



# Shoot proliferation, leaf anatomy and pigment content of *Eugenia dysenterica* growing in conventional and natural ventilation systems<sup>1</sup>

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10.1590/0034-737X201966050005

## ABSTRACT

Natural ventilation micropropagation systems differ from conventional systems by allowing gas exchange between the internal and external media of the culture flask. The objective of this work was to compare the conventional system of *in vitro* propagation of *Eugenia dysenterica* (Mart.) DC. with a natural ventilation system. For shooting, explants were inoculated into test tubes with conventional lids and natural ventilation lids (NV), and WPM medium supplemented with four concentrations levels of 6-benzilaminopurine (BAP). The position of explants in each plant was considered by identifying buds as more proximate to the root, or to the apex, or in an intermediate position. Plant growth in the natural ventilation system was superior to that in the conventional system, with a 448.53% increase in leaf number, 85.64% increase in chlorophyll *a* levels and 74.90% increase in chlorophyll *b* levels. Plants with intermediate buds exhibited better results. Foliar anatomical analysis revealed abnormalities in leaves grown in the conventional system such as “giant” stomata, which remain always open. Our results indicate that the use of natural ventilation improves the water loss regulation capacity in micropropagated *E. dysenterica* plants and may favor plants survival and growth after transference to the natural environment.

**Keywords:** cagaiteira; *in vitro* growth system; natural ventilation; plant tissue culture.

## INTRODUCTION

*Eugenia dysenterica* (Mart.) DC. (popularly known as cagaiteira) is a fruit tree species that occurs in Cerrado regions (Brazilian savanna) and has the potential for economic use. Exploited extractively, its main use is in the commercialization of its by-products such as ice cream, sweets and juices (da Silva *et al.*, 2017). The species also possesses medicinal properties, being used as an antioxidant (Takao *et al.*, 2015); a laxative, from fruit consumption (Silva *et al.*, 2015); and antidiarrheal, from leaf extract consumption (Galheigo *et al.*, 2016), among others (Lima *et al.*, 2010; Lima *et al.*, 2011; Souza *et al.* 2012).

The period of fruiting of *E. dysenterica* coincides with the beginning of the first rains after drought in Brazilian savannah regions. However, the seed viability period is considered short (approximately 50 days), which characterizes the seed recalcitrance. (Farias Neto *et al.*, 1991; Andrade *et al.*, 2003). Thus, the conventional production of seedlings of this species at any time of the year becomes infeasible, and requires alternative methods of vegetative propagation, such as micropropagation.

Micropropagation is a set of techniques that provides large-scale seedling production. If properly applied, micropropagation results in uniform individuals very similar to those from seeds and is up to a thousand times more productive than conventional propagation techniques.

Submitted on June 17<sup>th</sup>, 2019 and accepted on September 9<sup>th</sup>, 2019.

<sup>1</sup> This work is part of the first author's Master Dissertation.

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However, the application of conventional propagation to woody plants remains limited by the high cost of production, which is largely due to relatively low growth rates, significant losses due to contamination, lack of rooting and low percentages of *in vitro* plant survival after transfer to *ex vitro* (Kozai & Kubota 2001).

Thus, in addition to developing micropropagation methods, it is also necessary to improve these methods as a way to guarantee the propagation of plant material at any time of the year. Studies on propagation techniques for the native wood species of Brazil (including *E. dysenterica*) are still scarce (Dias *et al.*, 2012). This kind of propagation is a way to overcome difficulties in seed propagation and the cloning of genotypes of native forest species, thus making it possible to use the species for commercial purposes, as well as to help the conservation of forest genetic resources (Hawkes *et al.*, 2012). Considering the unbridled deforestation of the Cerrado domain (Brazilian savanna) (Brandão *et al.*, 2017), it is necessary to develop *in vitro* propagation protocols for this species in order to obtain *in vitro* conservation protocols in the future.

In the “heterotrophic” conventional micropropagation system, seedlings are stored in sealed flasks with low light intensity and no internal ventilation. Plants grown under this regime have several morphological disorders caused by the high relative humidity and the accumulation of gases inside the flasks. These morphological disorders include anatomical changes such as deformed stomata. Studies describe that the reduction of gas exchange in the culture flask may cause stomatal abnormalities, which may compromise the pigment content as well as stomatal conductance. (Monja-Mio *et al.*, 2015; Gago *et al.*, 2016). Other disorders related to the conventional system include: hyperhydricity, reduced leaves, premature death of explants and, consequently, high losses in acclimatization (Kozai & Kubota, 2005; Hazarika, 2006). Different cultivation systems have been used to increase the seedling nutritional quality, and the reduction of losses of micropropagated plants. The use of pigment quantification to monitor photosynthetic performance in plants is widespread (Baker, 2008). In the natural ventilation system, gas exchange is added to the system, thus improving the transpiration rate and, consequently, nutrient absorption. It is also known that rates of photosynthesis can be increased and significantly promoted by increasing CO<sub>2</sub> concentrations and by decreasing relative humidity in the culture vessel (Kozai & Kubota, 2005; Saldanha *et al.*, 2012). Considering that *E. dysenterica* has economic potential (Camilo *et al.*, 2013), it is necessary to develop protocols for *in vitro* propagation of the species aiming at large-scale seedling production. It also becomes necessary that these seedlings have morphological and nutritional quality, and thus, the

improvement of micropropagation techniques is necessary. This work had the objective of developing a shoot proliferation protocol for *E. dysenterica*, by comparing two *in vitro* growth conditions: a conventional and a natural ventilation system, and observing leaf anatomy and chlorophyll production.

## MATERIALS AND METHODS

### *Disinfection and germination*

*E. dysenterica* fruits were collected in October 2013 from the Coleção de Espécies Frutíferas do Cerrado of the Escola de Agronomia, da Universidade Federal de Goiás. Five hundred seeds were removed from the ripe fruits, washed and placed on absorbent paper for two days to eliminate excess moisture (Duarte, 2006). Seed coats were removed with the aid of a scalpel (Martinotto *et al.*, 2007) and the seeds were washed with running water for 20 minutes.

For decontamination, the seeds were placed in a sterilized flask and placed in a laminar flow chamber, where they were immersed in 70% alcohol for one minute. The seeds were then placed in a sterile flask with 0.5% sodium hypochlorite solution of active chlorine for 20 minutes, after which they were passed through a triple wash in autoclaved distilled water. Once decontaminated, the seeds were inoculated into test tubes containing 20 ml of WPM medium (Lloyd & McCown, 1980) supplemented with 3% sucrose (p/v) and 2.5% (p/v) of Gelsan®, and maintained in a growth room with a temperature of 25 ± 1 °C, luminosity of 42 μmol.m<sup>-2</sup>.s<sup>-1</sup> and a photoperiod of 16 h light / 8 h dark for at least ninety days and/or until the plants reached at least 100 mm in height with at least three axillary buds.

### *Shoot proliferation*

An experiment was performed with flasks with sealed lids (conventional system) and flasks with lids that allowed gas exchange with the external environment (natural ventilation system - NV). Each lid of the latter contained a 5.0 mm hole, that was covered with two layers of microporous tape and a layer of thread-sealing tape (PTFE tape). Lateral seals for both types of lids were made with two layers of PVC film (Saldanha *et al.*, 2012).

Under aseptic conditions, axillary and apical buds (0.5 mm diameter) were sectioned and inoculated in WPM medium supplemented with 3% sucrose (w/v) and exposed to treatments of four concentrations of 6-benzylaminopurine (BAP) [0.00 μM (control); 4.44 μM; 11.10 μM; and 17.76 μM] with thirty-two replicates. The juvenility of the tissue within each concentration of BAP was considered, and the position of the buds relative to the root and caulinar apex of the mother plant was identified. In this sense, each of the following types of buds had eight replicates:

- 1 - Axillary buds proximate to the root;
- 2 - Axillary buds intermediate between root and caulinar apex;
- 3 - Axillary buds more proximate to the caulinar apex;
- 4 - Apical bud.

The test tubes containing the explants were kept in a growth room under the same conditions of growth described previously for three months. The culture medium with the respective treatments was replaced every forty-five days. The height of the largest shoot, the number of shoots and the number of leaves were evaluated. When evaluating the height of the largest shoot in axillary buds, the shoot was considered from the axillary bud to the insertion of the last leaf.

#### *Leaf anatomy analysis*

In order to evaluate the influence of the conventional and natural ventilation systems on the development of the plants, a study of leaf anatomy was carried out. Leaves from buds type 2 and 3 were used. The material was fixed in FAA [37–40% formaldehyde, glacial acetic acid, and 70% ethanol (1:1:18, v/v/v)] and stored in 70% ethanol until processing. Part of this material was used for diaphanization (Johansen, 1940) and part for embedding in hydroxyethyl methacrylate (*Jung's Histo-resin* – Leica) according to the manufacturer's instructions.

The material embedded in Histo-resin (Leica Histo-resin-Leica™) was used to obtain cross-sections of 9- $\mu$ m thickness using a Leica RM2245 rotary microtome. These cross-sections were then subjected to toluidine blue (O'Brien *et al.* 1964) and were mounted in Vitral Incolor 500 Acrilex® varnish (Paiva *et al.*, 2006). The diaphanized material was placed in 0.1% aqueous safranin for 24 h, and mounted in hydrated glycerin.

The following characteristics were evaluated: total leaf blade thickness, thickness of palisade parenchyma, thickness of spongy parenchyma, stomatal frequency, stomatal index, diameter of the polar axis of the stomata and diameter of the equatorial axis of the stomata. Four replicates were used per treatment, with each replicate being considered a leaf taken from a plant. To determine thicknesses, 10 sections per leaf were used, while for stomatal analysis 20 quadrants per leaf were used.

#### *Chlorophyll and carotenoid contents*

Samples of 150 mg of leaves of *E. dysenterica* resulting from explants by conventional and natural ventilation systems were taken for the analysis of chlorophyll and carotenoids. The leaves were fragmented and conditioned in 2-mL Eppendorfs microcentrifuges. These were then immersed in 1.5 mL of acetone, sealed with Parafilm®, and

maintained in the dark under agitation (100 rpm) for 24 h at a temperature of 10 °C. The acetonic solution was removed from the tubes and subjected to spectrophotometric reading for the quantification of chlorophyll *a*, chlorophyll *b* and carotenoids. The calculation of the concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids was performed according to the equations of Lichtenthaler (1987). The experimental design was completely randomized with 2 treatments (two types of lids) and 10 repetitions.

#### *Statistical analysis*

The data were submitted to analysis of variance with a significance level of 5%. Data analysis was performed using R software (version 3.4.3) (R Core Team, 2017), while graphics were generated using Graph Pad Prism (version 5.01) (Radushev, 2007).

## RESULTS AND DISCUSSION

#### *Shoot proliferation*

In the conventional system, the best treatment as measured by the variables of height, number of shoots and number of leaves was WPM supplemented with 4.44  $\mu$ M of BAP with Type 2 buds (means of 42.00 mm, 4.33 shoots and 4.33 leaves, respectively) (Figure 1). In the natural ventilation system, the best result for the variable of height occurred in the treatment of 11.1  $\mu$ M of BAP with Type 2 buds (mean 20.66 mm), while for the number of shoots and number of leaves the best treatment was 17.76  $\mu$ M of BAP with Type 3 buds (means of 6.60 shoots and 9.20 leaves, respectively).

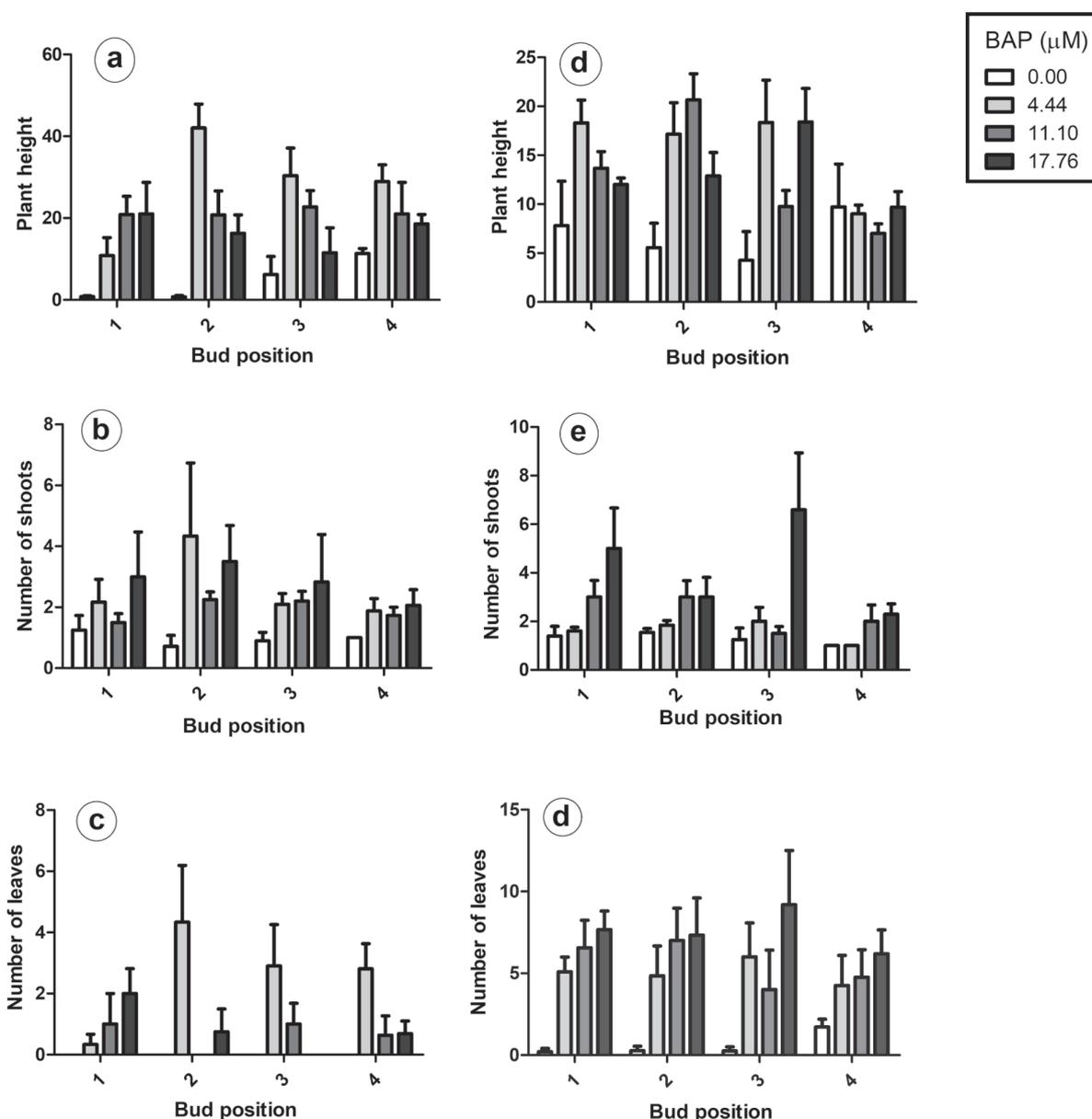
Shoots with lower mean height were reported in the natural ventilation system, compared with the conventional system. In conventional systems, the accumulation of gases such as ethylene occurs within the culture flask. Ethylene can inhibit or promote stem elongation (Suzuki & Kerbauy, 2006). In this second case, it is promoted by cell division, cell elongation, auxin transport, and auxin action. This occurs, for example, in rice, which showed a greater distance between the internodes when the stem was subjected to ethylene (Abeles *et al.* 1992; Pierik *et al.* 2006). Another factor to be considered is the fact that the growth rate of *E. dysenterica* is considered slow relative to other species (Souza *et al.*, 2002; Silveira *et al.*, 2013). It seems that the plants in flasks of the natural ventilation system probably had a tendency to behave in a manner similar to plants in normal conditions of *ex vitro* growth; the aerial part of the plants enters a state of latency for at least one year, and only after this period does it begin to grow slowly (Silveira *et al.*, 2013).

Reduced or absent leaves were observed in most of the explants in the conventional system, representing 76.37% of all the buds inoculated in this group. In contrast,

only 26% of the plants in the natural ventilation system did not produce leaves, with an increase of 448.53% in the number of leaves in relation to the number grown in the conventional system. Cytokinins can cause an increase in ethylene inside the culture flasks in heterotrophic systems (Abeles *et al.*, 1992; Chae, 2003), which can cause decrease on leaf limb, senescence (Pan *et al.*, 2016) and a decrease in chlorophyll production (Silva *et al.*, 2017). Thus, with the increase in gas exchange inside the culture flasks, the accumulation of ethylene likely did not occur, which favored the increase of leaves.

In the present study, shoots coming from intermediate positions (bud type 2 and 3) had higher means in the

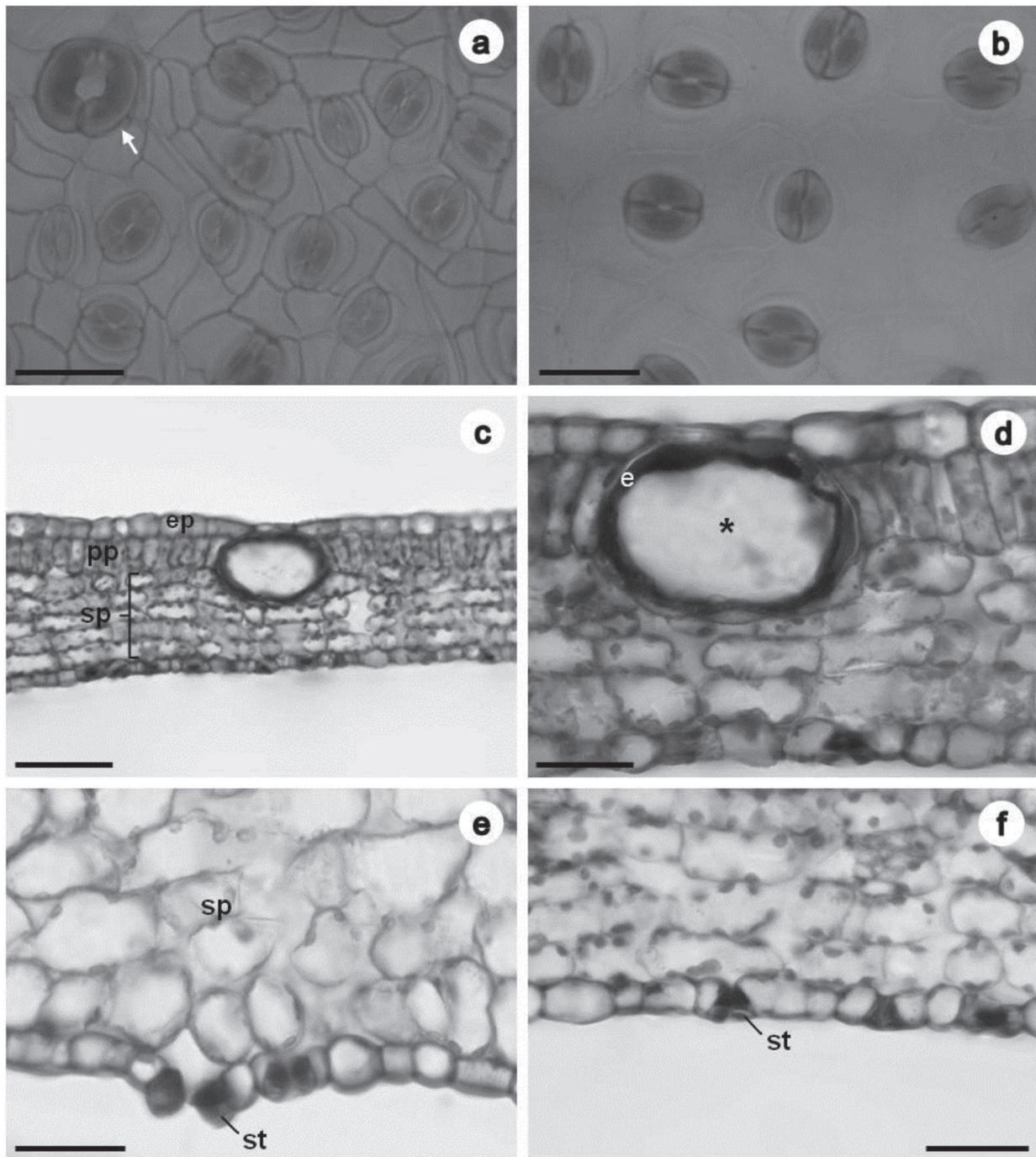
analyzed variables, in both the conventional system and the natural ventilation system (Figure 1). In shoot proliferation, juvenile explants generally have a better regeneration response under *in vitro* conditions than to mature explants (Ahuja, 1993). This is probably due to the fact that the highest concentrations of free auxins in plants are found in the apical meristems of the aerial part and in young leaves, therefore forming a gradient of concentration along the stem. (Taiz & Zeiger, 2010). Thus, the balance between endogenous and exogenous hormones and their interactions, probably contributed to promote positive results in intermediate buds. In addition, in the natural ventilation system with gas



**Figure 1:** Means for heterotrophic (a, b, and c) and mixotrophic (d, e, and f) systems for four levels of 6-benzylaminopurine (BAP). 1 – Axillary buds proximate to the root; 2 – Axillary buds intermediate between root and caulinar apex; 3 – Axillary buds more proximate to the caulinar apex; 4 – Caulinar apex. Asterisks (\*) indicate significantly different means by the Tukey test at 95% probability.

exchange, nutrients and growth regulators were probably better absorbed by the plant because of better transpiration (da Silva *et al.*, 2014). In a study involving guava micropropagation, the bud position influenced the shoot proliferation, leading to intermediate buds with a higher percentage of shoots (Shekafandeh & Khosh-Khui, 2008). Similarly, in another study involving passion-

fruit, the authors related that plants with intermediate buds also had higher mean plant height; however, the authors did not observe differences in plant leaf number (Faria *et al.*, 2007). Intermediate buds were superior in a study of *Corylus avellana* L., in which higher percentages of viability were reported, which consequently increased the proliferation of shoots (Hand *et al.*, 2016).



**Figure 2:** Leaf blade anatomy of *Eugenia dysenterica* under heterotrophic (a, c and e) and mixotrophic (b, d and f) systems. (a and b frontal view of the abaxial face. c-f transversal section of the internodal region). a and b Stomata on the abaxial face. a "Giant" stomata (arrow). c Dorsiventral mesophyll. d Secretory cavity (asterisk) with uniseriate epithelium. e "Giant" stomata projected above the other epidermal cells. f Stomata at the same level of ordinary epidermal cells. ep - epidermis; pp - palisade parenchyma; sp - spongy parenchyma; e - epithelium; st - stomata. Bars = 50 µm (a and b, d, e and f); 100 µm (c).

### Leaf anatomy

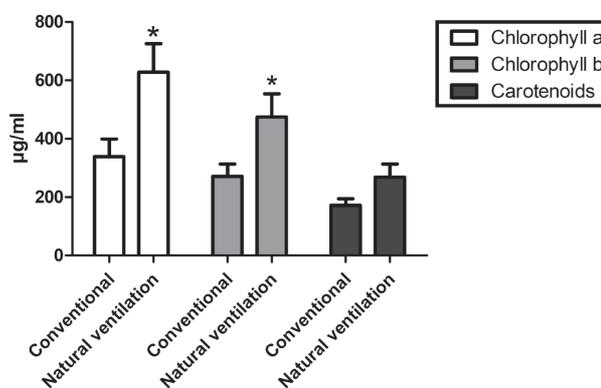
In both systems, leaves of *E. dysenterica* had a uniseriate epidermis covered by a thin cuticle and stomata on the abaxial face (Figures. 2a, 2b). Epidermal cells had slightly undulating anticlinal walls (Figures 2a, 2b), on both faces, and dorsiventral mesophyll with a single layer of palisade parenchyma and four to five layers of spongy parenchyma (Figure 2c). Tector trichomes occurred in both treatments. Secretory cavities (Figure 2d) with large lumens and uniseriate epithelium were observed in the mesophyll throughout the extension of the leaf blade.

*Eugenia dysenterica* cultivated *in vitro* possesses an organized tissue structure in both evaluated systems. However, plant leaves grown in containers from the conventional system may be thinner and have little differentiated mesophyll (Mohamed & Alsadon, 2010).

In the conventional system, some larger stomata (Figure 2a), called “giant” stomata were reported, with polar and equatorial diameters of approximately 70 x 55  $\mu\text{m}$ , respectively. These stomata contained fully opened ostioles and were different from the common stomata, as they projected above the other epidermal cells (Figure 2e). The remaining stomata of this system had mean polar and equatorial diameters of 36 x 28  $\mu\text{m}$ . In the natural ventilation system, all stomata were located at the same level as the ordinary epidermal cells (Figure 2f). The occurrence of abnormal stomata in micropropagated plants from heterotrophic systems are reported in some species, such as grapevine (Machado & Biasi 2011), apple (Chakrabarty *et al.*, 2006) and eucalyptus (Picoli *et al.*, 2008). In these species, these abnormalities are due to hyperhydricity, which causes chemical damage to the cell wall surrounding the stomata orifice, creating cell walls with less callose, that lack the characteristic orientation of cellulose microfibrils (Vasconcelos *et al.*, 2012). Thus, in culture conditions and factors associated with leaf hyperhydration seem to be linked to stomatal malfunction (remaining open) (Ziv & Ariel, 1994). Junior and Scherwinski-Pereira (2009) reported an increase in the size

of the stomata of *Tectona grandis* L. cultivated *in vitro*, which may be related to the lack of control in the mechanism of opening and closing of the stomata. Stomata that projected slightly above other epidermal cells were also observed in the leaves of *Cymbidium* Hort. grown *in vitro* (Mayer *et al.*, 2008).

The stomatal index and stomatal frequency were greater in the conventional system (Table 1). However, no differences were observed regarding the polar and equatorial diameters of the stomata, or the ratio between the two (Table 1). Additionally, no differences were reported in the thickness of the leaf blade, the palisade parenchyma or the spongy parenchyma (Table 1). For *in vitro* culture, the decrease in relative humidity with the higher gas exchange in the flask increased the transpiration rate and, consequently, the absorption of water and nutrients by the plant (da Silva *et al.*, 2014). In addition, the high relative humidity inside the culture flask reduces the development of functional stomata, which can lead to losses during acclimatization (Chandra *et al.*, 2010). Further work involving *in vitro* cultivated *E. dysenterica* hyperhydricity should be developed in the future to confirm the association of abnormal stomata with this condition.



**Figure 3:** Pigment content from leaves of *Eugenia dysenterica* under a heterotrophic system and natural ventilation. Asterisks (\*) indicate different means (within each group, represented by different colors) by the Tukey test at 95% probability.

**Table 1:** Stomata and tissue thickness for the leaf blade of *Eugenia dysenterica* DC

	Conventional system	Natural ventilation system
Stomatal frequency (3.3 mm <sup>2</sup> )	1054.4 ± 101.37 a*	678.4 ± 51.45 b
Stomatal Index	38.69 ± 2.22 a	31.62 ± 4.97 b
Stomatal - Polar length – PL (µm)	34.81 ± 1.99 a	37.72 ± 2.75 a
Stomatal - Equatorial length – EL (µm)	28.12 ± 3.89 a	28.66 ± 2.51 a
(PL)/(EL)	1.26 ± 0.12 a	1.33 ± 0.03 a
Leaf blade (µm)	194.07 ± 17.37 a	211.97 ± 20.24 a
Palisade parenchyma (µm)	102.65 ± 10.78 a	116.22 ± 15.87 a
Spongy parenchyma (µm)	53.78 ± 6.16 a	52.80 ± 3.63 a

\* Means followed by the same letters (in the row) do not differ from each other according to Tukey's test at 95% probability.

Although we report the presence of some abnormal stomata in plants from the conventional system, the high values reported for the ratio between polar diameter and equatorial diameter of the stomata (PD/ED ratio) in the two bottle-sealing conditions indicate functional stomata. According to Rocha (2005), the greater the relationship between PD and ED, the more ellipsoid the shape of the stomata, and thus the greater its functionality.

In general, a thin cuticle was observed in both culture systems. The cuticle protects the plant against excessive water loss and excess luminosity of solar radiation. However, micropropagated plants in heterotrophic systems usually have an undeveloped cuticle that is related to the high concentration of moisture inside the culture vessel (Johansson *et al.*, 1992).

### **Chlorophyll and carotenoid contents**

The natural ventilation system promoted an increase of 85.64% in chlorophyll a levels and 74.90% in chlorophyll b levels (Figure 3). This can be observed in the leaves of the treatments from the natural ventilation system, which presented intense green coloration (Figure 4).

Stomata play a very important role in regulating the entry and exit of water in plants, as well as the exchange of CO<sup>2</sup> (Jones, 1998). Most likely, the pigment

accumulation in plants of the natural ventilation system resulted from a more active photosynthetic machinery as the mixotrophic system allows for CO<sup>2</sup> interchanges stimulating photosynthesis (Morison, 1985; Anyia & Herzog, 2004). Chlorophyll increase was also observed in a strawberry (*Fragaria x Ananassa*) study using a natural ventilation system, a study in which the authors also reported root growth in plants grown under this system (Pandey *et al.*, 2015). In a study using *Pfaffia glomerata* (Spreng.), the authors reported that the natural ventilation system pigment synthesis tripled compared to the control, which was reflected in the nutritional quality of the plant (Saldanha *et al.*, 2012). The increase in pigment production was also observed in potato explants submitted to the natural ventilation system, in which a significant increase in explant dry mass was also observed compared to potatoes grown in a conventional system (Mohamed & Alsadon, 2010).

### **CONCLUSION**

It is possible to obtain an *in vitro* shoot proliferation protocol for *Eugenia dysenterica* with shoots above 20 mm in height and with a mean number of five leaves per stem. The natural ventilation system is superior at the moment in the formation of new shoots with regard to the number of shoots (greater), number of leaves per shoot (more), chlorophyll production and leaf anatomical characteristics.

The type of bud has an effective influence on micropropagation, with positive responses in plants with buds of intermediate position, regardless of the type of system — conventional or natural ventilation.

The occurrence of a greater number of stomata and the presence of stomata considered “giant” in the plants grown in the conventional system is probably associated with the difficulty of transpiration for these plants due to the high humidity accumulated in the internal environment of the flasks.

Our results indicate that the mixotrophic system can be used in future protocols for rooting *E. dysenterica*, which will contribute to the maintenance of the genetic resources of this species.

### **ACKNOWLEDGEMENTS, FINANCIAL SUPPORT AND FULL DISCLOSURE**

The authors thank Capes for their financial support in granting a master's degree.

### **CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.



**Figure 4:** *Eugenia dysenterica* shoots submitted to treatments with heterotrophic (left) or natural ventilation system (right). Bar = 1 cm.

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