random, obtained from 25 plants also chosen randomly, with a digital pachymeter. The 1000-seed weight was determined with a precision analytical balance and the moisture was measured using four samples of 25 seeds, based on the difference between fresh and dry weight after incubation at 105°C for 24 hours, according to the requirements of the Seed Analysis Rules (Brasil, 2009).

In vitro germination of the seeds

The seeds were disinfested in a sodium hypochlorite solution (2% active chlorine) and distilled water (2:1) for 20 minutes, followed by washing three times with autoclaved distilled water. Then they were placed in Petri dishes (100 mm x 20 mm) containing 15 mL of MS (Murashige & Skoog, 1962), MS $\frac{1}{2}$ or MS $\frac{1}{3}$ culture medium supplemented with 30 g L⁻¹ of sucrose and solidified with 2 g L⁻¹ of Phytigel®, with the pH being adjusted to 5.8 before autoclaving. The dishes with the seeds were kept for 60 days in a BOD chamber with 12 hours photoperiod, photon flux density of 22 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and one of four temperatures: 20°C, 25°C, 30°C or 35°C.

The germination was evaluated cumulatively by daily observation until stabilization of the process. The data were expressed as mean percentage of seeds germinated in each treatment. Seeds with emergence of the primary root were considered germinated (Pereira, Pereira, Rodrigues, & Andrade, 2008).

The germination percentage [G (%)] and germination speed index (GSI) were calculated by: $G (\%) = (N/A) \times 100$, where N = number of germinated seeds and A = total number of seeds; and $GSI = \sum (Gi/ni)$, where Gi = number of germinated seeds and ni = day of counting.

The experimental design was completely randomized in a 3 x 4 factorial scheme (MS mineral salts x temperature) with four repetitions, where each repetition was composed of 25 seeds, for a total of 100 seeds per treatment. The germination percentage and GSI data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test ($p < 0.01$), after transforming the percentage data into arcsine ($\sqrt{x/100}$). The analyses were conducted with the SAS program (SAS Institute Inc., 2010).

In vitro conservation

Plantlets with approximate length of 2 cm from the *in vitro* germination were established individually in test tubes (25 mm x 150 mm) containing 10 mL of either MS or MS $\frac{1}{2}$ medium with 2.0 g L⁻¹ of Phytigel® and supplemented with 15 g L⁻¹ or 30 g L⁻¹ of sucrose, where they remained in incubation conditions for 24 months at temperature of $25 \pm 2^\circ\text{C}$, light intensity of 22 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and photoperiod of 12 hours.

The experimental design was completely randomized in a 2 x 2 factorial scheme (MS mineral salts x sucrose concentration), with 20 repetitions, where each repetition consisted of one plantlet per tube. The following variables were quantified: plant height (cm); number of green leaves; number of senescent leaves; and number of roots after incubation for 24 months. Again, the data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test ($p < 0.01$), using the SAS program (SAS Institute Inc, 2010).

Micropropagation by inducement of sprouting of adventitious buds

Stem segments with average height of 0.5 cm were removed from 150 plants with approximate height of 4 cm, obtained from the *in vitro* germination experiment. The segments were established in Petri dishes (100 mm x 20 mm) containing 20 mL of MS culture medium along with 30 g L⁻¹ of sucrose, solidified with 2.0 g L⁻¹ of Phytigel®, supplemented with 0.5 μM of NAA (naphthalene acetic acid) and 0.0, 2.2, 4.4 or 6.6 μM of BAP (6-benzylaminopurine). The pH of the culture medium was adjusted to 5.8 before autoclaving at a temperature of 120°C for 20 minutes. The cultures were kept in a growth room with temperature of $25 \pm 2^\circ\text{C}$, photoperiod of 16 hours and photo flux density of 22 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

The multiplication was carried out in five subcultures, by longitudinal subdivision of the shoots whenever possible at intervals of 45 days. For each subculture, we evaluated the number of shoots, and measured the height only at the time of the last subculture. When the data were not normally distributed, they were log-transformed [$\log (x + 10)$].

The experimental design was completely randomized in a 4 x 5 factorial scheme (BAP concentration x subculture). Exponential regression analysis was used to evaluate the number of shoots. Each treatment was

composed of 6 repetitions, with each repetition consisting of 5 explants per dish. The analyses were conducted using the SAS program (SAS Institute Inc, 2010).

Acclimation of the micropropagated plants

About 160 plants were rooted in MS medium without growth regulator. For this purpose, plants with mean height of approximately 3 cm were transferred to plastic pots with capacity of 300 mL containing the commercial substrate Vivato® + vermiculite in the ratio of 8:2 (v v⁻¹). At the end of 30 days the number of surviving plants was counted.

Results and discussion

Biometry and *in vitro* germination of seeds

The *A. nahoumii* seeds are small (9.6 mm long, 0.9 mm wide and 0.6 mm thick), with a long brownish plumose appendage on one end, making them easily carried by the wind (Figure 1D). The 1000-seed weight was 1.69 g.

Biometric measurements of seeds are important to allow forming homogeneous groups, which can assure their physiological quality. Larger seeds are typically denser, with well-formed embryos and larger amounts of reserves, making them potentially more vigorous (Carvalho & Nakagawa, 2000). This vigor can translate into faster metabolic processes, resulting in more rapid and uniform emission of the primary root, faster growth and consequently more plants with larger initial size (Minuzzi, Braccini, Rangel, Scapim, Barbosa, & Albrecht, 2010).

The moisture of the seeds was approximately 13%, adequate for germination. However, this value is slightly above the level considered ideal for conservation of orthodox seeds. Seeds belonging to this category tolerate desiccation of 90% to 95%, retaining moisture of 5% to 10%, thus remaining viable for long periods (Ferreira & Borghetti, 2004; Silva et al., 2012; Barrozo et al., 2014).

The germination of the seeds started with emission of the cotyledon sheath with a distal root cap, between day 13 and 16 after sowing, and stabilized after around 24 days, regardless of the temperature and culture medium (Figure 1 E-F and Figure 2). These results demonstrate the homogeneity of the groups of seeds and thus their good physiological condition.

The analysis of variance demonstrated a significant interaction between the two factors studied for the two variables analyzed: *in vitro* seed germination and the germination speed index (GSI). The results of these interactions are reported in Table 1.

Table 1. *In vitro* seed germination percentage and germination speed index (GSI) of *Alcantarea nahoumii* seeds in different culture media and incubation temperatures.

Temperatures (°C)	Germination (%)		
	MS	MS½	MS½
20	87 aA	44 bA	36 bA
25	90 aA	45 bA	38 bA
30	54 aB	27 bB	21 bB
35	53 aB	23 bB	22 bB
CV (%)		8.37	
Germination Speed Index (GSI)			
20	1.15 aB	0.60 bA	0.45 bA
25	1.59 aA	0.62 bA	0.48 bA
30	0.73 aC	0.38 bB	0.27 bB
35	0.73 aC	0.30 bB	0.29 bB
CV (%)		35.02	

Means followed by the same lowercase letters in the rows and uppercase letters in the columns for each variable analyzed do not differ from each other by the Tukey test at 1% probability.

The germination varied between the temperatures and culture media. The highest percentages were obtained at 20°C and 25°C in MS medium, while higher temperatures and lower salt concentration in the medium reduced the germination significantly. For the germination speed index, the temperature of 25°C produced the best result, indicating the strong influence of temperature and culture medium on the dynamics of the seed germination process of this species.

The fact the germination occurred at all temperatures tested can be explained by the fact that *A. nahoumii* is a species that lives in a mountainous region (Martinelli et al., 2008), with large variations in

temperature every 24 hours. These conditions can be responsible for the plasticity exhibited in the seed germination test.

High germination rates were also reported for *Nidularium minutum* Mez (Carvalho, Hayashi, Braga, & Nievol, 2013) and *Vriesea incurvata* Gaudich (Pulido-Rueda, Milaneze-Gutierrez, & Negrelle, 2018), when grown in complete MS medium between 20°C and 27°C. The choice of the medium, physiological conditions of the seeds, their maturity and incubation conditions all had a significant influence on the germination of the seeds in the conditions tested (Zeng et al., 2013).

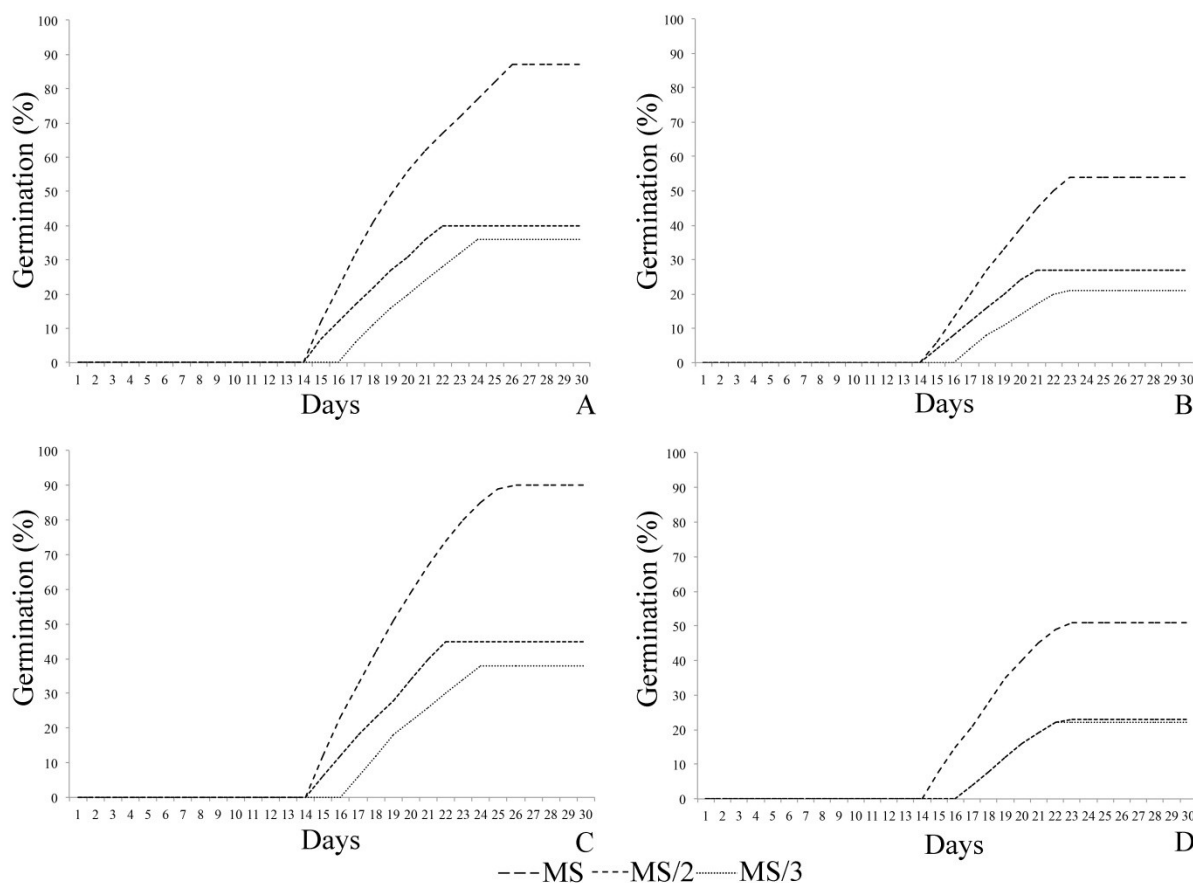


Figure 2. Germination percentage of *Alcantarea nahoumii* in relation to the number of days in different culture media (MS, MS $\frac{1}{2}$, MS $\frac{1}{3}$) and incubation temperatures: 20°C (A), 25°C (B), 30°C (C) or 35°C (D).

In relation to the germination speed index, species that naturally germinate in the winter favor lower temperatures in the laboratory. In natural conditions, plant germination is synchronized with the season of the year that is most favorable for the growth of young plants, thus improving the chances of survival and continued growth (Larcher, 2006). For bromeliads there are reports that the variation between the rainy and dry seasons has a strong influence on the flowering pattern, production and viability of the seeds and germination success (Wright & Calderon, 1995; Benzing, 2000).

As stated, *A. nahoumii* lives in a mountainous region with large temperature variations between day and night, but where daytime temperatures rarely are as high as 30°C and 35°C, evaluated in this study. This can explain the low germination rates in these conditions. On the other hand, it should be considered that *A. nahoumii* is an endangered species and its ability to germinate even in adverse conditions, albeit with low rates, is highly relevant for its survival.

In vitro conservation

The analysis of variance revealed a significant interaction of two of the variables analyzed: plant height and number of leaves. The number of roots and number of senescent leaves were not influenced by the treatments.

In general, the plants had normal morphological development, without the emission of lateral shoots after growth for up to 24 months (Figure 1G-H).

The MS medium with complete salts supplemented with 30 g L⁻¹ of sucrose promoted the greatest growth of the aerial part and highest number of leaves, i.e., it was the treatment that promoted the best plant development (Table 2). On the other hand, the plants in the treatment with half the concentration of salts in the MS medium had the smallest growth, suggesting a reduction of metabolism in those conditions, which is the objective of *in vitro* conservation. Nevertheless, it is important to differentiate the deceleration of metabolism from toxic effects, since these two effects are often confused, although we did not note any toxicity in this study.

Table 2. Height (cm) and number of green leaves of *Alcantarea nahoumii* plants conserved *in vitro* during 24 months in function of concentrations of salts and added sucrose in MS medium.

Sucrose	MS	MS½
	Plant height (cm)	
15 g	5.61 bA	5.11 bB
30 g	8.87 aA	7.22 aB
CV (%)	5.44	
	Number of green leaves	
15 g	1.70 bB	2.40 aA
30 g	3.20 aA	2.50 bA
CV (%)	25.99	

Means followed by the same lowercase letters in the rows and uppercase letters in the columns for each variable analyzed do not differ from each other by the Tukey test at 1% probability.

It is important to evaluate the entire set of variables before deciding on the best treatment, not just the size of the plants. One of the most important variables in *in vitro* conservation is the number of senescent leaves, since this is an indicator of a more advanced physiological state of the plant.

In this experiment, we did not observe a significant difference of the number of senescent leaves, revealing that despite the greater growth observed in the treatment with normal salt concentration in the MS medium and 30 g L⁻¹ of sucrose, there was no impairment or aging of the conserved plants. Thus, this treatment should be considered the best for *in vitro* conservation of *A. nahoumii*, since these plants have a better chance of performing well after acclimation, mainly due to the high number of leaves.

With respect to the number of roots, it did not vary among the treatments, with values between two (MS + 15 g L⁻¹ of sucrose) and three (MS + 30 g L⁻¹ of sucrose). The roots of plants grown *in vitro* are not always functional when the plants are transferred to *ex vitro* condition, sometimes requiring a cutting treatment to be renewed during the acclimation period (Souza et al., 2013). This step is delicate and requires great caution, since it involves a process of gradual adaptation of the plant to a different environmental condition. The plant leaves a heterotrophic regime and enters an autotrophic one.

In other bromeliad species, reducing the concentrations of salts and sucrose in MS culture medium has been used for *in vitro* conservation for a period between 12 and 24 months, such as in *Vriesea reitzii* (Rech Filho et al., 2005), *V. gigantea* Gaudich. and *V. philippocoburgii* Wawra (Droste, Silva, Matos, & Almeida, 2005), and *A. fasciata* and *A. miniata* Baker (Costa, Moreira, Souza, & Rocha, 2012). Besides being efficient, this strategy also reduces costs of *in vitro* cultivation. The difference of these results in relation to those found for *A. nahoumii* reveals the need for specific studies and careful evaluation of the results before deciding on the protocol to be adopted for a particular species. The period of 24 months without the need for subculture is considered very good for maintenance of *in vitro* germplasm banks. And finally, but not less relevant, the conserved plants, obtained from seeds of fruits collected at random, had good representativeness of the genetic diversity of the natural population, which should be considered in the establishment of a collection for the purpose of conservation.

Micropropagation by inducement of sprouting of adventitious buds

Propagation from seeds permits maintenance of the genetic variability of the population, which is relevant for *in situ* conservation programs for reintroduction into natural habitats (Pinto et al., 2010), as well as for *ex situ* conservation. This principle of using plantlets grown from seeds from a natural population should be followed when the intention is to produce seedlings in large scale, mainly when involving endangered species, as is the case of *A. nahoumii*. The differences that can be observed during the micropropagation process between plants do not act as limitations, nor are they determinants, in the commercial use of these plants. In other words, the morphological differences are not significant, so there is no justification for cloning these plants for commercial purposes.

Another advantage of using seeds to establish plants *in vitro* is the greater facility of disinfecting seeds compared to tissues extracted from native plants (Bellintani, Lima, Brito, Santana, & Dornelles, 2007).

We observed the satisfactory multiplication of shoots in all the treatments evaluated. However, the highest multiplication rates were in the treatments without the plant growth regulator or with the BAP concentration of 2.2 μM associated with 0.5 μM of NAA (Figure 1 I-J and Figure 3). Similar results using these concentrations have been obtained in multiplying *Cryptanthus sinuosus* L. B. Sm. (Arrabal, Amancio, Carneiro, Neves, & Mansur, 2002), *Aechmea blanchetiana* (Baker) L.B.Sm. and *A. distichantha* Lem. (Santa-Rosa, Souza, Vidal, Ledo, & Santana, 2013) and *A. setigera* Mart. ex Schult. f. (Vasconcelos, Leão, Raposo, & Fermino Junior, 2015).

The cytokinins, among other effects, act to stimulate the formation of shoots, mainly when associated with an auxin. However, the effects of the type and concentration of these growth regulators will vary with the species (Gana, 2011). The cytokinins work through cell receptors, and the cytokinin-receptor interaction can vary between plant species, where the process of molecular recognition of the cytokinins involves modifications of the side chains linked to adenine (Sakakibara, 2006).

For *A. nahoumii*, the treatment without regulator produced the largest number of shoots during the subcultures, suggesting that the presence of endogenous growth regulators favored this outcome. An inhibitory effect was also noted with rising concentration of BAP (Figure 3).

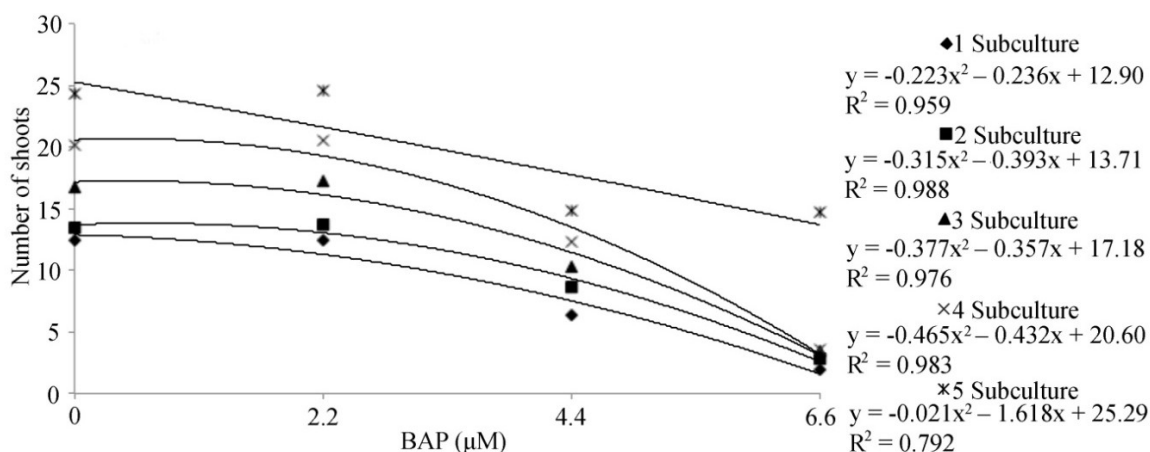


Figure 3. Number of shoots produced by *Alcantarea nahoumii* plants grown with different BAP concentrations in five subcultures.

With respect to the effect of the subcultures on the number of shoots, each time the shoots were transferred to a new culture medium, the number of adventitious shoots increased, even though the highest BAP concentrations were a limiting factor (Figure 3).

The inhibitory effect of high BAP concentrations can be noted in Figure 4, which presents the data on number of shoots per explant in relation to the size of the shoots obtained at each BAP concentration. The largest shoots were produced by the plants in the treatment without regulator, followed by the treatment with addition of 2.2 μM of BAP. The plants in the treatments with higher BAP concentrations only generated shoots shorter than 0.5 mm.

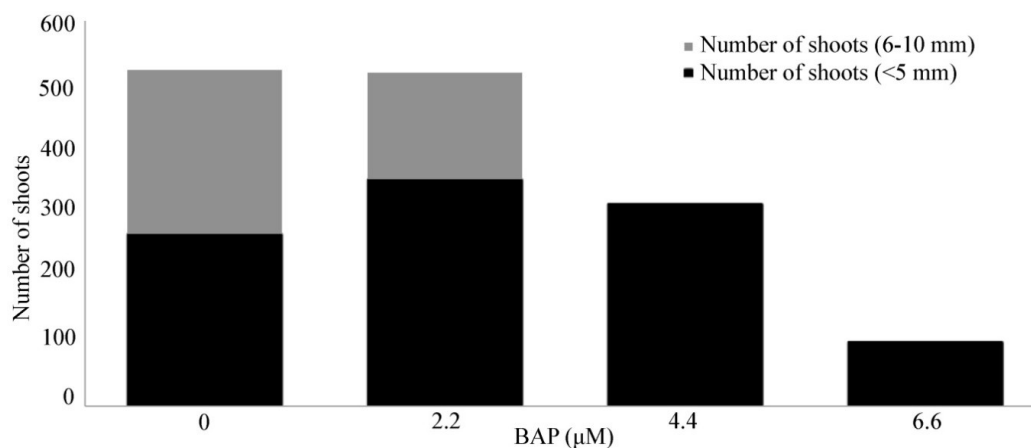


Figure 4. Total number of shoots classified by size in the treatments with different BAP concentrations at the end of five subcultures of *Alcantarea nahoumii*

After the fifth subculture, the number of shoots was recorded by size. In the treatment without growth regulator, there were 268 shoots with length equal to or smaller than 5 mm (51%) and 256 shoots with length between 6 and 10 mm (49%). In the treatment with 2.2 μM of BAP, there were 357 shoots (68%) with length between 6 and 10 mm. When the BAP concentrations were 4.4 μM and 6.6 μM , 100% of the shoots had size smaller than 5 mm (Figure 4).

Similar results regarding the use of growth regulators in the culture medium were reported in the micropropagation of *Aechmea ramosa* var. *ramosa* Mart. ex Schult. f. (Faria et al., 2018), in relation to the production of smaller shoots when grown in medium supplemented with high BAP concentration (6 μM). In a study of *A. blanchetiana* and *A. distichantha* (Santa-Rosa et al., 2013), the authors also observed an inhibitory effect when using the highest concentration of this growth regulator, and the largest number of shoots was produced by the plants grown in the medium containing the lowest BAP concentration (2.2 μM).

This behavior can be attributed to the presence of endogenous auxins and cytokinins, in concentrations sufficient to induce their development *in vitro* (Mallón, Rodríguez-Oubina, & González, 2011). Rosa et al. (2018), in a study of *A. blanchetiana*, found that the use of BAP at lower concentrations assured the break of apical dominance and formation of a larger number of shoots with lower degree of disorders of the photosynthetic apparatus.

The young regenerated *A. nahoumii* plants were acclimated and after 90 days the survival rate was 88%, demonstrating the viability of micropropagation for this species and its application to produce seedlings to serve the commercial market and also for conservation of the species in the wild (Figure 1K).

Conclusion

Temperatures from 20 to 25°C together with MS culture medium are most efficient parameters for germination of *A. nahoumii* seeds.

Alcantarea nahoumii plants can be conserved *in vitro* in MS culture medium supplemented with 30 g L⁻¹ of sucrose.

Alcantarea nahoumii plants can be multiplied in MS culture medium without growth regulator or when supplemented with 2.2 μM of BAP associated with 0.5 μM of NAA.

The acclimated plants have high survival rates.

Acknowledgements

We thank the support from the *Fundação de Amparo à Pesquisa do Estado da Bahia* (FAPESB), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (PQ - 304269/2018-2) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES 001, PNPD - 88882.315208/2019-01, PROCAD - 88887.124186/2014-00) for the scholarships granted.

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