

## Tissue culture for the conservation and mass propagation of *Vriesea reitzii* Leme and Costa, a bromeliad threatened of extinction from the Brazilian Atlantic Forest

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**Abstract.** The Brazilian Atlantic Forest biome is considered a hotspot of biodiversity. It is estimated that today the remaining primary vegetation covers only 7.5% of its original area. Bromeliad species are important components of this biome. Some of these species are endemic, like the highly endangered *Vriesea reitzii*. Tissue culture techniques have been often employed for the mass propagation and conservation of threatened bromeliad species. In the present work we describe a procedure for the micropropagation and *in vitro* conservation of *V. reitzii*. Seedling explants were cultured on MS and LPm liquid media supplemented with BA, NAA and GA<sub>3</sub>. The induction and multiplication of shoots were observed in the MS medium supplemented with NAA (2 μM) and BA (4 μM). The best conditions for maintenance and conservation of shoots were half-strength or MS medium. Shoot elongation was observed in MS medium supplemented with GA<sub>3</sub> (10 μM). MS medium supplemented with NAA (1 μM) and BA (2 μM) enabled an efficient proliferation system. The acclimatization of shoots longer than 2 cm resulted in 100% survival rate.

**Abbreviations:** BA – 6-benzyladenine; BM – basal medium; GA<sub>3</sub> – Gibberellic acid; LPm – von Arnold and Eriksson (1981) modified medium; MS – Murashige and Skoog (1962); NAA – α-naphthaleneacetic acid

### Introduction

The Atlantic Forest is a biome characterized by a high diversity of plant species. The occurrence of ca. 20,000 species is estimated, among which 6000 species are considered endemic, corresponding to ca. 3% of all endemic species in the world. It is also estimated that the remaining primary vegetation of this forest represents only 7.5% of the original extent (Heywood 1995; Myers et al. 2000).

The progressive process of devastation observed in this biome and its associated ecosystems caused an impressive reduction in its biodiversity. This loss was also observed in the epiphytic flora, rich in bromeliad species (Coffani Nunes 2002).

Bromeliads consist of a complex ecological subsystem that contributes to the stability of forest ecosystems. They are highly specialized and adapted to extreme climatic and oligotrophic conditions (Padilha 1978).

The Bromeliaceae family contains 51 genera and ca. 3500 species (Coffani Nunes 2002), all of them native to the Americas with the exception of *Pitcairnia feliciana*, which is native to Africa (Padilha 1978; Reitz 1983). The genus *Vriesea* consists of 257 species distributed in the Americas, from Mexico and Cuba to the south of Brazil and northeast of Argentina (Smith and Downs 1977).

In the Santa Catarina State, there are 31 species of *Vriesea*, many of them endemic and threatened with extinction, as is the case for *V. fosteriana*, *V. hieroglyphica* and *V. brusquensis* (Reitz 1983; Klein 1990).

*Vriesea reitzii* Leme and Costa (Figure 1a) is a bromeliad which occupies altitudes ranging from 750 to 1200 m in the states of Santa Catarina, Rio Grande do Sul and Paraná. Its name is a tribute to the so-called 'father of bromeliads,' the priest Raulino Reitz (Leme and Costa 1991). *V. Reitzii* lives in Brazilian mixed araucarian forest dominated by the Brazilian pine (*Araucaria angustifolia*). This ecosystem was severely devastated during the last century and its remnants are estimated at 1% of the original forest (Guerra et al. 2002). Associated to this ecosystem, *V. reitzii* is now threatened with extinction too.

*Vriesea reitzii* is morphologically similar to *V. philippocuburgi*, distributed in the same region, but living only at altitudes lower than 400 m (Baensch and Baensch 1994).

Bromeliad *ex situ* and *in situ* germplasm conservation programs employ different strategies, including genetic and ecological studies and habitat management. Recently techniques of tissue culture have been used as tools for germplasm conservation of rare or threatened species (Zornig 1996).

The utilization of *in vitro* techniques for germplasm conservation is of great interest in plant species (Costa Nunes et al. 2003). The *in vitro* conservation can be for medium and long periods. The conservation for a medium period is done by decreasing the growth of cultures. The long period conservation is done by cryopreservation techniques (Engelmann 1998).

The establishment of *in vitro* germplasm banks in developing countries has great importance, but these techniques must be associated with other plant genetic resources conservation practices (Engelmann 1997). The *in vitro* conservation techniques allow material exchanges among germplasm banks, and the germplasm keeps its sanitary conditions and viability during the transport (Ashmore 1998).

Bromeliad *in vitro* culture techniques are founded on the use of various competent morphological structures. *In vitro* cultures of *Vriesea fosteriana* produced protuberances which resulted in the proliferation of multiple shoots (Mercier and Kerbauy 1992). Similar structures formed from the basal leaf regions of *Dyckia macedoi* resulted in the development of adventitious buds (Mercier and Kerbauy 1993). In *Aechmea fasciata*, adventitious buds

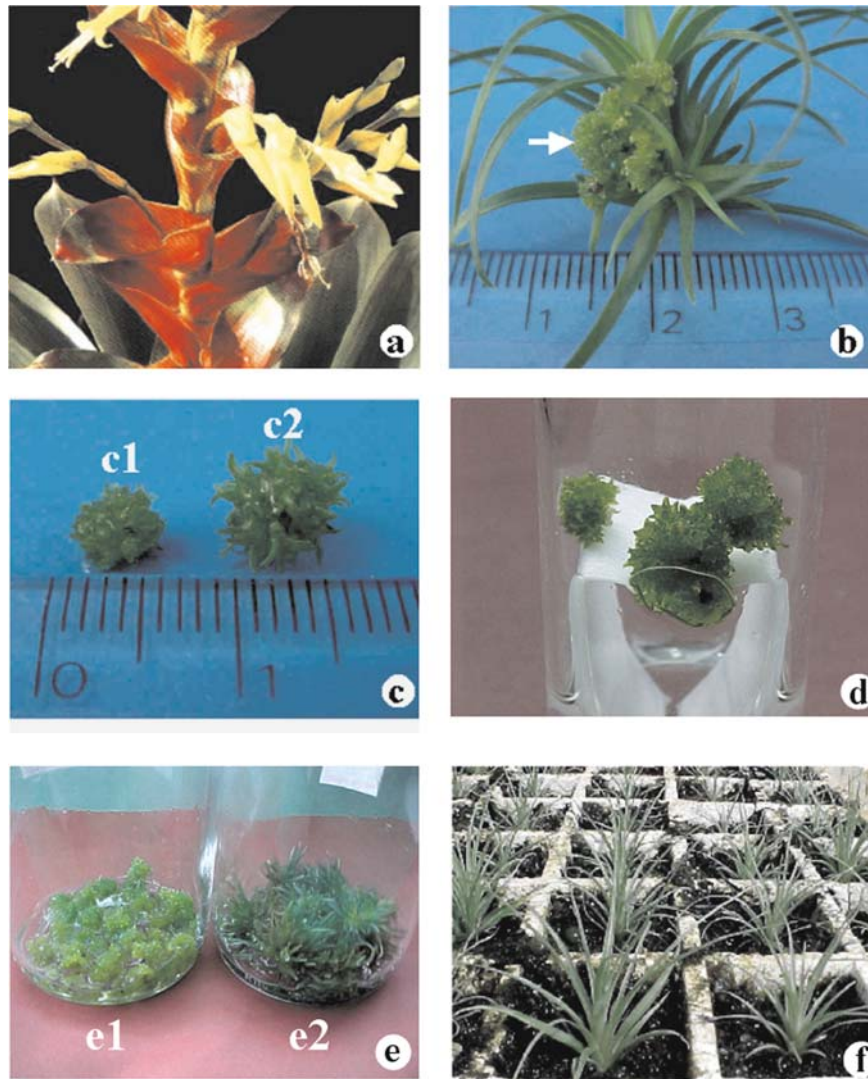


Figure 1. *Vriesia reitzii* LEME and COSTA. (a) Adult plant (source: Baensch and Baensch 1994). (b) *in vitro* protuberances developed in the presence of  $2 \mu\text{M}$  NAA and  $4 \mu\text{M}$  BA (arrow). (c) Shoot multiplication after 16 weeks in culture in BM supplemented with NAA ( $2 \mu\text{M}$ ) and BA ( $4 \mu\text{M}$ ) (c1), and shoot elongation in BM supplemented with  $\text{GA}_3$  ( $10 \mu\text{M}$ ) (c2). (d) *In vitro* shoot multiplication and conservation. (e) *In vitro* multiplication and elongation. (f) Acclimatization of plantlets.

differentiated from calluses induced from leaf explants (Vinterhalter and Vinterhalter 1994).

Methods for *in vitro* propagation of ornamental bromeliads have been reviewed by Mercier and Kerbauy (1997). There are also recent reports on the

propagation of *Cryptanthus sinuosus* (Carneiro et al. 1998), *Ananas comosus* (Guerra et al. 1999; Dal Vesco et al. 2001) and *Vriesea friburgensis* var. *paludosa* (Alves and Guerra 2001). In *Dickia distachia*, a bromeliad threatened with extinction, shoot cultures were induced from flower stalk explants (Daquinta et al. 1998).

Tissue culture techniques provide tools feasible for large scale propagation. The genetic fidelity depends on the choice of the explants. Using seeds as explants is a good strategy for conservation purposes. Somatic explants sampled from mother plants enable only cloning which is important for the capture and fixation of certain genetic gains, as in the ornamental plant production and breeding. In both cases productivity is higher than with conventional methods of propagation.

The overall aim of the present work was to establish protocols for the *in vitro* conservation and mass propagation of *Vriesea reitzii*.

#### **Material and methods**

Seeds of *V. reitzii* LEME and COSTA, were collected in the Lages county in the highland of Santa Catarina State, south Brazil. These seeds were immersed in 70% ethanol for 2 min; treated with 2% sodium hypochloride for 30 min and then rinsed three times in autoclaved water. Surface sterilized seeds were inoculated in 300 ml flasks with 25 ml of media containing MS inorganic salts (Murashige and Skoog 1962), Morel vitamins by Morel and Wetmore (1951), sucrose (3%) and agar (7 g l<sup>-1</sup>). The pH of the culture medium was adjusted to 5.8 before autoclaving. This medium is referred to as basal medium (BM). Culture conditions were the same as previously reported by Alves and Guerra (2001). The experimental design was in completely randomized blocks with four treatments. Each experimental unit consisted of five shoot clusters (0.05 g/tube), with 3–4 replicates. Data of increment in the size of shoots were recorded after 16 weeks in culture. Stages employed in tissue culture propagation included:

##### *Induction*

Cultivation of seedling explants cultured on liquid BM supplemented with 2  $\mu$ M NAA and 4  $\mu$ M BA. Protuberances which developed were used as explants in other experiments.

##### *Establishment and development*

Protuberances were inoculated in flasks of 300 ml containing 10 ml liquid BM supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M). Cultures were transferred to the same medium 10 weeks after inoculation.

### *Conservation*

Explants consisting of small shoots were inoculated in test tubes (25 × 150 mm) containing 10 ml of liquid culture medium over filter paper bridges and were submitted to the following treatments: (1) half strength BM (1/2 MS); (2) BM; (3) BM supplemented with NAA (1  $\mu$ M) and BAP (2  $\mu$ M); and (4) MS culture medium supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M). The test tubes were covered with Bellco® caps and sealed with parafilm®. Fresh mass (g) of shoots was recorded after 16 weeks in culture.

### *Elongation*

Shoots were incubated into test tubes (150 × 25 mm) containing liquid BM supplemented with 0, 5, 10 and 15  $\mu$ M GA<sub>3</sub> on filter paper bridges for 16 weeks, and subsequently subcultured to BM.

### *Multiplication*

Shoots 1.0–2.5 cm long previously elongated in liquid BM were inoculated in flasks of 300 ml, containing 25 ml of two basal salt formulations: MS (Murashige and Skoog 1962) or LPm (von Arnold and Erikson 1981) supplemented with NAA (0, 1 and 2  $\mu$ M) and BA (0, 2 and 4  $\mu$ M). The experimental design was in completely randomized blocks with six different treatments. The experimental units comprised three groups of three plants, with three replicates. Data of number of shoots, fresh mass and evolution of increment in height were recorded after 12 weeks in culture.

### *Acclimatization*

Shoots longer than 2 cm were transferred to trays with 72 cells (120 cm<sup>3</sup>) containing a mix substrate of 1:1 (v:v) of carbonized rice coat and Turfa Fertil® mineral supplement (N-4, P<sub>2</sub>O<sub>5</sub>-14, K<sub>2</sub>O-8). The trays were maintained in the greenhouse with controlled mist for a period of 9 weeks.

### *Statistical analysis*

Primary and transformed data were submitted to the Analysis of Variance (ANOVA) and to the Student–Newman–Keuls mean separation test (SNK 5%), according to Compton (1994).

## Results and discussion

### *Induction*

Yellow to green protuberances, consisting of meristematic clumps, similar to nodular calluses described in *A. comosus* by Teng (1996), were visually observed to arise from the basal region of seedling explants for 10 weeks cultivated on BM supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M) (Figure 1b).

### *Establishment*

Protuberances with yellow to green colour maintained in basal liquid MS culture medium supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M) resulted in the development of shoots after 10 weeks in culture.

### *Shoot conservation*

The MS culture medium supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M) resulted in a large increment in the production of fresh mass shoots ( $p < 0.05$ ) as compared to the initial inoculum (Table 1), after 16 weeks in culture (Figure 1c1, d). This could be an efficient strategy for large-scale micropropagation. However, the cultures maintained in liquid MS and MS half-strength revealed low multiplication rates (Table 1), which are suitable for *in vitro* conservation, since there is no need for frequent subcultures (Figure 1d, e1).

Similar responses observed in the present work were reported for *in vitro* cultures of *V. friburgensis* var. *paludosa* (Alves and Guerra 2001). In this case an enhancement of shoot regeneration was observed in BM or BM supplemented with NAA (2  $\mu$ M) and BA (4  $\mu$ M).

Table 1. Fresh mass (g) and increment (times) in fresh mass of *Vriesea reitzii* cultured in different compositions of culture media after 16 weeks in culture.

Culture media	Fresh mass (g)		Increment in mass (times)
	Initial	Final	
MS + NAA (2 $\mu$ M) + BA (4 $\mu$ M)	0.05	2.1 A	42.0
MS + NAA (1 $\mu$ M) + BA (2 $\mu$ M)	0.05	1.8 B	36.0
MS	0.05	1.1 C	22.0
1/2 MS	0.05	1.1 C	22.0
Mean		1.5	30.0
CV(%)	10.0		

Mean of four replicates, means followed by different letters are significantly different by SNK (test 5%).

The results of the present work showed that MS medium supplemented with NAA (2  $\mu\text{M}$ ) and BA (4  $\mu\text{M}$ ) was the more suitable culture medium composition for the multiplication of shoots. However, the maintenance and conservation of these shoots was enhanced in full or half-strength BM.

### *Elongation*

The presence of GA<sub>3</sub> in the basal culture media resulted in the uniform elongation of *V. reitzii* shoots, 10  $\mu\text{M}$  GA<sub>3</sub> being the level that promoted the highest elongation (Figure 1c2, e2). The shoots maintained in BM supplemented with GA<sub>3</sub> (10  $\mu\text{M}$ ), after two subcultures (16 weeks in total) in BM, were approximately 2.0 cm long.

### *Shoot multiplication*

The BM supplemented with NAA (1  $\mu\text{M}$ ) and BA (2  $\mu\text{M}$ ) provided the highest ( $p < 0.01$ ) proliferation rate (20.2 shoots/explant) after 12 weeks in culture (Table 2). The difference in culture media inorganic salts (MS or LPm) did not reveal statistical differences (SNK, 5%) in the number of shoots produced.

The use of culture media supplemented with BA and NAA has been associated with *in vitro* morphogenetic events in Bromeliaceae. Thus, in *Ananas comosus* the use of liquid MS culture medium supplemented with NAA (2.7  $\mu\text{M}$ ) and BAP (4.4  $\mu\text{M}$ ) resulted in the highest regeneration rate (Guerra et al. 1999). In *Cryptanthus sinuosus*, a high rate of proliferation was obtained in culture medium supplemented with 22  $\mu\text{M}$  BA (Carneiro et al. 1998). Using temporary immersion bioreactors, Daquinta et al. (1999) reported an increase

Table 2. Effect of culture media MS (Murashige and Skoog 1962), and LPm (von Arnold and Eriksson 1981) supplemented with NAA and BA on shoot number and fresh mass in *V. reitzii*, after 12 weeks in culture.

Culture media			Number of shoot/explant	Fresh mass Total (g)
Basal Medium	NAA ( $\mu\text{M}$ )	BA ( $\mu\text{M}$ )		
MS	1	2	4.5 A	6.0 AB
LPm	1	2	4.0 A	5.4 AB
MS	2	4	3.7 A	6.6 A
LPm	2	4	3.0 AB	3.5C
LPm	0	0	2.3 B	4.6 BC
MS	0	0	2.3 B	3.8 C
Mean			3.7	5.0
CV (%)			5.2 <sup>a</sup>	14.3

Mean of four replicates, means followed by different letters are significantly different by SNK (test 5%).

<sup>a</sup> Data transformed for analysis using  $\log(x + 2)$ ; CV (%), coefficient of variation.

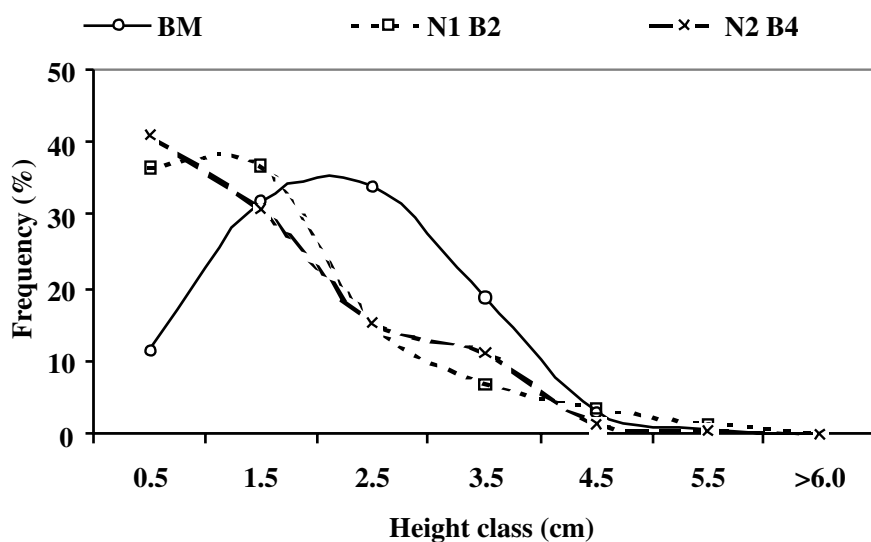


Figure 2. Frequency (%) of regenerated shoots as affected by the height class (cm) on BM\* supplemented with NAA (1 or 2  $\mu\text{M}$ ) and BA (2 or 4  $\mu\text{M}$ ), after 12 weeks in culture. Mean of three replicates; BM\*, basal medium; N1B2, NAA (1  $\mu\text{M}$ ) and BA (2  $\mu\text{M}$ ); N2B4, NAA (2  $\mu\text{M}$ ) and BA (4  $\mu\text{M}$ ).

in the regeneration rate of *Aechmea blumenavii*, *Cyrtanthus bromelioides* and *Neoregelia carolinae* in response plant growth regulators NAA, BA and paclobutrazol.

In the present work both the size and type of explants were determinant for the proliferation capacity and related features. Thus, when 0.5 cm long shoots were used as explants, the morphogenetic events leading to the regenerative pathway were associated with to a budding process (Figure 1, e1). When clusters of shoots 1.0–2.5 cm long (Figure 1, e2) were used as explants activation of axillary buds was observed, resulting in a wide distribution of shoot numbers and lengths in shoot clusters (Figure 2).

The highest frequency of shoot proliferation was observed in clusters containing shoots 1.5 cm long in BM supplemented with BA and NAA. On the other hand the use of culture media free of plant growth regulators promoted the growth and elongation of shoots. Single isolated, well elongated shoots manifested apical dominance and the induction of new buds in the basal region of such shoots.

#### Acclimatization

Shoots longer than 2.0 cm were acclimatized successfully when transferred to trays containing a mix substrate of 1:1 (v:v) carbonized rice coat and Turfa

Fertil<sup>®</sup> mineral supplement in the greenhouse with intermittent mist (Figure 1f). After 9 weeks the survival rate of plantlets was 100%.

The recovery and conservation of a broad genetic pool of wild and domesticated species are essential for the maintenance of plant biodiversity. Traditional *ex situ* conservation systems alongside *in situ* conservation make a fundamental contribution to the preservation of plant genetic resources (Lambardi and De Carlo 2003). According to these authors micropropagation can be considered the first approach towards *in vitro* germplasm preservation. The results of the present work show that this approach can be successfully employed for the conservation of *V. reitzii*. Other endangered bromeliad species from the Atlantic Forest biome are now candidates for the same approaches.

The results of the present work showed that the *in vitro* induction of shoots of *V. reitzii* was obtained in response to the supplementation of BA and NAA to the basal MS culture medium. The *in vitro* conservation and proliferation of these shoots was favored by the use of the MS full or half-strength liquid culture media free of plant growth regulators over filter paper bridges. The uniform elongation of shoots was achieved in liquid BM supplemented with GA<sub>3</sub> (10 μM). Our results show that tissue culture techniques can be successfully employed for the conservation and mass propagation of endangered bromeliad species, as is the case for *V. reitzii*.

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