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ORIGINAL ARTICLE

Anatomical and histological features of *Ilex paraguariensis* leaves under different in vitro shoot culture systems

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Abstract *Ilex paraguariensis*: (Aquifoliaceae) is an evergreen tree traditionally used to prepare a caffeine-rich infusion that has several medicinal properties. The in vitro propagation of this species has been studied as an alternative to conventional methods such as cuttings and seedlings. The in vitro culture environment consists of closed flasks with high relative humidity, reduced gas exchange and low luminosity conditions, which may induce physiological, anatomical and histological disruption in the cultivated plants. The occurrence of anatomical and histological alterations to the leaves of *I. paraguariensis* was examined in plants cultivated under greenhouse and different in vitro conditions. The variations in the microclimate inside the culture vessels generated by the different treatments tested affected the anatomy and histology of *I. paraguariensis* leaves by causing alterations in their architecture, thickness, vascularization and stomatal differentiation. A temporary immersion system was the best treatment for conserving the anatomical and histological features of the leaves. This treatment led to the lowest stomatal index and an extensive system of intercellular spaces that were similar to the characteristics developed under external environment conditions. Consequently, more than 80% of rooted plantlet transferred to pots were successfully rusticated. Plants from temporary immersion had greater photosynthetic rates due to a higher stomatal conductance.

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Introduction

Ilex paraguariensis (Aquifoliaceae) is a native plant of the subtropical region of South America, including southern Brazil, northeast Argentina and western Paraguay (Gottlieb et al. 2005), where it is largely cultivated to produce a popular tea-like infusion known as yerba mate. The chemical composition of the extract provides certain medicinal benefits, including antioxidant effects, weight loss and cholesterol reduction (Gugliucci 1996; Paganini Stein et al. 2005; Gambero and Ribeiro 2015). However, its recalcitrant characteristics have hindered the development of a suitable method for the propagation of selected genotypes with superior pharmacological or agronomic traits. In this regard, significant efforts have been made in recent years to propagate yerba mate via the induction of adventitious rooting from macro- and micro-cuttings using conventional techniques in nurseries (Tarragó et al. 2005, 2012) or in vitro tissue culture of nodal segments (Sansberro et al. 2000, 2001; Luna et al. 2013). To date, however, there is no method that can rapidly propagate adult plants on a large-scale.

The establishment of a useful method for vegetative propagation of mature trees is difficult due to the reduced rooting capacity of softwood cuttings. This morphogenetic process is associated with the environmental conditions surrounding the mother plants; that is, the material for cuttings is normally sourced from established stock plants exposed to seasonal changes and subjected to a variety of environmental stresses that influence growth and the ability to provide cutting material that forms adventitious roots

(Tarragó et al. 2012). Although the plant tissue culture technique is advancing in applications for the clonal propagation of medicinal, horticultural, agronomic crops and forest trees, its success at the commercial scale is constrained by the formation of aberrant plantlets and the low survival of regenerates during the transfer to field conditions (Isah 2015). Developing such a protocol is hampered by several physiological and developmental aberrations in the anatomy, histology and physiology of the plantlets attributed to the in vitro culture conditions of high humidity, low light levels and hetero- or mixotrophic conditions.

Considering these aberrations and the expected responses of leaf cells, the aim of this report was to see if internal leaf anatomy and stomatal structure of *Ilex paraguariensis* leaves subjected to in vitro cultures conditions is improved by the combinations of solid or liquid medium and diffusive or forced ventilation in the culture vessel headspace. To this end leaves originated from the different treatments have been compared with those produced *in vivo* under greenhouse conditions.

Materials and methods

Plant materials

Ilex paraguariensis St. Hil. cv SI-49 plants grown in greenhouse-maintained pots were the explant source for this study. Explants (1.5–2 cm long stem segments containing one axillary bud) were collected from young non-lignified branches, surface sterilized in 70% ethanol for 1 min and 1.5% NaOCl with 0.1% Triton® x-100 for 30 min and washed with several rinses of sterile distilled water (Luna et al. 2003). Afterwards, the explants were cultured in 11-ml glass tubes containing 3 ml of autoclaved (1.45 kg·cm^{-2} for 20 min) Murashige and Skoog (1962) medium (original concentration reduced to quarter-strength, ¼MS) and 3% sucrose with the pH adjusted to 5.8 prior to the addition of agar (0.65% agar A-1296, SIGMA® Chem. Co., USA). The shoots from nodal segments were grown for at least 30 days in a growth room at 27 ± 2 °C with a 14 h photoperiod (116 µmol m^{-2} s^{-1} PPFD, wavelength: 400–700 nm, from fluorescent lamps) before the experiment.

Treatments

Experiments were performed in a culture room under the environmental conditions described above. The established explants were transferred to 300 cc glass flasks (15 explants/recipient) containing 100 mL ¼MS with 3% sucrose and 0.75 mL L^{-1} Delcide™ TG (5-chloro-2-methyl-4-isothiazolin-3-one + 2-methyl-4-isothiazolin-3-one, 1.05 and 0.45%, respectively), according with our previously published protocol (Luna et al. 2013). The culture vessels were closed with polypropylene caps that contain two 50 mm nylon membrane filters of 0.22 µm pore size, which enabled gas exchange. Four treatments combining semisolid or liquid medium and ventilation were tested (Fig. 1): Semisolid medium with diffusive ventilation (SDV), continuous exposure to liquid medium with diffusive ventilation (LDV), continuous exposure to liquid medium with forced ventilation (LFV), and temporary immersion in liquid medium with forced ventilation (TI). Leaves harvested from plants grown in greenhouse-maintained pots were used as the reference environment (RE) group.

The principle of BIT bioreactors developed by Lorenzo et al. (1998) were used in this study with the pneumatic pumping system controlled by an automatic timer for regulating aeration and medium supply to the explants in the bioreactor. For the liquid-medium treatment, a polyurethane bead was placed at the bottom of each vessel to support the explant. For the forced ventilation system, air was injected with a pressure of 0.5 bar for 1 min every 4 h. For the temporary immersion programme, the explants were in contact with the medium for 1 min every 4 h. The cultures were incubated in a growth room in the same conditions described above. Each culture vessel containing 15 explants was considered an experimental unit, and the experiment was performed in triplicate. Treatments were randomly placed on the pneumatic line.

Leaf evaluation

Anatomical and histological parameters were recorded following completion of the experiment (30 days). For microscopic and morphometric analyses, the sixth fully expanded leaf from the primary explants cultured in vitro and leaves harvested from 30-day shoots of plants grown in the greenhouse (external environment) were collected on the same day and prepared according to the individual procedures. The leaf area (cm^2) was measured using a Li-3000A leaf area meter (Li-Cor Inc., USA).

For the architectural analysis, matured leaves were diaphanized and clarified by means of the protocol developed by Payne (1969) and modified by Vasco et al. (2014), and stained with safranin. The leaf architectural characters were described based on the terminology of the Manual of Leaf Architecture (Ash et al. 1999). The characters described were leaf size and shape, blade symmetry, petiole insertion, blade ratio, base and apex angles, base and apex shapes, margin type, and vein order.

For the anatomical and histological analysis, the leaf samples were fixed in a formalin:ethanol:acetic acid (FAA) solution. To unify the observations and corroborate the accuracy of the analysed characteristics, the leaf blade was

Fig. 1 Methods of propagation tested and analysis of morphological and anatomical parameters. **a** External reference environment(RE), **b** semisolid medium with diffused ventilation (SDV), **c** continuous exposure to liquid medium with diffused ventilation (LDV), **d** continuous exposure to liquid medium with forced ventilation (LFV), **e** temporary immersion in liquid medium with forced ventilation (TI)

divided into two parts at the midrib; one-half was used for observation by scanning electron microscopy (SEM), and the other half was used for analysis by optical microscopy (OM). For both fragments of the leaf, three independent observations were made. For the SEM analysis, the fixed samples were dehydrated using an acetone series, dried with CO_2 using the critical point technique and coated with gold and palladium. A JEOL scanning electron microscope (JLV 5800) operated at 20 kv (Universidad Nacional del Nordeste, Argentina) was used to examine and

photograph the lower epidermal surface and cuticle. For the OM examination, fixed materials were dehydrated using a Biopur© series (Gonzalez and Cristóbal 1997) and then embedded in paraffin. Transverse and paradermal serial Section 10–12 µm thick were stained with Safranin-Astra blue (CI 50240) and mounted in Canada balsam. Photomicrographs were taken using a Leica DM LV2 stereomicroscope equipped with a Canon Power Shot S50 camera and a Leica DM LB2 light microscope equipped with a Leica ICC50HD digital camera. The leaf thickness and palisade thickness was determined from the transverse sections of the leaf; while, stomatal densities, stomatal index and ostiole size were measurements from micrographs of the paradermal sections using ImageJ digital image processing software (Rasband 1997–2016).

Acclimatization

To study the gas exchange capacity of the plantlets, 40-ds-old elongated shoots from the temporary immersion system were isolated and subcultured into root-inducing medium composed of ¼MS plus indolebutyric acid (IBA, 7.5 µM) for 10 days; then, shoots were transferred to expression medium consisting of ¼MS without plant growth regulators for 30 days. This protocol was previously developed to induce in vitro rooting in *I. dumosa* nodal segments using Phytagel® as gelling agent (Luna et al. 2003). In this study, twenty elongated shoots were cultured per recipient of temporary immersion programme and the explants were in contact with medium for 1 min every 4 h. The cultures were incubated in a growth room in the same conditions described above. Finally, the 45-ds-old plantlets were transferred to 150 cc pots containing peat and controlled-release micro-fertilizer (Osmocote® 18-5-9, 0.6 mg cm^{-3} substrate). They were grown for 3 weeks in a growth chamber that maintained the day air temperature below 30 °C and the substrate temperature at 22–25 °C with a 12 h photoperiod (sunlight conditions reduced to 65%); the relative humidity ranged from 90 to 65%. The hardened plants were transplanted to 3200 cc pots filled with controlled-release micro-fertilizer and pine bark, which provides proper drainage and aeration; plants were grown under greenhouse conditions for 12 weeks. To compare the morphological and physiological responses of the in vitro plantlets, softwood cuttings (10–12 cm long with diameters ranging from 3 to 5 mm) of the same genotype were rooted under intermittent mist, according to a protocol developed by Tarragó et al. (2005).

The net photosynthesis rate (A), transpiration (E), and stomatal conductance (gs) were measured using a portable photosynthesis system (LI-6400, LiCor, NE, USA) with the following conditions: PPFD, 1500 mmol m^{-2} s^{-1}; airstreams, 350 mmol CO_2 mol^{-1}; leaf temperature, 27 °C; leaf-to-air vapour pressure deficit: 1.6 kPa. Measurements were taken at mid-day using the fifth and sixth fully expanded apical leaves from 90-ds-old in vitro plantlets and similarly aged rooted softwood cuttings. Sample data were calculated from three biological replicates.

Statistical analyses

All statistical analysis was carried out using ANOVA (GraphPad® Software, Version 7.0, San Diego, CA, USA) and Tukey tests ($P \leq 0.05$) to compare treatment differences.

Results

Leaf architecture

A descriptive analysis was performed following the terminology in the Manual of Leaf Architecture (Ash et al. 1999). Table 1 shows the morphology of the sixth leaf grown under different propagation systems after 30 days of incubation. In all cases, the size of the blades corresponded to the microphyll category, characterized by a leaf area between 2.25 and 20.25 cm^2.

In general, the use of liquid medium altered the obovate shape characteristics of the leaves grown under greenhouse conditions. In general, an ovate shape was observed in continuous liquid cultures provided with forced ventilation, an oblong shape was detected in liquid cultures with diffusive ventilation, and an elliptical form was evident under the temporary immersion system. In addition, the laminar length:width ratio, base and apex angles, base and apex shapes, and margin type varied in accordance with the surrounding environment. The laminar L:W ratio was highest for the leaves developed under temporary immersion, with leaves forming a cuneate shape on the laminar base.

Leaf vein pattern

The in vitro conditions tested did not affect the distribution pattern of the 1° leaf venation (Table 2; Fig. 2). Although the arrangement of the 2° vein category remained unchanged, the spacing of the 2° vein toward the laminar base was higher in the TI treatment, relative to the greenhouse and SDV treatments; the spacing was irregular in cultures with continuous liquid exposure (LDV and LFV). Similarly, the use of liquid media, either in continuous or temporary immersion forms, modified the distribution pattern of the 3° vein category without affecting the vein course. Interestingly, TI did not affect the 4° vein distribution with respect to the greenhouse environment, and

Table 1 Architecture characteristics of *Ilex paraguariensis* leaves subjected to different culture conditions

	External environment (RE)	Semisolid medium diffusive ventilation (SDV)	Continuous liquid medium diffusive ventilation (LDV)	Continuous liquid medium forced ventilation (LFV)	Temporary immersion (TI)
Laminar size	Microphyll	Microphyll	Microphyll	Microphyll	Microphyll
Laminar shape	Obovate	Obovate	Oblong	Ovate	Elliptic
Laminar symmetry	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical
Laminar L:W ratio	2.11	1.92	2.23	2.27	2.52
Base angle	Acute	Obtuse	Acute	Obtuse	Acute
Apex angle	Obtuse	Acute	Obtuse	Acute	Acute
Base shape	Decurrent	Decurrent	Decurrent	Decurrent	Cuneate
Petiole insertion	Marginal	Marginal	Marginal	Marginal	Marginal
Apex shape	Convex/rounded	Convex/acuminate	Convex	Convex/acuminate	Convex/straight
Margin type	Serrate	Crenate	Erose	Erose	Erose/entire

Table 2 Leaf vein pattern of *Ilex paraguariensis* shoots grown under different culture conditions

	External environment (RE)	Semisolid medium diffusive ventilation (SDV)	Continuous liquid medium diffusive ventilation (LDV)	Continuous liquid medium forced ventilation (LFV)	Temporary immersion (TI)
1° vein category	Pinnate	Pinnate	Pinnate	Pinnate	Pinnate
2° vein category	Festooned brochidodromous	Festooned brochidodromous	Festooned brochidodromous	Festooned brochidodromous	Festooned brochidodromous
Agrophic veins	Single	None	Single	None	None
Number of basal veins	2	2	2	2	2
2° vein spacing	Decreasing toward base	Decreasing toward base	Irregular	Irregular	Increasing toward base
2° vein angle	Decreasing toward base	One pair acute basal secondaries	Smoothly decreasing toward base	One pair acute basal secondaries	One pair acute basal secondaries marginal
Inter-2° veins	Weak	Strong	Weak	Weak to strong	Weak
3° vein category	Random reticulate	Random reticulate	Dichotomizing	Dichotomizing	Dichotomizing
3° vein course	Admedially ramified	Admedially ramified	Admedially ramified	Admedially ramified	Admedially ramified
3° (vein) angle to 1°	Varying	Varying	Varying	Varying	Varying
3° vein angle variability	Inconsistent	Inconsistent	Inconsistent	Inconsistent	Inconsistent
4° vein category	Irregular polygonal reticulate	Dichotomizing	Dichotomizing	Dichotomizing	Irregular polygonal reticulate
5° vein category	Dichotomizing	Dichotomizing	Dichotomizing	Dichotomizing	Dichotomizing
Areolation	Moderately developed	Well-developed	Moderately developed	Poorly developed	Poorly developed
Marginal ultimate (venation)	Incomplete loops	Incomplete loops	Incomplete loops	Incomplete loops	Incomplete loops

showed an irregular polygonal reticulate pattern. Finally, no alterations were noted in the 5° and marginal vein orders.

Leaf thickness, internal anatomy, and histology

The laminar thickness (Table 3) varied between 294 ± 21.4 µm (RE) and 160 ± 1.2 µm (SDV). The culture conditions generated by the temporary immersion system did not alter this parameter, and the leaves exhibited a thickness similar to the leaves from the external environment. By contrast, a significant reduction in laminar thickness was noted with respect to SDV ($P < 0.0001$), LDV ($P < 0.0001$) and LFV ($P < 0.001$).

The mesophyll of leaves originated under greenhouse conditions was clearly differentiated into palisade and spongy parenchyma (Fig. 3a). Under upper epidermis the mesophylls contain three layers of palisade which is composed of compactly arranged cylindrical and thin-walled small cells. The thickness of palisade parenchyma was higher in leaves originated under forced ventilation. The

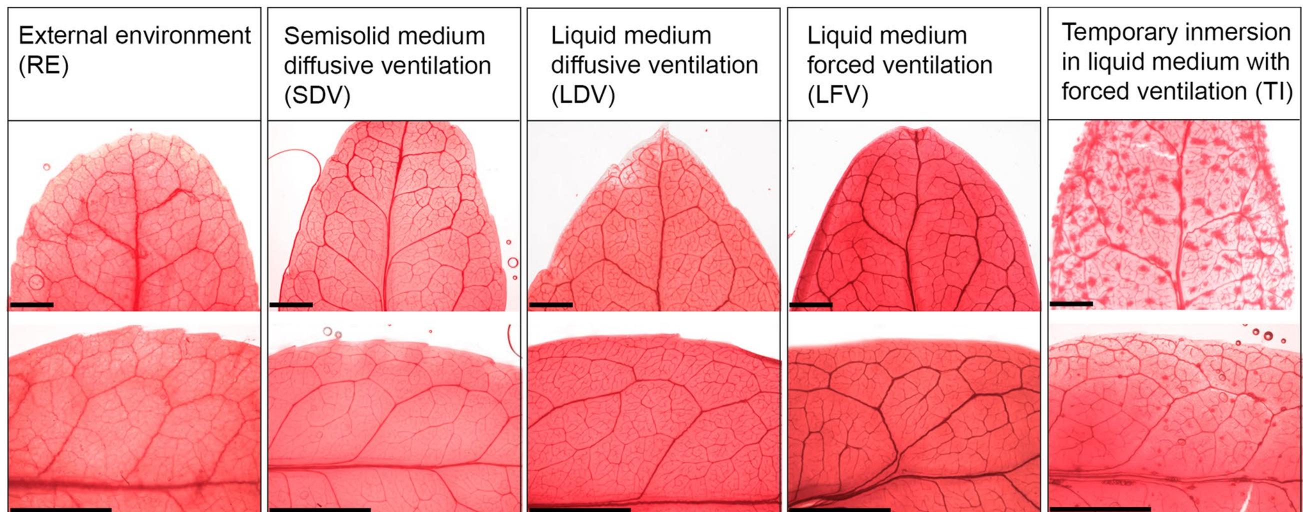

Fig. 2 Leaf venation of *Ilex paraguariensis* across different culture conditions. *Bars* indicate 1 mm

Table 3 Leaf thickness and stomatal index of *Ilex paraguariensis* from different systems of propagation

	External environment (RE)	Semisolid medium diffused ventilation (SDV)	Continuous liquid medium diffused ventilation (LDV)	Continuous liquid medium forced ventilation (LFV)	Temporary immersion (TI)
Leaf area (cm^2)	14.6 ± 0.9	3.0 ± 0.7	3.3 ± 0.2	3.6 ± 0.3	3.9 ± 0.2
Leaf thickness (µm)	294 ± 21.4 a	160 ± 1.2 c	191 ± 3.8 bc	222 ± 5.3 b	287 ± 7.8 a
Palisade thickness (µm)	52.4 ± 4.71 b	32.8 ± 3.33 d	28.0 ± 5.27 d	42.87 ± 7.30 c	63.0 ± 8.83 a
Stomatal density					
OM	1017 ± 7 ab	1710 ± 140 c	1138 ± 70 b	1845 ± 52 c	680 ± 60 a
SEM	192 ± 19 a	1173 ± 144 c	1024 ± 132 bc	1087 ± 71 c	554 ± 107 ab
Stomatal index	18.2 ± 0.7 ab	20.7 ± 1.5 b	18.5 ± 0.4 ab	22.2 ± 0.5 b	15.2 ± 0.8 a
Stomatal size (µm)					
Length	20.5 ± 0.2 a	19.3 ± 0.6 a	20.9 ± 0.5 a	21.3 ± 1.3 a	24.5 ± 1.8 a
Width	19.5 ± 0.3 a	17.6 ± 1.5 a	18.5 ± 0.2 a	20.7 ± 0.5 a	21.1 ± 0.2 a
Ostiole size (µm)					
Length	7.4 ± 0.4 a	6.9 ± 0.4 a	8.3 ± 0.3 a	8.1 ± 1.0 a	9.2 ± 0.5 a
Width	1.1 ± 0.1 a	4.4 ± 0.2 b	3.0 ± 0.3 c	4.0 ± 0.3 bc	3.5 ± 0.4 bc

All parameters were analyzed by using OM, except for the stomatal index quoted as SEM. Values are means ± standard error of the media (n = 30). Different letters indicate significantly different ($P < 0.05$) values

OM optical microscopy, *SEM* scanning electronic microscopy

spongy parenchyma is formed of circular or oval cells with conspicuous intercellular spaces. The expanded leaves of shoots grown in semi-solid medium with diffuse aeration (Fig. 3b) had a lesser number of spongy parenchyma cells, consequently, the intercellular spaces increased to form true air chambers. By contrast, the cells from palisade and spongy parenchyma of the expanded leaves subjected to LDV or LFV comprised a more closely pattern and smaller intercellular spaces (Fig. 3c, d). The expanded leaves originated under temporary immersion had a definite palisade and spongy mesophyll layers with large intercellular spaces; these cells were generally larger than those from the leaves of shoots grown in the SDV system.

Stomatal characteristics

The leaves of *Ilex paraguariensis* are hypostomatic (Fig. 4a–e) and have four types of typical stomata: anomocytic, cyclocytic, actinocytic, and tetracytic; which under the reference environment comprise 38.7, 14.8, 18.1, and 28.4%, respectively. Likewise, contain the giant type of stomata named D-type (Fig. 3f), which are markedly larger than normal ones. The D-type stomata

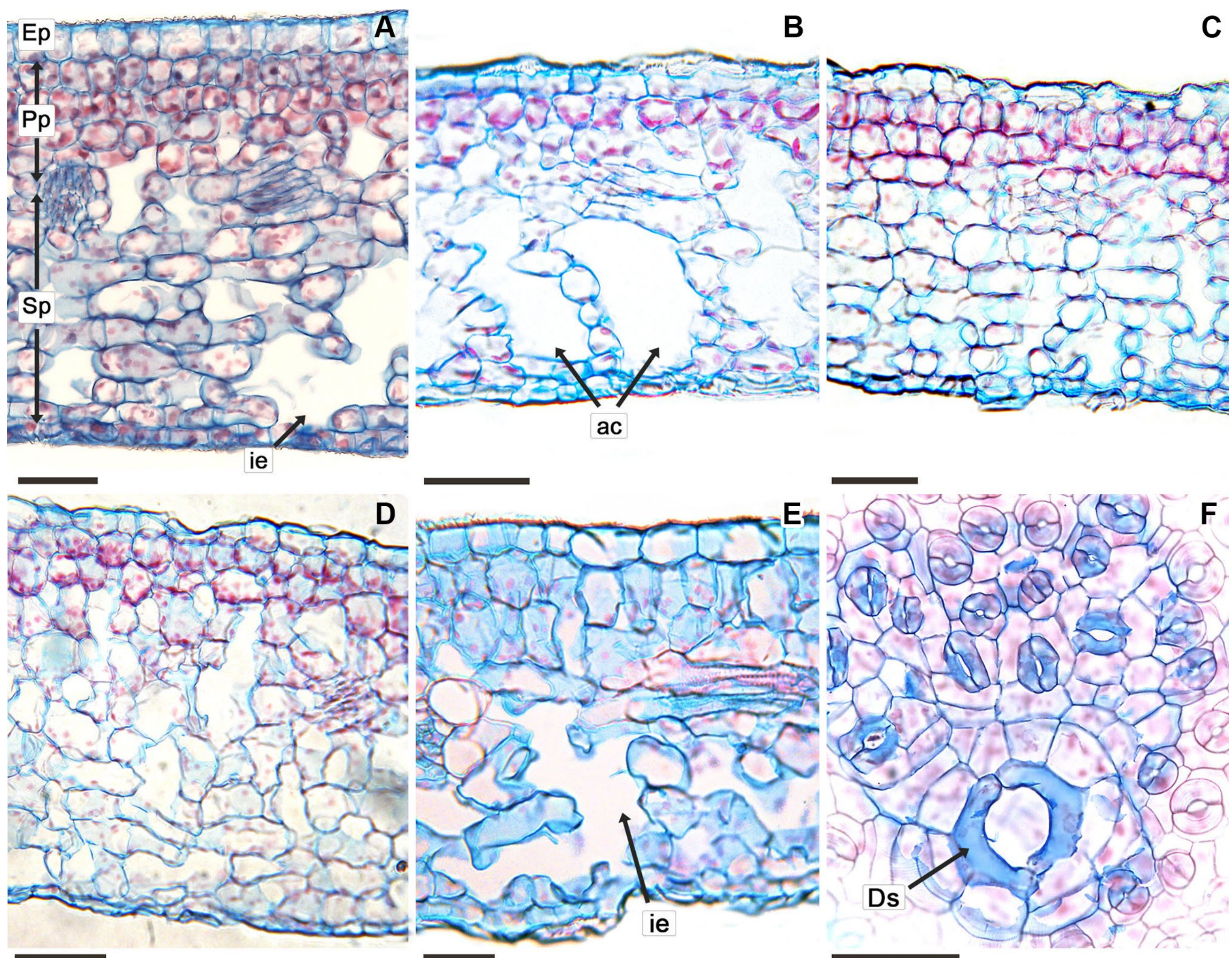

Fig. 3 Leaf blade anatomy of *Ilex paraguariensis* harvested from shoots grown under external environmental conditions **a** semisolid medium with diffused ventilation **b**, continuous exposure to liquid medium with diffused ventilation **c**, continuous exposure to liquid medium with forced ventilation **d**, and temporary immersion in liquid medium with forced ventilation **e**. Paradermal section showing typical and D-type stomata **f**. Ep, epidermis; Pp, palisade parenchyma cells; Sp, spongy parenchyma; ac, air chamber; ie, intercellular space; Ds, type D stomata. *Bar* indicates 50 µm

are characteristic of the genera *Ilex* (Korn and Frederick 1973; Spegazzini et al. 2002) and other species adapted to low sunlight and high relative humidity conditions. These large stomata are few in number, emerge early during development, and are relatively short-lived compared to typical leaf stomata. In this sense, Qi et al. (2017) conclude that its presence in the abaxial epidermis contribute significantly to stomatal conductance and transpiration during the early stages of the leaf ontogeny and possibly correlate with the adaptation of *Camellia* spp to more humid understory environment. In this report, we employed fully expanded leaf harvested from shoots grown either in vitro or in greenhouse conditions. Since, the developmental process of typical stomata is complete at this phenological stage, we assumed that the stomatal conductance from the D-type stomata per se become small in both cases. Consequently, the stomatal characterization was performed from the typical types.

The leaves from the reference environment contained 1017 ± 7 typical stomata per mm^2 of leaf area (Table 3) whereas under in vitro conditions, the stomatal density ranged from 680 ± 60 (TI) to 1845 ± 52 (LFV). Both the number of stomata per leaf area and the stomatal index were similar in TI and RE. Although no significant differences were observed between SDV and RE, the stomatal index significantly varied amongst the TI and SDV treatments ($P = 0.0024$). The environmental conditions inside the culture vessel did not affect the stomatal size. However, the higher moisture content promoted stomatal opening.

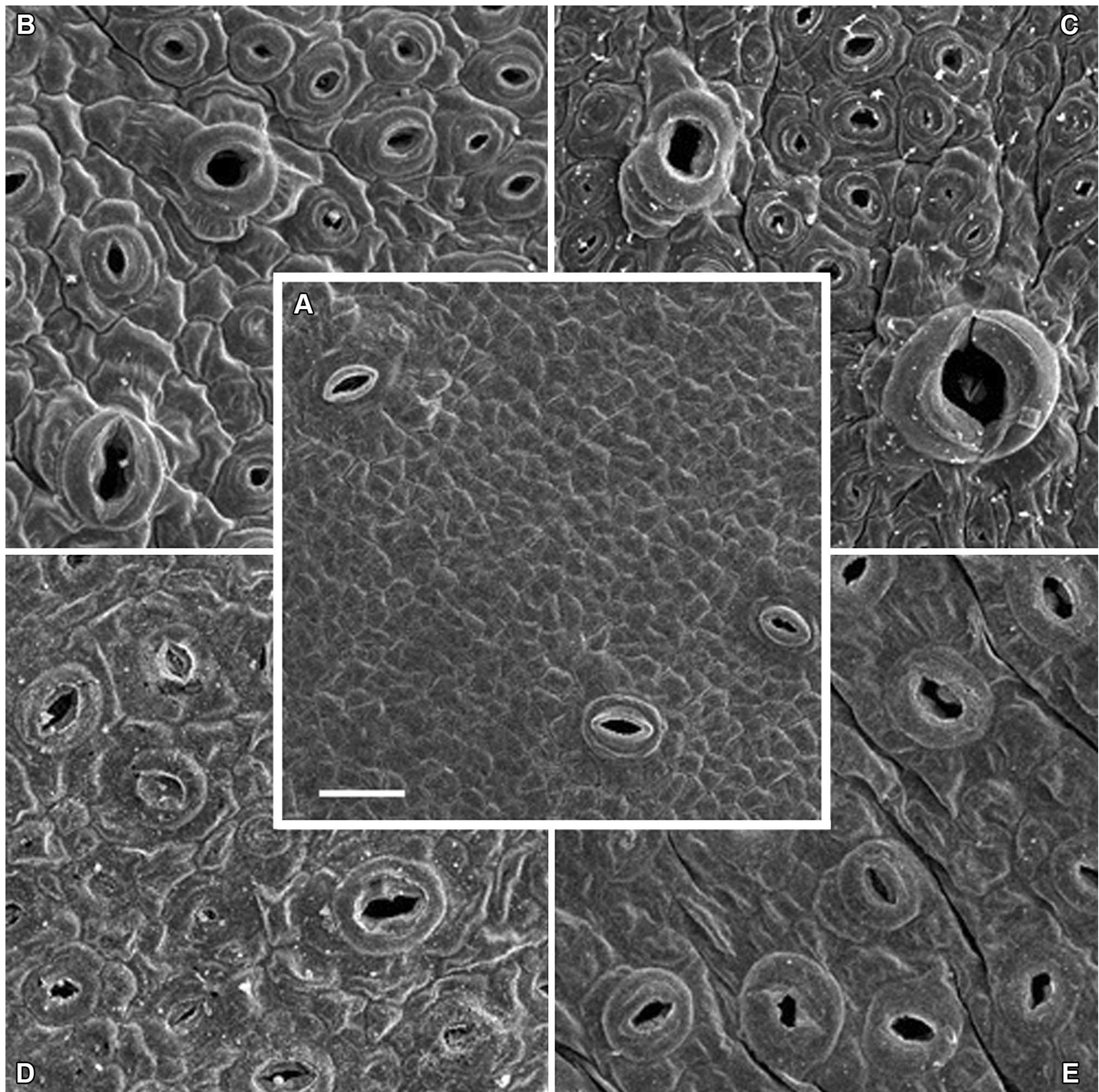

Fig. 4 Scanning electron microscopy images of the lower epidermis of the fifth leaf of *Ilex paraguariensis* harvested from shoots grown under external environmental conditions (**a**) semisolid medium with diffused ventilation (**b**), continuous exposure to liquid medium with diffused ventilation (**c**), continuous exposure to liquid medium with forced ventilation (**d**), or temporary immersion in liquid medium with forced ventilation (**e**). *Bars* indicate 20 µm

Physiological behaviour of micropropagated plants during post-acclimatization

To evaluate the performance of plants produced via the temporary immersion protocol, we analysed the gas exchange of acclimatized plantlets by comparing them with those obtained by the conventional propagation methods of adventitious rooting of softwood cuttings.

After 30 days of incubation, $36.3 \pm 4.2\%$ of in vitro shoots rooted and originated 5.3 ± 1.6 adventitious roots/rooted shoots through a direct pattern of root formation without callus proliferation. $83.3 \pm 6.7\%$ of rooted plantlet transferred to pots were successfully rusticated.

Plants from bioreactors had greater photosynthetic rates due to increased stomatal conductance and transpiration (Fig. 5a–c). Although the increased transpiration rate

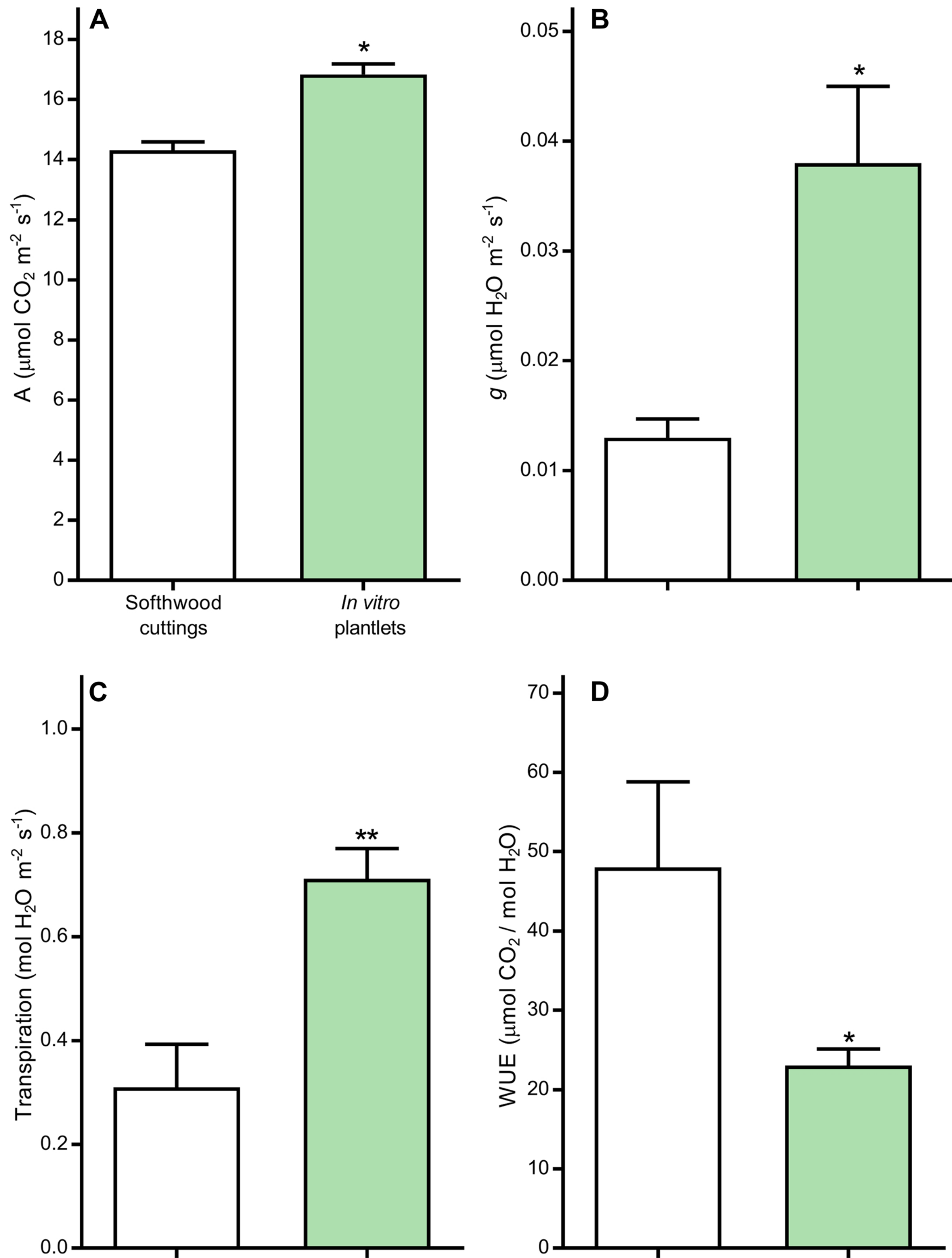

Fig. 5 Photosynthesis (**a**), stomatal conductance (**b**), transpiration (**d**) and water use efficiency of the sixth leaf of *Ilex paraguariensis* plants propagated by adventitious rooting of softwood cuttings and temporary immersion system. The values represent the mean (n = 3) ± SEM. *Asterisks* indicate significant differences at P < 0.05 (*) and P < 0.001 (**) from *t* test

negatively affected water use efficiency (Fig. 5d), it did not compromise the survival of the plants due to the supplemental irrigation during the nursery stage. Net photosynthesis increased from 14.3 ± 0.3 to 16.8 ± 0.3 µmol CO_2 m^{-2} s^{-1} ($p < 0.05$) while the transpiration rate increased from 0.31 ± 0.08 to 0.71 ± 0.06 mol H_2O m^{-2} s^{-1} ($p < 0.01$). Consequently, the water use efficiency declined from 48 ± 10.8 to 23 ± 2.1 ($p < 0.05$) for softwood cuttings and in vitro plantlets, respectively.

Discussion

Our results show that the environmental conditions inside the culture vessel affect the anatomy and histology of *Ilex paraguariensis* leaves by causing alterations to their architecture, thickness, vascularization and stomatal differentiation. On this subject, there is widespread consensus in the international scientific community that the morpho-anatomical modifications of a leaf in response to the environmental and nutritional changes constitute adaptive mechanisms that support leaf function in environments with wide variations in light condition, air temperature and humidity (Ishida et al. 2005; Reich et al. 2007; Carvalho Pereira et al. 2009).

The leaves grown under TI conditions are characterized by an elliptical shape, in contrast to the obovate form of leaves from plants subjected to greenhouse conditions. However, during the period of acclimation to the external environment, the plantlets grew natural obovate leaves.

In general, the thickness of the leaf blade decreased in poorly ventilated semi-solid cultures. Variations in leaf thickness are mainly attributed to differing proportions of mesophyll components, which are influenced by the surrounding environment (Caravita Abbade et al. 2009; Robinson et al. 2009). We found that the temporary immersion system does not affect laminar thickness; it produces leaves similar to those grown in the external reference conditions. The microclimate variation generated by the different treatments tested did not affect the structure of the palisade parenchyma cells. By contrast, the spongy parenchyma was greatly modified in response to the culture conditions of the SDV, LDV and LFV systems. The mesophyll of the SDV was compact with scarce intercellular spaces, whereas the mesophyll of the greenhouse group had spongy parenchyma composed of cells arranged in columns forming extended air chambers. Furthermore, the cross section of the TI leaves displayed an extensive system of intercellular spaces. This structure type may improve gas exchange, enabling the acclimatization process of in vitro plants (Toma et al. 2004; Yang and Yeh 2008).

Most of the stomatal types observed have been previously described in plants growing in natural environments, except the tetracyclic class (Baas 1975). Likewise, the literature has reported many other morpho-anatomical disorders in leaves grown in culture vessels, including alteration of shape, reduction of the size of stomata, substantial increase of stomatal density with loss of functionality (Hazarika 2006), and decrease of epicuticle and mesophyll thickness (Khan et al. 2003); together, these morpho-anatomical disorders negatively affect the water balance of plantlets during acclimatization to the external environmental conditions. Therefore, higher stomatal density is mainly associated with elevated relative humidity, reduced light intensity (Khan et al. 2003), accumulation of ethylene and high sucrose concentration in the culture medium (Jackson 2005; Kozai and Kubota 2005). In fact, the lower stomatal density of the *I. paraguariensis* leaves from the TI system than that of the leaves from the semisolid medium could be the result of intermittent exposure to the liquid medium and better ventilation. In effect, this technique has been successfully employed for the micropropagation of some woody species including *Eucalyptus* (McAlister et al. 2005), pistachio (Akdemir et al. 2014), and chestnut (Vidal et al. 2015). Finally, the acclimation of in vitro propagated plants to the ex vitro environment remains poorly understood and involves an understanding of the effects of in vitro culture conditions upon several parameters at different biological levels (Gago et al. 2014). In summary, the present results show that TI was the best treatment to conserve the lowest stomatal index and an extensive system of intercellular spaces in the leaves.

Conclusions

The obtained results show the feasibility of propagating *Ilex paraguariensis* from adult plants. The anatomical and histological characteristics of leaves grown under temporary immersion are similar to those grown in external environmental conditions and contribute to the acclimation of in vitro propagated plants to ex vitro conditions.

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