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Leaf histology of greenhouse plants, in vitro cultured and somatic embryo-derived plants of *Bactris gasipaes* Kunth

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ABSTRACT. *In vitro* cultures of peach palm (*Bactris gasipaes* Kunth) were established by somatic embryogenesis but some improvements in maturation and conversion steps are still needed. The aim of this study was to analyze morpho-anatomical differences in peach palm leaves from greenhouse cultured plants, *in vitro* plants developed from *in vitro* germinated seeds and somatic embryo-derived plants. Expanded leaves were prepared for histological analyses and scanning electron microscopy. No significant difference was found between *ex vitro* and *in vitro* cultured plants, but the somatic embryo-derived plants showed structural alterations of the leaves. The epidermal cells were elongated in shape, the mesophyll cells were thicker and the vascular bundle was not very developed. In somatic embryo-derived leaves the cuticle was thinner than in other leaves and epicuticular wax was present but poorly deposited. In *in vitro* cultured plants, the deposition of epicuticular wax on the leaves was irregular while in the greenhouse plants it was regular and abundant. These alterations in somatic embryo-derived leaves could hinder the acclimatization and development of peach palm plants so it is necessary to improve the protocol for somatic embryogenesis to produce better plants.

Keywords: Auramine O; Arecaceae; peach palm; heart-of-palm.

Histologia de folhas de *Bactris gasipaes* Kunth de plantas de casa-de-vegetação, cultivadas *in vitro* e obtidas por embriogênese somática

RESUMO. O cultivo de pupunha (*Bactris gasipaes* Kunth) *in vitro* foi estabelecido através de embriogênese somática; alguns melhoramentos nas fases de maturação e conversão, contudo, ainda são necessários. O objetivo deste trabalho foi analisar diferenças morfoanatômicas em suas folhas, cultivadas em casa de vegetação, germinadas *in vitro* e provenientes de embriogênese somática. Folhas expandidas foram preparadas para análise histológica e microscopia eletrônica. Houve diferenças significativas entre as plantas da casa de vegetação e as plantas obtidas por embriogênese somática. As células epidérmicas eram alongadas; a espessura da folha e do clorênquima era menor que nas outras; a cutícula era menos espessa, com baixa deposição de ceras. Os feixes vasculares estavam menos desenvolvidos. As folhas das plantas cultivadas *in vitro* e de embriogênese somática apresentavam estruturas pouco desenvolvidas, o que sugere a necessidade de uma melhoria na fase de conversão durante a embriogênese somática.

Palavras-chave: Auramina O; Arecaceae; palmito; pupunheira.

Introduction

The *in vitro* growth conditions are very different from greenhouse or field conditions, complicating the adaption of *in vitro* grown plants to the greenhouse. These conditions, such as low light irradiance and high humidity as well as culture medium composition, could determine the formation of morphologically, anatomically and physiologically abnormal plants (Hazarika, 2006; Pospísilová, Kadlecek, Haisel, & Plzáková, 1999). Thin cuticle, stomata alterations, thickness and differentiation of mesophyll tissues and a varied number and structure of chloroplasts could cause mortality in plants transferred to *ex vitro* conditions (Brainerd & Fuchigami, 1982; Dhawan & Bhojwani, 1987; Díaz-Pérez, Sutter, & Shackel, 1995).

Clonal propagation success is achieved when a large number of true-to-type plants are obtained on a large scale and at a low cost, with a high survival rate in field conditions. A study of the morpho-anatomical characteristics and adjustments of *in vitro* conditions and modifications during acclimatization are important for the success of propagation protocols (Isah, 2015).

In vitro somatic embryogenesis of peach palm (*Bactris gasipaes* Kunth) was already established and many factors affecting the induction of somatic embryos have been recognized (Steinmacher, Cangahuala-Inocente, Clement, & Guerra, 2007a; Steinmacher, Clement, & Guerra, 2007b; Steinmacher et al., 2007c). Moreover, in vitro multiplication using secondary somatic embryogenesis has been established (Steinmacher, Guerra, Saare-Surminski, & Lieberei, 2011). However, some problems have been found during maturation and conversion, leading to low rooting during acclimatization and slow development in the greenhouse. Batagin-Piotto, Almeida, Piotto, and Almeida (2012), studying peach palm plants cultured in vitro and acclimatized, did not observe anatomical differences that could explain these problems.

Plant regeneration capacity is essential for establishing an efficient culture protocol, so extensive studies are required to understand the plant response to culture conditions. With regard to palm species, few papers reported studies of the plant morphophysiology during conversion steps and for different culture types, mainly focusing on somatic embryo origin and ontogeny (Batagin-Piotto et al., 2012; Luis, Bezerra, & Scherwinksi-Pereira, 2010). These studies provide some insights into the process, which can lead to protocol improvement and better regeneration rates. Furthermore, the comparison between plants obtained by somatic embryogenesis and those grown from seeds may reveal some modifications related to the slow development of the plantlets after the acclimatization process.

In the present study we analyzed some morpho-anatomical characteristics of peach palm leaves of *in vitro* and greenhouse cultured plants and somatic embryo-derived plants, in order to observe differences related to *in vitro* culture and to the process of somatic embryogenesis.

Material and methods

Plant material and culture conditions

Peach palm seeds of spineless plants from Porto Velho, Rondônia, Brazil, were used for *in vitro* and *ex vitro* germination and for somatic embryogenesis induction.

In vitro germination

The endocarp that surrounds the seeds was removed and the kernels were surface-sterilized under aseptic conditions, by a five min immersion in 70% ethanol, followed by a 30 min immersion in a 10% sodium hypochlorite solution plus one drop of Tween 20® in each 100 mL (0,1% v/v). Afterwards the kernels were rinsed three times in sterile distilled water. Zygotic embryos were removed aseptically from the kernels and cultured in test tubes containing 10 mL of germination medium. This medium contained MS salts (Murashige & Skoog, 1962), Morel vitamins (Morel & Wetmore, 1951), 3% sucrose, 0.15% activated charcoal and 0.7% agar (Himedia®). The cultures were kept for 12 weeks at 25±2°C under cool-white fluorescent tubes with a photosynthetic photon flux density (PPFD) of 20 μmol $m^{-2} s^{-1}$ and a 16 hours photoperiod.

Somatic embryogenesis induction

The procedure was based on Steinmacher et al. (2011). Zygotic embryos obtained as described above were cultured in Petri dishes containing 30 mL of induction medium. This medium was composed of MS salts, Morel vitamins, 3% sucrose, 0.05% glutamine, 10 μM Picloram, 1 μM silver nitrate and 0.25% Gelzan (Sigma-Aldrich®). The cultures were kept in the dark until embryogenic masses appeared and then transferred to a conversion medium, the same as above, devoid of Picloram and silver nitrate and supplemented with 0.1% glutamine and 0.15% activated charcoal. The plantlets obtained after conversion were then cultured in a fresh medium (in vitro germination media) with transfer to a fresh medium every four weeks until plants reached approximately 10 cm height.

Greenhouse germination

Seeds were placed on seedling trays containing washed sand for 90 days until germination. Then the seedlings were transferred to 0.5 L plastic planter bags containing a mixture of soil and carbonized rice husk (1:1). The trays and plastic bags were kept in a greenhouse with a PPFD of approximately 100 μmol $m^{-2} s^{-1}$ and manually irrigated every day.

Histology

The first well-developed leaves, about 6 cm in length, were collected from about four-month-old *in vitro* plants and from somatic embryo-derived plants four months after conversion. In the case of greenhouse plants, the second opened leaf was collected from five-month-old plants. Some leaves were dissociated in a Franklin´s solution (1945) and stained with safranin solution (0,05%), mounted with glycerin and then photographed for stomata density calculation, determined in twelve quadrants of 1 mm^2 each. For other analyses, leaves were fixed in a FAA 50 solution (Johansen, 1940) and then infiltrated in a hydroxyethyl methacrylate resin (Leica) according to the manufacturer's instructions. Sections were acquired using a rotatory microtome, mounted on a slide, stained with 0.1% toluidine blue (O´Brien, Feder, & McCully, 1964) and then observed and photographed

in a photomicroscope (Olympus BX 41). Some sections were treated with 0.01% auramine O (Sigma-Aldrich®) diluted in water (Considine & Knox, 1979) in the dark and then immediately observed by fluorescence microscopy (UV filter, Leica DM 4000B). The morphological parameters were measured using ImageJ Software (Rasband, 2016), as well as chloroplast counting, using twelve quadrants of 1 mm^2. Seven replicates were used for each treatment, and each replicate consisted of six measurements. The data was subjected to ANOVA and the means compared by Tukey´s test at 5% significance, using Assistat software (Silva & Azevedo, 2016).

Scanning electron microscopic analysis

Samples were taken from the middle of the leaf of plants growing under different environmental conditions. They were fixed in FAA solution (Johansen, 1940) and then dehydrated in alcohol series, subjected to critical point drying (CPD 030, Leica®), followed by splutter gold coating (Balzers Union FL 9496 SCD030) and then examined using a Tescan Vega 3 scanning electron microscope. Leaves of five plants from each treatment were analyzed.

Results

The histology studies showed several anatomical differences between leaves of *in vitro* and greenhouse cultured plants and those from somatic embryo-derived plants (Figure 1). In greenhouse plants, the epidermis presented rounded cells in transversal section (Figure 1a; b). The cuticle was well-formed on the epidermis and on the stomata pore (Figure 1g). Above the epidermis, a hypodermis composed of large irregularly shaped cells without chloroplasts was present on both the abaxial and adaxial surfaces (Figure 1b). Both the epidermis and adaxial hypodermis showed significant differences when compared to leaves of somatic embryo-derived plants (Table 1). The mesophyll was composed of chlorenchyma with irregular rounded cells, thicker than the others (Table 1) with equidistant nonvascular fibrous bundles and small vascular bundles. The major veins were adaxially prominent, composed of xylem and phloem surrounded by a dense fiber sclerenchyma sheath. The vascular bundle was collateral, with protoxylem, metaxylem and two phloem strands (Figure 1a; b). The midrib was adaxially prominent, rounded, simple and multivascular. Furthermore, the leaves of greenhouse plants were significantly thicker than those of plants derived from *in vitro* germination and somatic embryogenesis (Table 1).

The epidermis of *in vitro* plants presented rounded cells in transversal section (Figure 1c). In some regions the cells had an irregular shape (Figure 1c; d). The cuticle was less evident than that of the greenhouse plant leaves (Figure 1h; Table 1). The hypodermis was more developed and uniform, with large cells. The mesophyll and chlorenchyma were more compact than in the greenhouse plants (Figure 1d; Table 1) and the major vein was similar to the greenhouse plants´major vein although it was less developed, without a fiber sclerenchyma sheath surrounding the bundle. The minor vascular bundles were less developed than those of greenhouse leaves (Figure 1d) and there was a lower number of chloroplasts in comparison with the leaves of the other treatments (Table 1). The leaves of somatic embryo-derived plants had a continuous epidermis, with irregular cells and an elongated shape (very extended and with a short diameter) (Figure 1e; f). The cuticle was less evident than in the greenhouse plant leaves (Figure 1i; Table 1). The hypoderm was present; the mesophyll was more compact than that of leaves from *in vitro* germination plants and showed disorganized chlorenchyma with large irregular cells and more intercellular spaces (Figure 1f). Interestingly, the number of chloroplasts was higher than in the *in vitro* plant leaves and similar to that found in greenhouse plant leaves (Table 1) The largest vascular bundle had fewer sclerenchyma fibers than in the leaves of greenhouse and *in vitro* germination plants and a small area was occupied by undifferentiated vascular elements.

Table 1. Morphological characteristics of *Bactris gasipaes* Kunth leaves from *in vitro* and greenhouse germination and from somatic embryogenesis plants.

Parameters	Somatic embryogenesis	In vitro	Greenhouse
Leaf thickness (µm)	95.81 b	109.08 b	124.34 a
Adaxial cuticle surface (µm)	0.781 b	1.004 ab	1.028 a
Abaxial cuticle surface (µm)	0.815 b	0.966 ab	1.068 a
Adaxial epiderm surface (µm)	9.513 b	10.808 ab	11.243 a
Abaxial epiderm surface (µm)	9.558 b	10.808 ab	11.56 a
Adaxial hypoderm surface (µm)	15.987 a	17.538 a	18.354 a
Abaxial hypoderm surface (µm)	14.199 b	17.963 ab	18.822 a
Chlorenchyma (µm)	52.233 b	57.286 b	69.,289 a
Chloroplasts ($n°mm^{-2}$)	16.315 a	114.263 b	160.961 a
Stomatal density on adaxial surface ($n°mm^{-2}$)	12.23 a	10.78 a	9.27 a
Stomatal density on abaxial surface ($n°mm^{-2}$)	60.36 a	59.76 a	58.83 a

Letters represent significant differences according to Tukey's test at 5% significance.

Under scanning electron microscopy differences were also observed in the epicuticular wax deposition on the leaves of the plants grown under different culture conditions. The greenhouse cultured plants had a higher epicuticular wax deposition on both adaxial and abaxial surfaces (Figure 2a) and it was not possible to observe the limit between the epidermal cells, mainly over the adaxial surface (Figure 2g). The deposition was in crusts and sometimes as a smooth fissured layer. The epicuticular wax of *in vitro* cultured plants was deposited as small irregular groups of platelets, distributed sparsely and unevenly over adaxial and abaxial (Figure 2b) leaf surfaces, mainly on the adaxial surface (Figure 2h). The epidermal surface of leaves presented alterations when compared with those of greenhouse plants, showing cells with high convexity (Figure 2d; e). The leaves of somatic embryo-derived plants lacked visible structured epicuticular wax. The epidermal surface was visible on both adaxial and abaxial (Figure 2c) surfaces and a smooth epicuticular wax deposition could be seen sparsely deposited (Figure 2f).

Discussion

The heterotrophic status of the plants, the high relative humidity and the low light intensity are the main factors that favor the induction of alterations of *in vitro* cultured plants, when compared to *ex vitro* conditions (Barry-Etienne, Bertrand, Vasquez, & Ettiene, 2002). These conditions are responsible for physiological and structural modifications of the plant. Therefore, differences in the plant origin (from *in vitro* germinated seeds or somatic embryo-derived) can alter anatomical and physiological characteristics unrelated to *in vitro* conditions.

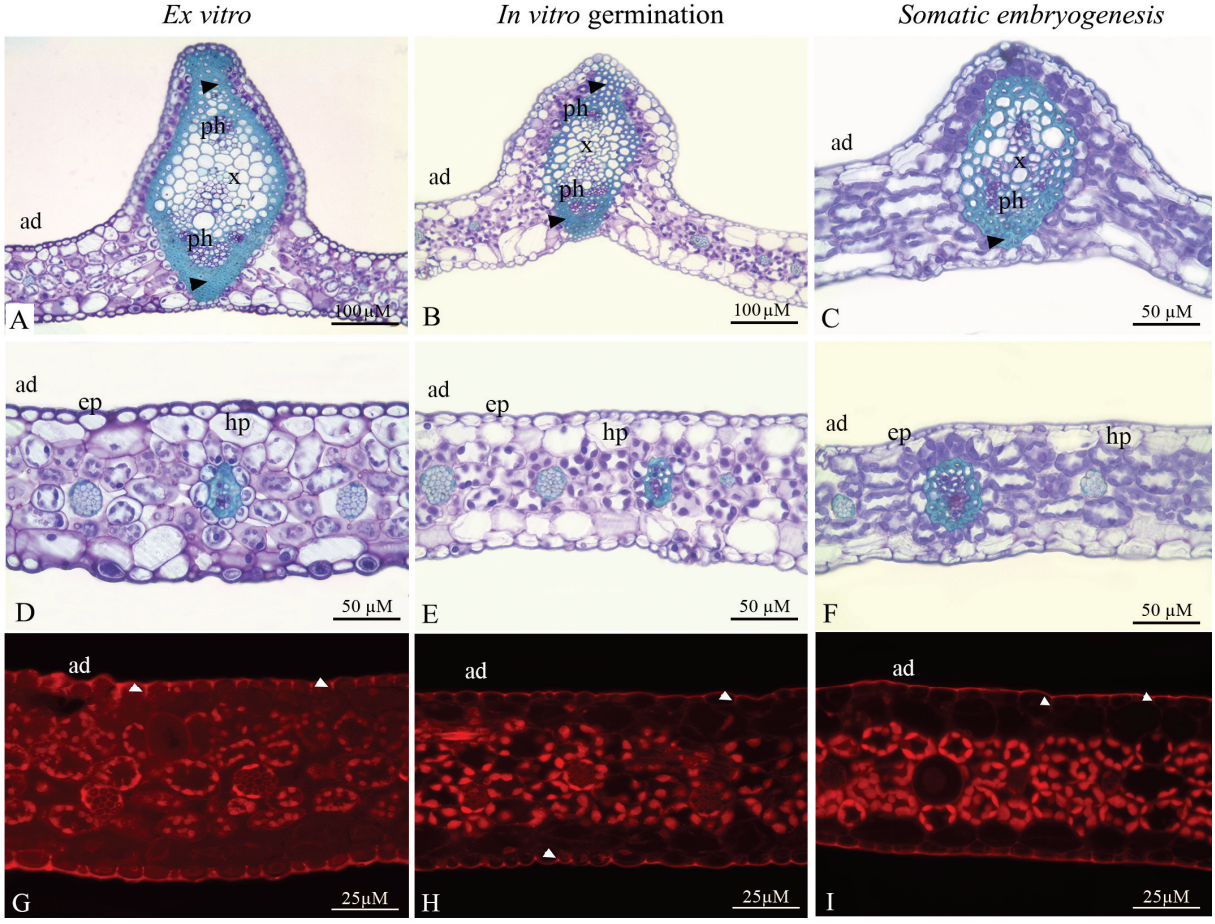

Figure 1. Transversal sections of leaves of *Bactris gasipaes* Kunth grown under different culture conditions. A, D Greenhouse plants, showing a well-developed vascular bundle (A) and a defined epidermis (D). B, E *In vitro* cultured plants, showing a vascular bundle with xylem (x) and phloem (ph) with some alterations. C, F Somatic embryo-derived plants, showing a malformed vascular bundle, with fewer fibers (fi). G, H, I Transversal section submitted to auramine O staining, showing the differences in cuticle development of greenhouse leaves (G), *in vitro* plant leaves (H) and somatic embryo-derived plant leaves (I). Note the presence of cuticular flanges (arrowhead) on anticlinal walls. ad= adaxial surface. ep= epidermis. fi= fibers. hp = hypodermis.

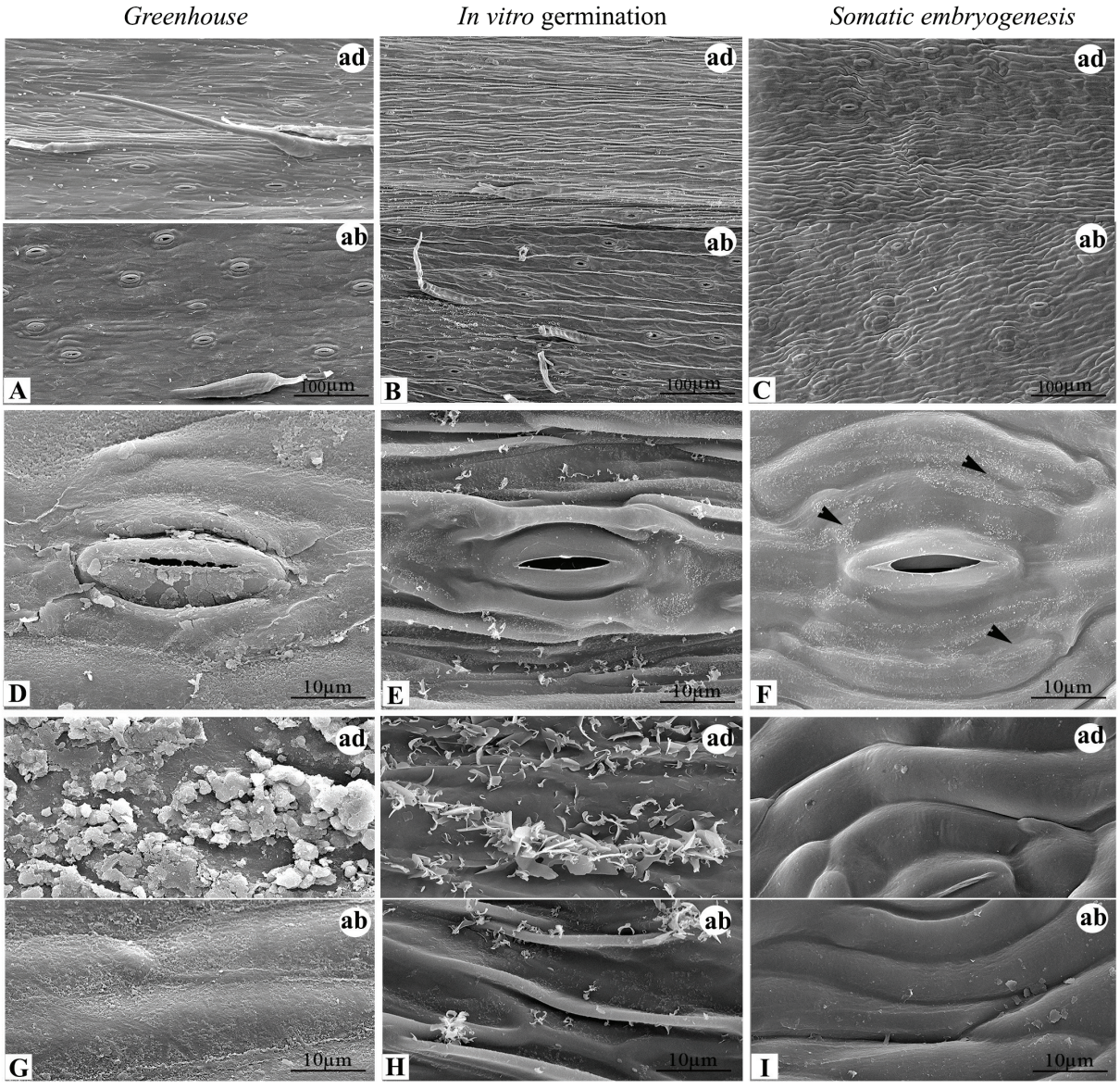

Figure 2. Scanning electron microscopies of *Bactris gasipaes* Kunth leaves grown under different environmental conditions. A Adaxial (ad) and abaxial (ab) surfaces of greenhouse plant leaves. B Adaxial (ad) and abaxial (ab) surface of *in vitro* plant leaves. C Adaxial (ad) and abaxial (ab) surface of somatic embryo-derived plant leaves. D Stomata on the abaxial surface of greenhouse plant leaves. E Stomata on abaxial surface of *in vitro* plant leaves. F Stomata of abaxial surface of leaves of somatic embryo-derived plants, showing a little wax deposition surrounding the stomata (arrowhead). G Detail of adaxial (ad) and abaxial (ab) surfaces of leaves from greenhouse plants. H Detail of adaxial (ad) and abaxial (ab) surfaces of leaves o *in vitro* plants. I Detail of adaxial and abaxial surfaces of leaves from somatic embryo-derived plants.

The morphological and anatomical attributes of somatic embryo-derived plants compared with those of normal zygotic seedlings provide the means to predict plant survival and growth after acclimatization and can lead to improvements in the culture protocol or in planting technique (Lamhamedi, Chamberland, Bernier, & Tremblay, 2000). In this study, the leaves of somatic embryo-derived plants had anatomical characteristics different from those of *in vitro* germination plants cultured under the same conditions. The leaves of somatic embryo-derived plants were less developed than the others, showing anatomical characteristics of younger plants. This fact may hinder post-acclimatization development and decrease the interest in peach palm propagation by somatic embryogenesis. Several histological studies showed that *in vitro* cultured plants presented less differentiated tissues if compared with greenhouse plants (Louro, Santiago, Santos, & Machado, 2003).

Anatomical characteristics of leaves are important functional and adaptive traits that determine plant capacity to survive in different environments, mainly due to implications for photosynthetic

potential (Scafaro, Von Caemmerer, Evans, & Atwell, 2001; Terashima, Hanba, Tholen, & Niinemets, 2011). In the present study, the leaf mesophyll structure of greenhouse and *in vitro* grown *Bactris gasipaes* plants was very similar, whereas the somatic embryo-derived plants presented structural differences in the mesophyll, epidermis, number of chloroplasts and cuticle width. Differences in the mesophyll cells such as shape, size and chloroplast content could result in plants with abnormal physiology, leading to low survival rates during acclimatization (El-Bahr, Ali, & Taha, 2003). Similarly, Batagin-Piotto et al. (2012) did not encounter differences between the leaf anatomy of peach palm plants cultured under three conditions: *in vitro* from stem apices, during *ex vitro* acclimatization and *ex vitro* from seeds.

In this study, the vascular bundle of somatic embryo-derived leaves exhibited structural alterations, but that of leaves from *in vitro* cultured plants did not differ from greenhouse plantlet leaves. However, El-Bahr, Ali, and Saker (2004) reported that seedlings of *Phoenix dactylifera* L. cv 'Zaghlool' obtained *in vitro* by somatic embryogenesis showed less developed vascular tissues than those from plants cultivated *ex vitro*.

The cuticle of somatic embryo-derived plants of *Bactris gasipaes* was less developed than in samples subjected to other treatments. This feature, in addition to the poor epicuticular wax deposition in these plants, could be indicative of their low survival. The cuticle and epicuticular waxes perform a number of physiological roles, notably preventing water loss, enabling the control of evapotranspiration by stomatal guard cells and providing an essential barrier to the entry of toxins and pathogens into the plant (Littlejohn et al., 2015). Furthermore, the cuticle is involved in signaling abiotic stress, regulating ABA biosynthesis and osmotic stress resistance and therefore allowing better plant survival under environmental changes, such as those occurring during acclimatization (Wang, Xiong, Li, Zhu, & Zhud, 2011).

Batagin-Piotto et al. (2012) did not encounter visible epicuticular wax deposition on the leaves of *in vitro* cultured and acclimatized plants of *B. gasipaes*. However, our study showed the presence of epicuticular wax on the leaves of *in vitro* grown plants. This result could be explained by our culture conditions and explant origin different from those used in the study of Batagin-Piotto et al. (2012).

The somatic embryogenesis protocol may be the cause of the poor development of peach palm plants. Several physiological and developmental problems may arise with the plants during the *in vitro* clonal propagation (Bairu & Kane, 2011). Adequate conversion and germination conditions, mainly meeting the nutritional and hormonal requirements of the species, could improve the development of plants.

Conclusion

The leaves of *in vitro* cultured plants did not present drastic morpho-anatomical alterations when compared to greenhouse plants. The structures of somatic embryo-derived plants were less developed than those of *in vitro* cultured plants, showing that the maturation and conversion phases need to be improved in order to produce plants with physiological and anatomical characteristics that both facilitate and accelerate their adaptation to greenhouse and field conditions.

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