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In vitro culture at low temperature and *ex vitro* acclimatization of *Vriesea inflata* an ornamental bromeliad

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ABSTRACT − (*In vitro* culture at low temperature and *ex vitro* acclimatization of *Vriesea inflata* an ornamental bromeliad). *In vitro* culture by seeds is a technique for preservation of threatened species because it may provide a large number of plants with genetic diversity. The bromeliad *Vriesea inflata* (Wawra) Wawra, an ornamental bromeliad, is extensively and illegally collected from the nature and must be preserved. It is possible to form plant threatened collections *in vitro* by reducing the temperature of culture, while occupying little space, with the consequent reduction of maintenance costs. This work evaluated the influence of temperature on *in vitro* growth and morphology of plants of *V. inflata*, with the aim of establishing a slow growth-rate and analyzing the *ex vitro* acclimatization. Seeds were germinated *in vitro* in Murashige and Skoog (MS) medium, with macronutrients reduced to 50% (MS/2). After three months the plants were transferred to flasks of new same medium and kept in two germination chambers with the temperature adjusted to 15 °C and to 28 °C. After 24 months the plants were subject to biometric, photosynthetic pigments content and anatomical analyses. Results showed that plants maintained at 15 °C were smaller than those at 28 °C. Nevertheless, there were no alterations in pigments content, anatomy. In both treatments there was a survival rate of 100%. This work showed that plants of this species can be kept *in vitro* at 15 °C with the aim of forming a slow-growth collection, thereby seeking its preservation, and can be transferred to growth at *ex vitro* condition to achieved 100% survival rate.

Key words - anatomy, Bromeliaceae, conservation, growth, photosynthetic pigments

RESUMO − (Cultivo *in vitro* em temperatura baixa e aclimatização *ex vitro* de *Vriesea inflata*, uma bromélia ornamental). O cutivo *in vitro* iniciado a partir de sementes pode ser utilizado para preservação de espécies ameaçadas porque contribui para o fornecimento de um grande número de plantas com diversidade genética. *Vriesea inflata* (Wawra) Wawra, uma bromélia ornamental, é extensivamente e ilegalmente coletada da natureza, sendo importante sua preservação. Por meio do cultivo *in vitro* é possível formar coleções de plantas ameaçadas, sob crescimento lento, ocupando pouco espaço, com redução dos custos de manutenção das culturas. Este trabalho avaliou a influência da temperatura sobre o crescimento e morfologia de plantas de *V. inflata* mantidas *in vitro,* com objetivo de diminuir o crescimento, além de verificar a aclimatização destas para a condição *ex vitro.* Sementes de *V. inflata* foram germinadas utilizando-se o meio de Murashige and Skoog (MS) com macronutrientes reduzidos à metade (MS/2). Após três meses as plantas foram transferidas para frascos contendo o mesmo meio e mantidos em câmaras de germinação, com temperaturas ajustadas para 15 °C e 28 °C. Após 24 meses essas plantas foram submetidas à analises biométricas, anatômicas e de pigmentos fotossintéticos. Verificou-se que as plantas mantidas a 15 °C foram menores que aquelas cultivadas a 28 °C. A taxa de sobrevivência foi de 100% e não houve alterações no conteúdo de pigmentos e na anatomia. Este trabalho mostrou que plantas dessa espécie podem ser mantida *in vitro* a 15 °C com o objetivo de se estabelecer uma coleção de crescimento lento visando à preservação, além de mostrar que essas plantas apresentaram uma taxa de 100% de sobrevivência quando transferidas para condições *ex vitro.*

Palavras-chave - anatomia, Bromeliaceae, conservação, crescimento, pigmentos fotossintéticos

Introduction

The Bromeliaceae form a group of 57 genera and around 3,086 species (Luther 2006). In various ecosystems, many bromeliads have been reduced in number or even eradicated due to habitat destruction as a result of anthropic action, such as increasing deforesting, and the occurrence of selective extraction (Rocha *et al.* 2004). The atributes of colorful bracts and flowers that can last for several months, and of leaves of high visual appeal, confer elements of a very highly appreciated esthetic value, much appreciated on the ornamental plant market, as occurs with the epiphyte species native of the Atlantic Rain Forest, *Vriesea inflata* (Wawra) Wawra. This bromeliad is widely commercialized in plant-sales outlets or even offered for commerce on the road-side by informal sales-personnel (Nunes 2002). Generally, and in order to supply plant markets, and with the additional possibility of quick and easy profit, the situation generates the appearance of specialized collectors operating on

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a commercial scale (Duran & Monteiro 2005). Thus, the development of conservation measures becomes necessary in order to avoid the extinction of the target species through illegal extraction.

In vitro culture of bromeliads could be a strategy for preservation and production of seedlings of phytosanitary quality on a large scale for ornamental plant commercialization and to be used for reintroduction programs of impacted areas (Silva *et al.* 2007, Bellintani *et al.* 2007, Rech-Filho *et al.* 2009, Silveira *et. al* 2009). Commercial *in vitro* culture techniques are mainly used for the production of selected genotypes, thus permitting the production of thousands of identical plants, over a short period of time, starting from just one explant (Hartmann *et al.* 2002). This type of culture can also be used for the conservation of germplasm with commercial and ecological interests (Sarasan *et al.* 2006). The micropropagation in the world's largest botanical garden – the Royal Botanic Gardens, Kew, in England – possesses an extensive *in vitro* collection which includes many plant species drawn from all over the world and considered under threat of extinction, whereby the dissemination of *in vitro* protocols becomes necessary in order to promote the continuous development and application of conservation techniques (Sarasan *et al.* 2006). The establishment of *in vitro* culture starting from seeds contributes to maintaining the genetic variability of a certain species, a necessary procedure in conservation programmes (Mercier & Nievola 2003).

The conservation of germplasm of plant species over a long span of time can be done through reducing growth without affecting the development, by the use of constant low temperatures (Jouve *et al.* 2000, Islam *et al.* 2005, Amoo *et al.* 2009). Nevertheless, it must consider that the adaptation of plants to variations of temperature in natural environments occurs seasonally. However, tropical plants do not subsist at low temperatures throughout all the life cycle, whereby an investigation of the adaptive capacity of these plants to a constant low temperature becomes important. Thus, for this type of *in vitro* preservation, it is necessary to analyze the survival rate, *in vitro* and after acclimatization. Gonçalves & Romano (2007) evaluated the survival and morphology of plants of *Drosophyllum lusitanicum* (L.) Link, a threatened species, and the results presented show that cultures can be conserved *in vitro* for eight months at 5 °C. Pretreatment of plants at low temperature (15 ± 2 °C) for 10 days before transfer to greenhouse conditions increased the survival percentage (Malik *et al.* 2009). Jouve *et al.* (2000) showed that poplar can be conserved *in vitro* more than one year at 10 °C, and Bekheet *et al.* (2002) showed that *Phoenix dactylifera* L. plants were successfully stored for 12 months at 5 °C. However, there are no reports about slow growth bromeliad preservation at low temperature.

Variations in anatomy, morphology and physiology can reflect the plants adaptability to the differences in the temperature changes (Klich 2000). Low temperatures also affect the size and shape of photosynthetic cells, amount of pigments, and consequently photosynthesis itself (Georgieva & Lichtenthaler 2006). The chlorophyll and other pigments can be influenced by temperature changes (Carter & Knapp 2001) and can indicate nitrogen utilization by plants (Bigot & Bocaud 1996, von Wiren *et al.* 1997).

There are few reports regarding studies on the influence of temperature in bromeliads cultivated *in vitro*. Nievola *et al.* (2005) observed that plants of the bromeliad *Ananas comosus* L. Merril obtained from an *in vitro* clone already presented a decrease in growth, as well as alterations in anatomy and pigments content when cultured for three months under a thermoperiod of 28 °C light/15 °C dark, compared to those kept at a constant temperature of 28 °C. According to Pierik (1987), the use of a temperature of 15 °C for tropical plants is adequate to produce a decrease in growth without causing plants damage.

The aim of the present work was to evaluate the influence of temperature on *in vitro* growth and morphology of the ornamental bromeliad *V. inflata*, aiming to establish a slow growth rate, and analyze the *ex vitro* acclimatization.

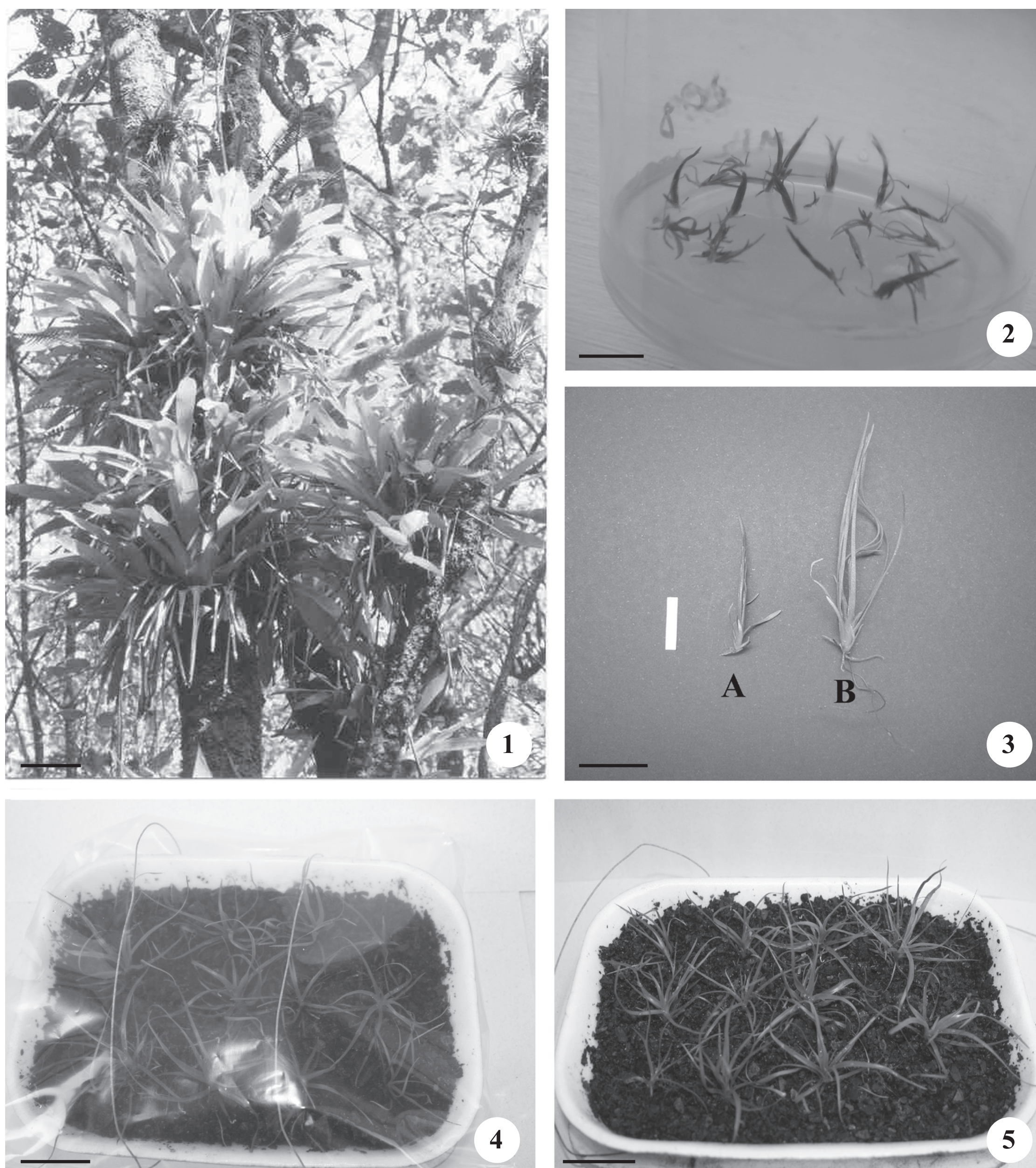
Material and methods

Seeds of *Vriesea inflata* (Wawra) Wawra were harvested from several plants (figure 1) from the Estação Biológica do Alto da Serra de Paranapiacaba-SP-Brazil (23°46'35.8" S and 46°18'42.9" W) and from specimens from different parts of Atlantic Forest present in the Bromeliaceae collection of the Instituto de Botânica, SP (Brazil).

Establishing *in vitro* culture – Seeds were sterilized for five minutes in ethanol (70%) and then were immersed in a solution of sodium hypochlorite 2% with drops of Tween 20 for one hour, followed by five rinses in sterilized water. Seeds were then inoculated in Petri dishes containing Murashige & Skoog (1962) medium (MS) with macronutrients reduced to 50% (MS/2), supplemented with 2% sucrose, 0.1 mg L^{-1} of thiamine-HCL, 100 mg L^{-1} of *myo*-inositol and 7 g. L^{-1} of agar (Bacto Difco) with pH adjusted to 5.8. Cultures were maintained in a growth room at 26 ± 2 °C and 55 µmol m^{-2} s^{-1} light photons provided by cool white lamps during 12 hours of light regime for three months (figure 2).

Experimental design of *in vitro* culture at different temperatures – Eighty three months old plants were distributed among eight flasks of 250 mL, with 10 plants in each one, with the same nutrient medium (40 mL of MS/2 medium) described above. Plant material was kept in two growth-chambers adjusted to 15 °C and 28 °C (figures 3A and 3B). After 24 months, 20 plants, from each temperature culture condition, were assigned to biometric analyses (length of the longest root, numbers of roots and leaves and length of the shoots), besides anatomy and amounts of fresh and dry masses of both roots and shoots, as well as the quantity of photosynthetic pigments in both group of plants. The experiment was carried out in a completely randomized design. The other 40 plants cultivated *in vitro* at 15 °C were used for acclimatization (figures 4 and 5).

Photosynthetic pigment content – Shoots were cut into small pieces and spread on a Petri dish containing humid



Figures 1-5. 1. Adult plants of *Vriesea inflata* at Estação Biológica do Alto da Serra de Paranapiacaba-SP-Brazil. 2. *In vitro* plantlets from seeds cultured in MS medium with macronutrients reduced to 50% for three months at 28 °C. 3. Growth of shoot at 15 °C (3A) and at 28 °C (3B) after two years. 4. First step of acclimatization of plants cultured at 15 °C (enclosing plants placed in tray in a plastic sack) for two months. 5. Plant growth in greenhouse for one month after the first step. Bar = 14 cm (5); 0.8 cm (6); 1 cm (7); 3.5 cm (8,9).

filter paper. Three replicates of 0.5 g of fresh mass of shoot were harvested for determination of photosynthetic pigments. Fresh leaf tissue (0.5 g) was homogenized with 5 mL of cold pure acetone. The homogenate was filtered (Whatman paper, number 1) and its solid residue was washed up to a final volume of 25 mL. Chlorophyll concentration of the filtrate was measured spectrophotometrically and calculated according to Lichtenthaler (1987): chlorophyll a + b = $7.05 \times A_{661.6} + 18.09 \times A_{644.8}$; carotenoid = $[1000 \times A_{470} - 1.90 \times (11.24 \times A_{661.6} - 2.04 \times A_{644.8}) - 63.14 \times (20.13 \times A_{644.8} - 4.19 \times A_{661,6})] / 214$

Anatomical analysis – The samples from both treatments were fixed in FAA (formalin: acetic acid: ethanol 70% – 1:1:18) (Johansen 1940). Fragments from the midrib of the most developed leaf were diafanized according to Strittmatter (1973), and were embebed in polyethylene glycol 2,000. The cross-sections of each fragment with 12 µm were stained with astra blue 1% and safranin 1% (9:1) and permanently mounted on microscopic slides with cover slips using Permount®. Measurements were obtained using a microscope equipped with a camera for image capturing and a semi-automatic measuring system (Olympus Model: BX41-BF-III) along with image analyses software: Image-Pro Express 4.0.1 – Media Cybernetics.

Experimental design of acclimatization – The 40 plants maintained *in vitro* at 15 °C for two years were transferred to *in vitro* conditions at 28 °C distributed in four flasks of 250 mL (with 40 mL of MS/2 medium). After one year *in vitro* at 28 °C, 20 plants were transferred to acclimatization. The first step of acclimatization consisted of enclosing plants for two months in trays, with substrate (fine commercial *Pinus* bark esterilized) and in a plastic sack, before transfer to grow in greenhouse (figure 4). After one month the survival and the number and length of leaves were determined (figure 5). The results were compared to the other 20 plants maintained *in vitro* condition. The experiment was carried out in a completely randomized design.

Data analysis – The means were compared using the *t*-test.

Results and discussion

Table 1 shows the slow growth of *V. inflata* cultured at 15 °C, without alterations in the tissues and in the pigments as those grown at 28 °C. A survival rate of 100% of plants cultured in both treatments was observed during the two years of culture. Plants kept at 15 °C presented significantly smaller length and less dry mass of both, roots and shoots, in relation to those kept at 28 °C, thus demonstrating the influence of low temperature on growth. Similar results were observed by Pérez *et al.* (2001) with *Festuca arundinaceae* Schreb. Cv. Tima, in which the drop in temperature from 25 °C to 12 °C caused a reduction in growth and in the leaf area of plants.

A reduction in growth, as a result of the influence of temperature in the bromeliad *Ananas comosus* (L.) Merr., was reported by Nievola *et al.* (2005). These authors showed that the external and internal morphology of the plants had been altered depending on the temperature of culture. The leaves of plants kept at 15 °C during the dark period were thicker (had more dry mass) although

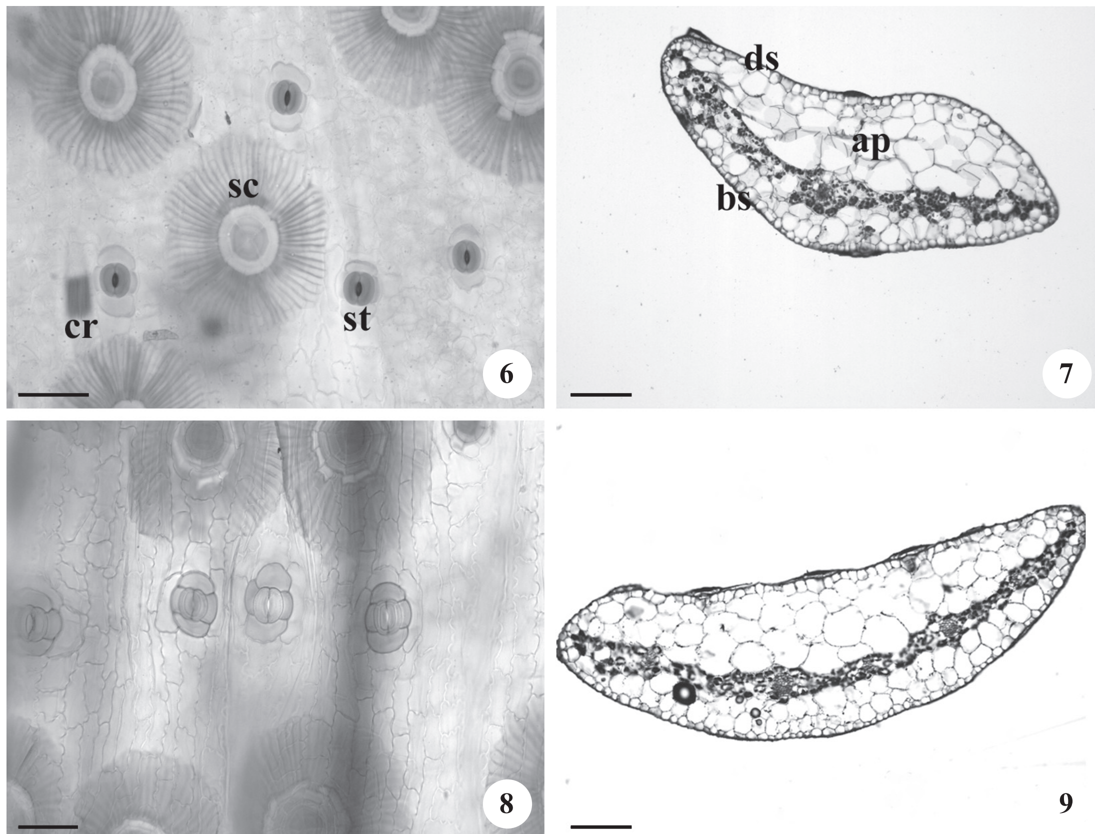
Table 1. Growth parameters, photosynthetic pigments and survival of *Vriesea inflata* cultured at 15 °C (*n* = 20) and 28 °C (*n* = 20) after two years. Means with the same letter within the same line are not significantly different at the 5% level.

		15 °C	28 °C
		$\bar{x} \pm s$	
Root	Quantity of roots (nº)/plant	4.33 ± 0.81a	3.12 ± 0.64a
	Lenght (cm)	0.87 ± 0.33b	2.80 ± 0.56a
	Fresh mass (mg)/plant	1.2 ± 0.1b	5.00 ± 0.01a
	Dry mass (mg)/plant	0.28 ± 0.03b	1.5 ± 0.1a
Shoot	Quantity of leaves (nº)/plant	8.77 ± 0.66a	9.50 ± 0.90a
	Lenght (cm)	2.04 ± 0.19b	3.66 ± 0.43a
	Fresh mass (mg)/plant	16.7 ± 1.3b	41.9 ± 4.9a
	Dry mass (mg)/plant	2.8 ± 0.4b	4.3 ± 0.8a
	Chlorophyll a+b (µg of pigment g FM^{-1})	848.64 ± 109.18a	727.25 ± 224.32a
	Carotenoids (µg of pigment g FM^{-1})	213.47 ± 18.45a	218.77 ± 43.34a
	Quantity of cells of aquiferous parenchyma (nº)	68.3 ± 1.4b	102.0 ± 2.0a
		%	
Survival rate		100 %	100 %

they were smaller in comparison to those cultured at 28 °C. The result of greater dry mass was associated to an increase in the number of cell layers in plants cultured under lower temperatures during the dark period.

For the species *V. inflata* studied herein, plants cultured at different temperatures presented the same structures and possessed the same tissues. Plants from both treatments presented elongated cells, rectangular and sinuous cell wall in both abaxial and adaxial surfaces (figures 6 and 8). In cross-section, cells presented uniseriate epidermis on the abaxial and adaxial surfaces (figures 7 and 9). Below the adaxial surface cells, there were three layers of aquiferous parenchyma, followed by a spongy parenchyma with two more layers of aquiferous parenchyma (figures 7 and 9). The number of cells correlates to larger values of dry mass. The highest value of dry mass was observed for plants kept at 28 °C, which has 102 cells of aquiferous parenchyma, whereas plants cultured at 15 °C, the average value was 68 cells of this parenchyma (figures 7 and 9), correlating to the lower values of dry mass observed for plants of the latter treatment.

Therefore, an analysis of biometry associated to anatomy showed that *V. inflata* plants cultured at 15 °C displayed a reduction in growth in comparison to those maintained at 28 °C, mainly besause of the number of cells in leaves. Nevertheless, there was no significant alteration in chlorophylls and carotenoids content between the two temperature treatments (table 1), thus suggesting that plants kept at 15 °C assimilated the nitrogen into pigments at the same rate (µg of pigments g FM^{-1}) as those at 28 °C, indicating cold acclimatization. The plants of *Malva neglecta* Wallr. characterized in winter exhibited higher rates of photosynthesis than in the summer with no change in carotenoid to chlorophyll ratio (Verhoeven *et al.* 1999). Thus, the higher value of dry mass that occurred in plants kept at 28 °C might not be related to chlorophyll tissue but rather to an increase in the number of reserve tissue cells (aquiferous parenchyma), thereby resulting in a higher capacity to store water. According to Evert (2006), cells of this tissue are specialized in storing water. They are bulky, with a large vacuole and thin walls and generally lacking in chloroplasts.



Figures 6-9. Photomicrographs of leaves of *Vriesea inflata*. 6,8. Frontal view, abaxial surface. 7-9. Cross-sections. 6-7. Plants cultured at 15 °C. 8-9. Plants cultured at 28 °C (ap = aquiferous parenchyma; bs = abaxial surface; cr = crystals; ds = adaxial surface; sc = scales; sp = spongy parenchyma; st = stomata; uv = vascular unit). Bar = 50 µm (6, 8); 100 µm (7, 9).

In frontal view, anatomical analysis showed scamiform trichomes and tetracytic stomata (Evert 2006). In plants kept at 28 °C, stomata are more highly developed than in plants at 15 °C. At both treatments no abnormality were observed in stomata structure. This result shows that the *in vitro* condition did not induce alterations in stomata morphology, contrary to that observed in *Wrightia tomentosa* (Roxb.) Roem. & Schult. (Joshi *et al.* 2006). There was no difference in the amount of raphyde crystals in both treatments.

In spite of the smaller size of *V.inflata* plants kept at 15 °C, it was noted that the amount of leaves did not differ significantly, there being, therefore, no alteration in the general aspect of the plant (table 1). Pérez *et al.* (2001) noted that the drop in temperature from 25 °C to 12 °C in the culture of fescue gave rise to inhibition of growth as expressed in leaf area. Clarkson *et al.* (1986), when working with *Lolium perenne* L., noted that in plants cultured at temperatures lower than 25 °C, less roots were formed when compared to those raised at 25 °C. This result was related to the fact that when temperature increased, the number of roots also increased, thus contributing to greater nutrient absorption at higher temperatures, thereby intensifying plant growth. Therefore, in relation to *V. inflata* plants studied in the present work, no difference was identified in relation to the number of roots in plants cultured in both treatments, however, length, dry and fresh masses increased at plants at 28 °C. Probably, these roots are important for fixation of bigger-sized shoot epiphyte bromeliad. These results are in accordance with the epiphytic habit of this species in nature, whose main roots' function is fixation (Medina 1974).

Ex vitro acclimatized plants had 100% survival after three months (table 2), and no noticeable morphological abnormalities in tissue cultured plants were observed even after three months of growth under greenhouse condictions. Results of this study provide a practical method to enhance commercial production and germplasm conservation of this plant. The use of seeds in this work allows the maintenance of the genetic diversity observed in the natural populations, as observed with the bromeliads *Dyckia distachya* Hassl. micropropagated (Pompelli & Guerra 2005) and *Tillandsia eizii* L. B. Smith (Pickens *et al.* 2003), among others.

Interestingly, studies have shown that the transfer of plants cultured under *in vitro* conditions to growth in greenhouse, may be favored by the use of low temperatures, as commented by Campostrini & Otoni (1996). According to Tadesse *et al.* (2000), at lower temperatures (17 °C) there was an increase in leaf area in *Solanum tuberosum* L., which could be related to the higher production of structural carbohydrates related to the process of climate adjustment. In this sense, the use of low temperatures could induce a type of resistance in the plant necessary at the phase of climate adjustment to *ex vitro* conditions. Verhoeven *et al.* (1999) observed that in *Pinus ponderosa* Douglas ex Lawson & C. Lawson, the adjustment to a winter climate involved lower photosynthetic capacity and an increase in carotenoids rate in relation to that of chlorophylls and, on the other hand, *Malva neglecta*, presented higher photosynthesis rates in the winter than in the summer, and no alteration in the carotenoids/chlorophylls rate with the drop in temperature.

Table 2. Rate of survival and growth of *Vriesea inflata* (Wawra) Wawra plants (n = 40) maintained *in vitro* at 15 °C for two years and transferred to *in vitro* conditions at 28 °C (Treatment I) and plants, from one year *in vitro* at 28 °C, acclimatizated for three months (Treatment II). Means with the same letter within the same line are not significantly different at the 5% level.

	Treatment I	Treatment II
	%	
Survival rate after acclimatization	100%	100%
	$\bar{x} \pm s$	
Length of leaves (cm)	4.70 ± 0.46b	6.87 ± 0.31a
Quantity of leaves (nº)/plant	16.33 ± 3.21a	17.00 ± 0.81a

The present study was a source of information regarding *in vitro* growth of the bromeliad *V. inflata* at two temperatures. The establishment of *in vitro* culture starting from seeds harvested at natural environment (figures 1 and 2) contributes to maintaining the genetic variability of *V. inflata*. Results observed for plants kept at 15 °C (figure 3A) and 28 °C (figure 3B) showed reduced growth at 15 °C. It is concluded that this species can be maintained *in vitro* at this temperature, with the aim of forming a collection of slow-growth plants for preservation, and they can be acclimatized (figures 4 and 5). This knowledge contributes to the future mounting of a germplasm bank containing plants of the same species from different populations.

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References

AMOO, S.O., FINNIE, J.F. & STADEN, J.V. 2009. effects of temperature, photoperiod and culture vessel size on adventitious shoot production of *in vitro* propagated *Huernia hystrix*. Plant Cell Tissue Organ Culture 99:233-238.

BEKHEET, S.A., TAHA, H.S. & SAKER, M.M. 2002. *In vitro* long-term storage of date palm. Biologia Plantarum 45:121-124.

BELLINTANI, M.C., LIMA, C.C., BRITO, A.L., SANTANA, J.R.F. & DORNELLES, A.L.C. 2007. Estabelecimento *in vitro* de *Orthopytum mucugense* e *Neoregelia mucugensis*, bromélias endêmicas da Chapada Diamantina, Bahia – Brasil. Revista Brasileira de Biociências 5:1101-1103.

BIGOT, J. & BOUCAUD, J. 1996. Short-term responses of *Brassica rapa* plants to low root temperature: effects on nitrate uptake and its translocation to the shoot. Physiology Plantarum 96:646-654.

CAMPOSTRINI, E. & OTONI, W.C. 1996. Aclimatização de plantas: abordagens recentes. http://www.cnph.embrapa.br/laborato/biocel/abctp25.htm (acesso em 23/12/2009).

CARTER, G.A. & KNAPP, A.K. 2001. Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. American Journal of Botany 88:677-684.

CLARKSON, D.T., HOPPER, M.J. & JONES, L.H.P. 1986. The effect of root temperature on the uptake of nitrogen and the relative size of the root system in *Lolium perenne.* I. Solutions containing both NH_4^+ and NO_3^-. Plant, Cell and Environment 9:535-545.

DURAN, S. & MONTEIRO, K. 2005. Jardim de luxo sustenta tráfico de plantas. www.biodiversityreporting.org (acesso em 23/12/2009).

EVERT, F.R. 2006. Esau's plant anatomy. John Wiley & Sons, New Jersey.

FUNDAÇÃO BIODIVERSITAS. 2008. Lista da flora brasileira ameaçada de extinção. http://www.biodiversitas.org.br/florabr/lista_florabr.pdf (acesso em 23/12/2009).

GEORGIEVA, K. & LICHTENTHALER, H.K. 2006. Photosynthetic response of different pea cultivars to low and high temperature treatments. Photosynthetica 44:569-578.

GONÇALVES, S. & ROMANO, A. 2007. *In vitro* minimum growth for conservation of *Drosophyllum lusitanicum*. Biologia Plantarum 51:795-798.

HARTMANN, T.H., KESTER, D.E., DAVIES, F.T. & GENEVE, R.L. 2002. Hartmann & Kester's plant propagation: principles and practices. 7th ed., Prentice Hall, New Jersey.

ISLAM, M.D.T., DEMBELE, D.P. & KELLER, E.R.J. 2005. Influence of explant, temperature and different culture vessels on *in vitro* culture for germplasm maintenance of four mint accessions. Plant Cell, Tissue and Organ Culture 81:123-130.

JOHANSEN, D.A. 1940. Plant microtechnique. McGraw-Hill, New York.

JOSHI, P., JOSHI, N. & PUROHIT, S.D. 2006. Stomatal characteristics during micropropagation of *Wrightia tomentosa.* Biologia Plantarum 50:275-278.

JOUVE, L., FRANCK, T., GASPAR, T., CATTIVELLI, L. & HAUSMAN, J.F. 2000. Poplar acclimatization to cold during *in vitro* conservation at low non-freezing temperature: metabolic and proteic changes. Journal of Plant Physiology 157:117-123.

KLICH, M.G. 2000. Leaf variations in *Elaeagnus angustifolia* related to environmental heterogeneity. Environmental and Experimental Botany 44:171-183.

LICHTENTHALER, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *In* Methods in enzymology (L. Packer & R. Douce, eds.). Academic Press, London, v.148, p.350-382.

LUTHER, H.E. 2006. An alphabetical list of bromeliad binomials. 10th ed., The Bromeliad Society International, Sarasota.

MALIK, S., KUMAR, R., VATS, S.K., BRUSHAN, S., SHARMA, M. & AHUJA, P.S. 2009. Regeneration in *Rheum emodi* Wall.: a step towards conservation of an endangered medicinal plant species. Engineering in Life Sciences 9:130-134.

MEDINA, E. 1974. Dark CO_2 fixation, habitat preference and evolution within the Bromeliaceae. Evolution 28: 677-686.

MERCIER, H. & NIEVOLA, C.C. 2003. Obtenção de bromélias *in vitro* como estratégia de preservação. Vidália 1:57-62.

MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

NIEVOLA, C.C., KRAUS, J.E., FRESCHI, L., SOUZA, B.M. & MERCIER, H. 2005. Temperature determines the occurrence of CAM or C_3 photosynthesis in pineapple plantlets grown *in vitro.* In Vitro Cellular & Development Biology Plant 41:832-837.

NUNES, J.V.C. 2002. Bromélias. *In* Sustentável Mata Atlântica (L.L. Simões & C.F. Lino, eds.). Editora Senac, São Paulo, p.119-132.

PÉREZ, P., MORCUENDE, R., MOLINO, I.M., PUENTE, L.S. & MARTINEZ-CARRASCO, R. 2001. Contrasting responses of photosynthesis and carbon metabolism to low temperatures in tall fescue and clovers. Physiology Plantarum 112:478-486.

PICKENS, K.A., AFFOLTER J.M. & WETZSTEIN H.Y. 2003. Enhanced seed germination and seedling growth of *Tillandsia eizii in vitro.* Hortscience 38:101-104.

PIERIK, R.L.M. 1987. *In vitro* culture of higher plants. Martinus Nijhoff Pyblishers, Dordrecht.

POMPELLI, M.F. & GUERRA, M.P. 2005. Micropropagation enables the mass propagation and conservation of *Dyckia distachya* Hassler. Crop Breeding and Applied Biotechnology 5:117-124.

RECH-FILHO, A., DAL VESCO, L.L. & GUERRA, M.P. 2009. Adventitious shoots from nodule cluster cultures of *Vriesea reitzii*: an endemic and endangered bromeliad from atlantic forest. Ciência Rural 39:909-912.

ROCHA, C.F.D., COGLIATTI-CARVALHO, L., NUNES-FREITAS, A.F., ROCHA-PESSÔA, T.C., DIAS, A.S., ARIANI, C.V. & MORGADO, L.N. 2004. Conservando uma larga porção da diversidade biológica através da conservação de Bromeliaceae. Vidália 2: 52-68.

SARASAN, V., CRIPPS, R., RAMSAY, M.M., ATHERTON, M.M., PRENDERGAST, G. & ROWNTREE, J.K. 2006. Conservation *in vitro* of threatened plants: progress in the past decade. In Vitro Cellular & Development Biology Plant 42:206-214.

SILVA, A.L.L., DORNELLES, E.B., BISOGNIN, D.A., FRANCO, E.T.H. & HORBACH, M.A. 2007. Micropropagation of *Dyckia agudensis* Irgang & Sobral – an extinction threatened bromeliad. Iheringia 62:39-43.

SILVEIRA, D.G., SOUZA, F.V.D., PELACANI, C.R., SOUZA, A.S., LEDO, C.A. & SANTANA, J.R.F. 2009. Micropropagation and *in vitro* conservation of *Neoglaziovia variegata* (Arr. Cam.) Mex, a fiber producing bromeliad from Brazil. Brazilian Archives of Biology and Technology 52:923-932.

STRITTMATER, C.G.D. 1973. Nueva tecnica de diafanizacion. Boletín de la Sociedad Argentina de Botánica 15:126-129.

TADESSE, M., LOMMEN, W.J.M. & STRUIK, P.C. 2000. Effects of *in vitro* treatments on leaf area growth of potato transplants during acclimatization. Plant Cell, Tissue and Organ Culture 61:59-67.

VERHOEVEN, A.S., ADAMS III, W.W. & DEMMIG-ADAMS, B. 1999. The xanthophyll cycle and acclimatization of *Pinus ponderosa* and *Malva neglecta* to winter stress. Oecologia 18:277-287.

VON WIREN, N., GAZZARRINI, S. & FROMMER, W.B. 1997. Regulation of mineral nitrogen uptake in plants. Plant and Soil 196:191-199.