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Leaf anatomy plasticity of *Acca sellowiana* (O. Berg) Burret *in vitro* cultured in natural ventilation system and *ex vitro* acclimatized

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Abstract

Acca sellowiana (O. Berg) Burret is a fruit species native to southern Brazil and Uruguay. Micropropagation techniques have been developed in order to scale-up the mass propagation of new varieties. However, micropropagated plants display anatomical, morphological and physiological changes, which may negativelly, affect their initial development in the acclimatization phaseand compromise the entire process. The objective of the present work was to characterize changes in leaf anatomy in order to identify the plasticity of *A. sellowiana* submitted to different *in vitro* culture conditions (conventional and natural ventilation system) and also in the acclimatization process (*ex vitro*). Stomatal density, stomatal length, pore length and chlorophyll content were enhanced in the natural ventilation system. The dimensions of the stomata and stomatal pore, the mesophyll tissues, the thickness of the cuticle and both faces and the perimeter of the xylem increased in the transition from *in vitro* to *ex vitro* culture. Micropropagation systems of *A. sellowiana* promote structural changes in leaves. The stomatal density, xylem area perimeter, palisade and spongy parenchyma showed plasticity indicating greater adaptability in water and light relationships in the transition from *in vitro* to *ex vitro* culture.

Index terms: Feijoa; gas exchange; micropropagation; leaf histology; stomata.

INTRODUCTION

The Atlantic Forest biome in Brazil has a high frequency of native species with potential use. Among them, the feijoa or goiabeira serrana – *Acca sellowiana* (O. Berg) Burret stands out for its high value of bioactive compounds from fruits and food products (Vuotto et al., 2000). *A. sellowiana* is native to southern Brazil, Argentina, Uruguay and Paraguay (Ramírez; Kallarackal, 2017), belonging to the Myrtaceae family which has a predominantly pantropical and subtropical distribution, concentrated in the Neotropical region and in Australia. Feijoa fruit is sweet/sour and highly aromatic. The pulp near the skin is a bit gritty and that around the seeds is juicy (Zhu, 2018). Feijoa fruit is commonly eaten fresh, and also a range of feijoa based products has been developed. They include ice cream, chocolate, candy, smoothie, wine, breadspread, jam, yogurt, muffin, puree, juice, and soon (Zhu, 2018).

For its fruit and as ornamental tree, feijoa has been adapted for cultivation in other parts of the world such as USA, France, Italy, Turkey, Iran, Australia, and New Zealand (Tuncel; Yilmaz, 2015). In Brazil the cultivation of feijoa is still incipient (Weston, 2010). Sexual propagation by seeds is the main method for obtaining seedlings, but the genetic variability and quality of the seedlings formed constitute a limitation to the expansion of the culture (Gomes et al., 2015). Methods of conventional vegetative propagation based on grafting and cutting show unsatisfactory results, thus plant tissue culture techniques comprise important tools in order to mass propagate new varieties recently released in South Brazil (Ciotta et al., 2018). Several protocols for micropropagation based on

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shoot proliferation (Bhojwani; Mullins; Cohen, 1987; Dal Vesco; Guerra, 1999; Oltramari; Dal Vesco; Pedrotti, 2000), or somatic embryogenesis (Pavei et al., 2018; Stefenon et al. 2020) of feijoa have been established. In developed somatic embryogenesis protocols, there is still no control for the mass production of plantlets, requiring further adjustments. In micropropagation protocols based on the development of lateral shoots, the development of plantlets occurs, however the multiplication rates are still low and adjustments are necessary. The knowledge of anatomical modifications in *A. sellowiana* plants in the micropropagation processes (*in vitro* and *ex vitro* phases) will further in the development of more efficient protocols.

In vitro culture environment may generate anatomical, morphological and physiological changes in plants, harming the acclimatization of micropropagated plants (Luna et al., 2017). Shoots developed *in vitro* are exposed to a controlled microenvironment, organized to generate minimal stress (Hazarika, 2006). The development of methods that allow better plant growth and less disorder in physiology and anatomy is necessary to reduce acclimatization losses (Mahendra et al., 2020). Different *in vitro* culture systems have been used to increase the photosynthetic capacity and hardening of plants. Among them, the use of lids on bottles that allow gas exchange is called natural ventilation system (Kozai; Kubota, 2001). This system reduces the relative humidity inside the bottle, increases gas exchange with the external atmosphere, and reduces water availability (Silva et al., 2014).

Leaves developed *in vitro* have asimplified mesophyll and vascular system when compared to leaves developed in a greenhouse (Hazarika, 2006). The stomata of *in vitro* plants are significantly related to the photosynthesis capacity and the acclimatization process (Aliniaeifard et al., 2020). Pospísilová et al. (2007) mention that the stomata of plants under *in vitro* culture system are abnormal, more rounded, reduced in number and unable to function, resulting in plants more susceptible to dehydration in the acclimatization phase. At the time of transfer to the soil, the plants present an excess of water loss due to poor functioning of stomata, reduced formation of epicuticular waxes and cuticular membrane (Zobayed; Armstrong; Armstrong, 2001).

The plasticity of anatomical structures during *in vitro* culture process and subsequent acclimatization (*ex vitro*) helps to understand the success of micropropagation (Lando et al., 2016). Species with a higher plasticity index are more likely to survive in different environments, due to their ability to develop morphological, physiological and also biochemical adaptations (Valladarres; Sanchez-Gomez; Zavala, 2006). Despite the existence of *A. sellowiana* micropropagation protocols, comparative studies of leaf structures do not exist in the literature.

The objective of the present work was to characterize changes in leaf anatomy in order to identify the plasticity of *A. sellowiana* submitted to different *in vitro* culture conditions (conventional and natural ventilation system) and also in the acclimatization process (*ex vitro*).

MATERIAL AND METHODS

Plant material

Seeds and plantlets of *Acca sellowiana* (O. Berg) Burret were obtained from the germplasm collection of the Agricultural Research and Extension Company of Santa Catarina (EPAGRI), in the county of São Joaquim, Santa Catarina State, South Brazil.

The seeds were immersed for 2 min in 70% ethanol (v/v), followed by immersion for 20 min in sodium hypochlorite (NaOCl) (2.0-2.5% active chlorine). Subsequently, they were washed in distilled water and sterilized three times to remove excess NaOCl. The seeds were then inoculated in 250 mL flasks with 30 mL MS culture medium (Murashige; Skoog, 1962) containing 30 g L^{-1} sucrose (Vetec, Fortaleza, CE, Brazil). The pH of the culture medium was adjusted to 5.8 ± 0.1 before addition of gelling agent (2.0 g L^{-1} Phytagel – Sigma, St. Louis, MO, USA). The culture medium was sterilized by autoclaving at 121 °C for 15 min at 151.9 kPa pressure. Cultures were maintained in a growth room at 25 ± 3 ºC, with white fluorescent lamps (50 µmol m^{-2} s^{-1}), with a photoperiod of 16 hours.

Micropropagation

In vitro systems of conventional culture (CS) were used, consisting of flasks with closed Polyvinyl chloride (PVC) lids, and a natural ventilation system (NV) (Figure 1a, 1b and 1c), consisting of flasks

with lids having a rubber orifice, with a hole 1 mm in diameter filled with hydrophilic cotton and a millipori filter with 22 µm porosity, model BioSama (Samavidros, Santo André, SP, Brazil).

Nodal segments with two nodes (about 2 cm) were excised from young plants with 60 days of germination and inoculated in flasks with a capacity of 250 mL containing 30 mL MS culture medium (Murashige; Skoog, 1962) with 30g L^{-1} sucrose (Vetec, Fortaleza, CE, Brazil) and 2.0 g L^{-1} Phytagel (Sigma, St. Louis, MO, USA) for shoot multiplication.

Microshoots developed in the multiplication medium (about 2.5 cm) after 45 days were transferred to *ex vitro* rooting, according to the methodology of Oltramari, Dal Vesco and Pedrotti (2000). Microshoots basal region was immersed in a 100 µM solution of indolebutyric acid (IBA) for 60 minutes. Subsequently, the microshootings were transplanted into polypropylene pots with lids, containing as substrate a mixture of vermiculite and carbonized rice husk, in a ratio of 1:1 (v/v), kept in a growth room for pre-acclimatization (Figure 1c).

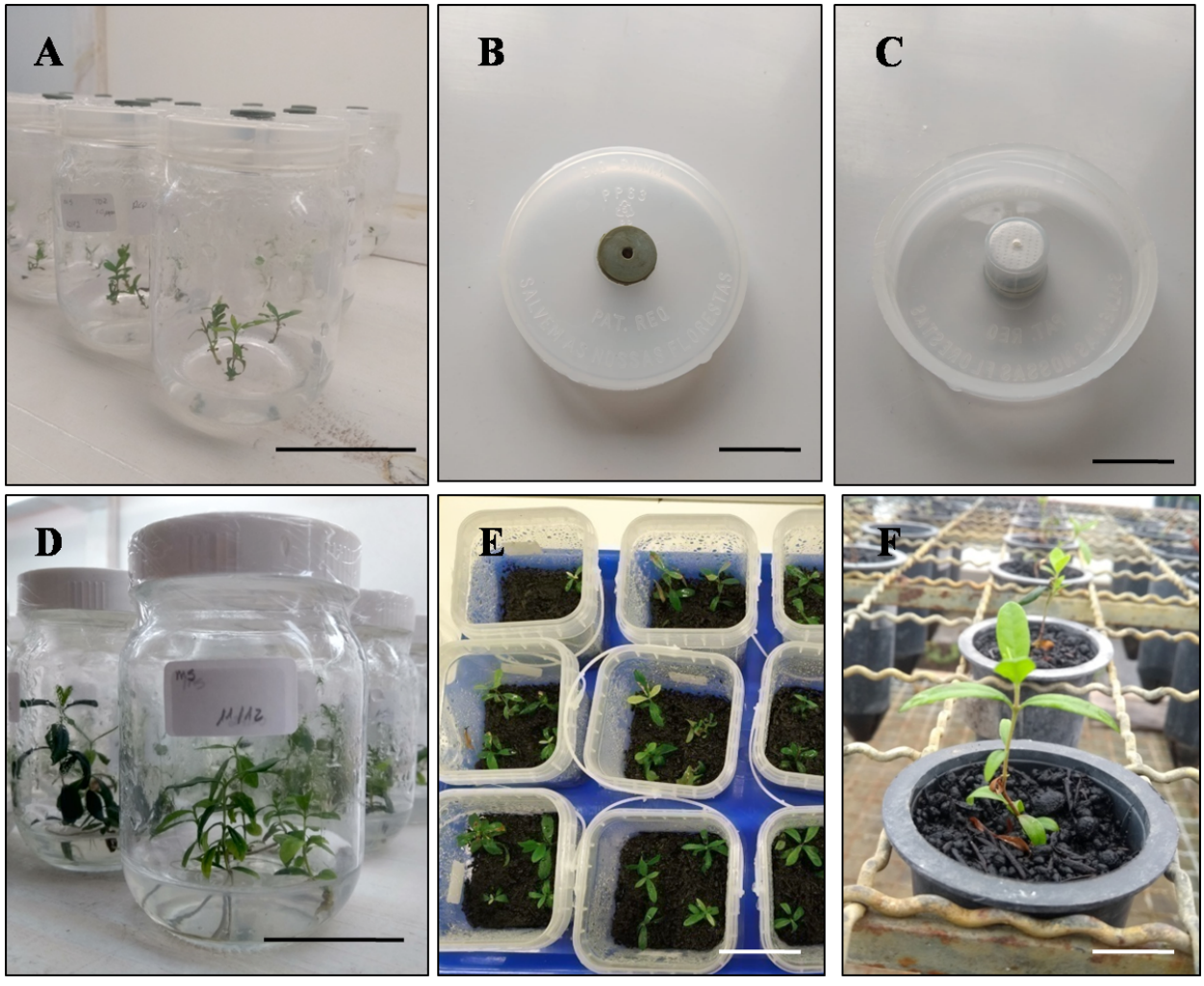

Figure 1: Micropropagation of *Acca sellowiana* (O.Berg) Burret. A. Microshoots in an *in vitro* natural ventilation system. B. Top view of the lid with natural ventilation hole. C. Bottom view of cover with rubber hole and filter in natural ventilation system. D. Microshoots developed in a conventional *in vitro* culture system. E. Plants regenerated in pre-acclimatization with substrate. F. Plants regenerated in *ex vitro* acclimatization. Bars A=3 cm; B-C= 2 cm; D= 3 cm; E-F= 1 cm.

The regenerated plants were transferred to the greenhouse (Figure 1f), with automatic irrigation and placed in plastic tubes (280 cm^3) containing commercial substrate (Mecplant, Telêmaco Borba, PR, Brazil). After 90 days of acclimatization, the random removal of plants was carried out with subsequent collection of their leaves for morphoanatomical studies. The greenhouse is located in Curitibanos, Santa Catarina, Brazil, under coordinates 27°16'60''S and 50°35'07''W, level 987m, and a humid subtropical Cfb climate with mild summers.

For *in vivo* culture, young plants (aged 6 months), from germinated seeds in a nursery, were kept in plastic tubes (280 cm^3) containing commercial substrate (Mecplant, Telêmaco Borba, PR, Brazil) under greenhouse conditions, with automatic intermittent irrigation. Leaf collections were carried out on healthy leaves, from the second and third nodes.

Leaf anatomy

Comparisons of anatomical and morphometric characteristics were performed between leaves *in vitro* CS and NV conditions, and between *ex vitro* and *in vivo* leaves in CS. Fragments of the middle third of leaf blades (0.5 cm^2) were fixed in FAA 70 (Johansen, 1940) and preserved in 70% ethanol. For the analysis of stomatal density, stomatal pore size and guard cells, temporary slides were performed, observed in frontal view. For the analysis of cuticle thickness, free-hand cross sections with the aid of a razor blade were performed and identified with Sudan IV (O'Brien; McCully, 1981). For the analysis of epidermis thickness on both sides, and the thickness of the palisade and spongy parenchyma permanent slides were prepared by means inclusion in historesin (Leica Historesin® Kit) and sectioning of the blocks in a manual rotating microtome (Leica®, RM2125RT, Buffalo Grove, IL) with 5 µm thickness. Slides were stained with toluidine blue (O'Brien; McCully, 1981), and evaluated in a light microscope (Olympus BX53F) with a digital image capture system (Cell Sens Standard® software). Ten replicates were performed for each type of treatment.

Quantification of leaf chlorophyll and carotenoids

The evaluation of chlorophyll *a*, *b*, total chlorophyll and total carotenoid content in leaves were performed according to the methodology described by Hiscox and Israelstam (1979) for CS and NV. Samples of 100 mg fresh leaves were used for extraction with dimethylsulfoxide (DMSO) during two hours at 65 °C, without maceration. Aliquots were used for reading in microplate UV-visible spectrophotometry (SpectraMax® Paradigm® Multi-mode Detection Platform) taking into account the optical density measured at 480 nm, 649 nm and 665 nm. To estimate the content of chlorophylls and carotenoids, the equations of Wellburn (1994) were used. Five replicates were performed for each type of treatment.

Phenotypic plasticity index

The phenotypic plasticity index was calculated for each anatomical and physiological parameter, according to the methodology of Valladares, Sanchez-Gomez and Zavala (2006), based on maximum and minimum means, expressed by the equation: PI= (M-m)/M. Where: PI= plasticity index; M= maximum mean; m= minimum average.

Experimental design and statistical analysis

The experimental design was completely randomized, consisting of ten replicates (plants) for each treatment of the anatomical analyzes and five replicates for the quantification of photosynthetic pigments. The first experiment consisted of *in vitro* culture in two ventilation systems (CS and NV) and the second experiment in conventional *in vitro* culture, *ex vitro* and *in vivo*. The data were submitted to analysis of variance and the means were comparedby the Tukey test at the 5% level of significance, using the R statistical software (Team, 2016).

RESULTS AND DISCUSSION

In vitro culture conventional system and natural ventilation

The stomatal density differed between treatments, with a higher average of stomata in the natural ventilation system when compared to the conventional micropropagation system (Table 1).

In *Annona glabra* L., higher stomatal density was also recorded in natural ventilation system, which was associated to the lower humidity inside the flasks (Deccetti et al., 2008). According to Lima-Brito et al. (2016), high stomatal density increases the conductance of gases, thus preventing photosynthesis from being limited under different environmental conditions. In the present work, the natural ventilation system resulted in increased gas exchange, thus favoring acclimatization.

Table 1: Density and stomatal dimensions in leaves of *Acca sellowiana* (O. Berg) Burret in conventional and natural ventilation system in *in vitro* culture. Abbreviations: CS= Conventional micropropagation system; NV= Natural ventilation system.

	Stomatal density (est.mm^{-2})	Stomata width (µm)	Stomata lenght (µm)	Stomatal pore width (µm)	Stomatal pore lenght (µm)
CS	597 ± 113 b	12.7 ± 0.5 a	15.7 ± 0.6 b	5.4 ± 0.6 a	9.9 ± 0.6 b
NV	711 ± 112 a	12.9 ± 0.6 a	16.3 ± 0.6 a	5.8 ± 0.6 a	10.7 ± 0.6 a
CV %	24.8	5.5	5.2	15.5	8.4

Different lowercase letters on the vertical indicate statistically significant differences by ANOVA – F.Mean ± SD (Standard deviation).

The width of the stomata and stomatal pore (Table 1) did not show statistical differences, however, the length of the stomata and stomatal pore were greater in a natural ventilation system. Similar results were observed in the *in vitro* culture of *Eugenia dysenterica* in a natural ventilation system, with larger stomata length dimensions (Silveira et al., 2019). An increase in pore length was recorded in *Juglans regia* L. in an *in vitro* culture system with a reduction in the relative humidity inside the flasks with the use of saline osmotic agent (Asayesh et al., 2017). The elliptical shape of the stomata demonstrates its functionality; the more rounded the stomata, and the less functional it became (Khan et al., 2003). In this sense, the closer the environment is to the natural one (*ex vitro*), the more important this functionality becomes, preventing the desiccation of the micropropagated material. Plant stomata under the conventional *in vitro* culture system are malformed, more rounded, reduced in number and unable to function, making the plants more susceptible to dehydration in the acclimatization phase (Pospísilová et al., 2007). *A. sellowiana* stomata are distributed exclusively on the abaxial surface (Figure 2 a-b), defining them as hypostomatic leaves. The stomata have subsidiary cells arranged radially around the guard cells, classifying them as the actinocytic type, as described by Hosney et al. (2018), and are arranged above the level of other ordinary epidermal cells (Figure 2d).

Plants grown under a natural ventilation system presented stomata with quick closing in environments outside the culture flasks with reduced relative humidity (Deccetti et al., 2008). The present study shows the characteristic of less rounded *A. sellowiana* stomata in the natural ventilation cultivation system, suggesting better functionality. Larger stomatal pore dimensions and stomatal density in a natural ventilation system should constitute a strategy of *A. sellowiana* to promote increased carbon dioxide uptake and stomatal transpiration.

The thickness of the leaf blade, epidermal cells on both faces did not show significant differences (Table 2). The palisade and spongy leaf parenchyma (Figure 2c and d) in a conventional system were thicker when compared to the natural ventilation system. The *in vitro* cultured of *Dianthus caryophyllus* L. showed similar results to the present work, with chlorophyll mesophyll cells occupying a larger area in normal plants grown in a non-ventilated environment (Majada; Tadeo; Sanches-Tmés, 2000). However, in *Eugenia dysenterica* leaves in a natural ventilation system, the thickness of the mesophyll and parenchymal tissues did not vary (Silveira et al., 2019).

In the present work, under natural ventilation system the cuticle layer was thicker on the abaxial surface than in the conventional system (Table 2). These results are in accordance to those observed on cauliflower (Zobayed; Armstrong; Armstrong, 2001) and clove (Majada; Tadeo; Sanches-Tmés, 2000), where the authors found thinner cuticular layers in a conventional culture system. The thicker cuticle layer in ventilated environments was attributed to the low relative humidity inside the bottle (Zobayed; Armstrong; Armstrong, 2001).

The xylem perimeter in *A. sellowiana* was smaller in plants grown in natural ventilation system. The data found in the present study differ from those obtained for *Dianthus caryophyllus* L. in which the cultivation in a ventilation system promoted an increase in the number of cell layers of xylem and phloem (Majada; Tadeo; Sanches-Tmés, 2000).

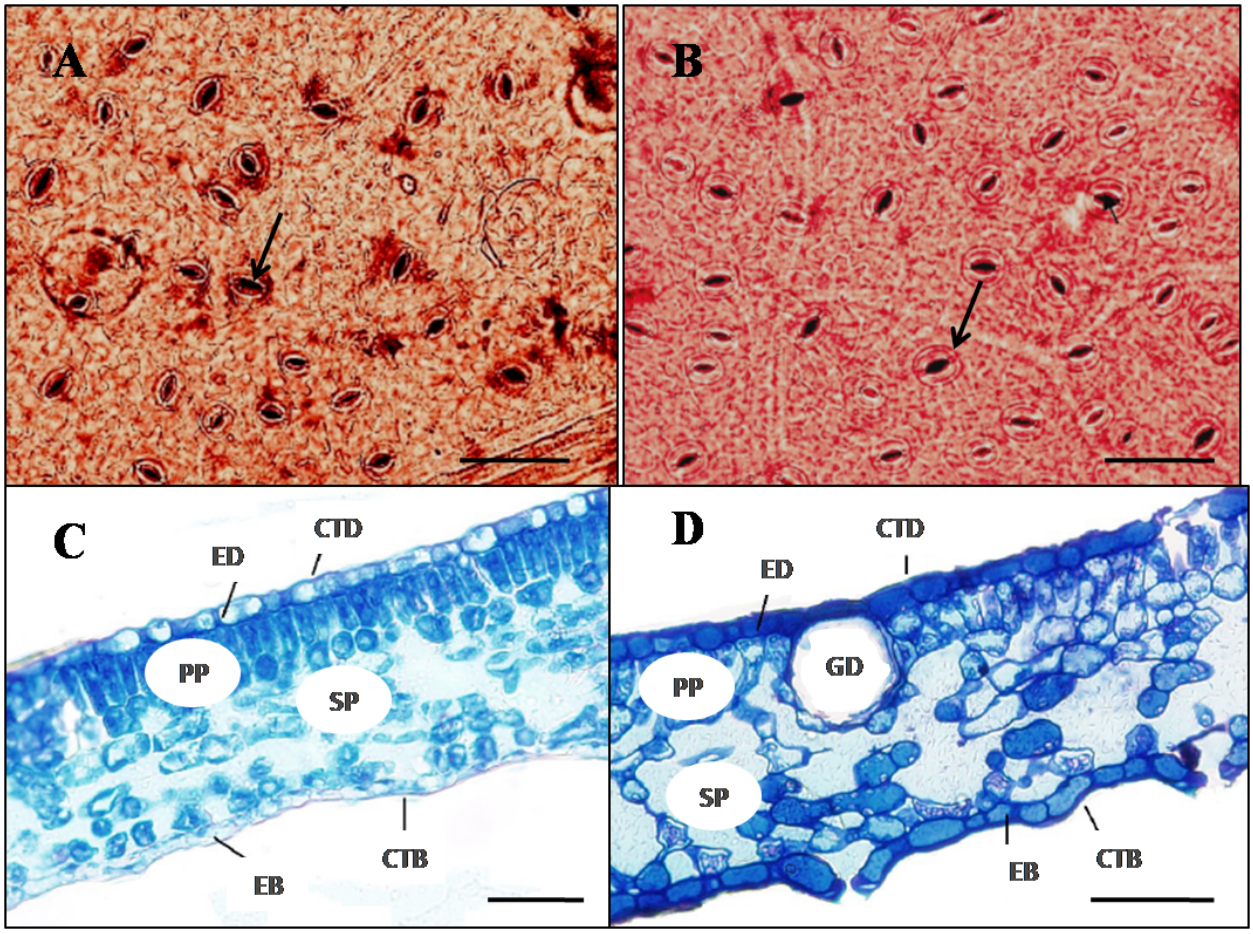

Figure 2: Leaf blades in *in vitro* culture of *Acca sellowiana* (O. Berg) Burret. **A.** Frontal view of abaxial face in paradermal section in the conventional *in vitro* culture system; arrow indicating stomata. **B.** *In vitro* natural ventilation system; arrow indicating stomata. **C.** Cross section in the conventional *in vitro* culture system. **D.** Natural ventilation system. Abbreviations: ED= epidermis on adaxial face; CTD= cuticle on adaxial face; EB= epidermis on abaxial face; CTB= cuticle on abaxial face; PP= palisade parenchyma; SP= spongy parenchyma; GD= glandular duct. Bars a-b= 40 µm; c-d= 20 µm.

Table 2: Morphometric characteristics of leaf tissues of *Acca sellowiana* (O. Berg) Burret in conventional system and natural ventilation system in *in vitro* culture. Abbreviations: CS= Conventional micropropagationsystem; NV= Natural ventilation system; LBT= Leaf blade thickness; ABET= Abaxial epidermis thickness; ADET= Adaxial epidermis thickness; PPT= Palisade parenchyma thickness; SPT= Spongy parenchyma thickness; ADCT= Adaxial cuticle thickness; ABCT= Abaxial cuticle thickness; XP= Xylem perimeter.

	LBT (µm)	ABET (µm)	ADET (µm)	PPT (µm)	SPT (µm)	ADCT (µm)	ABCT (µm)	XP (µm)
CS	63.8 ± 7.8 a	4.7 ± 0.8 a	6.2 ± 1.3 a	15.6 ± 2.9 a	38.5 ± 6.6 a	1.2 ± 0.3 a	0.9 ± 0.2 b	1365 ± 451 a
NV	59.9 ± 13.7 a	5.2 ± 1.4 a	6.9 ± 1.9 a	13.9 ± 2.8 b	34.0 ± 10.7 b	1.4 ± 0.6 a	1.1 ± 0.4 a	967 ± 394 b
CV %	16.5	19.9	21.4	16.6	21.6	33.3	29.7	39.9

Different lowercase letters on the vertical indicate statistically significant differences by ANOVA-F. Mean ± SD (Standard deviation).

The results obtained in the present study with the natural ventilation system showed smaller palisade and spongy parenchyma, were also correlated with increased stomatal density and larger stomata size, which was associated with greater water loss in this culture system. According to Majada, Tadeo and Sanches-Tmés (2000), inside the ventilated flasks there are gradients in the humidity of the air. This loss of water could lower the turgor pressure of the cells, causing a smaller volume (thickness) and perimeter of the xylem, as well as a smaller number of cells formed during its morphogenesis. The changes in anatomy observed in feijoa leaves can be justified in terms of the environment that regulates the flux of water (Fahn, 1982).

The chlorophyll *a* content in the natural ventilation system showed higher concentration as compared to the conventional system (Figure 3), while the chlorophyll *b* content, total chlorophylls and total carotenoids were not statistically different. Similar results were observed in *Eugenia dysenterica* (Silveira et al., 2019), and potato (Mohamed; Alsadon, 2010), in which higher synthesis of chlorophylls in a natural ventilation system was reported. Accumulation of pigments in plant leaves under a natural ventilation system is a result of greater photosynthetic activity, considering that the greater uptake of CO_2 stimulates photosynthesis (Anyia; Herzog, 2004).

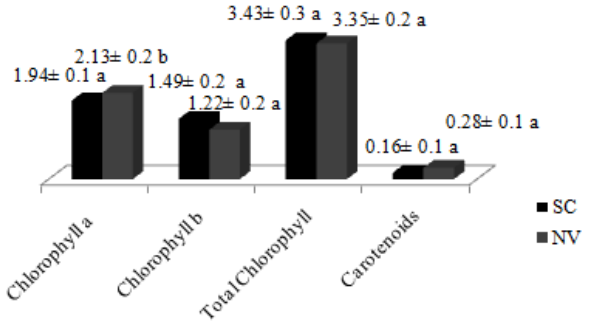

Figure 3: Concentration of photosynthetic pigments in *Acca sellowiana* (O.Berg) Burret leaves in conventional system and natural ventilation system in *in vitro* culture. Values in mg g^{-1} FM. Different letters indicate significant differences by the ANOVA- F. (Mean± SD).

In vitro culture in conventional system, *ex vitro* and *in vivo*

Stomatal density was higher in *ex vitro* and *in vivo* cultures as compared to *in vitro* cultures in the conventional system (Table 3) (Figure 4a, b and c). The stomatal density in the transition from *in vitro* to *ex vitro* conditions reveals high plasticity in plants (Asayesh et al., 2017). Several studies compared the effects of acclimatization after *in vitro* culture, and similar results with increased stomatal density in *ex vitro* acclimatization and *in vivo* plants were recorded in *Azadirachta indica* (Rodrigues et al., 2020), *Piper aduncum* (Maciel et al., 2014), and *Bactris gasipaes* (Batagin-Piotto et al., 2012). In the present study, the acclimatized plant revealed stomata density similar to the *in vivo* plant, demonstrating the plant's ability to adapt to the new environment and favor survival in the micropropagation process.

Stomatal width, length and pore length were smaller in *ex vitro* as compared to *in vitro* culture, and the stomatal pore width was not altered in the transition from *in vitro* to *ex vitro* conditions (Table 3). The ability to control water loss by reducing stomatal size in the transition from *in vitro* to *ex vitro* culture is critical for survival (Pospísilová et al., 2007), and some species showed similar responses to those obtained in this study regarding the reduction in stomata dimensions in *ex vitro* culture, as recorded in six *Annona* species (Santana et al., 2008), and two *Piper* species (Maciel et al., 2014). In the present study the transition from *in vitro* to *ex vitro* culture induced a leaf adaptation in order to increase stomatal density and reduce stomatal size. The reduction in stomatal dimensions should increase the resistance to the passage of water through transpiration, increasing the capacity to capture carbon dioxide and avoiding water stress.

In the present work, epidermis thickness on the abaxial and adaxial face was smaller in *in vitro* culture than in the other cultures (Table 4). The dimensions of the epidermal layers are related to the environmental conditions (Javelle et al., 2011), which may vary with the increase in luminosity (Dickison, 2000). The increase in epidermal cells thickness and cuticle in *ex vitro* culture in the present work should help to reduce light radiation transmittance to the mesophyll, maintaining adequate levels for photosynthetic processes.

Our results showed that leaf blade thickness, mesophyll, palisade and spongy parenchyma were smaller in *in vitro* culture, and greater in leaves in *ex vitro* and *in vivo* cultures (Table 4). Prominent intercellular spaces are one of the characteristics that can be found in the parenchyma of *in vitro* cultured plants (Campostrini; Otoni, 1996). Also,

Table 3: Density and stomatal dimensions of *Acca sellowiana* (O. Berg) Burret leaves *in vitro*, *ex vitro* and *in vivo* culture.

	Stomatal Density (est. mm^{-2})	Stomata Width (µm)	Stomata Lenght (µm)	Stomatal Pore Width (µm)	Stomatal Pore Lenght (µm)
In vitro	597 ± 113 b	12.7 ± 0.5 a	15.7 ± 0.6 a	5.4 ± 0.6 a	9.9 ± 0.6 a
Ex vitro	1141 ± 281 a	12.1 ± 0.8 b	14.8 ± 0.7 b	5.0 ± 0.8 ab	9.1 ± 0.9 b
In vivo	1232 ± 301 a	11.9 ± 0.7 b	15.5 ± 0.7 a	4.5 ± 0.7 b	9.9 ± 0.7 a
CV%	32.4	5.6	6.6	21.4	13.6

Different lowercase letters on the vertical indicate statistically significant differences by the Tukey test at 5%. Mean ± SD (Standard deviation).

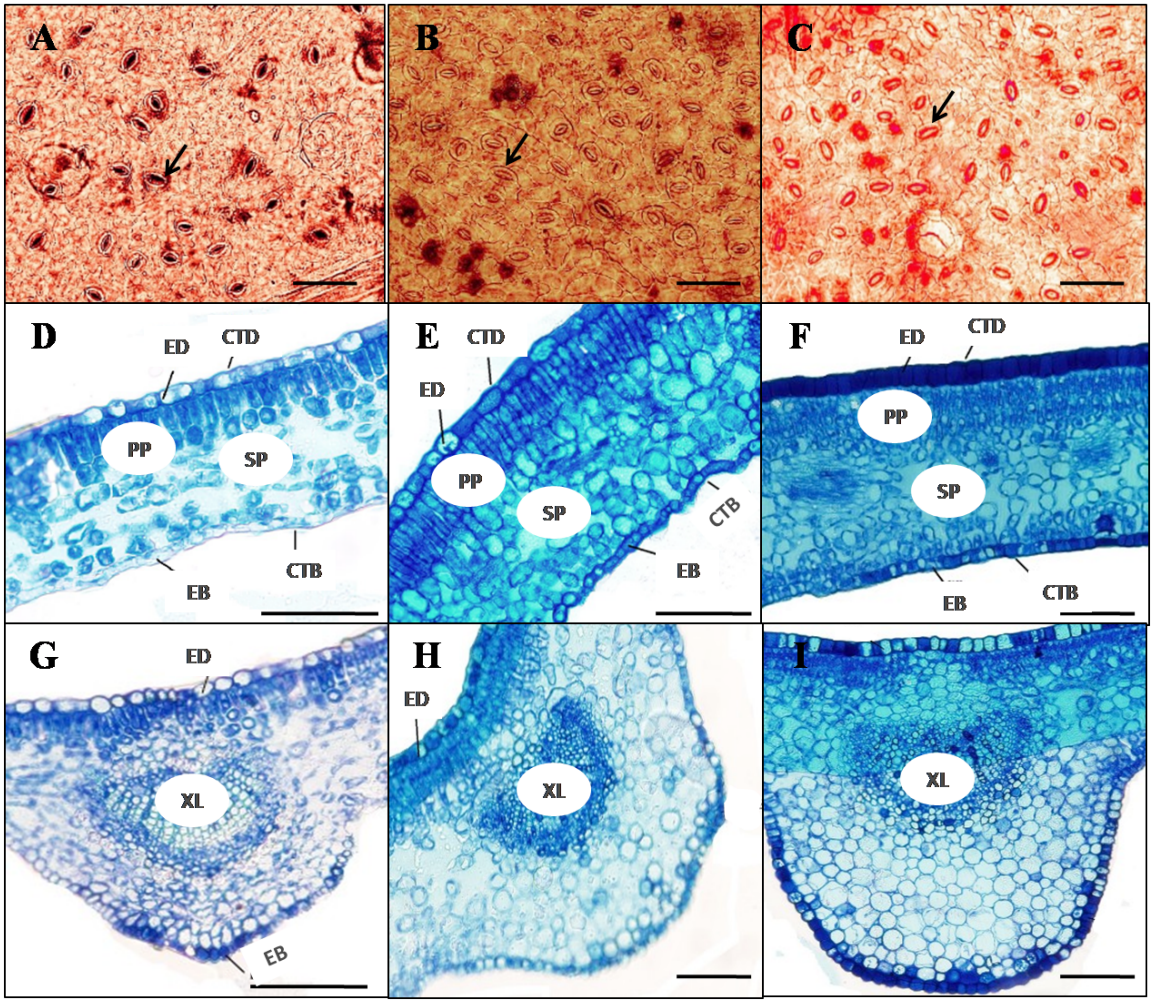

Figure 4: Leaf anatomical characteristics of *Acca sellowiana* (O.Berg) Burret *in vitro*, *ex vitro* and *in vivo* culture. A. Frontal view in paradermal section *in vitro*. B. Frontal view in paradermal section *ex vitro*.C. Frontal view in paradermal section *in vivo*; arrow indicating the stomata. D. Cross-section in the intercostal region *in vitro*. E. Cross-section in the intercostal region *ex vitro*. F. Cross-section in the intercostal region *in vivo* cultures. G. Cross section of the midrib region *in vitro*. H. section of the midrib region *ex vitro*. I. Cross section of the midrib region *in vivo* cultures. Abbreviations: ED= epidermis on the adaxial face; CTD= cuticle on the adaxial face; EB= epidermis on the abaxial face; CTB= cuticle on the abaxial face; PP= palisade parenchyma; SE= spongy parenchyma; XL=xylem. Bars A-C= 40 µm; D-I= 50 µm.

in the present work *ex vitro* and *in vivo* culture, the mesophyll tissues were more compacted (Figure 4). Literature refers to the compactation of the palisade parenchyma due to the effects of luminosity, and in the development of the palisade parenchyma, and the elongation of these cells can help its photosynthetic capacity (Oguchi; Hikosaka; Hirose, 2003). The extension of these chlorophyll cells results in closer proximity to the chloroplasts in the plasma membrane, increasing the diffusion of carbon dioxide (Flexas et al., 2012).

The palisade parenchyma in leaves *in vitro* culture was composed of a stratum of poorly elongated and compacted cells (Figure 4d). In *ex vitro* and *in vivo* cultures, the palisade parenchyma was 2-3 stratified, with more elongated cells in the adaxial-abaxial direction and more compacted (Figure 4e and f). The cuticle was observed in all treatments, although in *ex vitro* culture it was more expressive than in other cultures (*in vitro* and *in vivo*).

The cuticle thickness on the adaxial and abaxial faces was greater in *ex vitro* culture and smaller *in vitro* culture (Table 4). This reduction may be linked to the high humidity inside the bottle (Batagin-Piotto et al., 2012). The cuticle is of essential importance in the pre-acclimatization process, helping to control water loss in transpiration (Yeats; Rose, 2013). *In vivo* culture, cuticle thickness on both sides was smaller than in *ex vitro* culture. The results of the present work indicate that switching from *in vitro* environment to *ex vitro* one causes an abrupt change in humidity and solar radiation, resulting in a morphophysiological adjustment to compensate for these different abiotic factors.

Xylem perimeter area (Figure 4g, h and i) was smaller in leaves in *in vitro* culture and larger in *ex vitro* culture. Environmental factors can directly affect the dimensions and the arrangement of vascular elements and these changes are directly related to some stress (Alves; Angyalossy-Alfonso, 2000), such as the transition from *in vitro* to *ex vitro* culture. In the present study when the plant moved from *in vitro* culture, which is an environment with saturated relative humidity, to the *ex vitro* condition, there was an increase in xylem perimeter area. The increase in xylem vessels number contributes to the increase in the assimilation and transpiration capacity of the plant, which can help the plant's productivity (Noblin et al., 2008).

Phenotypic Plasticity Index

The analysis for the phenotypic plasticity index (PI) in *A. sellowiana* leaves (Table 5) indicated that the stomatal density and xylem perimeter area showed the highest index (0.52), while the stomatal pore length had the lowest index (0.003). Among the values related to stomata, stomatal density and stomatal pore width had the highest indexes (0.52 and 0.16, respectively). Regarding to tissue thickness, the xylem perimeter (0.52), mesophyll, palisade and spongy parenchyma showed the highest indexes, the last three with a value of 0.48.

In plant micropropagation studies, the analysis of tissue phenotypic plasticity has not been reported. In studies of young plant growth at different levels of luminosity of *Eucalyptus dunnii* and *Eucalyptus benthamii*, also Myrtaceous, the highest phenotypic plasticity index occurs in stomatal density, palisade and spongy parenchyma and leaf blade (Bireahls; Fermino Junior, 2020). According to Valladarres, Sanchez-Gomez and Zavala (2006) are considered very plastic parameters with PI above 0.6 and non-plastic parameters close to zero.

Table 4: Leaf blade thickness, epidermal cells, mesophyll, palisade and spongy parenchyma, cuticle and xylem area perimeter for *Acca sellowiana* (O.Berg) Burret leaves *in vitro*, *ex vitro* and *in vivo* culture. Abbreviations: LBT= Leaf blade thickness; ABET= Abaxial epidermis thickness; ADET= Adaxial epidermis thickness; MT= Mesophyll thickness; PPT= Palisade parenchyma thickness; SPT= Spongy parenchyma thickness; ADCT= Adaxial cuticle thickness; ABCT= Abaxial cuticle thickness; XP= Xylem perimeter.

	LBT (µm)	ABET (µm)	ADET (µm)	MT (µm)	PPT (µm)	SPT (µm)	ADCT (µm)	ABCT (µm)	XP (µm)
In vitro	63.8 ± 7.8 b	4.7 ± 0.8 b	6.2 ± 1.3 b	53.0 ± 7.3 b	15.6 ± 2.9 b	38.5 ± 6.6 b	0.9 ± 0.1 b	1.2 ± 0.1 b	1365 ± 251 c
Ex vitro	116.6 ± 22.3 a	6.6 ± 1.2 a	8.4 ± 1.3 a	102.0 ± 22.3 a	29.6 ± 9.9 a	72.9 ± 16.9 a	1.0 ± 0.1 a	1.4 ± 0.2 a	1700 ± 422 b
In vivo	120.9 ± 17.8 a	6.7 ± 1.1 a	10.3 ± 1.2 a	100.0± 20.3 a	30.0 ± 7.3 a	73.5 ± 15.0 a	0.8 ± 0.1 b	1.1 ± 0.1 b	2858 ± 438 a
CV %	30.4	19.4	24.4	32.8	38.0	33.0	22.2	20.8	39.3

Different lowercase letters on the vertical indicate statistically significant differences by the Tukey test at 5%. Mean ± SD (Standard deviation).

Table 5: Phenotypic Plasticity Index - PI of morphometric characteristics in leaves of Acca sellowiana (O. Berg) Burret developed *in vitro*, *ex vitro* and *in vivo* culture.

Parameters evaluated	PI
Xylem perimeter area	0.52
Stomatal density	0.52
Mesophyll	0.48
Palisade parenchyma	0.48
Spongy parenchyma	0.48
Leaf blade	0.47
Adaxial epidermis	0.40
Abaxial epidermis	0.29
Adaxial cuticle	0.22
Stomatal pore width	0.16
Abaxial cuticle	0.13
Stomatal width	0.06
Stomatal lenght	0.01
Stomatal pore lenght	0.003

In this context, in leaves of *A. sellowiana* parameters such as stomatal density (0.52), xylem perimeter area (0.52), followed by mesophyll, palisade and spongy parenchyma with an index of 0.48 both, can be considered plastic tissues. The high plasticity demonstrated in these tissues in *A. sellowiana* leaves indicates greater adaptability in the aspects of water and light availability in the transition from *in vitro* to *ex vitro* culture. Species with a higher plasticity index are more likely to survive in different environments, due to their ability to develop morphological, physiological and also biochemical adaptations (Valladares; Sanchez-Gomez; Zavala, 2006).

CONCLUSIONS

The natural ventilation system promotes morphophysiological adjustments in the plant that should favor the transition from *in vitro* to *ex vitro* culture (acclimatization). The contents of chlorophyll *a* in leaves in the natural ventilation system in *in vitro* culture indicate an important physiological adjustment in the resumption of photosynthetic activity for *ex vitro* transition and full autotrophy.

The dimensions of the stomata and stomatal pore, the mesophyll tissues, the thickness of the cuticle and both faces and the perimeter of the xylem increased in the transition from *in vitro* to *ex vitro* culture.

Micropropagation systems of *A. sellowiana* promote structural changes in leaves. The stomatal density, xylem area perimeter, palisade and spongy parenchyma are considered plastic tissues, indicating greater adaptability in water and light relationships in the transition from *in vitro* to *ex vitro* culture.

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