# *In vitro* multiplication of *Vriesea scalaris* E. Morren (*Bromeliaceae*)

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ABSTRACT - *Vriesea scalaris* is an ornamental bromeliad. The overall aim of this research was to establish conditions for *in vitro* multiplication and growth. Seeds were disinfected and *in vitro* germinated on MS medium containing 0, 0.5, 1, 2, or 4.5 µM BAP (6-benzylaminopurine) or KIN (6-furfurylaminopurine or kinetin). Rooting was achieved on half strength MS supplemented with 0, 2.5, 5, or 10 µM IBA (indole-3-butyric acid). Some seedlings (thirty-day-old) were kept on MS medium in the presence of 10 $mL.L^{-1}$ B5 vitamins and others in the absence. Leaf explants were cultured on MSL medium (MS salts and vitamins, 30 $g.L^{-1}$ sucrose, 0.15 $g.L^{-1}$ ascorbic acid, 0.4 µM BAP, 0.5 µM NAA (alpha-naphthaleneacetic acid) and solidified with 6 $g.L^{-1}$ agar) and submitted to the treatments: MSL medium (control); MSL + 0.06$g.L^{-1}$ $NH_4NO_3$; MSL + 0.25$g.L^{-1}$ hydrolyzed casein or MSL + 2$mL.L^{-1}$ Fuji vitamins. BAP was more suitable than KIN for shoot multiplication. IBA at the concentrations used induced low rooting, the absence of IBA promoted a better rooting rate. The B5 vitamins promoted an increase in multiplication rate. The results of these experiments demonstrated the viability of the *in vitro* multiplication for this species. Seedlings and leaf explants showed the same multiplication rate (ca. 8 shoots per explant). These protocols can be used for *in vitro* propagation and for *in vitro* germplasm conservation.

Key words: organogenesis, bromeliads, tissue culture, plant growth regulators.

RESUMO - **Multiplicação *in vitro* de *Vriesea scalaris* E. Morrem (*Bromeliaceae*).** *Vriesea scalaris* é uma bromélia ornamental. O objetivo desta pesquisa foi estabelecer condições para multiplicação e crescimento *in vitro*. Sementes foram desinfestadas e germinadas *in vitro* em meio MS contendo 0; 0,5; 1; 2 ou 4,5 µM de BAP (6-benzilaminopurina) ou KIN (6-furfurilaminopurina ou cinetina). O enraizamento *in vitro* foi induzido em meio MS adicionado de 0; 2,5; 5 ou 10 µM de IBA (ácido indolbutírico). Plântulas com 30 dias foram cultivadas na presença ou ausência de 10$mL.L^{-1}$ de vitaminas B5. Explantes foliares foram cultivados em meio MSL (MS sais e vitaminhas, 30 $g.L^{-1}$ de sacarose, 0,15 $g.L^{-1}$ de ácido ascórbico, 0,4 µM de BAP, 0,5 µM de ANA (ácido naftalenoacéito) e solidificado com 6 $g.L^{-1}$ agar), meio MSL + 0,06$g.L^{-1}$ de $NH_4NO_3$; MSL + 0,25$g.L^{-1}$ de caseína hidrolisada ou MSL + 2$mL.L^{-1}$ de vitaminas Fuji. O BAP foi mais satisfatório do que KIN para a multiplicação dos brotos. O enraizamento *in vitro* foi obtido na ausência de IBA. As vitaminas B5 promoveram um aumento na taxa de multiplicação de brotos. Plântulas e explantes foliares apresentaram a mesma taxa de multiplicação, cerca de 8 brotos por explante. Os resultados destes experimentos demonstraram a viabilidade da multiplicação *in vitro* para esta espécie, podendo ser utilizados para a propagação *in vitro* e conservação de germoplasma *in vitro*.

Palavras-chave: organogenêse, bromélias, cultivo de tecidos, reguladores de crescimento.

## INTRODUCTION

*Vriesea scalaris* E. Morren belongs to the Bromeliaceae family and Tillandsioideae subfamily. It is an ornamental plant found in small populations in the Atlantic Forest due to the difficulty of its vegetative reproduction, because few offshoots are formed and they grow very slowly (Reitz, 1983). The Red List of Endangered and Threatened Species of Rio Grande do Sul State, Brazil considers this species vulnerable (Sema, 2005).

Although *Vriesea* Lindl. consists of 257 species (Smith & Downs, 1977), *in vitro* culture has only been studied in some species including: *V. splendes* (Brongn.) Lem. and *V. heliconioides* (Kunth) Hook. Ex Walp. (Mekers, 1977; Pierik *et al*., 1984); hybrid *V. ´poelmannii'* (Hosoki & Asahira, 1980); *Vriesea* sp. (Mekers & Van Onsem, 1983); *Vriesea* spp. (Kukulczanka & Czastka, 1989); *V. fosteriana* L. B. Smith (Mercier & Kerbauy, 1992*); V. hieroglyphica* (Carrière) E. Morrem (Mercier & Kerbauy, 1994); *V. friburgensis* var. *paludosa* (L. B. Smith) L. B. Smith (Alves & Guerra, 2001); *V. gigantea* Gaudich., *V. philippocoburgii* Wawra (Droste *et al*., 2005); *V. reitzii* Leme & Costa (Rech-Filho *et al*., 2005; Alves *et al*., 2006; Rech-Filho *et al*., 2009), and *V. cacuminis* L. B. Smith (Mendes *et al*., 2007).

Conventional methods for bromeliad propagation are not efficient for plant multiplication. Therefore, *in vitro* multiplication promotes a high rate of shoots produced with a good sanitary quality (Mercier & Kerbauy, 1995). Moreover, there are other applications for these *in vitro* techniques, as *in vitro* conservation of bromeliads, but it is necessary that micropropagation protocols are developed previously (Carneiro & Mansur, 2004). On the other hand, through the multiplication of seedlings obtained *in vitro*, a large number of genetically homogeneous plants can be obtained (Droste *et al*., 2005), which is an exigency of ornamental plants consumers.

The overall aim of this study was to establish conditions for *in vitro* multiplication and growth of *V. scalaris*.

## MATERIAL AND METHODS

All Cultures were kept in a growth chamber at the temperature of 25° ± 2° C and 16 hours of photoperiod under a light intensity of 14.3 $\mu M.m^{-2}.s^{-1}$ produced by white fluorescent bulbs.

Seeds were immersed in 70% ethanol during three minutes, rinsed twice in sterilized distilled water, followed by immersion in commercial bleach (1% NaOCl) for 10 min, and rinsed again three times with distilled sterilized water. The seed papus of the seeds was mechanically removed prior to inoculation on the germination medium. The germination medium consisted of distilled water solidified with 6 $g.L^{-1}$ agar and pH was adjusted to 5.7 prior to autoclaving. The germination percentage was evaluated after 10 days of *in vitro* culture.

In order to induce adventitious shoot formation, seeds were germinated under treatments with cytokinins, BAP (6-benzylaminopurine) or KIN (6-furfurylaminopurine or kinetin) in the concentrations of 0, 0.5, 1, 2, or 4.5 µM, added to the basal medium. This medium was MS, salts and vitamins (Murashige & Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose and 2 $mL.L^{-1}$ Fuji vitamins (2.5 $g.L^{-1}$ nicotinic acid, 2.5 $g.L^{-1}$ pyridoxine chloridrate, 0.5 $g.L^{-1}$ thiamine chloridrate, 50 $g.L^{-1}$ inositol, and 1 $g.L^{-1}$ glycine). Medium was solidified with 6 $g.L^{-1}$ agar and the pH adjusted to 5.7 prior to autoclaving. Shoot number per explant was evaluated after 120 days of *in vitro* culture.

Shoots were isolated from clumps (originated from seeds) and cultivated on a half strength MS medium supplemented with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar and pH adjusted to 5.7 prior to autoclaving. The treatments were 0, 2.5, 5, or 10 µM IBA (indole-3-butyric acid). IBA was tested to accelerate rooting. The rooting percentage was evaluated after 45 days of *in vitro* culture.

Seedlings at 30 days were transferred to MS medium (salts and vitamins) supplemented with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar, with the objective to evaluate the effects of adding 10 $mL.L^{-1}$ B5 medium vitamins (Gamborg *et al*., 1968) on the *in vitro* growth, and compare it with other medium without B5 vitamins. The final fresh mass (g), the final shoot number, and fresh mass increment (quotient between final fresh mass and initial fresh mass) were evaluated after 120 days of *in vitro* culture.

In the experiments of shoot induction in leaf explants, seedlings (ca. 1 cm tall) obtained by *in vitro* germination on MS medium as described above, were used as a source for leaf explants. These explants were cultured on a MS salts and vitamins, 30 $g.L^{-1}$ sucrose, 0.15 $g.L^{-1}$ ascorbic acid, 0.4 µM BAP, 0.5 µM NAA (alpha-naphthaleneacetic acid) and solidified with 6 $g.L^{-1}$ agar, this medium was called MSL. The treatments were: MSL; MSL + 0.06 $g.L^{-1}$ $NH_4NO_3$; MSL + 0.25 $g.L^{-1}$ hydrolyzed casein, and MSL + 2 $mL.L^{-1}$ Fuji vitamins. The survival percen-

tage and the final shoot number was evaluated after 60 days of *in vitro* culture.

The experimental design was completely randomized with five replicates of five explants. The data was submitted in a normality analysis for the Lilliefors´s test and, analysis of variance (ANOVA) followed by regression analysis (data from quantitative treatments) or Tukey's test (data from qualitative treatments), both at a $P<0.05$. All statistical analyses were done following the procedures of the software GENES (Cruz, 2001). Variables from counting were transformed to $\sqrt{x+0.5}$ and variables from percentage were transformed to arcsin $\sqrt{x/100}$.

## RESULTS AND DISCUSSION

The germination started seven days after *in vitro* inoculation on a germination medium. The germination efficiency reached 60%. In comparison with the *in vitro* germination of other *Vriesea* species this rate is not very high. It can be related to the germination medium, as the MS medium had a high concentration of salts. For *V. hieroglyphica*, the best germination rate (90%) was obtained on KC medium (Knudson, 1946) with a salt concentration at 25% full strength. This modified medium had a lower concentration of salts than MS medium (Mercier & Kerbauy, 1994).

BAP promoted the adventitious shoot proliferation at the basal region of the seedlings after 30 days of *in vitro* culture. Similar results were found in experiments with *Dyckia distachya* Hassler where the seed germination and adventitious shoots were induced on a culture medium supplemented with BAP (Pompelli & Guerra, 2005). In cultures of *V. scalaris*, BAP was more suitable than KIN with respect to the shoot number per explant (Fig. 1). In the presence of BAP, the highest multiplication rate was close to 8 shoots per explant, whereas in the presence of KIN only two shoots were produced per explant. However, there were no significant differences among the results obtained in the presence of KIN (0-4.5 µM). This is in agreement with results found in experiments with *V. hieroglyphica*, in which KIN had no effect on proliferation (Mercier & Kerbauy, 1994). The results obtained with BAP containing media followed a positive linear regression, which indicated that concentrations higher than 4.5 µM can increase the multiplication rate.

The most suitable BAP level (4.5 µM) for *V. scalaris* produced only ca. 8 shoots per explant. However, in agreement with results obtained by regression analysis (Fig. 1), it is possible that elevated concentrations of BAP can promote higher multiplication rates, whereas in *V. fosteriana* cultivated in KC medium and supplemented with 8.9 µM BAP and 2.7 µM NAA produced ca. 22 explants per seedling (Mercier & Kerbauy, 1992).

In the absence of plant growth regulators, seedlings of *V. scalaris* produced 1-2 shoots per explant (Fig. 1). Similar results were found in *Vriesea hieroglyphica* cultured on KC medium in the absence of growth regulators, where each embryo yielded 3 to 7 plantlets (Mercier & Kerbauy, 1994). However, when bromeliad seeds are germinated in a medium supplemented with cytokinins, more shoots from each embryo are produced (Fischer & Zimmer, 1988). In the case of *V. hieroglyphica* the result can be associated to the genetic characteristics of this species, since in other *Vriesea* species, such as *V. fosteriana* (Mercier & Kerbauy, 1992), *V. gigantean*, and *V. philippocoburgii* (Droste *et al*., 2005), each seed yielded only one plant, when cultivated in a KC medium.

The results obtained with IBA showed a negative quadratic regression (Data not shown). The best result for rooting was 40% obtained in the absence of IBA. Nevertheless, the lowest rooting can be related with the largest number of adventitious shoots on the isolated shoots. These adventitious shoots proliferation in *V. scalaris* could have been promoted by cytokinin endogenous residues originated from multiplication medium supplemented with cytokinins. For example, for *V. fosteriana*, the addition of 0.54 µM NAA to MS medium was necessary to avoid adventitious proliferation as well as re-establish apical growth of the shoots (Mercier & Kerbauy, 1992).

The supplementation of 10 $mL.L^{-1}$ B5 vitamins to the culture medium promoted an increase in the fresh mass of the seedlings, in the final shoot number and in the final fresh mass (data not shown). The supplementation of B5 vitamins promoted a significant increment of the adventitious shoot proliferation ($P<0.05$), varying among 1 to 2 shoots per explant, whereas in the control only one shoot was produced. Similar results were found in *Passiflora edulis* Sims, where the culture medium supplemented with the B5 vitamins induced higher shoot number per explant (Ribas *et al*., 2002). For some species cultivated *in vitro*, there is need to increase the concentration of the vitamins, and for others it is necessary to increase other types of mixture patterns (Caldas *et al*., 1998).

Leaf explants did not survive on the MSL medium without supplementation; these explants

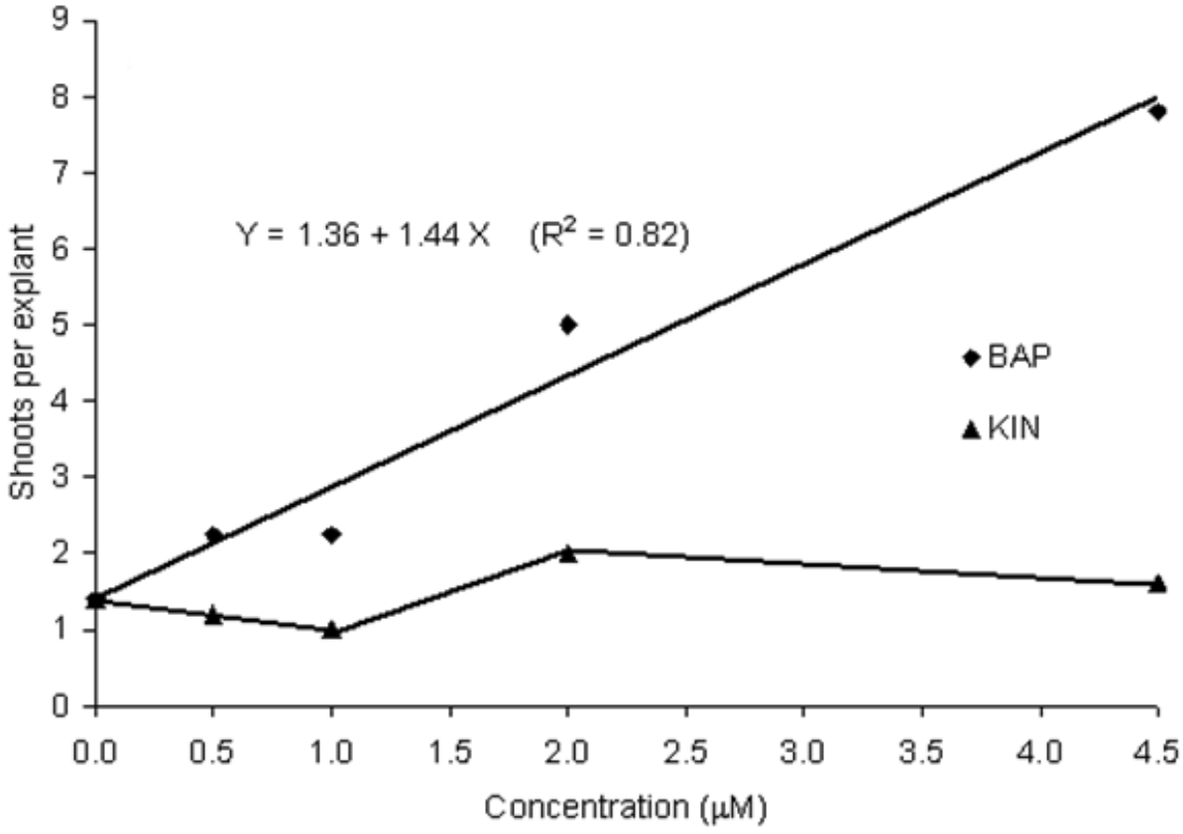


**Fig. 1.** Effect of 6-benzylaminopurine (BAP) and kinetin (KIN) on number of shoots per explant of *Vriesea scalaris* seedlings after 120 days of *in vitro* culture.

also did not survive with the supplementations of: 0.25 $g.L^{-1}$ hydrolyzed casein or 2 $mL.L^{-1}$ Fuji vitamins. Nevertheless, addition of $NH_4NO_3$ to the culture medium allowed the leaf explant survival; shoots were formed at the leaf base, the shoot number per explant reached 8. Similar results were found in *Dyckia maritima* Baker, where the higher levels of $NH_4NO_3$ concentration on the culture medium increases the multiplication rate (Da Silva *et al*., 2005). $NH_4NO_3$ concentrations can influence endogenous levels of plant growth regulators, as demonstrated in *Eleusine coracana* (L.) Gaertn. (Poddar *et al*., 1997), and some bromeliads (Mercier & Kerbauy, 1998).

In conclusion, KIN is not efficient, while the concentration of 4.5 µM BAP is suitable for *in vitro* multiplication of *V. scalaris*. IBA at the concentrations used in this investigation induced less rooting. Rooting occurs in the absence of IBA. Seedlings cultivated in the presence of B5 vitamins increase *in vitro* shoot multiplication. Supplementation of 0.06 $g.L^{-1}$ $NH_4NO_3$ on the MSL medium induces higher multiplication rates. Seedlings and leaf explants showed the same multiplication rate (ca. 8 shoots per explant). The results of these experiments demonstrated the viability of the *in vitro* multiplication for this species; this protocol can be adapted for *in vitro* germplasm conservation.

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