**In vitro culture of Vriesea cacuminis L.B. Sm. (Bromeliaceae): an endemic species of Ibitipoca State Park, MG, Brazil**

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**ABSTRACT** – This study aimed to establish a protocol for the micropropagation of *Vriesea cacuminis*. After the *in vitro* introduction from seeds, plantlets were propagated in medium supplemented with BA or GA, combined with NAA. The microcuttings were rooted in medium with NAA, IAA or IBA. The results showed that the cultures were efficiently established *in vitro*. Medium supplemented with 15 µM BA + 4.5 µM NAA provided the highest propagation rates. In response to 15 µM GA₃ + 1.5 µM NAA it was possible to obtain both shoots and roots. The highest microcutting rooting was found in response to the medium supplemented with NAA at 0.2 µM. After six months *in vitro*, the plantlets were acclimatized. This study’s results showed that micropropagation is an efficient tool for *in vitro* genetic variability conservation and for large-scale multiplication of *V. cacuminis*, an endangered and endemic bromeliad.

**Keywords:** biodiversity conservation, acclimatization, micropropagation

**RESUMO** – Cultivo *in vitro* de *Vriesea cacuminis* L.B. Sm. (Bromeliaceae): uma espécie endêmica do Parque Estadual do Ibitipoca, MG, Brasil. Este estudo teve como objetivo estabelecer um protocolo para micropropagação de *Vriesea cacuminis*. Após introdução *in vitro* a partir de sementes, plântulas foram propagadas em meio suplementado com GA₃ ou BAP combinado com ANA. Microestacas foram enraizadas em meio de cultura suplementado com ANA, AIA ou AIB. As culturas foram eficientemente estabelecidas *in vitro*. O meio suplementado com 15 µM BA + 4,5 µM de ANA produziu taxas de propagação elevadas. Em resposta a 15 µM de GA₃ + 1,5 µM de ANA obteve-se brotos e raízes. Maior enraizamento das microestacas foi observado em resposta ao meio contendo ANA a 0,2 µM. Plântulas foram aclimatizadas após seis meses de cultivo *in vitro*. Os resultados deste estudo mostraram que a micropropagação é uma ferramenta eficiente para a conservação *in vitro* da variabilidade genética e para a multiplicação em larga escala de *V. cacuminis*, uma bromélia endêmica e ameaçada de extinção.

**Palavras-chave:** conservação da biodiversidade, aclimatização, micropropagação

**INTRODUCTION**

The Ibitipoca State Park, located in Lima Duarte, in the southern of Minas Gerais State, Brazil, was established in 1973. In the last decade, tourism has grown to more than 40,000 people visiting the area each year. The park presents different vegetation types, of which the *campos rupestres* (dry, rocky grasslands) are distinguished by the occurrence of endemic bromeliads (Rodela 1998). The common problems related to the intensive visitations on the *campos rupestres* are the development of erosion on trails and the extractivism of the visual appealing plant species (Versieux & Wendt 2006).

*Bromeliaceae* is one of the most important families of the Brazilian flora, accounting for around 40% of the described species (Martinelli et al. 2008). An important center of the bromeliads diversity is in the southeastern of Brazil, being the Atlantic Forest the ecosystem that has the highest biological richness and the highest level of specific endemism (Forzza & Wanderley 1998, Martinelli 2008).

The Vriesea cacuminis L.B. Smith is a rare bromeliad found only in two locations in Minas Gerais State, Brazil: the Ibitipoca State Park, a conservation unit, and at Serra Negra, an unprotected area (Versieux 2011). The species occurs as rupicola in campos rupestres, essentially at elevations above 1400 m (Versieux & Wendt 2006, Monteiro & Forzza 2008). Because of its endemicity and threat of environmental degradation, this species was included on the Brazilian list of endangered plants as vulnerable (Martinielli & Moraes 2013). In order to enable the in vitro variability preservation as well as large-scale multiplication of V. cacuminis, this study aimed to develop a protocol for micropropagation and ex vitro acclimatization of this species.

MATERIAL AND METHODS

Plant material and in vitro establishment

Seeds of the Vriesea cacuminis L.B. Smith (Bromeliaceae) were collected in mature infructescences from a naturally developed specimen at Ibitiopia State Park (21º40'15" - 21º43'30"S and 43º52'35" - 43º54'15"W), Minas Gerais, Brazil, under license from regulatory agencies. The seeds were disinfested in a bleach solution from a naturally developed specimen at Ibitipoca State Park (5% v/v) and maintained in field conditions under periodical watering (twice a week, until field capacity), without further fertilization. At the end of this phase, the survival rate of the transplanted plants was registered. After 30 days, the explants were evaluated regarding their shoot and root number and shoot number longer than 2 cm.

In the analysis of in vitro rooting, explants from culture media containing BA or GA, were inoculated on MS basal medium, supplemented with 0, 0.1, 0.2, 0.3 or 0.4 µM of NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA), with 10 replications each, since these plant growth regulators are the auxins most widely employed aiming at cell division and root induction in the in vitro systems (Krikorian 1995). The cultures were maintained in a growth room under the same conditions as reported for the previous assays. The cultures were also kept in test tubes (15 x 2.5 cm) containing 15 mL of culture medium. After 90 days of in vitro growth, the explants were evaluated regarding their root number and root length.

Acclimatization

The plantlets obtained in in vitro rooting phase were acclimatized in a shade environment using polystyrene trays covered with transparent plastic, totally enclosed, to simulate the moist environment found inside of the test tubes. Plantmax Hortaliças HT® (Eucatex Agro, SP) was used as an acclimatization substrate. After 30 days, the trays, without a plastic cover, were transferred to the greenhouse and kept under a microsprinkler irrigation system (twice a day, for 5 min) for five months. Then the fully acclimatized plants were transplanted in 5 L vessels containing a substrate prepared with vegetable-soil/manure/sand/coconut fiber dust in the ratio of 4:3:2:1 (v/v) and maintained in field conditions under periodical watering (twice a week, until field capacity), without further fertilization. At the end of this phase, the survival rate of the transplanted plants was registered.

Statistical Analyses

The data were normalized by use of and subjected to analysis of variance (ANOVA). Polynomial regression was used in accordance with residual requirements in the essays related to multiplications and rooting experiments. The averages were clustered by the Scott-Knott test at 5% of probability, using the SAEG program (version 9.1).

RESULTS

In vitro establishment and shoot propagation

The in vitro establishment of the V. cacuminis cultures
In vitro culture of *Vriesea cacuminis* L.B. Sm. (*Bromeliaceae*): ... 

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**Figs. 1A-D.** *In vitro* culture of *Vriesea cacuminis*. A. In multiplication media supplemented with GA$_3$ and NAA, 180 days after inoculation. (Treatments: 1. 0 μM GA$_3$ + 0 μM NAA; 2. 0 μM GA$_3$ + 1.5 μM NAA; 3. 0 μM GA$_3$ + 3 μM NAA; 4. 0 μM GA$_3$ + 4.5 μM NAA; 5. 5 μM GA$_3$ + 0 μM NAA; 6. 5 μM GA$_3$ + 1.5 μM NAA; 7. 5 μM GA$_3$ + 3 μM NAA; 8. 5 μM GA$_3$ + 4.5 μM NAA; 9. 10 μM GA$_3$ + 0 μM NAA; 10. 10 μM GA$_3$ + 1.5 μM NAA; 11. 10 μM GA$_3$ + 3 μM NAA; 12. 10 μM GA$_3$ + 4.5 μM NAA; 13. 15 μM GA$_3$ + 0 μM NAA; 14. 15 μM GA$_3$ + 1.5 μM NAA; 15. 15 μM GA$_3$ + 3 μM NAA; 16. 15 μM GA$_3$ + 4.5 μM NAA). B. In multiplication media in response to BA and NAA, 180 days after inoculation. (Treatments: 1. 0 μM BA + 0 μM NAA; 2. 0 μM BA + 1.5 μM NAA; 3. 0 μM BA + 3 μM NAA; 4. 0 μM BA + 4.5 μM NAA; 5. 5 μM BA + 0 μM NAA; 6. 5 μM BA + 1.5 μM NAA; 7. 5 μM BA + 3 μM NAA; 8. 5 μM BA + 4.5 μM NAA; 9. 10 μM BA + 0 μM NAA; 10. 10 μM BA + 1.5 μM NAA; 11. 10 μM BA + 3 μM NAA; 12. 10 μM BA + 4.5 μM NAA; 13. 15 μM BA + 0 μM NAA; 14. 15 μM BA + 1.5 μM NAA; 15. 15 μM BA + 3 μM NAA; 16. 15 μM BA + 4.5 μM NAA). C. and D. In rooting media, explants precultured in media with GA$_3$ or BA, respectively, in response to NAA, IAA or IBA, 140 days after inoculation. (Treatments: 1. control; 2. 0.1 μM NAA; 3. 0.2 μM NAA; 4. 0.3 μM NAA; 5. 0.4 μM NAA; 6. 0.1 μM IAA; 7. 0.2 μM IAA; 8. 0.3 μM IAA; 9. 0.4 μM IAA; 10. 0.1 μM IBA; 11. 0.2 μM IBA; 12. 0.3 μM IBA; 13. 0.4 μM IBA). Scale bars = 1 cm.

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was followed by the assessment of fungal and/or bacterial contaminations, and mainly, by the recording of seed germinations. The germination began five days after the *in vitro* seed inoculations, reaching a maximum germination percentage (95%) after 15 days. The culture contaminations were low, with less than 2% of microbiological infection.

In the analysis of the GA$_3$ and NAA effects on the shoot number and on the shoot number larger than 2 cm a significant difference was not found among the treatments (Table 1). Regarding the rooting, the analysis showed significant effects for the interaction between these growth regulators. Although we have found differences regarding to the control, increases in NAA concentration did not result in significant increases in the root number up to 10 μM GA$_3$. However, at the higher GA$_3$ level, the addition of 1.5 μM NAA promoted a higher root number than those found in response to the other treatments with NAA (Fig. 1A; Fig. 2A).

In the absence of BA, increases in NAA concentrations did not result in a significant increase in shoot numbers (Table 2; Fig. 1B). In contrast, under BAP effects, the shoot multiplications were improved in response to the increment in the NAA concentration, especially at the highest ones. The increase in BAP concentrations resulted
in an adjustment of a quadratic polynomial regression in response to all NAA concentration (Fig. 2B). The highest propagation rate (9.3 shoots/explant) was found in response to 15 μM BA + 4.5 μM NAA. Regarding to the shoots number larger than 2 cm, no significant effects were observed for interaction between BA and NAA, although, compared to the control, there were significant responses to the addition of NAA, in the medium without BA (Table 2; Fig. 2C). For the rooting, when compared to the control, significant effects were found only in the absence of BA (Table 1; Fig. 2D).

**Root formation and plantlets acclimatization**

In the absence of auxins, explants from the culture media supplemented with GA₃ did not demonstrate a suitable rooting, which was also observed for those from the media with BA (Table 3; Fig. 1C-D). The addition of NAA in the culture media stimulated the rooting more efficiently than other auxins. Improvement in the rooting was found at 0.2 and 0.4 μM NAA, respectively for plantlets from the culture media supplemented with GA₃ and BA (Fig. 2E-F). The root size was also evaluated. Regardless of their origin, explants from the culture media containing
Table 1. Average shoot number, shoots larger than 2 cm and root number of *V. cacuminis* cultured *in vitro* in response to different combinations of GA$_3$ and NAA, after 90 days of treatment.

<table>
<thead>
<tr>
<th>GA$_3$ (µM)</th>
<th>shoot number</th>
<th>shoots larger than 2 cm</th>
<th>root number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA (µM)</td>
<td>NAA (µM)</td>
<td>NAA (µM)</td>
</tr>
<tr>
<td>0</td>
<td>1.3 aA*</td>
<td>1.0 aA</td>
<td>1.2 aA</td>
</tr>
<tr>
<td>5</td>
<td>1.0 aA</td>
<td>1.1 aA</td>
<td>1.2 aA</td>
</tr>
<tr>
<td>10</td>
<td>1.3 aA</td>
<td>1.0 aA</td>
<td>1.2 aA</td>
</tr>
<tr>
<td>15</td>
<td>1.1 aA</td>
<td>1.0 aA</td>
<td>1.2 aA</td>
</tr>
</tbody>
</table>

* For each parameter, means followed by the same small (in lines) or capital (in arrows) letters are not different according to Scott-Knott’s test at 5% of probability.

Table 2. Average shoot number, shoots larger than 2 cm and root number of *V. cacuminis* cultured *in vitro* in response to different combinations of BA and NAA, after 90 days of treatment.

<table>
<thead>
<tr>
<th>BA (µM)</th>
<th>shoot number</th>
<th>shoots larger than 2 cm</th>
<th>root number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA (µM)</td>
<td>NAA (µM)</td>
<td>NAA (µM)</td>
</tr>
<tr>
<td>0</td>
<td>1.3 aC</td>
<td>1.0 aB</td>
<td>1.1 aA</td>
</tr>
<tr>
<td>5</td>
<td>2.7 ba</td>
<td>2.3 ba</td>
<td>7.6 aA</td>
</tr>
<tr>
<td>10</td>
<td>3.1 ba</td>
<td>4.0 ba</td>
<td>6.2 aA</td>
</tr>
<tr>
<td>15</td>
<td>3.0 ca</td>
<td>4.4 ba</td>
<td>9.3 aA</td>
</tr>
</tbody>
</table>

* For each parameter, means followed by the same small (in lines) or capital (in arrows) letters are not different according to Scott-Knott’s test at 5% of probability.

Table 3. Average root number in explants of *V. cacuminis*, precultured *in vitro* in media containing GA$_3$ or BA, in response to different concentrations of NAA, IAA or IBA, 90 days after the beginning of the experiment.

<table>
<thead>
<tr>
<th>from GA$_3$ culture media</th>
<th>from BA culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>NAA</td>
</tr>
<tr>
<td>0.0</td>
<td>1.0 aB*</td>
</tr>
<tr>
<td>0.1</td>
<td>1.6 aB</td>
</tr>
<tr>
<td>0.2</td>
<td>2.3 aA</td>
</tr>
<tr>
<td>0.3</td>
<td>2.3 aA</td>
</tr>
<tr>
<td>0.4</td>
<td>2.0 aA</td>
</tr>
</tbody>
</table>

* For each previously growth regulator utilized (BA or GA$_3$), means followed by the same small (in lines) or capital (in arrows) letters are not different according to Scott-Knott’s test at 5% of probability.

Table 4. Average root length (cm) in explants of *V. cacuminis*, precultured *in vitro* in media containing GA$_3$ or BA, in response to different concentrations of NAA, IAA or IBA, 90 days after the beginning of the experiment.

<table>
<thead>
<tr>
<th>from GA$_3$ culture media</th>
<th>from BA culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>NAA</td>
</tr>
<tr>
<td>0.0</td>
<td>0.1 aB*</td>
</tr>
<tr>
<td>0.1</td>
<td>1.5 aA</td>
</tr>
<tr>
<td>0.2</td>
<td>1.4 aA</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5 aA</td>
</tr>
<tr>
<td>0.4</td>
<td>1.4 aA</td>
</tr>
</tbody>
</table>

* For each previously growth regulator utilized (BA or GA$_3$), means followed by the same small (in lines) or capital (in arrows) letters are not different according to Scott-Knott’s test at 5% of probability.

**DISCUSSION**

The disinfection procedures used in this study were quite effective from the *V. cacuminis* seeds, with a very low fungal and bacterial contamination (less than 5%). Some species of the genus *Vriesea* produces seeds with a low germination capacity (Mercier & Kerbauy 1995, Droste et al. 2005, Silva et al. 2009) while in others the...
germination reaches values close to 100% (Droste et al. 2005, Silva et al. 2008). The germination found in this study from the V. cacuminis seeds were quite high (95%), showing that, at least for this species, the micropropagation is an efficient and useful propagation method, because the in vitro germination ensures a substantial amount of aseptic explants for subsequent in vitro culture phases and the maintenance of the genetic variability (Mercier & Kerbauy 1997).

The propagation rate found in response to 15 μM BA + 4.5 μM NAA was close to those observed by Fitchet (1990), studying pineapple micropropagation, and by Silva et al. (2009), with the Vriesea scalaris, species which a high BA concentration promoted extensive shoot proliferation, despite the shoot, as observed in this study, have become small and compact. The gibberellins are associate with the in vitro explant elongation in bromeliads (Rech Filho et al. 2005, Dal Vesco & Guerra 2010) and this effect was also noticed in this study for V. cacuminis by the high number of shoots larger than 2 cm.

In bromeliads, the basal part of the leaves shows vascular elements that may have competent cells for re-differentiation when activated by plant growth regulators (Hosoki & Asahira 1980). Several studies show that in bromeliads, the in vitro regenerative route in response to cytokinins follows a specific pattern associated with nodular cultures (NC) (Dal Vesco et al. 2011). NCs are globular in form, translucent to yellowish in color, and compact in texture. Under appropriate conditions, the NCs develop radially into a number of small shoots called microshoots, which elongate to form shoots (Guerra & Dal Vesco 2010). This morphogenetic pattern seems to be a common characteristic in the Bromeliaceae and recurrent in Vriesea genus (Rech Filho et al. 2005, Alves et al. 2006, Guerra & Dal Vesco 2010). Morphogenic structures with regenerative potential similar to those found in this study for the V. cacuminis were also observed for different Brazilian bromeliads, Vriesea friburgensis var. paludosa (L.B. Sm.) L.B. Sm. (Alves & Guerra 2001), Vriesea fosteriana L.B. Sm. (Mercier & Kerbauy 1997) and Vriesea reitzii Leme & Costa (Rech Filho et al. 2005). Based on the regenerative competence of the NCs, they constituted an important source to the mass production of plantlets at a low cost, mainly when associated with the use of bioreactors (Guerra & Dal Vesco 2010).

The new adventitious roots formation in the in vitro multiplication phase allows the achievement of complete V. cacuminis plantlets for ex vitro acclimatization, increasing the efficiency of the micropropagation procedure. According to Kochiba et al. (1974), the GA₃ action on rooting is due that stimulates the emergence and/or the development of a meristematic root zone, allowing the emergence of new adventitious roots. In this study, the positive effects of GA₃ on rooting were observed when associated to NAA. Although some species develop adventitious roots stimulated only by endogenous auxin, the additional gibberellins requirement is common for stimulating the rhizogenesis (Hartmann et al. 2002). The importance of NAA on in vitro rooting was also found by Nilssen & Sutter (1990), showing that this auxin is more stable in the culture than IAA and IBA (Mercier & Kerbauy 1992, Silva et al. 2008, 2009). The analysis of the results presented in Tables 2 and 3 showed that rooted plantlets can be produced using culture media supplemented with 0.2 μM NAA or even 15 μM GA₃ + 1.5 μM NAA, since the root number in these treatments was the same (2.3 roots/explant). It should be emphasized, however, that the characteristics of the plantlets obtained after the acclimatization was better when they were previously cultured in the presence of GA₃. Although the root number was low in the evaluated conditions, the rooting founded here were similar to those achieved with another Vriesea species (Mercier & Kerbauy 1995, Droste et al. 2005, Rech et al. 2005).

The survival of ex vitro conditions depends on the features of plantlets produced in vitro (Hartmann et al. 2002). In this work, the survival to acclimation was quite high, with average of 95% for plantlets from culture media containing GA₃ and 85% for plantlets from BA, what is commonly observed for most species of bromeliads grown in vitro (Mercier & Kerbauy 1995, Rech et al. 2005, Alves et al. 2006, Rech Filho et al. 2009, Guerra & Dal Vesco 2010). Although the explants (seeds) used to establish the in vitro cultures of the V. cacuminis does not allow obtaining clones, the plantlets produced in vitro are suitable for maintenance of genetic variability in field conditions, which is important for wild species.

The results of this study allowed for the establishment of an efficient protocol for in vitro propagation and the maintenance of the genetic variability of the V. cacuminis, an endangered and endemic bromeliad.

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