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Original Paper
Leaf anatomy micromorphometry plasticity and histochemistry of *Azadirachta indica* during acclimatization

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Abstract
Environmental conditions of grow can modify leaf structure and metabolite production. Neem plants produce a high amount of medicinal metabolites and contain biopesticide terpenoids with low toxicity. However, the high genetic variation and the low material quality, besides the environmental modifications warn to the need of biotechnological techniques to ensure the production of high quality metabolites. The aim was to investigate leaf structural and histochemical characteristics of *Azadirachta indica* grown *in vitro*, *in vivo* and acclimatized condition. It was found anatomical differences among the environments, with higher leaf thickness associated to *in vivo* conditions, as well as were more evenly distributed stomata. Those modifications did not qualitatively affect the production of medicinal metabolites and biopesticides. Terpenes and tannins were observed in specialized cells called idioblasts, located in the mesophyll and in the midrib region, respectively. Thus, in a qualitative approach, we can affirm that the different environments do not modify metabolites production. Increased production of these bioactive compounds could be achieved by isolation and *in vitro* culture of idioblasts as a new source of research in plant biotechnology.
Key words: idioblasts, leaf structure, neem, tannins, terpenoids.

Resumo
Condições ambientais de crescimento podem modificar a estrutura foliar e a produção de metabólitos. Plantas de nim produzem uma quantidade elevada de metabólitos medicinais e contém terpenóides biopesticidas com baixa toxicidade. Entretanto, a alta variação genética e a baixa qualidade do material, além das modificações do ambiente alertam para a necessidade de técnicas de biotecnologia para certificar a produção de metabólitos de elevada qualidade. O objetivo foi investigar a estrutura foliar e características histoquímicas de *Azadirachta indica* cultivadas *in vitro*, *in vivo* e em condições de aclimatização. Foram encontradas diferenças anatômicas entre os ambientes, com maior espessura foliar e estômatos mais uniformemente distribuídos associados a condições *in vivo.* Essas modificações não afetaram a produção de metabólitos e biopesticidas. Terpenos e taninos foram sintetizados em células especializadas chamadas de idioblastos, localizadas no mesofilo e na região da nervura central, respectivamente. Assim, em uma abordagem qualitativa, pode-se afirmar que os diferentes ambientes não modificaram a produção de metabólitos. O aumento da produção destes compostos poderia ser relizada por meio do isolamento e cultivo *in vitro* de idioblastos como nova fonte de pesquisa em biotecnologia vegetal.
Palavras-chave: idioblastos, estrutura foliar, nim, taninos, terpenóides.

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Introduction

After the transfer of plants from cultures *in vitro* to the greenhouse or field, substantial changes in leaf morphology and anatomy are observed, including characteristics of the epidermis, the thickness of cuticle and leaves, the differentiation of mesophyll, the number and structure of stomata and chloroplasts (Pospíšilová *et al*. 1999; Sáez *et al*. 2012; Mani & Shekhawat 2017), the structure and amount of epicuticular wax on the top surface of the leaves, the number of trichomes and the intercellular spaces of the mesophyll (Fanourakis *et al.* 2013; Shekhawat & Manokari 2018). Furthermore, the leaf structure is highly sensitive to environmental changes, as well as the composition, production sites and reserve medicinal metabolites and biopesticides (Zheng *et al.* 2010; Batagin-Piotto *et al.* 2012) such as essential oils, phenols and flavonoids (Santos *et al.* 2013).

Different environmental factors and conditions, such as atmospheric carbon concentration, temperature, relative humidity and photosynthetic active radiation during acclimatization of some species, may modify anatomical features, in the same way that changes medicinal metabolites and biopesticide production in this species (Sidhu *et al*. 2003; Arora *et al*. 2016). In this context, it is important to use anatomical analyses to characterize structural changes in plants caused by environmental conditions and in the survival rate of transplanted plantlets related to abnormal morphogenesis, stomatal malfunction (Apóstolo *et al.* 2005; Shekhawat & Manokari 2018) and variations in the production of bioactive compounds (Murthy *et al*. 2014). The type and location of secretory structures are also focus of studies, once it can help in the identification of species and serve as quality control parameters for pharmaceutical manufacturing (Kolb & Muller 2004).

Azadirachta indica A. Juss, a tree belonging to Meliaceae family (Allan *et al*. 1999), produces more than 300 secondary metabolites, a third of which are triterpenoids (plant limonoids) with different biological effects and commercial applications (Dai *et al*. 2001; Rodrigues *et al*. 2012). The best known are the active ingredients azadirachtin (insecticide and fungicide), nimbin (spermicidal and anti-inflammatory action in vertebrates) and salannin (insect repellent) (Dai *et al.* 2001). The remarkable bioinsecticide terpenoids against a wide range of agricultural pests and the advantage of being environmentally harmless have increased interest in this class of compounds (Campos *et al*. 2016).

The increasing importance of this species as the source of the medicinal metabolites and biopesticide terpenoids and tannins demands extensive research on growth conditions acting in processes of cell differentiation, aiming to increase the production of metabolites of high economic value. Considering that the extraction of terpenoids from Neem plants has problems of variability in content because of the genetic variability and lower quality of plant material (Kumar & Parmar 1997), biotechnological techniques can ensure the regular supply of high quality homogeneous raw material and increase the production of this highly valuable active metabolite, as research into *in vitro* growing conditions and plant cell culture, or even the enhancement biosynthesis in cell suspension culture using chemical elicitors, is ongoing (Rodrigues *et al*. 2014).

The aim of this study was to examine the structural differences in the leaves of *A. indica* grown *in vivo* and *in vitro* conditions, as well as acclimatized plants; moreover, this work investigates the presence of terpenoids, tannins, lipids, mucilage and their respective cell types in the accumulation of these medicinal metabolites and biopesticides in leaves of Neem developed in these three environments.

Materials and Methods

Plant material and acclimatization

Seeds of *A. indica* were donated by the Plant Nursery Nim Brasil (São José do Rio Preto, São Paulo, Brazil). After germination of the seeds and further plant development in 8 L vases containing substrate, 20 individual plants were cultivated for 8 months under greenhouse conditions and subjected to regular pruning and fertilization (NPK 4-14-8) every 60 days. This allowed for the growth of donor plants and biological sources for experimentation. The seeds or plant material was identified by the botanist Dr. Marcelo Rodrigues UFTM/Brazil, and explant specimen vouchers were deposited in the germplasm of the Tissue Culture Laboratory/BIOAGRO/UFV/Brazil under the supervision of Dr. Wagner C. Otoni.

For *in vitro* propagation of shoots, nodal segments containing one axillary bud were excised and immediately immersed in distilled water after collection. Stem segments were cut to 20 mm lengths and again immersed in distilled

water. Explants were subsequently disinfected by immersion in 70% (v/v) ethyl alcohol for 1 minute under continuous agitation, followed by placement in a 50% (v/v) bleach solution containing 5% sodium hypochlorite and three drops of Tween 20 per 100 ml for 5 minutes, in sequence; then, samples were rinsed three times with sterile distilled water. A second disinfection step was implemented in the laminar flow cabinet by immersing the tissues again in 70% (v/v) ethyl alcohol for 1 minute and then rinsing three times with sterilized deionized water under continuous agitation. Finally, nodal segments were placed in glass flasks containing MS-based medium (pH 5.8) (Murashige & Skoog 1962), supplemented with 3% (w/v) sucrose, 1.0 g L^{-1} PVP (polyvinylpyrrolidone), 0.6% (w/v) agar Merck® Brazil, 0.5 mg L^{-1} 6-benzyladenine (BA), 0.5 mg L^{-1} kinetin (KIN), and 0.05 mg L^{-1} naphthaleneacetic acid (NAA).

Subcultures were carried out monthly into 370-mL glass flasks (50 × 120 mm) containing 40 mL of culture medium. The flasks were sealed with autoclavable rigid polypropylene caps with two 10-mm diameter ventilation holes, each covered with a disk of Fluoropore hydrophobic membrane, 0.22 µm pore size (PTFE; MilliSeal® Air Vent, Tokyo, Japan). The cultures were stored at 27 ± 2 °C with a 16-hour photoperiod and 36 µmol m^{-2} s^{-1} irradiation provided by two 20 watt fluorescent lamps (Osram, Brazil).

Roots developed during *ex vitro* acclimatization in plastic cups containing 1:1 (v/v) ground coconut fiber and eucalyptus bark compost (Eucatex, MA). The cups were covered with transparent plastic bags and incubated in a growth chamber at 25 ± 1 °C and 14 µmol m^{-2} s^{-1} irradiance initially, gradually increasing to 75 µmol m^{-2} s^{-1} for 20 days. After this period, they were transferred to a greenhouse under natural conditions for 30 days, in order to acclimatize the plants. The bags were progressively opened and then totally removed within the initial 10 days in the greenhouse.

The plant material used for this work was: (i) leaves from donor plants that will be called throughout this paper as *in vivo* plants; (ii) leaves from *in vitro* plants; and (iii) leaves from plants after the acclimatized period. Regardless the environment, the choice of the leaves was based on the development of this organ, in order to standardize the comparisons made through the measurements. Thus, the youngest fully expanded leaves from the fourth node from the apex were collected for analyses. The material *in vivo* was collected from new branches 30 days after the pruning of stems near the base (average 45 cm height). *In vitro*-grown leaves were collected at the end of the third subculture, after 75 days in culture, and acclimatized leaves were collected after 50 days (20 days under controlled conditions plus 30 days in a greenhouse under natural conditions).

Scanning electron microscopy

Samples of approximately 50 mm^2 from the middle of the leaf blades from the three environmental conditions were fixed in solution (2.5% glutaraldehyde + 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2) for 72 hours (Karnovsky 1965). After dehydration through a graded series of ethyl alcohol, followed by critical point drying in a Bal-Tec CPD 030 (Bal-Tec, Balzers, Liechtenstein), the samples were mounted on stubs and coated with gold using an FDU010 sputter coater (Bal-Tec, Balzers, Liechtenstein). Examinations and photography were carried out using a Leo 1430VP scanning electron microscope (Zeiss, Cambridge, England).

Light microscopy

Leaves were sampled from five plants per environment with 3 replicates. Leaf sections (approximately 100 mm^2) were cut from the middle portion of the leaf blades and fixed in FAA_{50} (formaldehyde, acetic acid and 50% ethyl alcohol; 1:1:18 v/v) (Johansen 1940). Samples were dehydrated in an ethanol series (10 to 95%) and embedded in methacrylate resin (Historesin Leica® Instruments, Heidelberg, Germany). Thin sections (5–8 µm thick) were cut in a rotary automatic microtome (RM 2155, Leica Microsystems Inc., Deerfield, USA), stained with toluidine blue at pH 4.0 (O'Brien *et al.* 1981) and mounted in Permount. Structural analysis and photography were carried out in an Olympus AX70TRF light microscope (Olympus Optical, Japan) with a U-Photo camera system.

Histochemistry

Samples of fresh leaves were obtained from the three environmental conditions and it was performed the same procedure as for the structural analysis. Microtome cross sections were used for histochemical tests to detect total lipids with Sudan black B (Pearse 1980), terpenoids with Nadi reagent (David & Carde 1964), tannins with vanillin hydrochloric acid (Mace & Howell 1974) and mucilage with tannic acid/ferric chloride

III (Pizzolato & Lillie 1973). Control sections were performed simultaneously, according to the recommendations of the respective authors.

Experimental design and data analysis

The micromorphometric analysis of the leaf in *A. indica* included the measurements of the thickness of adaxial (ADS- µm), abaxial epidermis surface (ABS- µm) and mesophyll (ME- µm), and cross-sectional leaf area (LA- µm²), using Image-Pro-Plus software version 4.5 (Media Cybernetics, Silver Spring, MD, USA). Five measurements of thickness of adaxial, abaxial epidermis surface and mesophyll were recorded per sample. Thus, for each treatment, 45 samples × 3 parameters × 5 measurements were evaluated, resulting in 225 measurement points for each parameter evaluated, totaling 675 measurement points per treatment. Leaf area in cross-section was measured in 15 samples × 3 replications, totaling 45 measurement points for each treatment. The experiment was arranged in randomized block design, and data were analyzed by analysis of variance with means compared by Tukey's test at a 5% probability level using GENES software, Windows/2004.2.1 (Cruz 2001).

Results

Structural characterization

The leaves from the three conditions presented anomocytic stomata, but the SEM analysis showed qualitative differences in epidermis characteristics according to the grown environment. The stomata in the abaxial surface of *in vivo* plants were more evenly distributed in the leaf surface (Fig. 1a,b), when compared to leaves cultivated *in vitro* and acclimatized conditions (Fig. 1c-f). Epidermal cells of *in vivo* plants, including stomata had irregular edges (Fig. 1a). On the other hand, leaves of plants cultivated *in vitro* had a regular surface with a clear contour of anticlinal epidermal cell walls (Fig. 1c) with stomata slightly raised above the other epidermal cell and ostioles more open than in other environments (Fig. 1d). Inversely, stomata of leaves of acclimatized plants were positioned slightly below the other epidermal cell (Fig. 1e,f).

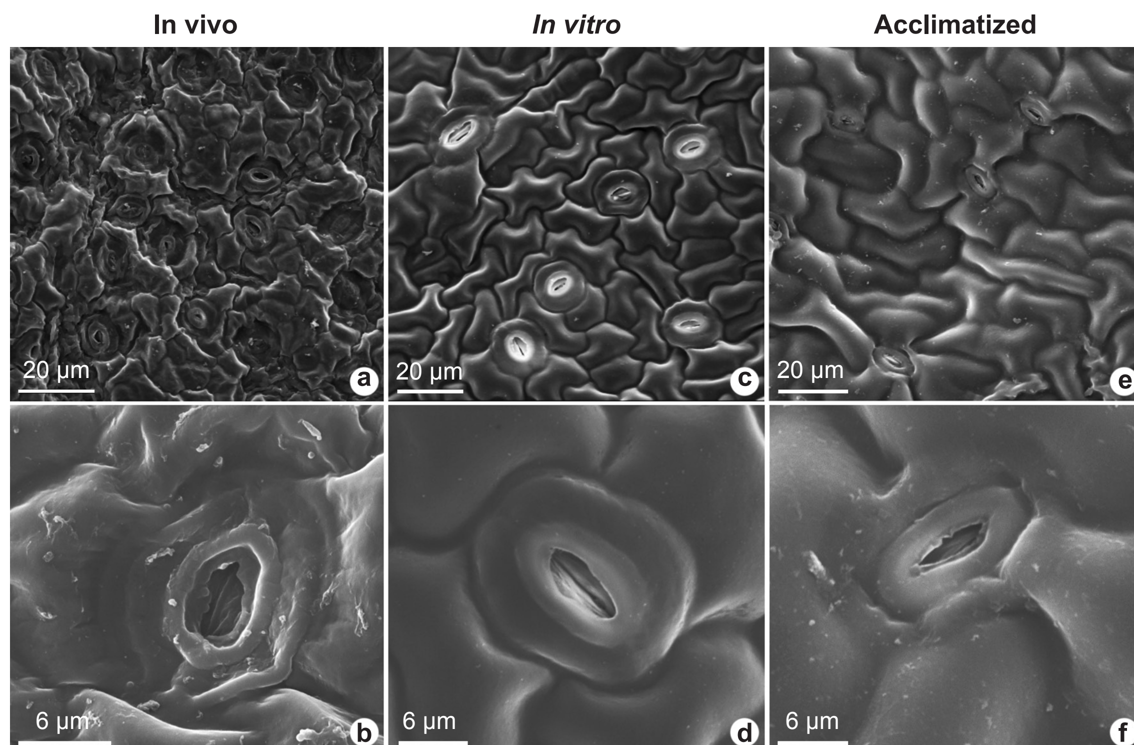

Figure 1 – Scanning electron micrographs of leaf abaxial surface of *A. indica* grown in three different environments. a-f. Details of epidermal cells and stomata – a,b. *in vivo* plants; c,d. *in vitro* plants; e,f. acclimatized plants.

Leaf cross sections showed that the plants from the three environments were hypostomatic. The epidermis on both surfaces was uniseriate and the mesophyll was dorsiventral with one layer of palisade parenchyma and three to four layers of spongy parenchyma. Leaves of *in vivo* plants, grown in the greenhouse, had mesophyll cells with a compact arrangement (Fig. 2a), whereas *in vitro* and acclimatized plants had mesophyll with larger intercellular spaces (Fig. 2b,c). The midrib region contained collateral vascular bundles in plants from all environments (Fig. 2d-f), but they were underdeveloped in acclimatized plants (Fig. 2f). The abaxial surface of *in vitro* plants was more prominent (Fig. 2e). Mesophyll and epidermal cells of *in vivo* plants had denser cytoplasm because of

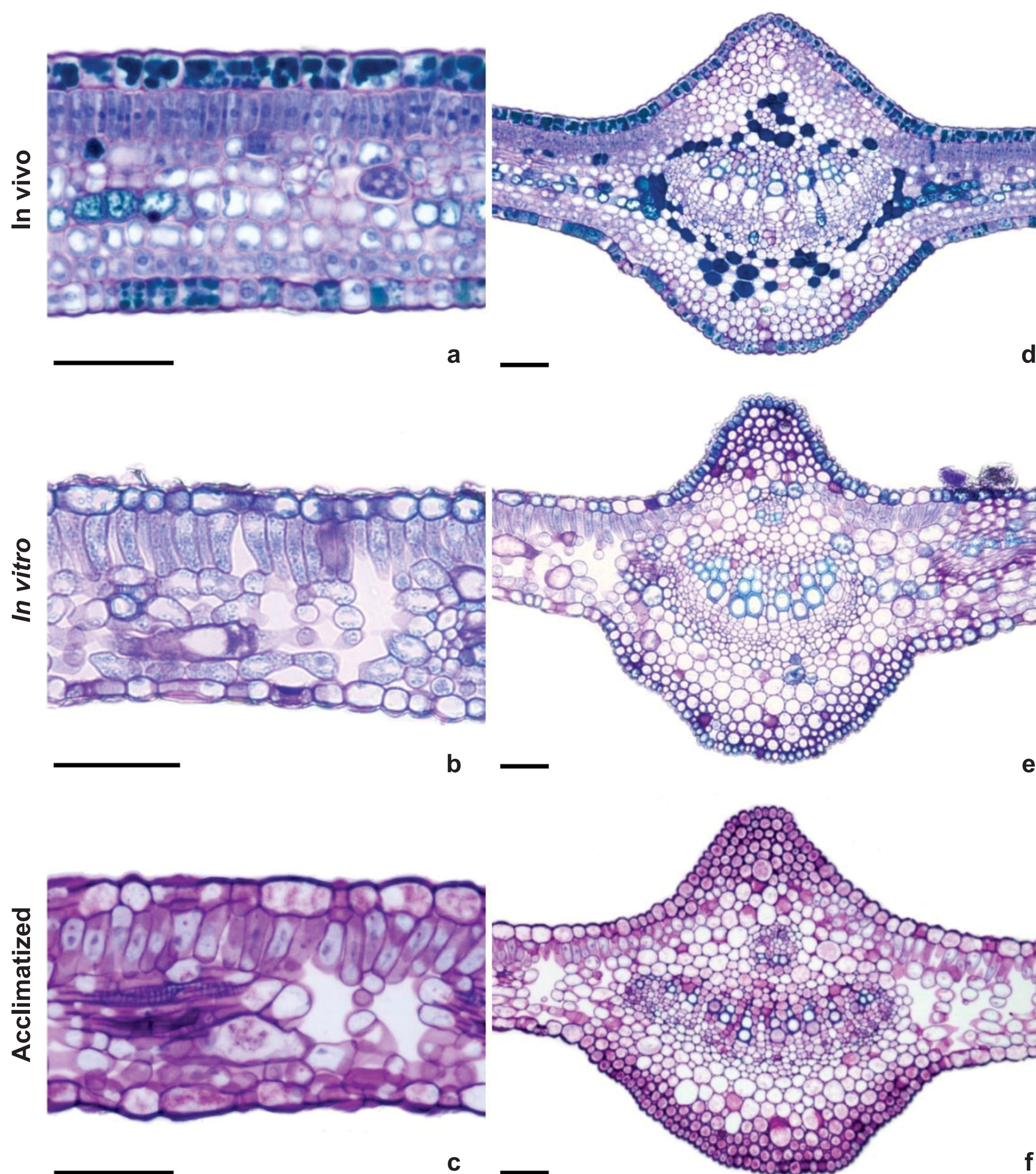

Figure 2 – a-f. Leaf cross sections of *A. indica* grown in three different environments – a-c. middle portion of the leaf blade collected at 30, 50 and 75 days of cultivation, respectively; d-f. midrib region collected at 30, 50 and 75 days of cultivation, respectively. Scale bar = 50 µm.

the higher content of phenolic compounds and the greater number of chloroplasts (Fig. 2d).

Idioblasts occurred in the mesophyll cells of plants from all environments (Fig. 2d-f). The idioblasts occurred singly or in small groups and were endowed with different morphological features in relation to the cells that form the surrounding tissue due to the activity of reserve metabolites. With the use of the Toluidine blue dye, *in vivo* leaves contained idioblasts near the central vein and large greenish compound phenolics, indicating tannins, when compared to the leaves of other environments.

The different growth conditions had a significant effect on the quantitative anatomical characteristics evaluated. The cross-sectional thickness of the adaxial (ADS) and abaxial (ABS) leaf surfaces of *in vivo* (ADS = 15.750 μm; ABS = 12.325 μm) and acclimatized plants (ADS = 13.840 μm; ABS = 11.690 μm) were not significantly different, but both were significantly different from plants grown in *in vitro* environment (ADS = 11.462 μm; ABS = 9.825 μm) (Tab. 1).

The thickness of the mesophyll was significantly different among the three environments: plants grown *in vivo* had the highest mean (186.470 μm), followed by acclimatized (132.876 μm) and *in vitro* (68.465 μm) plants. Cross-sectional area of the leaf was also significantly different among the three environments, with the highest means recorded for plants grown *in vivo* (2364.365 μm^2), followed by acclimatized plants (2236.520 μm^2) and plants grown *in vitro* (2152.630 μm^2).

Histochemical analysis

Regardless the growth environment, histochemical tests indicated an accumulation of compounds in epidermal cells, mesophyll idioblasts, and midrib region which are represented by the images from the acclimatized treatment (Fig. 3a). The positive reaction to NADI reagent, which forms indophenol blue by oxidation, indicated the presence of terpenoids (Fig. 3b). This reaction occurred in the mesophyll, in large, rounded idioblasts accumulating metabolites. In Fig. 3c, details of the leaf midrib region of control plants are shown. The presence of total lipids in cells of the midrib, mesophyll and epidermis was confirmed by the positive reaction to Sudan black B, which detects lipids in the liquid phase at room temperature (Fig. 3d). The positive reaction to vanillin hydrochloric acid indicated the accumulation of tannins in idioblasts in the midrib region and epidermal cells (Fig. 3e). However, the staining reaction with tannic acid-ferric chloride for mucilage was negative for leaf tissues of *A. indica* from all the environmental conditions assessed.

Discussion

Leaves of *A. indica* grown *in vivo* generally had thicker epidermal cells, and the stomata were below the other epidermal cells, creating a moist microclimate around the stomatal cells. These morphological traits, associated with the location of the stomata - hypoestomatic, can restrict transpiration and promote water retention in leaf tissues (El-Hawary *et al*. 2013). Although it was expected that leaves from *in vitro* conditions had a higher stomatal density (Asayesh *et al*. 2017; Luna *et al*. 2017), qualitatively it was not observed in Neem plants. However, stomata that developed in leaves of plants grown *in vitro* usually remained open and located at the same level or above the other epidermal cells due to the high value of

Table 1 – Micromorphometric measurements of leaf structural parameters for *A. indica* grown under *in vivo* and *in vitro* conditions, as well as acclimatized plants, with mean values of the height of the epidermal cells of the adaxial surface (ADS); height of the epidermal cells of the abaxial surface (ABS); thickness of the mesophyll (palisade and spongy parenchyma) (ME); and leaf area in cross-section (LA).

	Structural Parameters			
	ADS	**ME**	**ABS**	**LA**
In vivo	15.750 a	186.470 a	12.325 a	2.364.365 a
In vitro	11.462 b	68.465 c	9.825 b	2.152.630 c
Acclimatized	13.840 a	132.876 b	11.690 a	2.236.520 b

Means ± standard errors followed by the same letter in the same column are not significantly different by Tukey's test at 5% probability.

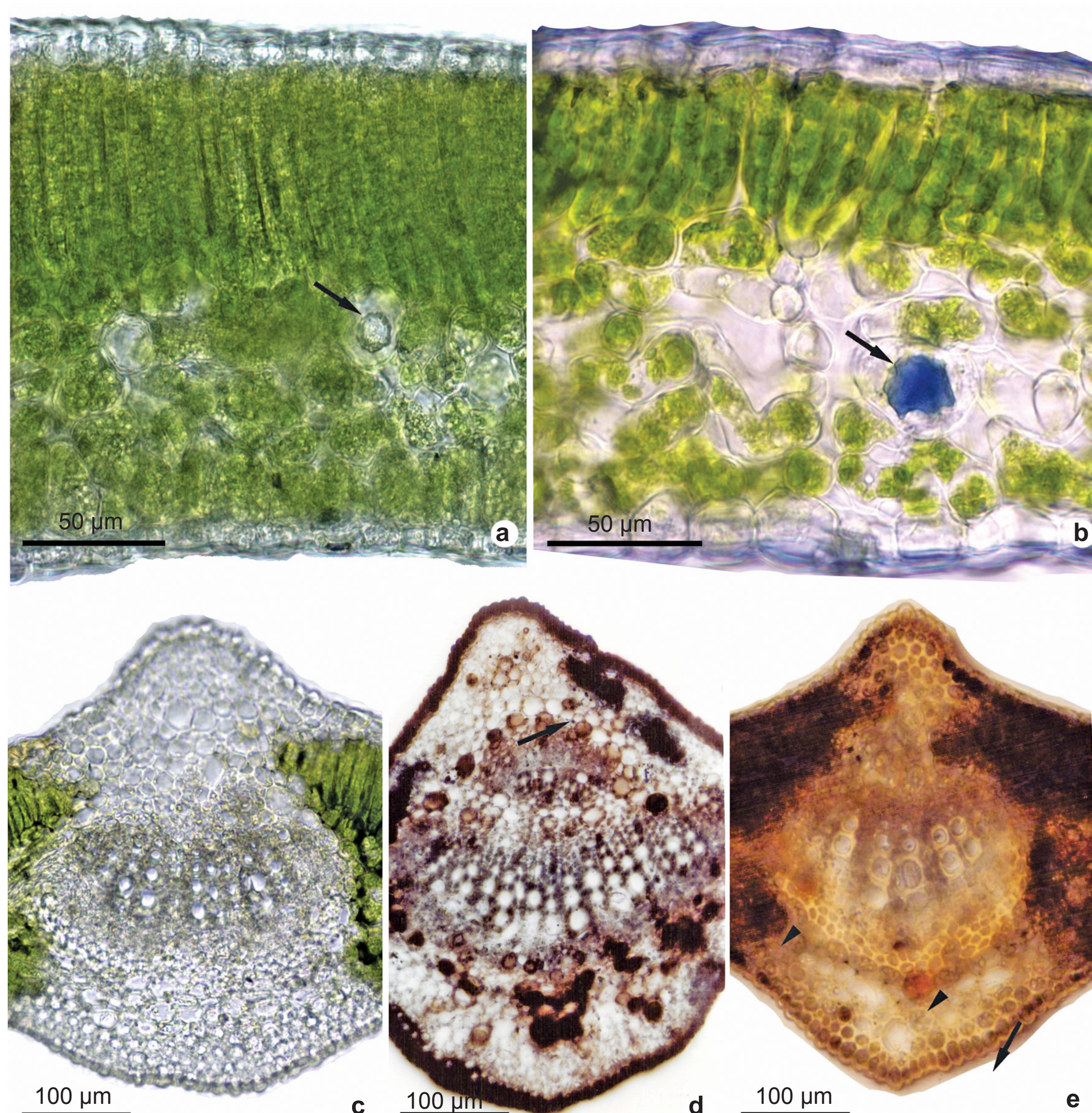

Figure 3 – a-b. Leaf cross sections of *A. indica* after 50 days of acclimatization – a. region between the midrib and the leaf margin without staining with the Nadi reagent (arrow indicates translucent idioblasts containing metabolite to be identified); b. region between the midrib and the leaf margin stained with the Nadi reagent (arrow indicates the positive reaction for essential oil (terpenoids) within idioblasts). c-e. cross section of the midrib – c. detail of the leaf midrib region (control); d. positive reaction with Sudan black B (arrows indicate black-stained general lipids within idioblasts and epidermal cells); e. positive reaction with vanillin hydrochloric acid (arrows indicate red-stained tannins within idioblasts and epidermal cells).

relative humidity inside the glass tube. Similar results were already found for *Vicia faba* when grown *in vitro*, in which leaves had a lower stomatal density with a higher pore aperture (Aliniaeifard *et al*. 2014). Thus, plant growth can be slowed in an environment with high relative humidity due to poor training and functionality of the stomata (Drake *et al.* 2013; Fanourakis *et al.* 2013). The reduced ability to control leaf water loss when plants are subjected to transplantation conditions increased evaporative demand *ex vitro* and the death rate of individuals. Furthermore, the water supply

may be a limiting factor due to the low hydraulic conductivity of the roots (Pospíšilová *et al.* 1999; Franks & Farquhar 2007; Arve *et al.* 2013).

The leaf mesophyll of *A. indica* grown *in vivo* showed more elongated and juxtaposed cells of the palisade layer than *in vitro* leaves. A lower leaf thickness is a response usually observed for plants grown inside poorly ventilated *in vitro* conditions (Dousseau *et al.* 2008; Luna *et al*. 2017), which is due to the quantity and quality of light used in these conditions (Wang *et al*. 2015). The same trend was observed in *A. indica* after 50 days of acclimatization, since the increase in the mesophyll thickness is probably related to external factors such the modification of radiation and composition of atmospheric gases. These characteristics were also observed in the leaves of yellow-ipe (*Tabebuia chrysantha*) after 60 days of acclimatization (Dousseau *et al.* 2008). This morphophysiological plasticity resulting from information contained in the plant genome allows the survival of heterotrophic status caused by cultivation *in vitro* with non-functional stomata in addition to autotrophic status with functional stomata by activating their photosynthetic machinery in the *in vivo* culture system (Zheng *et al.* 2010).

The vascular bundles in the midrib of *A. indica* grown *in vitro* were less developed than in the leaves of *in vivo* plants, which had large collateral vascular bundles; this was also reported for leaves of *Cymbidium* Hort. (Orchidaceae) cultivated under similar conditions (Mayer *et al.* 2008). The *in vitro* plants had poorly developed vascular tissue with smaller midribs and phloem; vascular bundles can be adjusted to ration water with lower hydraulic conductivity (Franks & Farquhar 2007; Fanourakis *et al.* 2013).

Another striking characteristic of *A. indica* was the presence of idioblasts accumulating metabolites in the midrib region and in the mesophyll observed in plants grown in the three conditions. Although this study did not evaluate the quantitative content of the reserve compounds in leaves, in a qualitative framework, there was production of reserve metabolites in the idioblasts. Histochemical tests revealed the presence of terpenoids (Nadi reagent), total lipids (Sudan black B), and tannin (vanillin-hydrochloric acid) in the mesophyll, epidermal cells, and midrib region of *A. indica* leaves from plants grown in the three environments. Thus, our results are consistent with previous findings for other medicinal plants, such as *Lavandula viridis*, where no qualitative changes were observed in the production of essential oil depending on the microclimate conditions of plant cultivation by histochemical analysis (Santos *et al.* 2013).

The large and rounded idioblasts in the mesophyll of leaves of *A. indica* have a clear function of storage of secondary metabolites, since staining with Nadi reagent for terpenoids was positive in these cells. This substance that generally have a defensive role against invading fungi and insects (Dai *et al.* 2001; Tofela *et al*. 2017) were already described to be stored in vegetative structures as idioblasts (Martel *et al*. 2014). The presence of tannins were associated to idioblasts near to the midrib and epidermis of *A. indica* by the reaction with vanillin-hydrochloric acid, evincing their function as a defense mechanism against herbivory and the invasion of pathogenic microorganisms and viruses, as has also been reported for *Spondias dulcis* Forst. F (Sant'Anna-Santos *et al.* 2006). It has been described the presence of tannin compounds associated to idioblasts near the central vein (El-Hawary *et al*. 2013), and to epidermal cells (Ilmiah *et al*. 2018).

The structure and histochemistry of leaves of *A. indica* indicate that this species is a producer of active ingredients. More studies with the quantification of those compounds are required; however our results indicate that this species is able to maintain the production of secondary metabolites, especially terpenoids, in spite of the growth environment. In this context, we can indicate this cellular framework to select and isolate idioblasts cells to produce these compounds. Thus, biotechnological approach can help in the production of metabolites with economic importance.

Conclusion

Terpenoids and tannins are synthesized in specialized cell idioblasts of leaves of *A. indica*. Although the atmospheric changes of the different environments influence cell differentiation and anatomical plasticity, in a qualitative approach, leaves from all environments kept the production of secondary metabolites. Thus, our results indicate that the use of biotechnological techniques can be explored in order to increase the production of extracts of commercial value by isolating and culturing these special cells.

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