



Original Paper

Influence of test tube sealing on the morpho-anatomy and ultrastructure of leaves of *Aechmea bromeliifolia* (Bromeliaceae) grown *in vitro*¹

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Abstract

In vitro grown plants may have abnormal structural and physiological features. However, the type of the sealing material used in tissue culture may minimize such abnormalities. This study evaluates the influence of the type of sealing of test tubes on the anatomical and ultrastructural features of leaves of *Aechmea bromeliifolia* (Rudge) Baker (Bromeliaceae), an ornamental bromeliad native to Brazil, grown *in vitro*. Three types of sealing were used: rigid polypropylene cap (PC), polyvinyl chloride film (PVC), and PC coupled with a microporous membrane (PM). Seedlings germinated in a greenhouse were also studied for comparison. Plants grown in test tubes sealed with PM were more similar to those from the greenhouse, as far as the pattern of stomatal opening, the presence of starch grains, and the organization of the internal membrane system of the chloroplasts is concerned. Plants cultivated in test tubes sealed with PC had higher stomatal density and the chloroplasts had large areas without thylakoids in the stroma. Plants grown in test tubes sealed with PVC had few or no starch grains. These results suggest that microporous membrane used coupled with PC sealing provided natural ventilation, thus contributing to a better plant development.

Key words: chloroplast ultrastructure, *in vitro* environment, plant tissue culture.

Resumo

Plantas desenvolvidas *in vitro* podem apresentar características estruturais e fisiológicas pouco funcionais. Contudo, o tipo de vedação dos recipientes utilizados na cultura de tecidos pode minimizar tais características. Este estudo avaliou a influência do tipo de vedação dos tubos de ensaio sobre a anatomia e a ultraestrutura das folhas de *Aechmea bromeliifolia* (Rudge) Baker (Bromeliaceae), uma bromélia ornamental nativa do Brasil, desenvolvidas *in vitro*. Foram utilizados três tipos de vedação: tampa rígida de polipropileno (PC), filme de policloreto de vinila (PVC) e PC coberta com uma membrana microporosa (PM). Para efeitos de comparação, plantas provenientes de sementes germinadas em telado também foram estudadas. As plantas desenvolvidas em tubos vedados com PM se assemelharam mais às desenvolvidas em telado, quanto à abertura dos estômatos, a presença de grãos de amido e a organização do sistema interno de membranas dos cloroplastos. Nas plantas cultivadas em tubos vedados com PC, houve maior densidade estomática e os cloroplastos ficaram com grandes áreas sem tilacóides no estroma. Na condição PVC, pouco ou nenhum grão de amido foi encontrado. Esses resultados sugerem que a membrana microporosa, adicionada ao orifício da PC, proporcionou uma vedação com ventilação natural, contribuindo para o melhor desenvolvimento das plantas.

Palavras-chave: ambiente *in vitro*, cultura de tecidos vegetais, ultraestrutura dos cloroplastos.

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Introduction

In vitro culture techniques are widely used for the propagation of many plant species, resulting in rapid multiplication and pathogen-free plants that are genetically identical to the original ones (Chen 2004). The *in vitro* environment is characterized by dim light, high relative humidity, low CO₂ concentration during the photoperiod, high ethylene concentration, and restricted air movement inside the culture vessel (Kozai & Smith 1995). Such features affect the anatomy and physiology of *in vitro* grown plants, resulting in absent or reduced cuticle, dysfunctional stomata, and poorly developed photosynthetic apparatus. As a consequence, *in vitro* grown plants may be vulnerable when exposed to the *ex vitro* environment (Hazarika 2006).

Some materials used for sealing, such as screw caps, aluminum foils, transparent films (e.g., Parafilm®), and polypropylene caps, may restrict gas exchange between the culture vessel and the outer atmosphere (Zobayed *et al.* 2000; Zobayed 2008). The sealing material used for *in vitro* culture has significant influence on plant morpho-anatomy, thus affecting its growth (Gonçalves *et al.* 2008; Ribeiro *et al.* 2009). Nonetheless, only a few studies Majada *et al.* (2002), Lucchesini *et al.* (2006) and Sáez *et al.* (2012a) have evaluated the effect of the different types of sealing by using ventilated and non-ventilated vessels, on the ultrastructure of plants grown *in vitro*.

It is a common practice for culture vessels be kept completely sealed to avoid contamination by microorganisms (Kozai & Kubota 2005; Kozai 2010). However, in order to enhance gas exchange and to promote normal plant growth, the vessels must be ventilated (Zobayed *et al.* 2000). Sealing materials that are more permeable can reduce the relative humidity inside the culture vessel, enabling an increased transpiration, and a higher uptake of water and nutrients by the plants (Xiao *et al.* 2011).

Given that the *in vitro* culture conditions are closely related to plant growth and that the sealing material may improve plant development therefore facilitating the acclimatization phase, herein we evaluate the effect of the type of test tube sealing on the morpho-anatomy and ultrastructure of leaves of *Aechmea bromeliifolia* (Rudge) Baker (Bromeliaceae) seedlings. *A. bromeliifolia* is a bromeliad native to Brazil with a huge ornamental potential, which is distributed in Central America, Northwest of South America and from North

to South of Brazil. (Luiz-Santos & Wanderley 2012). The *in vitro* propagation of this species has currently been investigated by the Plant Tissue Culture Laboratory of the Federal University of Goiás (UFG) aiming at avoiding the exploitation of natural populations from the state of Goiás, Brazil. Therefore, in the present study, we compared the growth of plants obtained from seeds using three types of test tube seal with that of plants grown from seeds in a greenhouse.

Material and Methods

Plant material and cultivation conditions

Seeds of *A. bromeliifolia* were collected from an individual plant from the Bromeliads Collection of the Empresa de Assistência Técnica, Extensão Rural e Pesquisa Agropecuária do estado de Goiás (EMATER-GO), Brazil. The examined material was deposited in the herbarium of the Federal University of Goiás: BRAZIL. GOIÁS: Mossâmedes, Reserva Ecológica Professor José Ângelo Rizzo, 25.IV.2009, T.H.S. Sampaio and A.M. Teles 05 (UFG).

In vitro culture was initiated by placing the seeds in test tubes containing 20 mL half strength basal MS medium (Murashige & Skoog 1962) supplemented with 30 g L⁻¹ sucrose, pH 5.7 ± 0.1, and solidified with 7.0 g L⁻¹ agar. The total volume of the test tubes was 55 mL. The plants were kept in a growth room at 25 ± 1 °C with a photoperiod of 16 h, and photosynthetic photon flux density (PPFD) of 43.23 μmol m⁻² s⁻¹ provided by two cool-white fluorescent lamps. Plants grown *in vitro* were approximately 9 weeks old when collected for the analyses.

For greenhouse cultivation, seeds (n = 40) were sown in polyethylene terephthalate (PET) bottles as described by Paula & Silva (2004). The substrate consisted of a mixture of sand and soil (2:1) which was covered with Plantmax® (Aitkens, Glasgow, UK). The PPFD inside the greenhouse was of 350 μmol m⁻² s⁻¹ obtained from natural light and water was provided manually whenever needed. Plants grown *ex vitro* were approximately 10 weeks old when collected for the analyses and were about 1–2.5 cm high (aerial part).

Sealing of test tubes

The test tubes used for *in vitro* culture were sealed with three types of lids: rigid polypropylene cap (PC), single-layered polyvinyl chloride film

(PVC), and PC with 5 mm-diameter holes covered with a membrane composed of two layers of microporous tape (Cremer[®], São Paulo, SP, Brazil) and a single layer of polytetrafluoroethylene film (Amanco[®], Grupo Mexichem, São Paulo, SP, Brazil) (PM) (Saldanha *et al.* 2012). The PPF was measured inside the test tubes at the height of 6 cm, resulting in 42.34, 42.32, and 41.09 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the tubes sealed with PC, PVC, and PM, respectively. We used one seed per tube and 40 test tubes per treatment.

Morpho-anatomical and ultrastructural characterization

For anatomical analyses, the plants were collected at 8 am and the leaves were fixed in FAA (formaldehyde, acetic acid, and 50% ethanol; 1:1:18, by volume) for 48 h (Johansen 1940) and stored in 70% ethanol. The middle regions of the most expanded leaves were used for all the analyses. Leaf samples were dehydrated in an ethanolic series (Johansen 1940) and embedded in Leica[®] historesin (Leica Microsystems Nußloch GmbH, Heidelberg, Germany) following manufacturer's recommendations. Cross-sections (12- μm thick) of the samples were obtained using a rotary microtome and stained with toluidine blue (O'Brien *et al.* 1964), followed by a double stained with 0.1% basic fuchsin and 0.3% astra blue (1:3) (Kraus *et al.* 1998). Analysis of the surface view of the epidermis was performed using leaf samples that were cleared with a 10% sodium hydroxide solution for 30 min and then were immersed in 10% commercial sodium hypochlorite (2–2.5% active chlorine) until they were completely cleared and stained with 1% aqueous safranin solution (Johansen 1940). To confirm the presence of cuticle, Sudan III (Johansen 1940) was used in freehand cuts of fresh leaf samples.

The scanning electron microscope analysis and photography of fresh leaf samples were carried out using a JSM IT300LV scanning electron microscope (JEOL USA, Inc., Peabody, MA, United States) at the Centro Regional para o Desenvolvimento Tecnológico e Inovação, UFG.

For ultrastructural analysis, leaf fragments (1.0 mm^2) were fixed for 24 h in 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate buffer, pH 7.2, containing picric acid. Samples were washed in cacodylate buffer, post-fixed in 1% osmium tetroxide in the cacodylate buffer for 2 h. Subsequently, leaf fragments were dehydrated in acetone series and embedded in Epon resin

EMbed-812 (Electron Microscopy Sciences, Industry Road, Hatfield, PA, United States) (Cotta-Pereira *et al.* 1976). Ultra-thin transverse sections were stained with 2% uranyl acetate solution (20 min), washed in distilled water, immersed in 2% lead citrate solution (6 min), and washed in double distilled water. Samples were analyzed using a JEM 2100 transmission electron microscope (JEOL USA, Inc., Peabody, MA, United States), equipped with an Energy Dispersive X-ray Spectrometer (EDS) (Thermo Fisher Scientific Inc., Waltham, MA, United States). The area of the chloroplasts was measured using the ImageJ software (National Institute of Health, Bethesda, MD, United States). For this analysis, the samples of all treatments were collected at the same time of the day (2 pm).

The anatomical analyses were carried out using five repetitions for each treatment. For cuticle and ultrastructural analysis, three repetitions of each treatment were used. The stomata index (SI) was calculated according to the formula of Salisbury (1927), $\text{SI} (\%) = [S/(S+E)] \times 100$, where S is the number of stomata per unit area and E the number of epidermal cells at the same unit area, these two parameters were counted in 10 field of 0.25- mm^2 per leaf.

For the height of the aerial part and number of leaves, 12 plants of each treatment were used.

Statistical analysis

The experimental design was completely randomized, and the data were submitted to analysis of variance (ANOVA) by using STATISTICA version 7.0 (StatSoft Inc., Tulsa, OK, United States), where significant main effects were obtained and compared by performing the Tukey's test ($\alpha = 0.05$).

Results

The *in vitro* grown plants had longer aerial parts and a larger number of leaves compared to those cultivated in the greenhouse (Tab. 1). However, the leaves of plants cultivated *in vitro* were membranaceous, whereas the leaves of those grown in the greenhouse became thicker and rigid. Plants grown in tubes sealed with PC and PM showed similar heights (Tab. 1).

The same basic anatomical structure was found in all analyzed leaves: multicellular and uniseriate glandular trichomes (Fig. 1a) at the margin of the leaf (Fig. 1b), one-layered epidermis with thin cuticle; tetracytic stomata found on

Table 1 – Leaf parameters of *A. bromeliifolia* grown in greenhouse or under *in vitro* conditions using three different test tube seals (PC = rigid polypropylene cap; PVC = polyvinyl chloride film; PM = PC covered with a microporous membrane).

Parameter	Greenhouse	<i>In vitro</i>		
		PC	PVC	PM
Length of the aerial part [cm]	1,67 ± 2.61 c	6,22 ± 2.10 a	4.67 ± 1.00 b	7.70 ± 1.39 a
Number of leaves	4.91 ± 0.51 b	7.16 ± 1.26 a	6.66 ± 1.72 a	6.50 ± 1.00 a
Leaf thickness [μm]	1260.43 ± 133.85 a	772.42 ± 129.94 b	710.73 ± 135.33 b	814.46 ± 63.59 b
Mesophyll thickness [μm]	1130.23 ± 129.49 a	679.41 ± 126.06 b	612.72 ± 127.25 b	722.54 ± 64.17 b
Aquifer parenchyma thickness [μm]	464.52 ± 55.96 a	249.57 ± 52.62 b	199.39 ± 52.67 b	228.60 ± 55.81 b
Chlorenchyma thickness [μm]	669.84 ± 114 a	431.57 ± 93.53 b	416.09 ± 91.66 b	493.22 ± 35.39 b
Upper epidermis thickness [μm]	87.51 ± 9.86 a	57.39 ± 5.63 b	62.70 ± 7.23 b	59.12 ± 9.64 b
Lower epidermis thickness [μm]	53.90 ± 3.89 a	42.57 ± 6.77 b	41.84 ± 6.32 b	43.72 ± 6.37 ab
Polar diameter (PD) [μm]	62.55 ± 7.07 a	65.05 ± 6.39 a	66.01 ± 2.65 a	65.19 ± 3.12 a
Equatorial diameter (ED) [μm]	57.06 ± 3.92 a	50.79 ± 2.62 ab	52.58 ± 8.14 ab	46.81 ± 3.05 b
PD/ED [μm]	1.10 ± 0.10 b	1.28 ± 0.09 ab	1.28 ± 0.17 ab	1.40 ± 0.05 a
Stomatal density [mm ²]	49.36 ± 6.93 bc	81.04 ± 14.40 a	52.96 ± 5.30 b	32.88 ± 7.62 c
Stomatal index [%]	10.41 ± 1.17 ac	10.45 ± 1.61 a	8.06 ± 0.79 bc	7.16 ± 1.47 b
Chloroplast area [μm ²]	12.29 ± 0.43 a	10.56 ± 1.42 a	12.98 ± 3.34 a	14.95 ± 0.90 a

Means ± SDs, *n* = 5 (for the height of the aerial part and number of leaves: *n* = 12). Different letters between lines indicate that means are significantly different (*p* ≤ 0.05) using the Tukey's test.

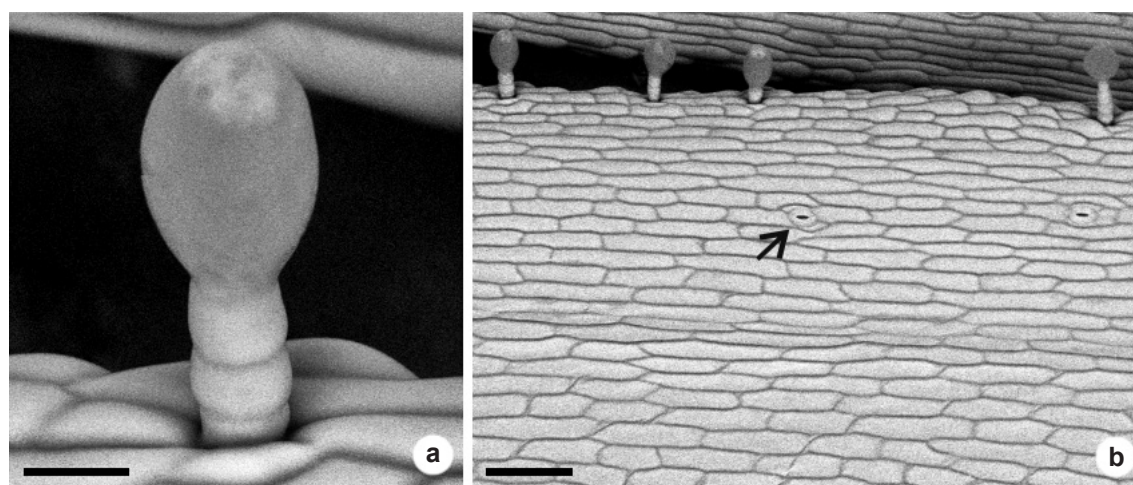


Figure 1 – a-b. Scanning electron micrographs of the adaxial leaf surface of *A. bromeliifolia* grown in test tubes sealed with rigid polypropylene cap covered with microporous membrane – a. detail of a multicellular uniseriate glandular trichome; b. glandular trichomes on the leaf margin and stomata restricted to the leaf margin (arrow). Scale bars: a = 20 nm; b = 100 nm.

the abaxial surface and on the margins of the adaxial surface (Fig. 1b) at the same level of other epidermal cells; heterogeneous mesophyll with aquifer parenchyma and chlorenchyma (Fig. 2a,b); collateral vascular bundles with fiber poles associated with the xylem and phloem (Fig. 2c-f). However, some differences were noticed on the aperture of stomata ostioles. Plants developed in the greenhouse had completely closed stomata (Fig. 2g), while plants grown *in vitro* had either open stomata in tubes sealed with PC and PVC (Fig. 2h,i), and completely closed in tubes sealed with PM sometimes with very reduced aperture (Fig. 2j).

The total thickness of the leaf, mesophyll, aquifer parenchyma, and chlorenchyma, as well as the height of the epidermal cells on the adaxial surface were significantly higher in plants developed in the greenhouse when compared to the *in vitro* grown plants, which did not differ statistically (Tab. 1). In addition, the epidermal cells on the abaxial surface were not significantly different in the leaves of plants developed in the greenhouse, and those grown in the tubes sealed with PM when compared to plants cultivated in the tubes sealed with PC and PVC (Tab. 1). Overall, the vascular bundles located at the leaves edges had more fibers associated with the xylem under all culture conditions (Fig. 2c-f). However, in the leaves of the plants grown in tubes sealed with PVC, the walls of these fibers were visually thinner (Fig. 2e).

The polar diameter of the stomata did not differ significantly under any culture conditions (Tab. 1). Nevertheless, the equatorial diameter was smaller in plants developed in tubes sealed with PM compared to those grown in the greenhouse. Consequently, the ratio between the polar diameter and the equatorial diameter was greater in the plants cultivated in tubes sealed with PM, whereas no significant differences were found for this parameter in the plants *in vitro*. The stomata of the plants grown under all treatments were elliptical (Tab. 1).

The stomatal density was significantly higher in leaves of plants grown in tubes sealed with PC compared to those obtained under all culture conditions. However, a stomatal index of leaves of plants grown in tubes sealed with PC was not significantly different to plants grown in greenhouse (Tab. 1).

The chlorenchyma cells of plants obtained in all treatments had typical organelles (Fig. 3a-f). We observed large central vacuoles, with other

organelles adjacent to the cell walls (Fig. 3a). Mitochondria had well-developed crests (Fig. 3b-f), and were found associated to chloroplasts. The nuclei had conspicuous nucleoli and a similar shape in the cross-section of the leaf (Fig. 3b,c,e,f). Chloroplasts did not differ significantly in area among the treatments (Tab. 1). However, the shape of the chloroplasts, the organization of the internal membrane system, the number of starch grains, and the lipid bodies were different among the treatments.

The chloroplasts of plants grown in the greenhouse were oval, and had a less appressed arrangement of thylakoid membranes and a high amount of starch grains and plastoglobules, both large-sized (Fig. 4a). The leaves of plants developed *in vitro* had homogeneously small plastoglobules (Fig. 4b-d) and a more appressed arrangement of thylakoid membranes when compared to the plants grown in the greenhouse (Fig. 4a-d). In plants grown in tubes sealed with PC, the chloroplasts were more elongated, and had large areas without thylakoids filled with the stroma (Fig. 4b). In plants developed in tubes sealed with PVC, the chloroplasts were semicircular, and the internal membrane system was irregularly arranged in some regions (Fig. 4c). Fewer chloroplasts contained starch grains in plants developed in tubes sealed with PVC and PC and, when present, they were smaller and less numerous (Figs. 3e; 4c). In tubes sealed with PM, the chloroplasts ranged from semicircular to oval, had large starch grains (Fig. 4d) and, in some regions, a less appressed arrangement of thylakoid membranes.

Discussion

In vitro culture influenced the morphology of leaves of *A. bromeliifolia*, which were more elongated, slender and delicate when compared to those grown under greenhouse conditions. This result corroborates those previously reported (Johansson *et al.* 1992; Aoyama *et al.* 2012). In response to *in vitro* environment, plant physiology and morphology are significantly different from those of the plants grown in natural environments (Kozai 2010). The thicker leaves developed in the greenhouse indicate increased cell expansion, since no significant difference was observed in the number of cell layers in the leaves of all analyzed plants. This result is probably related to the intensity of light inside the greenhouse. In fact, several studies have reported thickening of leaves due to cell expansion under higher light

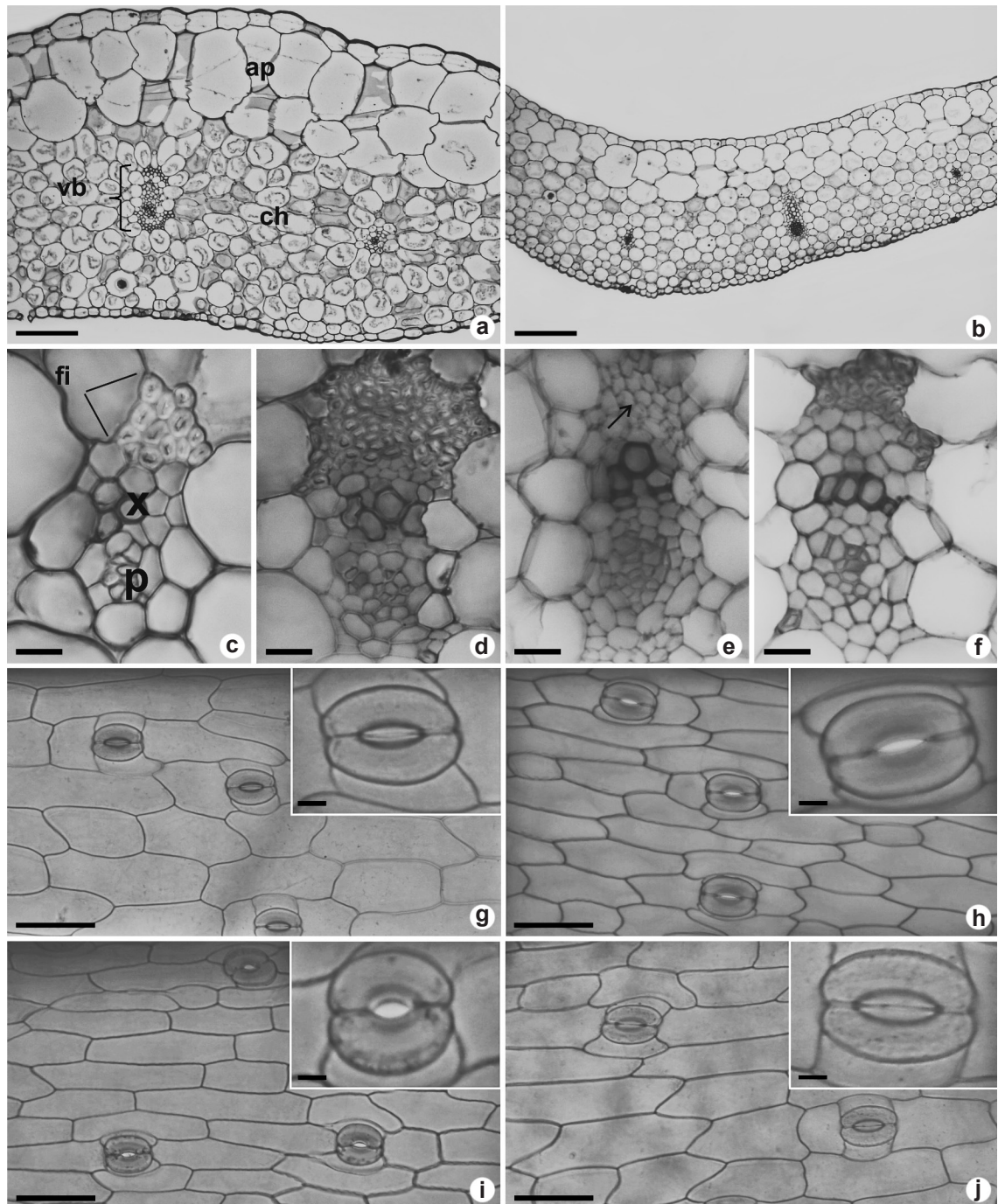


Figure 2 – a-j. Light microscopy of the cross-sections (a-f) and abaxial surface (g-j) of the middle region (a,b,g-j) and margin (c-f) of *A. bromeliifolia* leaves – a,c,g. leaves of plants developed in greenhouse; b,d,h. leaves of plants grown in test tubes sealed with rigid polypropylene cap; e,i. leaves of plants developed in test tubes sealed with polyvinyl chloride film; f,j. leaves developed in test tubes sealed with rigid polypropylene cap covered with a microporous membrane – a. anticlinally elongated cells of the aquiifer parenchyma; b. isodiametric cells of the aquiifer parenchyma; c,d,f. vascular bundles with thick wall fibers; e. vascular bundles evidencing fibers with thin walls (arrow); g,j. stomata with closed ostiole; h,i. stomata with open ostiole. (ap = aquiifer parenchyma; vb = vascular bundle; ch = chlorenchyma; fi = fibers; x = xylem; p = phloem). Scale bars: a,b = 250 μm ; c-f = 50 μm ; g-j = 100 μm (detail of the stomata = 25 μm).

intensity (Voltan *et al.* 1992; Hanba *et al.* 2002; Lima Jr. *et al.* 2006; Fernandes *et al.* 2014), with improved photosynthetic efficiency (Kim *et al.* 2005). According to Hazarika (2006), the delicate structure of plants developed *in vitro* makes them more vulnerable when exposed to *ex vitro* conditions.

The leaves of *A. bromeliifolia* were anatomically similar under all culture conditions,

and they are also similar to that reported for leaves of other species of *Aechmea*, grown under natural conditions (Proença & Sajo 2004; Souza *et al.* 2005; Silva & Scatena 2011). Nonetheless, we observed that some characteristics in adult plants of *A. bromeliifolia* are different from those of young plants. The leaves of the adult plants are hypostomatic, the epidermis has peltate trichomes, the mesophyll has aeration channels continuous

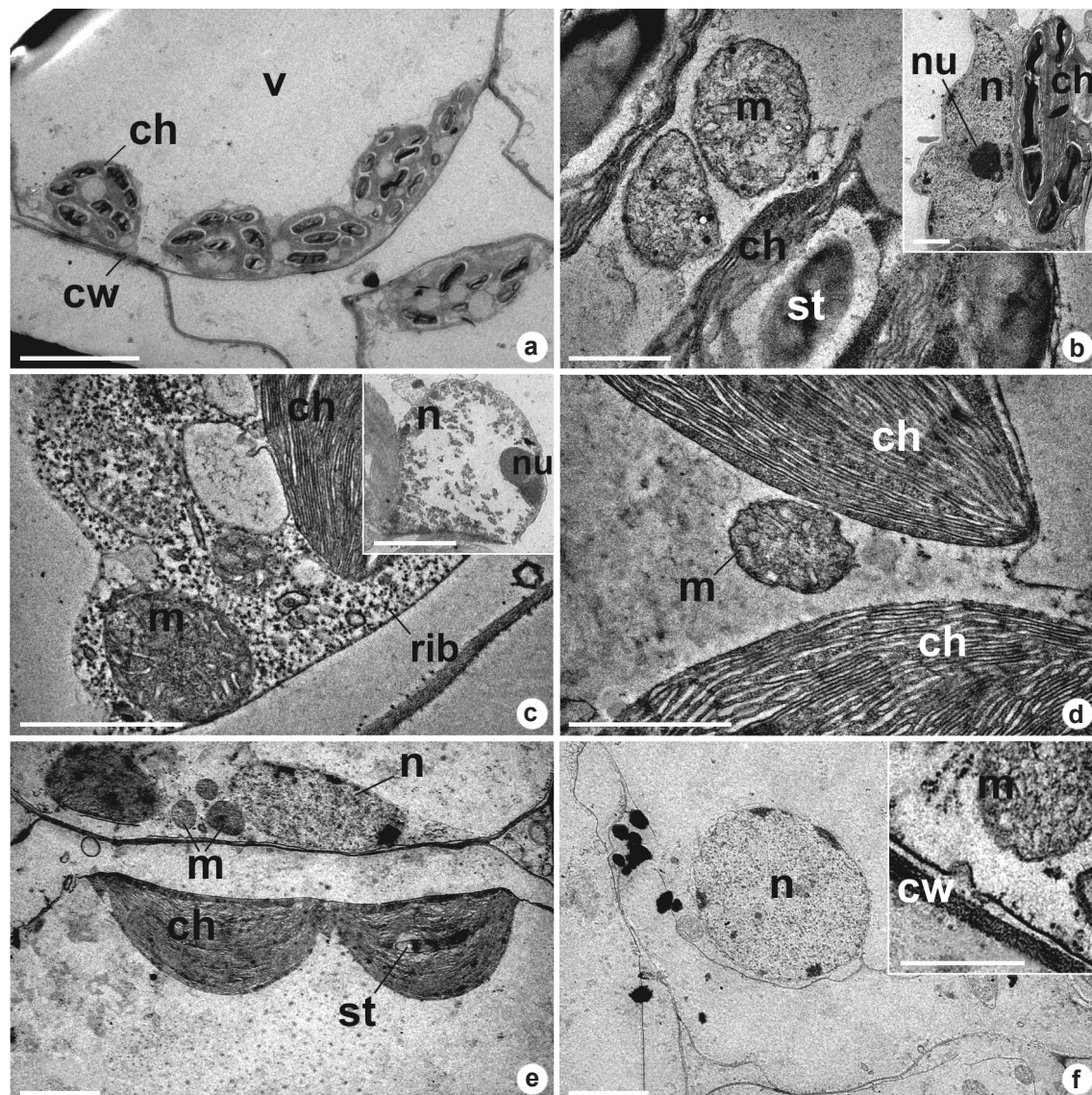


Figure 3 – a-f. Transmission electron micrographs showing cross-sections of the chlorenchyma of *A. bromeliifolia* leaves, evidencing typical structure and organelles of plant cells – a,b. plants grown in greenhouse; c. plants grown in test tubes sealed with rigid polypropylene cap; d,e. plants grown in test tubes sealed with polyvinyl chloride film; f. plants grown in test tubes sealed with rigid polypropylene cap with microporous membrane. (v = vacuole; ch = chloroplast; cw = cell wall; m = mitochondrion; st = starch grain; n = nucleus; nu = nucleolus; rib = ribosome). Scale bars: a = 5 μm ; b = 0.5 μm (detail = 1 μm); c,d = 1 μm ; e,f = 2 μm (detail = 0,5 μm)..

to the substomatic chambers with bractiform cells, and groups of extravascular fibers dispersed throughout the chlorenchyma (Proença & Sajo 2004). In addition, Proença & Sajo (2004) do not report the occurrence of glandular trichomes.

Despite the anatomical similarities, some characteristics differed between the leaves of *A. bromeliifolia* cultivated *in vitro*. The stomata of leaves of *A. bromeliifolia* grown both in a greenhouse and in tubes sealed with PM were closed by the time of collection (daytime period). Considering that *A. bromeliifolia* is a crassulacean acid metabolism (CAM) species (Griffiths & Smith 1983; Scarano *et al.* 2002), it is expected that stomata remain closed during the day under natural conditions. In contrast, the leaves of plants grown in tubes sealed with PC and PVC had opened stomata by the time of collection. The

microporous membrane used in the PM sealing type allows greater amount of gas exchange per hour than other sealing types without membrane (Saldanha *et al.* 2012), and this probably favors the proper functioning of *A. bromeliifolia* stomata.

The leaves of *A. bromeliifolia* grown *in vitro* had elliptical stomata, similar to what was observed in leaves grown in a greenhouse. However, this result is not common, since the stomata of plants grown *in vitro* are usually rounded, which is often associated with low functionality (Khan *et al.* 2003; Afreen 2005), and incapacity to fully close when stimulated (Khan *et al.* 1999). The elliptical stomata found in *A. bromeliifolia* are believed to have good functionality since they are characteristically found in plants grown under photoautotrophic conditions (Khan *et al.* 2003).

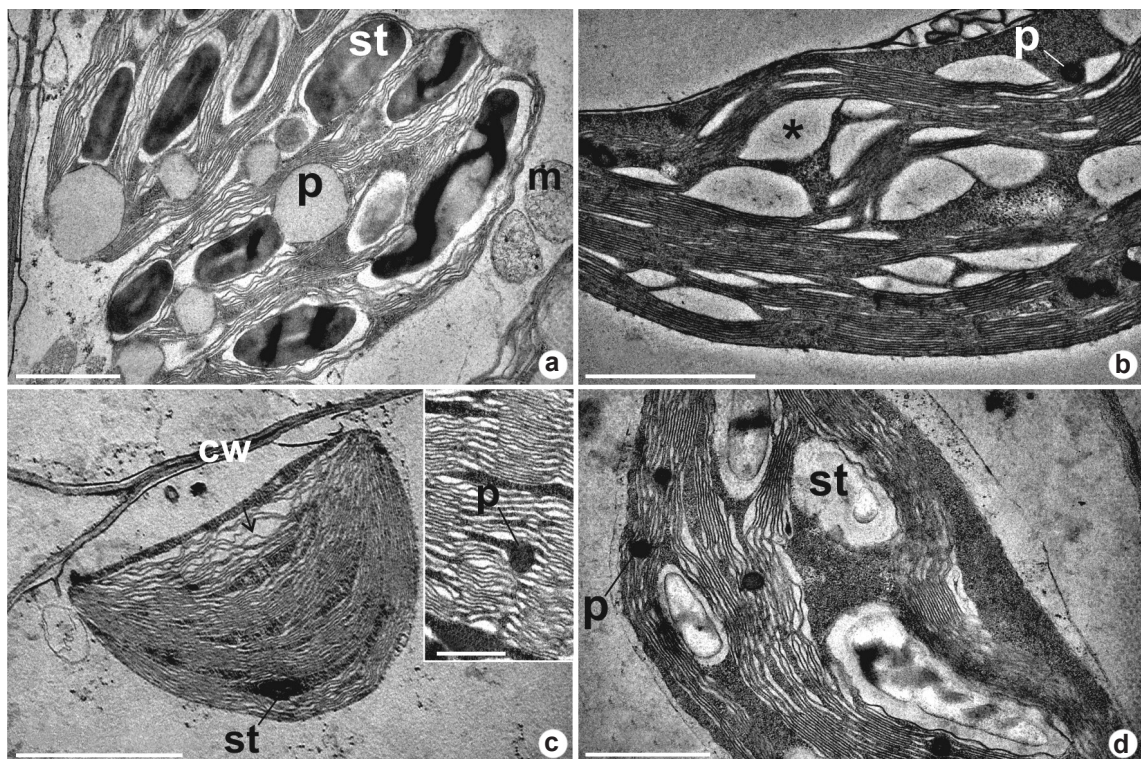


Figure 4 – a-d. Transmission electron micrographs showing cross-sections of the chlorenchyma of *A. bromeliifolia* leaves, evidencing the chloroplasts – a. plants grown in greenhouse, chloroplasts with a large number and size of starch grains and lipid bodies; b. plants grown in test tubes sealed with rigid polypropylene cap, evidencing areas in the stroma without thylakoids (*); c. plants developed in test tubes sealed with polyvinyl chloride film, chloroplast with internal membrane system arranged irregularly in some regions (arrow), detail of appressed thylakoids; d. plants grown in test tubes sealed with rigid polypropylene cap with microporous membrane, evidencing chloroplasts with a large number of starch grains and internal membrane system less appressed in some regions. (st = starch grain; p = plastoglobuli; m = mitochondrion; cw = cell wall). Scale bars: a,b,d = 1 μ m; c = 2 μ m.

Plants developed in tubes sealed with PC had leaves with higher stomatal density. However, stomatal index of leaves from this condition was not significantly different from that observed in greenhouse leaves. This result suggests that the increase of stomatal density occurred due to a decrease on the expansion of ordinary epidermal cells. Greater stomatal density has been reported to occur due to high relative humidity in tubes with less ventilation (Zobayed *et al.* 2001; Mohamed & Alsadon 2010). Thus, the high number of stomata in plants grown in hermetically sealed tubes may be a strategy to enhance leaf transpiration, thus leading to higher uptake of water and nutrients.

The fibers associated with the vascular bundles in leaves of plants grown in tubes sealed with PVC were not very thick and this may provide less mechanical support for the plants during the process of acclimatization.

The *in vitro* environment affected the organization of chloroplasts of *A. bromeliifolia*, different from other organelles that were similar to those of plants grown in the greenhouse, as reported by Rodrigues *et al.* (2014). The most frequent changes in the ultrastructure of leaf cells of plants cultivated *in vitro* are observed in the chloroplasts, which vary in shape, size, organization of the internal membrane system and number of starch grains (Wetzstein & Sommer 1982; Lee *et al.* 1985; Majada *et al.* 2002; Sáez *et al.* 2012b; Kapchina-Toteva *et al.* 2014; Stefanova *et al.* 2015).

Little appressed thylakoid membranes are typical of chloroplasts exposed to high light intensity (Yano & Terashima 2001; Fernandes *et al.* 2014). The less appressed arrangement of thylakoid membranes in the chloroplasts of plants grown in the greenhouse may be associated with the formation of large starch grains which are due to high light exposure. Similarly, in the chloroplasts of *Liquidambar styraciflua* L. exposed to high light intensity, grana were poorly organized as a result of the presence of starch (Lee *et al.* 1985).

The chloroplasts of *A. bromeliifolia* grown in tubes sealed with PC have large areas without thylakoids and the chloroplast development in tubes sealed with PVC have irregularly arranged inner membranes. The abnormal development of chloroplasts has been reported in plants grown in non-ventilated vessels (Majada *et al.* 2002). Several authors have reported changes in the ultrastructure of chloroplasts in response to ethylene (Toyama 1980; Fukuda & Toyama 1982; Fan *et al.* 2013), which is a volatile plant hormone that accumulates

due to lack of ventilation. In addition, chloroplasts of *A. bromeliifolia* grown *in vitro* have small and few plastoglobules. According to Sáez *et al.* (2012b) these features are associated to the lower oxidative stress in the *in vitro* environment when compared to the greenhouse conditions.

In conclusion, the analysis showed that the *in vitro* environment influences the texture and thickness of the leaves, the functionality of stomata, and the ultrastructure of chloroplasts. However, plants grown in tubes sealed with PM were similar to those developed in the greenhouse, especially considering stomata opening, occurrence of starch grains and organization of the internal membrane system of chloroplasts.

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