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Structural analysis of the foliar epidermis during acclimatization of *Aechmea blanchetiana* (Bromeliaceae) *in vitro* cultured

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ABSTRACT

The aim of this experiment was to study the anatomical alterations in the foliar epidermis of micropropagated ornamental bromeliad (*Aechmea blanchetiana*), during the acclimatization period. Structural characteristics of the abaxial epidermis of leaves of seedlings *in vitro* cultured were analyzed under photonic microscopy. The analysis was undertaken in plants at 0, 7, 28, 49, 70 and 91 days of acclimatization. Usual techniques were employed to prepare the material to obtain the semi-permanent laminae of the paradermal sections. The stomatal density was determined in the leaf medium zone. The studied parameters showed significant anatomical alterations on the first weeks of acclimatization, when the epidermal cell wall thickness, the stomata density and scale area and density showed a considerable decrease compared to the *in vitro* plantlets. After 49 days the leaves presented characteristics of an adult plant, which probably indicates the end of the acclimatization period.

Keywords: *Aechmea blanchetiana*, anatomy, micropropagation, Bromeliaceae.

RESUMO

Análise da epiderme foliar durante a aclimatização de bromélia micropropagada

O objetivo do trabalho foi estudar as alterações anatômicas na epiderme de folhas de bromélia ornamental (*Aechmea blanchetiana*) micropropagadas, durante o período de aclimatização, no qual ocorre grande perda de plantas, e o início da produção comercial em estufa. Realizou-se a análise, em microscopia fotônica, das características estruturais da epiderme abaxial de folhas da espécie, provenientes de mudas cultivadas *in vitro*, nas plantas aos 0, 7, 28, 49, 70 e 91 dias de aclimatização. Foram utilizadas as técnicas usuais no preparo do material para obtenção das lâminas semipermanentes de secções paradérmicas. A densidade estomática foi determinada na região mediana da folha. Os parâmetros estudados evidenciaram alterações anatômicas significativas nas primeiras semanas, sendo que a espessura das paredes celulares epidérmicas, a densidade dos estômatos, e a área e densidade das escamas apresentaram diminuição considerável nas primeiras semanas de aclimatização. A partir dos 49 dias, as folhas apresentaram características da planta adulta, possivelmente indicando o término do período de aclimatização.

Palavras-chave: *Aechmea blanchetiana*, anatomia, micropropagação, Bromeliaceae.

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Tissue culture is a technique for propagation of plants, in which plant part/tissue is inoculated into nutrient medium favoring shoot and root production, which are acclimatized and transferred to soil. Quality seedlings of economically important species can be produced on large scale or in the quantity required, using this technique (Bhoite & Palshikar, 2014).

Micropropagated plants require acclimatization period (Jeon *et al.*, 2005); however, the acclimatization phase, when plants are transferred to conditions external to the laboratory (*ex vitro*) may be limiting for some species (Silveira *et al.*, 2013). For *in vitro* culture, plants grow in special conditions, with reduction of gas exchange, high relative humidity, low light intensity and utilization of existing carbohydrate in the culture medium as a source of energy. These factors may cause inhibition of photosynthesis, presence of abnormal stomata, greater accumulation of reserves or biomass, thin cuticle, fragile roots, making it difficult to acclimatize and causing high losses when transferring plants to *ex vitro* conditions (Sciutti & Morini, 1993; Pospíšilová *et al.*, 1999).

Under *ex vitro* conditions, micropropagated plants are exposed to natural environment, with higher or lower temperatures, depending on the season, shorter photoperiod in relation to *in vitro* culture, water deficit, lower concentrations of CO_2 and nutrients, which increases the necessity of roots adapted to the new condition (Maciel *et al.*, 2002; Hazarika, 2003). Plants at the acclimatization stage need adaptation to the new environment with functional leaves and roots, which were modified by *in vitro* development (Dewir *et al.*,

2005).

Anatomical changes which occur during acclimatization are variations on the quantities of stomata, abundance and diameter of scales and the quantities of chloroplasts present in new leaves (Pospíšilová *et al.,* 1998). Lima Junior *et al.* (2006), studying micropropagated seedlings of *Cupania vernalis,* observed that, at different levels of irradiance, favorable anatomical changes to better seedling development occurr under *ex vitro* conditions. Barboza *et al.* (2006) observed variations in stomatal frequency, shape and sinuosity of cell walls, cuticle thickness, among others, in plants of *Ananas comosus* (pineapple), retained *in vitro* and at acclimatization, attributing these variations to phenotypic plasticity of the species.

In literature, few studies on micropropagation and acclimatization of plants of Bromeliaceae family are found. However, due to its use in fruticulture (Barboza *et al.*, 2006), its ornamental potential (Rodrigues *et al.*, 2004), medicinal (Rech Filho, 2004) and ecological importance, the *in vitro* multiplication becomes necessary. The genus *Aechmea* with 250 species is the largest genus in subfamily Bromelioideae (Luther, 2008). *Aechmea blanchetiana* is a herbaceous, perennial, rhizomatous, robust species with 60-90 cm long, may present an epiphyte or terrestrial habit and has been widely used in landscaping (Lorenzi & Mello-Filho, 2001).

This work aimed to test the hypothesis that adaptive changes in *A. blanchetiana* leaf epidermis, grown *in vitro,* occur during acclimatization.

MATERIAL AND METHODS

Plants of *A. blanchetiana* were obtained through *in vitro* seed germination in MS medium (Murashige & Skoog, 1962), supplemented with 30.0 g/L sucrose, 6.0 g/L agar and pH adjusted to 5.8, inside a growth room under irradiance of 16.2 μmol/m²/s and in 12-hour photoperiod at 24-28°C.

The plants, 30 individuals with 60 days of *in vitro* culture and 3.0 cm in length, were removed from the flasks, washed with deionized water and transplanted into polystyrene trays, containing Pine bark decomposed as substrate. The plants were grown for 91 days in a greenhouse with transparent polyethylene plastic cover under irradiation of 170 μmol/m²/s, daily average temperature of 27.5ºC, microsprinkler irrigation system (NaanDanJain® Modular flow 141 L/H Microsprinkler) in two daily irrigation regimes of 30 minutes.

Comparative analysis of leaf structure was carried out by randomly collecting four plants on periods of 0, 7, 28, 49, 70 and 91 days of acclimatization and leaf blade fragments of adult individuals (approximately five years) grown under environmental conditions of plant acclimatization.

The whole plants were fixed in FGAA (Lersten & Curtis, 1988) for 48 hours and stored in alcohol 70%. For the analyzes of epidermis, the middle region fragments, selected from external totally expanded leaves were diaphanized according to Franklin (1945), modified by Berlyn & Miksche (1976), using hydrogen peroxide solution 30 volumes and glacial acetic acid (1:1). The material remained in this solution for 24 hours and, subsequently, washed in distilled water and subjected to staining with astra blue and safranin 1% (Bukatsch, 1972). For each leaf, two semipermanent laminae were mounted in glycerin 50%, a total of 24 laminae per treatment, including the adult plant.

In paradermic sections, stomata and scale densities, thickness of the anticlinal walls of epidermal cells and first leaf area expansion from the periphery to the center of the tank of *A. blanchetiana* were evaluated. Stomata and scale densities were determined by counting in an area of 500x500 μm, projected on a flat surface with the aid of a Zeiss microscope with projection system. Sixty fields were evaluated in order to determine the stomata density and thirty fields for the determination of the scale density.

The thickness of epidermal cell walls and scale area were obtained with an image-capturing system and UTHSCSA ImageTool Version 3.0 software, using the micrometric scales provided by Image Plus software as parameters.

The experiment was carried out on a randomized complete block design, with six treatments (collections at 0, 7, 28, 49, 70 and 91 days) and five plots; considering that for stomata density 60 replications were performed, for trichomes density, 30 replications and for scale area and wall thickness, 15 replications. The data were subjected to variance and regression analysis based on polynomial model, applying the test F. Acclimatization period function was adjusted to polynomial equation using as criteria for model selection, significant effect by F test at 5% probability and correlation coefficients using SANEST statistical software.

RESULTS AND DISCUSSION

During acclimatization period, no death of plants was observed, probably due to the fact that *Aechmea blanchetiana* is considered to be robust, fibrous, perennial, tolerant to direct sunlight exposure, easy handling and also easy to be cultivated (Kanashiro *et al.*, 2007). This plant can be found in nature both in epiphytic life-form in a shaded environment or terrestrial form in full sun (Gilman & Robert, 1999). *A. blanchetiana* adult plants (first expanded leaf from the periphery toward the interior of the tank) showed an average of 10.6 stomata/mm², scale area of 23,123.5 μm², an average of 0.70 scales/mm², anticlines walls of epidermal cells thickness of 9.2 μm.

The leaf is hypostomatic, this characteristic being common to bromeliads, mostly for genus *Aechmea* (Proença & Sajo, 2004). On the abaxial surface of leaves, multiseriate hairs and scale-type trichomes were observed, on different developmental stages (Figures 1A to 1F), whereas in the analysis of adult plant only scales at the final development stage were observed (Figure 1B). The analysis of multiseriate hairs showed that they are the precursors of peltate scales, which explains their absence on adult plants.

Scales weren't found in plants harvested at 7 and 28 days, once the authors considered scales those which

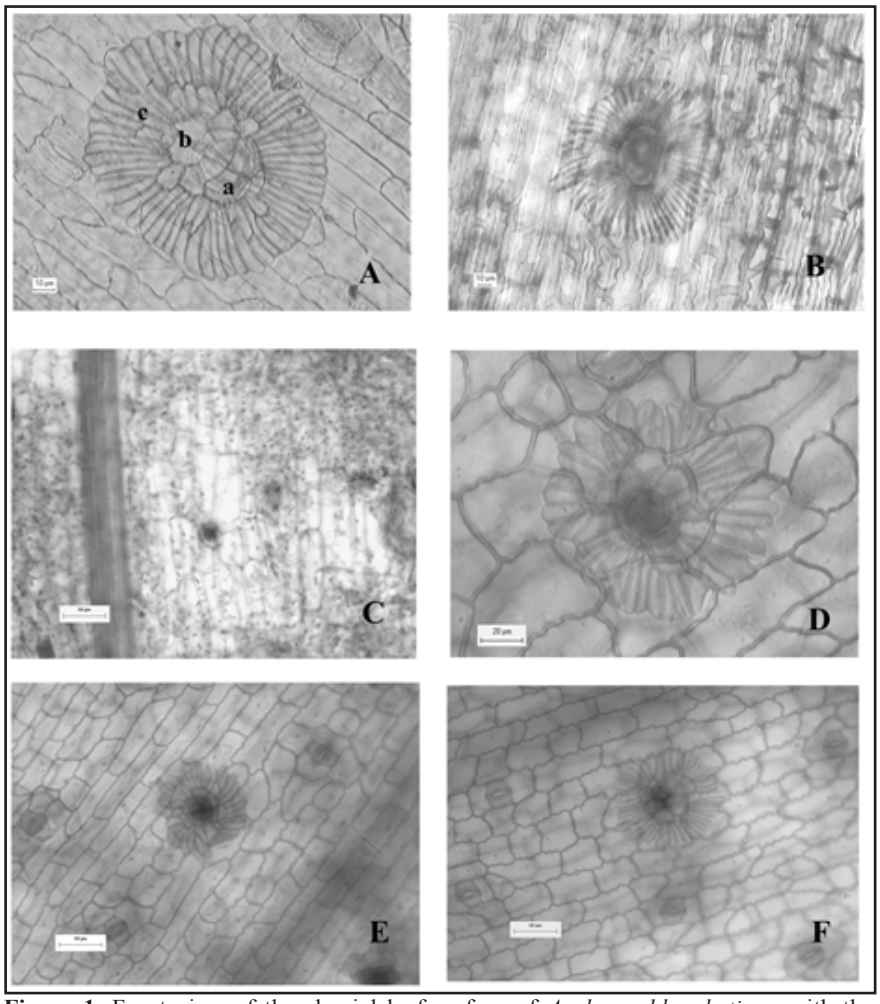

Figure 1. Front view of the abaxial leaf surface of *Aechmea blanchetiana* with the epidermal cells and trichomes displayed; A: plant sampled after removal of the culture medium (time zero). The scales with shield are formed by four or more central cells that fit in the circle (a), the central cells are surrounded by cells with irregular morphology (b), Externally to these cells are peripheral cells with thin walls and radial disposition (c); B: adult plant; C: precursor fell of plant scale in plants acclimatized during 7 days; D: plant acclimatized during 49 days and, plant acclimatized during 70 days; F: plant acclimatized during 91 days {vista frontal da superfície abaxial da folha de *Aechmea blanchetiana*, onde são visualizadas células epidérmicas comuns e tricomas do tipo escamas; A: planta coletada logo após a retirada do meio de cultura (tempo zero). As escamas com escudo são formadas por quatro ou mais células centrais que se encaixam no formato de círculo (a). As células centrais são circundadas por células de morfologia irregular (b). Externamente a estas células, estão as células periféricas com paredes delgadas e disposição radial (c); B: planta adulta; C: pelo precursor da escama em planta aclimatizada durante 7 dias; D: planta aclimatizada durante 49 dias; E: planta aclimatizada durante 70 dias; F: planta aclimatizada durante 91 dias}. São Paulo, IBt, 2009.

presented developed peripheral cells. Barboza *et al.* (2006) observed uniserate hair, in plants of *Aechmea nudicaulis*, in the first and second leaf, 15 days after the beginning of germination. For *A. blanchetiana* scales totally developed were observed in plants acclimatized for 49 days. The scales of plants harvested at 49, 70, 91 and adult, shield formed by four or more cells; where in the central cells are surrounded by irregular cells and, radial arrangement (Figure 1A). The authors observed variation on the number of cells in the shield and in the peripheral cells, also described by Proenca & Sajo (2004) who studied several species of *Aechmea.*

Plants collected *in vitro* (time zero) showed scales under the developmental phase with central square cells surrounded by a number of incomplete cells. Plants collected at 49 days showed the central cells with triangular shape (Figure 1D), with two series of cells, the pericentral cell being complete and the cells with a rectangular sinuous shape and the subperipheral incomplete and irregularly shaped. The scales of the plants collected at subsequent times were morphologically similar to an adult plant (Figure 1B), with four central cells triangular shaped and with thin walls, surrounded by two series of cells with also thin walls, the first (pericentral) with rectangular shaped, the second (subperipheral) with sinuous squared shape cells. Externally to these cells, the peripheral cells with thin walls and radially elongated form the shield (Figures 1E and 1F).

The stomata are tetracitic type and present on the abaxial surface of leaves (Figures 2A and 2F) and, mostly occur at the same level as the other cells (Proença & Sajo, 2004). Plants collected in periods of 49 and 91 days had the stomata among the epidermal depressions. The position of the stomata cells is generally related to the environment, indicating the adaptive change of the acclimatized plants to the *ex vitro* condition (Alquini *et al*., 2003).

The regular arrangement of stomata, forming longitudinal rows, and its presence only on abaxial epidermis, assisting in prevention of water loss, are characteristics of the genus (Aoyama & Sajo, 2003; Sousa *et al*., 2005). Brazilian species of *Aechmea* subg. *chevaliera* present the stomata partially covered by scales (Sousa *et al*., 2005), characteristic present only on the adult plant of *A. blanchetiana* (Figures 2E and 2F).

The epidermal cells of plants grown *in vitro* (time zero) (Figure 3A) and after 28 days of acclimatization (Figure 3B) showed rectangular and elongated shape, thin walls and they were practically rectilinear. The seedlings evaluated after 49, 70 and 91 days of acclimatization showed rectangular, isodiametric and sinuous epidermal cells similar to those observed in adult-plant leaf (Figures 3C and 3D). Presence of sinuous walls is common in some species of the genus *Aechmea* evaluated by Sousa *et al.* (2005) and this characteristic was

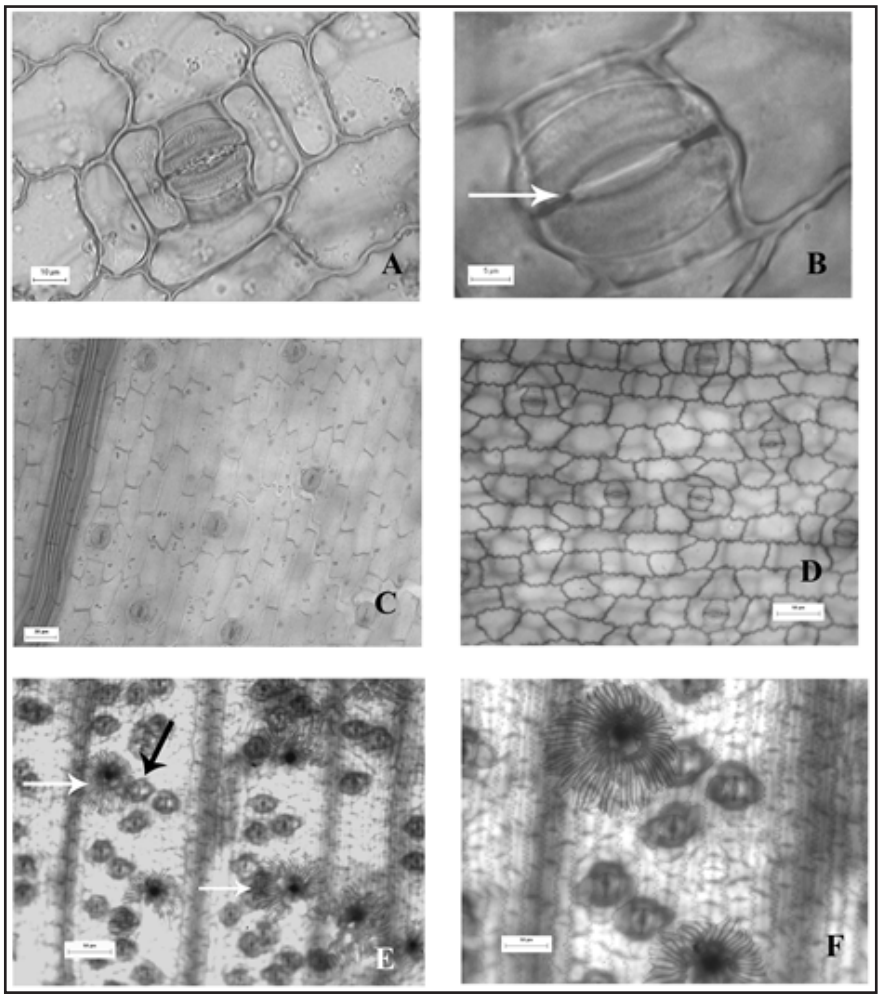

Figure 2. Front view of the abaxial leaf surface of *Aechmea blanchetiana*. A: stomata on plant sampled after 91 days of acclimatization; B: detail of stomata of plant acclimatized during 49 days, arrow shows the thickened wall of the pore; C: stomata of plant after 49 days of acclimatization; D: stomata in plants after 91 days of acclimatization and the sinuous anticlinal walls of the epidermal cells; E: adult plant, black arrows indicate stomata and light arrows indicate scales; F: adult plant with details of the overlapping scales and stomata (vista frontal da superfície abaxial da folha de *Aechmea blanchetiana*. A: estômato em planta coletada após 91 dias de aclimatação; B: detalhe do estômato da planta aclimatizada por 49 dias, seta indica parede espessada do poro; C: estômatos em planta após 49 dias de aclimatização; D: estômatos em planta após 91 dias de aclimatização e sinuosidade das paredes anticlinais nas células epidérmicas; E: planta adulta, setas pretas indicam estômatos e setas claras indicam escamas; F: planta adulta, com detalhe da sobreposição das escamas e estômatos). São Paulo, IBt, 2009.

striking during the acclimatization process of *A. blanchetiana* seedlings.

The scales of *A. blanchetiana*, observed in frontal view, are located in the epidermal depressions, longitudinally located with irregular distribution in the adult plant, as for the acclimatized seedlings of 49, 70 and 91 days. Characteristic also observed by Sousa *et al.* (2005) on bromeliads of *Aechmea* genus. The asymmetrical shield extension of epidermal scales in species of bromeliads of genus *Tillandsia* possibly increases the contact area between the shell and the fluids, increasing the uptake of water and nutrients diluted in these fluids, of the atmospheric environment in the form of dew, fog or rain (Scatena & Segecin, 2005). The increases of scale area (Figure 4D) can be related to the new environment, whereas the presence of a high number of scales in the seedlings *in vitro* (time zero) is due to the scale properties which eliminate salt excess in MS medium in the *in vitro* culture (Benzing, 2000; Larcher, 2000). According to Proença & Sajo (2004), the highest density of scales is related to water uptake, which is stored along the leaf on the aquifer parenchyma in enlarged foliar sheath, providing water accumulation at the base of the rosette in plants of *Aechmea* genus.

The stomatal density in *A. blanchetiana* varied throughout the acclimatization period, decreasing in relation to the plants collected at time zero and the adult plant. Plants collected after 91 days of acclimatization showed stomatal density about two times lower, than the adult plant. Plants collected during the acclimatization period showed about four stomata per mm^2 (Figure 4B) and twice in adult. Pospíšilová et al. (1998), evaluating the stomatal density in leaves of *Liquidambar styraciflua*, *Vaccinium corymbosum* and *Nicotiana tabacum* grown *in vitro,* obtained results similar to those obtained in this study and suggested that changes are due to the time required for species produce leaves adapted to the new environment. The stomatal frequency reveals consonance with *in vitro* and *in vivo* environments, considering that the low autotrophic activity and low stomatal efficiency imposed by conditions *in vitro* are related to the decrease in the number of stomata per area unit (Barboza *et al.*, 2006).

The scale density on the leaf surface of plants at time zero, 7 and 28 days after acclimatization is lower than for acclimatized plants between 70 and 91 days and for adult plants (Figure 4D), showing an increase of the epidermal appendages along the acclimatization period. After 49 and 70 days of acclimatization an increase in the total scale area was observed, and at 91 days this value almost doubled, showing foliar morphological adaptations to acclimatization process. These results are in accordance with Aoyama *et al.* (2012), in which seedlings of *Alcantarea imperialis* (Imperial bromeliad) after 60 days of *in vitro* culture could already be acclimated, contributing to the reduction of cultivation time and optimization of commercial production. The scales are able to maintain a condensed water

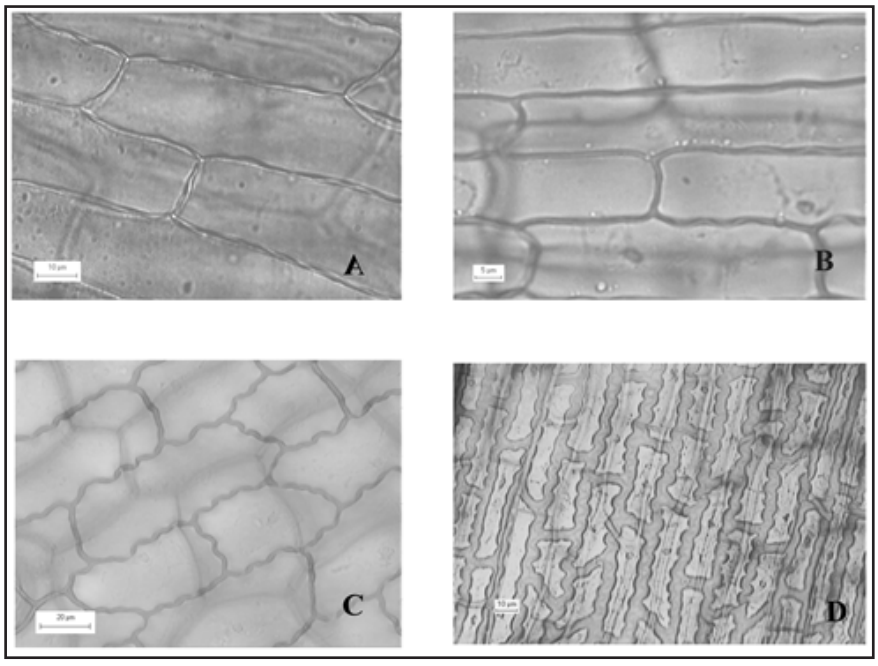

Figure 3. Front view of the abaxial leaf surface of *Aechmea blanchetiana*, showing the thickness of the wall of epidermal cells at different times and on adult plant; A: Immediately after the plant removal from the culture medium *in vitro* (time zero); B: after 28 days of acclimatization; C: after 49 days of acclimatization; D: adult plant {vista frontal da superfície abaxial da folha de *Aechmea blanchetiana,* mostrando a espessura da parede das células epidérmicas em diferentes tempos e na planta adulta; A: logo após a retirada do meio de cultura *in vitro* (tempo zero); B: após 28 dias de aclimatização; C: após 49 dias de aclimatização; D: planta adulta}. São Paulo, IBt, 2009.

vapor atmosphere around the leaf and regulate the temperature of the leaves by the reflection of solar radiation (Larcher, 2000). The increase in number and area of scales restricts water losses and reduces the temperature of the plant and it may be related to the adaptation of the plant to the new environment. During acclimatization, new leaves of fig (*Ficus carica*) produced *ex vitro* showed transitional anatomy comparing to *in vitro* and field plants; thus, according to this study, morphological, anatomical and probably physiological abnormalities of plants grown *in vitro* can be repaired during the period and proper conditions of acclimatization (Chirinéa *et al.*, 2012).

An increase in sinuosity and thickness of the epidermal cell walls was noticed (Figure 4C) after 49, 70 and 91 days of acclimatization, these values being higher than in plants in acclimatization at 7 and 28 days. According to Sousa *et al.* (2005), thickened epidermal cell walls are xeromorphic characteristics and, these characteristics were observed in four

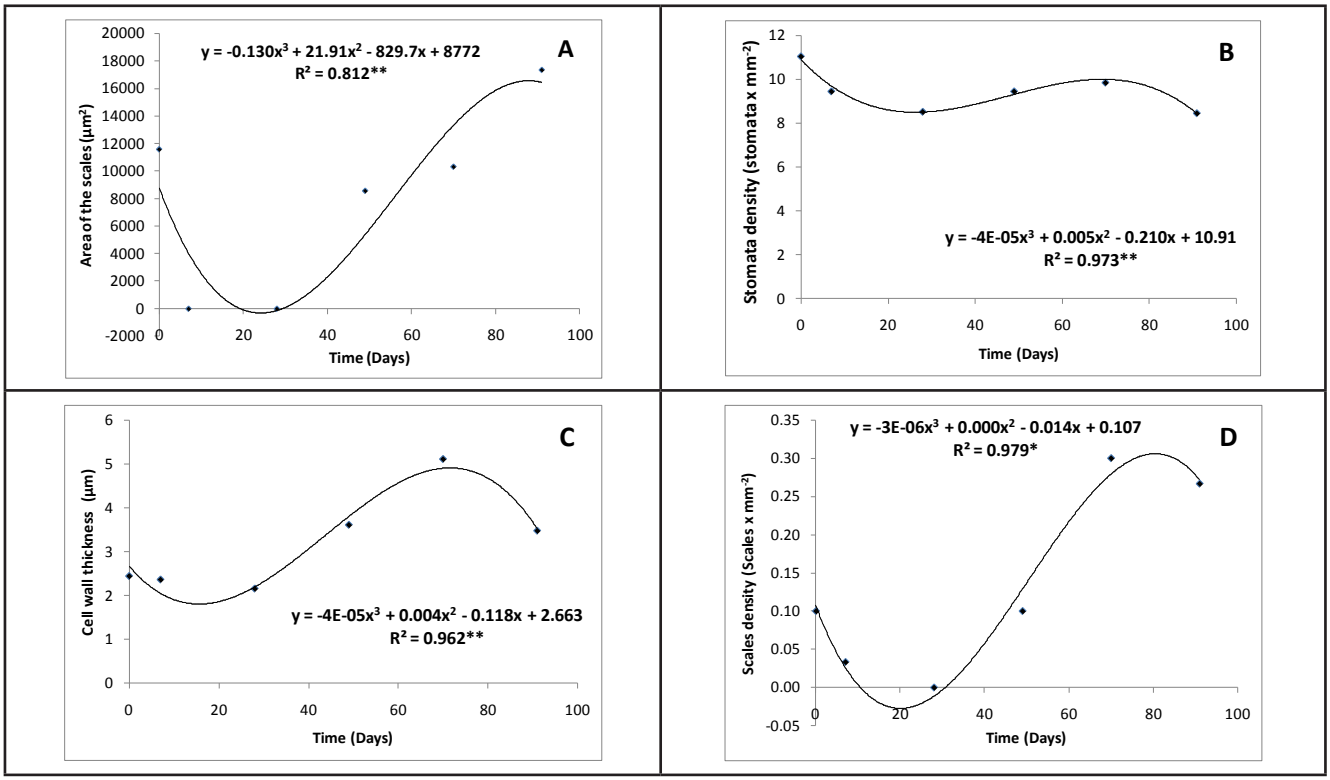

Figure 4. Scale area (µm²) – A; Stomata density (stomata/mm²) – B; cell wall thickness (µm) – C and scales density (scales/mm²) of *A. blanchetiana* on acclimatization at 0, 7, 28, 49, 70 and 90 days (**p≤0.1, *0,1≤p≤0.5) {área das escamas (µm²) – A; Densidade dos estômatos (estômatos/mm²) – B; Espessura das paredes (µm) – C e Densidade das escamas (escamas/mm²) de *A. blanchetiana* em aclimatização aos 0, 7, 28, 49, 70 e 90 dias (**p≤0,1, *0,1≤p≤0,5)}. São Paulo, IBt, 2009.

different species of genus *Aechmea*. Xeromorphic plants show adaptive structures against water loss, such as thickness increasing of epidermal cell wall and cuticle, higher stomatal density, large amount of scales and adaptations in the operation of stomatal apparatus (Alquini *et al.*, 2003). The thickness of epidermal walls possibly acts decreasing water evaporation from the tissue, preventing the collapse of the cells by wilting and ensuring species survival under drought (Scatena & Segecin, 2005).

The results obtained and evaluated in this study showed that *A. blanchetiana* develops some xeromorphic characteristics as an increase in amount and size of scales, and an increase of thickness and sinuosity of epidermal walls which help the adaptation to *ex vitro* condition. The species has natural hardiness which allows its survival until the emergence of the first leaf adapted to the new environment, occurring after 49 days, when the main morphological changes were observed.

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