Original Article

THE EFFECTS OF *IN VITRO* CULTURE ON THE LEAF ANATOMY OF *Jatropha curcas* L. (Euphorbiaceae)

EFEITOS DA CULTURA IN VITRO NA ANATOMIA FOLIAR DE Jatropha curcas *L. (Euphorbiaceae)*

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ABSTRACT: *Jatropha curcas* (Euphorbiaceae) is an evergreen tree traditionally used in popular medicine, and also as a promising source for bio-fuel production. The *in vitro* propagation of this species has been studied as an alternative to conventional methods such as cutting and seedling. *In vitro* culture environment consists on closed flasks, with high relative humidity, reduced gas exchanges and artificial temperature and luminosity conditions. These conditions may induce physiological and anatomical alterations in cultivated plants. The occurrence of anatomical alterations on the leaves of *J. curcas* was examined in plants cultivated both *in vitro* and *ex vitro*, under greenhouse conditions. The stomatal index was higher on the leaves from greenhouse plants. Mesophyll thickness did not differ, but the greenhouse leaves presented an additional palisade layer, which reflects the environmental influence on cell division. The cells of the chlorophyllous parenchyma of young plants grown in greenhouse conditions have larger chloroplasts than those of the plants grown *in vitro.* The chloroplasts of mature leaves are similar in height, but the mitochondria are smaller. Current results indicate that the leaves of *J. curcas* respond distinctly to both environments. It is necessary to adjust the abiotic conditions *in vitro* to avoid precocious senescence, diagnosed by chloroplasts and mesophyll degradation.

KEYWORDS: Bio-fuel. Cytology. Leaf anatomy. Ultrastructure.

INTRODUCTION

Anatomical characteristics are mostly a reflex of the environmental conditions in which plants have developed and are the result of a complex process that reveals the phenotypical plasticity of these organisms (EHRENDORFER, 1973; REEVE; SHERMAN, 1993). Anatomical abnormalities are common on plants grown *in vitro* (RIGHETTI et al., 1993), and the process of transference to greenhouse conditions generates substantial changes on the leaves, especially on the shape and distribution of epidermal cells, thickness and differentiation of mesophyll tissues, and number and structure of chloroplasts (POSPISILOVA et al., 1999). *In vitro* cultured tissues are maintained in small sealed flasks, which generally have high relative humidity, constant temperature, low active photon flux density, elevated CO_2 fluctuation, high sugar, salt and plant hormone contents in the medium (AITKEN-CHRISTIE et al., 1995). Depending on the flask sealing, there is also a low level of air exchange between the external and internal environments (CHEN, 2004), which may result in the accumulation of gaseous components in the flask or test tube headspace.

The modification of headspace gas concentration and air exchange affects the growth and morphogenesis of several species such as quince and eucalyptus in different *in vitro* culture systems (MARINO; BERARDI, 2004; ZOBAYED, 2006), getting plant conditions to be either heterotrophic or nearly photoautotrophic (INFANTE et al., 1989; RIGHETTI et al., 1993; SERRET et al., 1996; GONÇALVES et al., 2008). Considering this physiological condition and the expected responses of leaf cells, we comparatively analyzed the leaves of *Jatropha crucas* from plants grown in greenhouse and *in vitro* conditions. This analysis should indicate necessary adjustments to optimize *in vitro* conditions for a better propagation ratio of this species.

MATERIAL AND METHODS

Plants of *Jatropha curcas* were cultivated in greenhouse (*ex vitro* conditions) on Alegria Farm, Araçuaí, MG, and micropropagated at Phoneutria Biotechnology and Services Ltd. *Ex vitro* analyses were conducted with 45 days old plants, ~ 40 cm tall, obtained from seeds, and *in vitro* analyses were conducted with 30 days old shoots regenerated from

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cotiledonary leaves according to Sardana et al. (2000). Young leaves from the third internode below the shoot apex and fully expanded mature leaves were sampled from both treatments.

Samples for anatomical analyses were fixed in Karnovsky's fixative (KARNOVSKY, 1965), modified to phosphate buffer 0.1M, pH 7.2, for 24 hours, dehydrated in ethanolic series, and embedded in glycol methacrylate (Leica® Embedding Kit). Transverse and longitudinal sections, 6-8µm thick, were made in rotary microtome, and stained with Toluidine blue O (O'BRIEN; MCCULLY, 1981). Epidermal fragments were obtained with Jeffrey's mixture (JOHANSEN, 1940), and were stained with 0.5% safranin (JOHANSEN, 1940).

The thickness of the leaf tissues, and the stomatal index (CUTTER, 1986) on abaxial epidermal surface were evaluated on three leaves of five individuals. All measurements were made on 40 fields per sample using a drawing tube coupled to a light microscope. The cytometry was performed on five fine sections prepared according to standard procedures for ultrastructural analysis. The samples were fixed in 4% Karnovsky (KARNOVSKY, 1965), modified to phosphate buffer 0.1M,pH 7.2, for 24 hours, post-fixed in 1% osmium tetroxide in phosphate buffer 0.1M, dehydrated in ethanolic series (JOHANSEN, 1940) and embedded in Araldite® (LUFT, 1961). Samples were sectioned in an ultramicrotome Reichert-Jung, Ultracut, contrasted in uranyl acetate and lead citrate (REYNOLDS, 1963), and analyzed on a Transmission Electron Microscope ZEISS EM 109. Organelles were measured on photos from transmission electron microscopy. All data were submitted to analysis of variance (ANOVA) and means were compared by Tukey test ($p \leq 0.05$) through XLSTAT 2008 software.

RESULTS

Leaf anatomy

The young and mature leaves of *Jatropha curcas* are glabrous and dorsiventral, with a prominent midrib vein. The leaves from plants cultivated *in vitro* are hypostomatic, while those cultivated *ex vitro* are amphistomatic. The epidermis is one-layered with straight anticlinal cell walls, and its cells are square-shaped over the mesophyll, and round-shaped over the vascular bundles. The cuticle is inconspicuous. Paracytic stomata are randomly distributed on internervural regions of the abaxial epidermal surface on both groups of plants. More rarely, anomocytic ones are observed on the internervural regions of the epidermal surfaces of the leaves cultivated *ex vitro*.

The young leaves from plants grown *in vitro* have round-shaped epidermal cells, one layered palisade parenchyma undergoing differentiation, and five layers of chlorophyllous parenchyma which originates the spongy parenchyma. Minor vascular bundles are observed interspaced to the median layers of the mesophyll (Figure 1a). The young leaves of plants grown *ex vitro* have column-shaped epidermal cells, one layered palisade parenchyma undergoing differentiation, and five cell layers that originates the spongy parenchyma. Procambial strands differentiate from the middle layers of the mesophyll (Figure 1b).

The expanded leaves of plants grown *in vitro* have one-layered epidermis with round-shaped cells, 1-2 layered palisade parenchyma, and 7-8 layered spongy parenchyma. Mature minor vascular bundles are observed in the middle of the mesophyll, on the interface between palisade and spongy parenchyma (Figure 1c). The expanded leaves grown *ex vitro* have one-layered epidermis with round-shaped cells, slightly elongated periclinally on the adaxial surface. The mesophyll presents one layered palisade parenchyma and 8-9 layered spongy parenchyma with prominent air spaces (Figure 1d). Laticifers and crystalliferous idioblasts may be observed throughout the mesophyll and the cortex of the midrib on both treatments.

Young and mature leaves are anatomically similar, but cell differentiation was qualitatively more evident on the leaves of plants cultivated *ex vitro*.

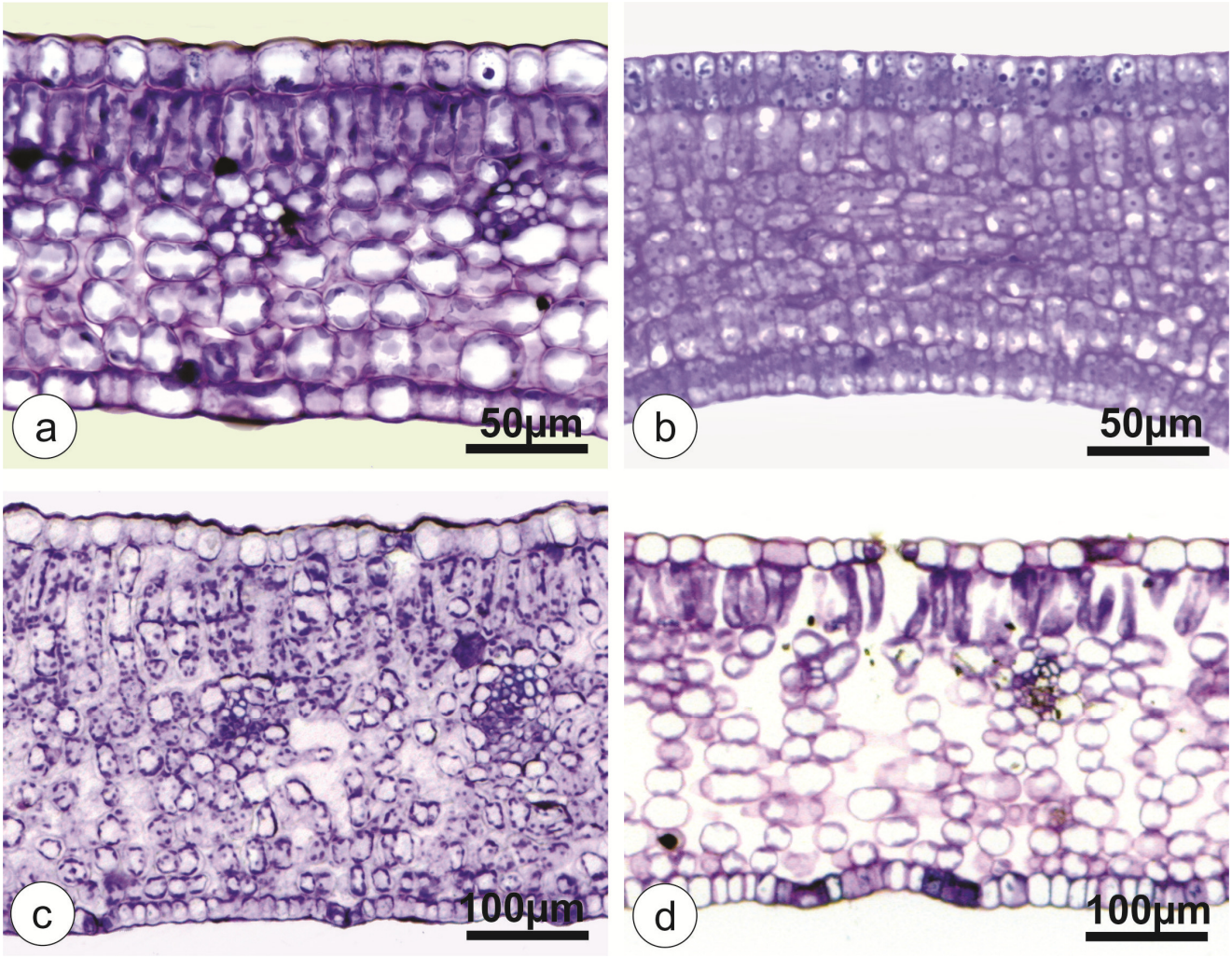

Figure 1. Young and mature leaves of *Jatropha curcas* cultivated *in vitro* and *ex vitro.* a-b –Young leaves; c-d – Mature leaves. a- *In vitro* cultured leaf with reduced intercellular spaces; b- *Ex vitro* cultured leaf with reduced intercellular spaces; c- *In vitro* cultured leaf with little air spaces; d- *Ex vitro* cultured leaf, with dorsiventral structure and many intercellular spaces.

Cytometrical and histometrical analysis

Leaf tissues from *ex vitro* and *in vitro* plants are similar in thickness, but stomatal index was higher in *ex vitro* leaves (Table 1). The thickness of the mesophyll did not differ, but the leaves from plants grown *ex vitro* present a two layered palisade parenchyma.

Table 1. Thickness of the tissues and stomatal index from mature leaves grown in *in vitro* and in *ex vitro* conditions of *Jatropha curcas.* (average ± standard deviation)

Tissues thickness	Thickness (µm)	
	In vitro	*Ex vitro*
Epidermis (adaxial surface)	23.9 ± 3.4 a	22.7 ± 2.9 a
Palisade parenchyma	70.9 ± 4.9 a	61.1 ± 16.7 b
Spongy parenchyma	84.7 ± 12.6 a	81.1 ± 15.5 a
Epidermis (abaxial surface)	16.3 ± 3.5 a	16.7 ± 4.5 a
Mesophyll	162.7 ± 23 a	166.9 ± 15.2 a
Stomatal index	4.1 ± 0.4 b	12.5 ±0.6 a

Values followed by equal letters at the same line do not differ significantly by Tukey test ($p< 0.05$)

Cell organelles from young leaves were structurally similar to those of mature leaves. *In vitro* cultured leaves have cells with large vacuoles, conspicuous nuclei with nucleoli, lamellate

chloroplasts and numerous mitochondria both for the young (Figures 2a-c) and mature leaves (Figures 2d-f). *Ex vitro* cultured leaves have cells with large nuclei with conspicuous nucleoli, large vacuoles, numerous mitochondria and intensely lamellate chloroplasts in both young (Figures 3a-c) and mature leaves (Figures 3d-f).

The dimensions of the organelles are similar except for chloroplasts which were larger in mature leaves when compared to young ones either in *ex vitro* or in *in vitro* cultures (Table 2).

Table 2. Cytometric analysis of *in vitro* and *ex vitro* cultivated tissues of young and mature leaves of *Jatropha curcas.* (average ± standard deviation)

	Mature leaves		Young leaves	
	Area (µm²)			
	In vitro	*Ex vitro*	*In vitro*	*Ex vitro*
Chloroplasts	2.120 ± 0.970 c	6.646 ± 3.142 a	3.757 ± 1.560 b	4.046 ± 1.584 b
Mitochondria	0.394 ± 0.174 a	0.346 ± 0.265 ab	0.182 ± 0.084 c	0.238 ± 0.094 bc

Values followed by equal letters at the same line do not differ significantly by Tukey test (p< 0.05)

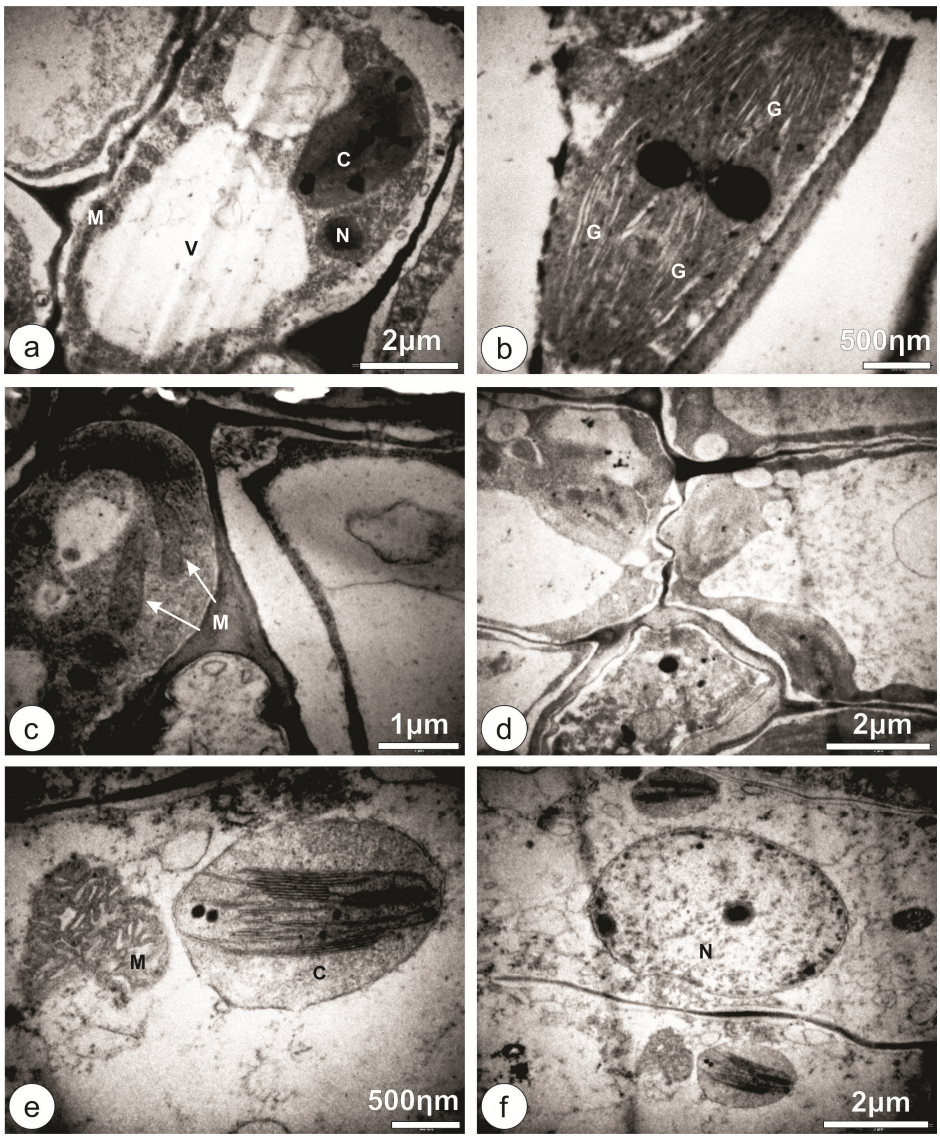

Figure 2. Transmission electron microscopy of young and mature leaves of *Jatropha curcas* cultivated *in vitro.* a-c – Young leaf tissues; d-f –Mature leaf tissues. a-General view of the cell containing large vacuole (V), chloroplast (C), mitochondria (M) and nucleus (N); b – Detail of chloroplast with *grana* (G); c – Abundant mitochondria in the cytoplasm of the cell; d - Cells with large vacuoles, and chloroplasts; e – Detail of chloroplast with *grana* and associated mitochondria; f – Detail of the nucleus with conspicuous nucleolus.

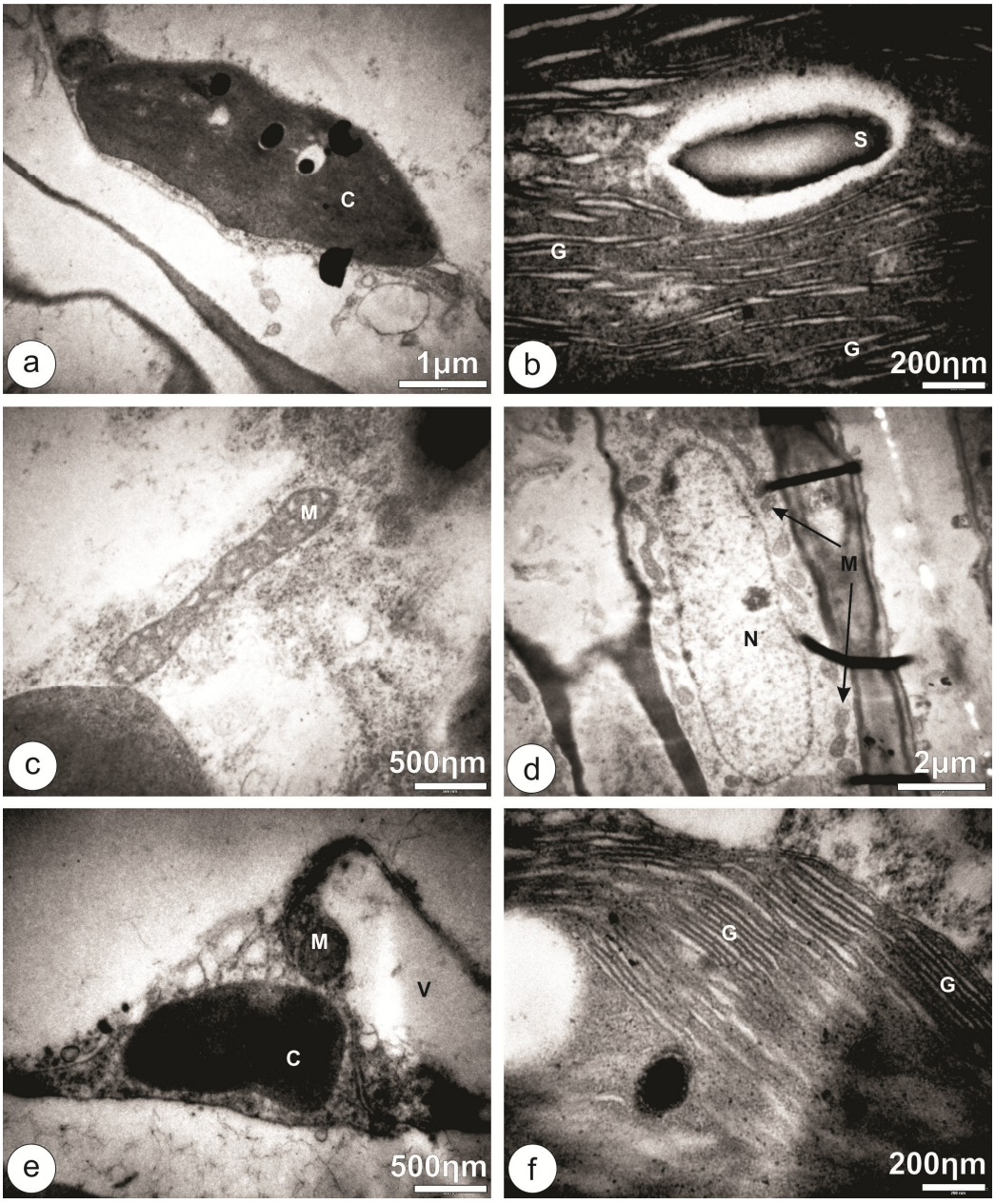

Figure 3. Transmission electron microscopy of young and mature leaves of *Jatropha curcas* cultivated *ex vitro.* a-d –Young leaf tissues; e-f –Mature leaf tissues. a – Detail of chloroplast (C); b – Detail of intense formation of *grana* (G) and primary starch grain (S) in the chloroplast; c – Detail of large mitochondria; d – Detail of the nucleus (N) surrounded by many mitochondria; e – General view of the cell with chloroplast (C), vacuole (V) and mitochondria (M); f – Detail of intense formation of *grana* in the chloroplast.

DISCUSSION

Leaves from the plants of *J. curcas* cultivated *ex vitro* and *in vitro* are anatomically similar either in young or mature stages. The observed anatomical patterns are similar to those described by Gupta (1985) namely, thin cuticle, paracytic and anomocytic stomata on both leaf surfaces, and one layered palisade parenchyma, except for the inhibition of stomata differentiation on the adaxial surface of the leaves of *in vitro* cultivated plants.

The *in vitro* conditions promoted stomata absence on adaxial epidermis, and higher stomatal index. These features should be consequence of the high relative humidity and external sucrose offering of the *in vitro* conditions. The triggering for stomata development involves a 'default' fate of some cells of the meristemoid cells (TRICKER et al., 2012), which is blocked on the adaxial surface of the leaves of plants of *J. curcas* cultivated *in vitro*. Royer

(2001) found a correlation between atmospheric CO2 levels and stomatal density and SI of fossil samples, and suggested that plants are capable of a short-term plastic response and a longer-term genetic adaptation, and that both are determined by the prevailing conditions during growth. In current analysis, the leaves of *J. curcas* present a short-term plastic response to the relative high humidity of the *in vitro* conditions, which indicates the low necessity of CO2 assimilation once the sucrose input is artificially guaranteed. Also, the increasing in SI should compensate this blockage of stomata differentiation on one of the epidermal surfaces, maintaining the levels of gases exchange.

The light irradiance inside the flasks used for *in vitro* cultivation is reduced, and can affect the development of the photosynthetic apparatus. Leaves cultivated develop under high irradiance, as in the *ex vitro* conditions, present higher photosynthetic capacity when compared to those cultivated under shadow conditions (NIINEMETS; TENHUNEN, 1997). The differentiation of an additional palisade parenchyma layer on the leaves of plants cultivated *ex vitro* corroborates this anatomical response. The maintenance of a one-layered palisade parenchyma on leaves of plants cultivated *in vitro* may be related to the exogenous supplement of sucrose, as proposed by Hazarika (2006) for poorly developed photosynthetic apparatus. This tendency is also observed in the midrib region where an average of 3 cell layers may be observed in addition to three collenchyma cell layers.

Cell divisions were inhibited in *in vitro* cultivated tissues probably because of the flask sealing, which induce low gas exchanges and ethylene production. This kind of cell response has been previously described by Fidelis et al. (2000) on leaves of *Brosimum gaudichaudii* (Moraceae) cultivated *in vitro*. Also, Hazarika (2006) observed that underdeveloped palisade tissues is characteristic of *in vitro* cultivated leaves, indicating a pattern of reduced cell division, development, or differentiation for *in vitro* grown plants, as observed for *J. curcas*.

The thickness of the spongy parenchyma does not vary between leaves of *J. curcas* in both analyzed conditions; however, tissues from the *in vitro* cultivated leaves are more compact. This higher compactness suggests a reduced degree of leaf expansion when compared to *ex vitro* cultivated leaves. This feature observed on the leaves of *J. curcas* is different those of leaves from *in vitro* cultivated plants of plum trees (BRAINERD; FUCHIGAMI, 1981) and *Brosimum gaudichaudii* (FIDELIS et al., 2000), which presented increased air spaces in mesophyll when compared to leaves of plants grown under field conditions or inside greenhouses (HAZARIKA, 2006). The comparative cytological analysis of the leaves of *J. curcas* on both treatments evidenced chloroplasts 3-fold larger on the tissues of mature leaves cultivated *ex vitro.* The difference in size is related to the plastids differentiation under proper light condition (VOTHKNECHT; WESTHOFF, 2001), which was not promoted by the *in vitro* conditions. Hence, the minor chloroplasts observed on leaves from plants cultivated *in vitro* can be a diagnostic feature of the precocious senescence of these leaves. Within the same age and treatment, mitochondria size was maintained which is probably related to external sucrose offering, which could indicate high metabolism rates even in young leaves, besides the inhibition of the development of the photosynthetic apparatus (HAZARIKA, 2006).

Investigation around the conditions of cultivation environment and its control are essential to stimulate the development of *in vitro* anatomical characteristics coherent to those expected for plants cultivated *ex vitro,* which can decrease the mortality rate during adaptation to field conditions and also provide high quality plants. The similarity among the evaluated growth conditions are in accordance with an appropriate *in vitro* propagation protocol for *J. curcas*. Current comparative structural analyses of leaves of *J. curcas* evidenced differences on palisade parenchyma thickness, but the maintenance of the *in vitro* and *ex vitro* parameters in statistically similar values. The diagnostic features, such as lower stomatal index, and smaller chloroplasts and mitochondria of the plants cultivated *in vitro*, indicate possible problems during the acclimatization process. Little modifications on the protocols, such as an increasing in gas exchanges should provide better *in vitro* developmental conditions for the plants, possibly resulting in an increase in dry weight (GONÇALVES et al., 2008) improving the morphogenesis. Also, alteration in the size of culture flasks and the type of sealing lids should positively influence the internal conditions (NAVATEL; BOURRAIN, 1994; NGUYEN et al., 1999; ZOBAYED et al., 2001a; MARINO; BERARDI, 2004; GONÇALVES et al., 2008; RIBEIRO et al., 2009), and consequently plant structure.

Current results reinforced the proposal of using anatomical diagnosis to help adjusting culture conditions aiming to improve survival rate and quality of micropropagated plants.

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RESUMO: *Jatropha curcas* (Euphorbiaceae) é uma espécie arbórea sempre-verde usada tradicionalmente na medicina popular e com potencial para produção de biodiesel. A propagação *in vitro* desta espécie tem sido avaliada como alternativa aos meios convencionais de estaquia e plantio. O ambiente *in vitro* consiste em frascos de vidro fechados, com umidade relativa elevada, trocas gasosas reduzidas, e condições artificialmente controladas de temperatura e luminosidade. Tais condições podem induzir anormalidades fisiológicas e anatômicas nas plantas cultivadas. A ocorrência de alterações anatômicas foi avaliada em folhas de *J. curcas* cultivadas *in vitro* e em casa de vegetação visando diagnosticar os efeitos do ambiente *in vitro* sobre o desenvolvimento das plantas. O índice estomático foi maior nas folhas de plantas crescidas em casa de vegetação. A espessura do mesofilo não apresentou alterações, mas as folhas das plantas crescidas em casa de vegetação apresentaram uma camada adicional de parênquima paliçádico. Citologicamente, as células do parênquima clorofiliano de plantas jovens crescidas em casa de vegetação possuem cloroplastos maiores do que aqueles das plantas crescidas *in vitro.* Nas plantas maduras, os cloroplastos apresentam tamanhos similares. As mitocôndrias possuem tamanhos reduzidos nas plantas maduras *ex vitro*. Nossos resultados mostram que as folhas de *J. curcas* respondem distintamente aos dois ambientes. Ajustes nas condições abióticas *in vitro* são necessários para evitar a senescência precoce, diagnosticada pela desintegração dos cloroplastos e consequentemente do mesofilo. Current results indicate that the leaves of *J. curcas* respond distinctly to both environments. It is necessary to adjust the abiotic conditions *in vitro* to avoid precocious senescence, diagnosed by chloroplasts and consequent mesophyll degradation.

PALAVRAS CHAVE: Biodiesel. Citologia. Anatomia foliar. Ultraestrutura.

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