Influence of flask sealing and activated charcoal on the morphogenesis and leaf anatomy of *Annona glabra* (Annonaceae) cultured *in vitro*

José Raniere Ferreira de Santana[1*], Renato Paiva[2], Lenaldo Muniz de Oliveira[1], Flávia Dionísio Pereira[3], Daniela Garcia Silveira[1] & Cristina Ferreira Nepomuceno[1]

[1] Laboratório de Cultura de Tecidos da Unidade Experimental Horto Florestal, Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, C.P. 252-294, 44031-460, Feira de Santana, Bahia, Brasil.
[2] Departamento de Biologia, Universidade Federal de Lavras, C.P. 37, 37200-000, Lavras, Minas Gerais, Brasil.
[3] Instituto Federal Goiano, Rodovia Sul Goiana, Km 01, Zona Rural, 75900-000, Rio Verde, Goiás, Brasil.

Abstract – This study evaluated the effects of the type of tube closure and the use of activated charcoal in the morphogenesis and leaf anatomy of *Annona glabra* (Annonaceae). Nodal segments of *A. glabra* were inoculated in WPM medium supplemented with 30 g l^{-1} sucrose and five concentrations of activated charcoal (0, 1, 2, 3 and 4 g l^{-1}). During *in vitro* culture, nodal segments were kept in test tubes closed in one of three ways: plastic cap sealed with PVC wrap, plastic cap without a PVC wrap and cotton plug. The results showed that the shoots obtained in tubes closed with a plastic cap without a PVC wrap or sealed with a cotton plug had increased growth compared to those obtained in tubes sealed with a plastic cap and a PVC wrap. The presence of activated charcoal caused a reduction of leaf abscission and influenced the anatomical development of the shoots in the tubes sealed with a cotton plug and sealed with a plastic cap without a PVC wrap. The highest means for leaf tissue thickness were obtained from the shoots kept in closed tubes with a plastic lid and a PVC wrap in the absence of activated charcoal.
Additional key words: *in vitro* hardening, micropropagation, tissue culture.

Resumo (Influência do fechamento dos frascos e do carvão ativado na morfogênese e anatomia foliar de *Annona glabra* (Annonaceae) cultivada *in vitro*) **–** Este trabalho buscou avaliar os efeitos do tipo de fechamento dos tubos e da utilização de carvão ativado na morfogênese e anatomia foliar de *Annona glabra* (Annonaceae). Segmentos nodais de *A. glabra* foram inoculados no meio de cultura WPM suplementado com 30 g l^{-1} de sacarose e cinco concentrações de carvão ativado (0, 1, 2, 3 e 4 g l^{-1}). Durante o cultivo *in vitro*, os segmentos nodais foram mantidos em tubos de ensaio e estes foram fechados de três formas: com tampa plástica envolvida com película de PVC, tampa plástica sem película de PVC e tampão de algodão. Os resultados mostraram que as brotações obtidas em tubos fechados com tampa plástica sem película de PVC ou fechados com tampão de algodão promoveram maior crescimento em relação às obtidas em tubos fechados com tampa plástica e película de PVC. A presença do carvão ativado promoveu a redução da abscisão foliar e influenciou no desenvolvimento anatômico das brotações dos tubos fechados com tampão de algodão ou vedados com tampa plástica sem película de PVC. As maiores médias para a espessura dos tecidos foliares foram obtidas nas brotações mantidas em tubos fechados com tampa plástica e película de PVC na ausência de carvão ativado.
Palavras-chave adicionais: aeração *in vitro*, cultura de tecidos, micropropagação.

Annona glabra L. (Annonaceae) is a fruit species that has been extensively researched due to its favorable agronomic characteristics, serving as a grafting stock for several cultivated Annonaceae (Oliveira et al. 2007) due to its phytochemical properties, with antibacterial, antifungal, insecticidal and cytotoxic effects (Padmaja et al. 1995; Oliveira et al. 2008). However, the commercial exploitation of this species as a rootstock for cultivated Annonaceae, and as a source of bioactive compounds or even for the recovery of degraded areas of vegetation has been limited by the difficulty of obtaining healthy specimens, especially in large quantities (Hoffmann et al. 1996).

*Corresponding author: raniere@uefs.br
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Clonal propagation via *in vitro* culture has been a viable alternative for the multiplication of several species (e.g., Azevedo et al. 2008; Castro et al. 2008; Schiavinato et al. 2008; Victorio et al. 2008), including *A. glabra* (Oliveira et al. 2007; Santana et al. 2008a,b). However, obtaining an efficient protocol for *in vitro* multiplication of Annonaceae on a commercial scale is still a challenge. Several studies have been conducted in order to reduce or eliminate a variety of problems found during *in vitro* culture of these species, such as endogenous contamination of the explants (Santana et al. 2006) and the high rate of early leaf abscission (Oliveira et al. 2007).

Lemos & Blake (1996) reported that the cause of higher leaf abscission is the accumulation of ethylene in the tissues in the confined *in vitro* environment, thus impairing the

shoot strength and growth of Annonaceae. Ethylene is a gas that acts as a phytohormone, playing an important role in regulating the intrinsic senescence of the whole plant. Its autocatalytic synthesis is highly stimulated by exogenous factors, such as fungal infections and/or bacterial infections, mechanical damage, water, thermal and saline stress and also by phytohormones (Winkler et al. 2002). *In vitro*, the production and action of this gas in growth containers directly affect the response of the explants, both positively and negatively. Nepomuceno et al. (2009) reported that the accumulation of ethylene is increased by some types of closures used on containers not allowing gas exchange with the external environment.

Activated charcoal has also been used successfully with some species, acting as an inhibitor of ethylene action. Concentrations between 0.1 and 2% (w/v) of activated charcoal have been described as beneficial for many species (Arena & Pastur 2001; Hazra et al. 2002; Fráguas 2003), promoting the adsorption of exudates released by the explant, which cause toxic effects on it. In addition to adsorbing exudates, activated charcoal has the property of reducing the availability of auxin in the culture medium, indirectly reducing the synthesis of ethylene (George 1993).

The reduction of leaf abscission is something that should be pursued, so that the micropropagation of this species can be carried out on a commercial scale, optimizing protocols for *in vitro* propagation of existing *A. glabra*. Therefore, our objective was to study the effect of test tube closure type and the use of activated charcoal in the morphogenesis and leaf anatomy of shoots of *A. glabra* cultivated *in vitro*.

Material and Methods

Cultivation of mother plants. Two-year-old plants of *A. glabra* were kept in a greenhouse under photosynthetic active radiation of 130–170 μmol m^{-2} s^{-1}, obtained by the combination of grolux and cool white lamps, 16-h photoperiod, without temperature control. The phytosanitary control of the mother plants was performed using the fungicide Benlate® at a concentration of 2 g l^{-1} one week before the collection of explants. The shoot apex was pruned for induction and growth of new shoots, from which the nodal segments were removed.

Disinfestation of nodal segments. The nodal segments taken from mother plants remained in a container with running water for 20 min and were then placed in a laminar flow hood and immersed in 70% alcohol for 1 min, followed by a soaking in sodium hypochlorite (1% available chlorine) with a few drops of detergent for 15 min. Finally, they were washed five times in distilled and autoclaved water.

***In vitro* cultivation.** Three types of test tube closures were evaluated (plastic cap sealed with a PVC wrap, plastic cap without a PVC wrap and cotton wool plug) and five concentrations of activated charcoal (0, 1, 2, 3 and 4g l^{-1}). WPM (Lloyd & McCown 1981) supplemented with 3% sucrose, solidified with 7 g l^{-1} agar, pH 5.7 and sterilized at 120ºC was used.

Nodal segments 15–20 mm long containing a single bud were inoculated in a test tube (25 × 150 mm) containing 20 ml of culture medium and then kept in a growth chamber at 25 ± 3ºC, photosynthetic active radiation of 45–55 mmol m^{-2} s^{-1} and a photoperiod of 16 h. After 45 days of culture, the average number of shoots, percentage of responsive explants, shoot length (mm), number of expanded leaves, length of the longest leaf (mm), leaf abscission and the percentage of explants with roots were evaluated.

The experimental design was completely randomized in a 3 × 5 factorial design (types of closure × concentrations of activated charcoal) with four replicates per treatment; each replication consisted of 10 tubes.

Anatomical study. Anatomical studies were based on microscopic examination of transverse free-hand sections of 45-days old plants. The studies were performed at the mean position of the first newly expanded leaflet from the apex of the plant, which possesses a defined anatomical structure and maximum metabolic activity (Fahn 1990). For each treatment, four plants were taken at random and, from each plant, segments of 0.5 cm^2 were extracted from the intermediate region of a leaf. These segments were cleared in 50% solution of sodium hypochlorite, and then washed in distilled water, neutralized in acetic acid : 500 and mounted in glycerin 50%. The dye used was an astra blue-safranin mix, according to Bukastsh (1972). Measurements were performed on the cross-sections to determine thickness of the abaxial and adaxial epidermis, and palisade, spongy parenchyma, and limb thickness, using a calibrated ocular micrometer scale. Sixteen measurements of each treatment were performed from the cross sections, which were taken using an Olympus model BX-60.

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Statistical analysis. The experiments were statistically evaluated by analysis of variance, and the means compared by the Tukey's test. Data on percentages were transformed into arcsine $\sqrt{\%}$ and the numbers count on $\sqrt{x+1}$. The data were analyzed using SISVAR (Ferreira 2003).

Results and Discussion

The growth of axillary buds to form vigorous shoots with no callus formation was observed in all treatments. It was also observed that the growth of axillary buds was not dependent on the supply of activated charcoal in the culture

medium, although it was highly influenced by the closure of the tubes (Figure 1).

The variable average number of shoots, percentage of responsive explants and length of the longest shoots showed similar trends, with the best results obtained in treatments of cotton plug or a non-PVC plastic cover (Figure 1A–C). Similar results were obtained by Nepomuceno et al. (2009) in culture *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb.) Altschul (Leguminosae), finding increases in shoot length when the tubes were closed with a cotton plug in comparison to PVC film. Mohamed & Alsadon (2010), working with the *in vitro Solanum tuberosum* L. (Solanaceae), observed that the plants had longer shoot length, as well as a greater number of nodules, in fully enclosed containers. However, these authors argue that cultivation in conditions that favor gas exchange results in better quality plants which will in turn help *ex vitro* establishment.

For the number of expanded leaves, no significant difference was found between the types of closure used. Tubes closed without PVC promoted on average 2 leaves per shoot, while the tubes sealed with a cotton plug or closed with PVC film promoted averages of 1.9 and 1.6, respectively (Figure 1D). These results differ from those reported by Nepomuceno et al. (2009), who observed a significant increase in the number of *Anadenanthera colubrina* leaves when the culture containers were closed with a plastic cap without a PVC wrap or a cotton wool plug.

As for the variable length of the longest leaf, the best results were obtained in tubes that were closed with a cotton plug and PVC-free caps (16.5 and 14.2 mm), respectively (Figure 1E), with no statistical difference between them. These results corroborate those found by Nepomuceno et al. (2009), who observed that the leaves of *Anadenanthera colubrina* were larger and more expanded when the tubes were closed with a cotton plug. The same occurred in culture of *Solanum tuberosum* when they were ventilated

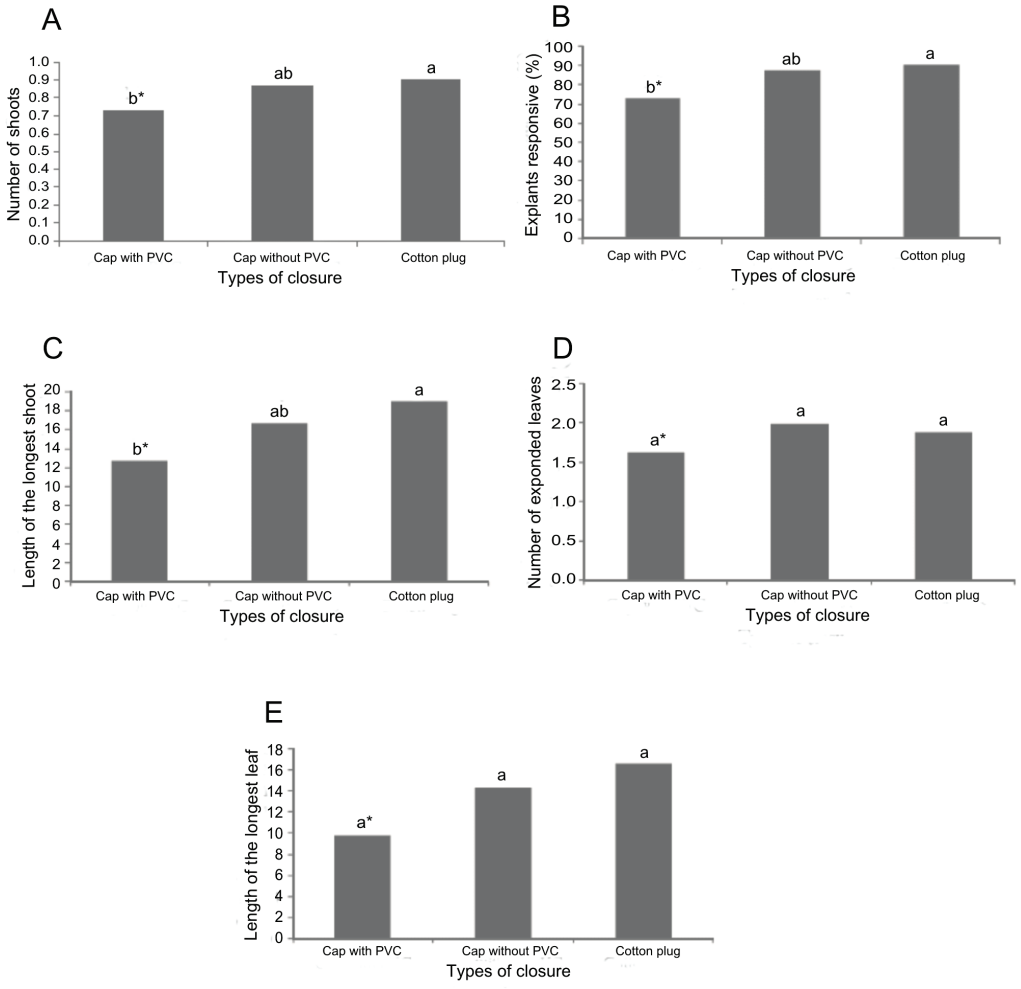

Figure 1. *In vitro* morphogenesis of *Annona glabra* as a function of the type of flask closure: **A-** average number of shoots; **B-** percentage of responsive explants; **C-** length of the longest shoot (mm); **D-** number of expanded leaves; **E-** length of the longest leaf (mm). * means followed by same letter do not differ by Tukey test.

(Mohamed & Alsadon 2010).

These results show the need for aeration in the culture flasks to obtain shoots with more developed aerial parts. According to Gonçalves et al. (2008), containers with closures that allow aeration of the cultures *in vitro* favor greater water loss than completely closed containers (restricted gas exchange), which can lead to changes in the characteristics and concentration of culture medium and therefore affect the plant growth.

For the leaf abscission variable, in the absence of activated charcoal, a significant loss of leaves in plants grown in tubes closed with plastic and PVC was observed, the abscission being controlled through the use of 2 g l^{-1} activated charcoal (Figure 2A). In contrast, leaf abscission was not observed in plants grown in tubes with closures that allowed gas exchange, which clearly shows that the accumulation of ethylene may restrict the *in vitro* cultivation of this species. Thus, plants from tubes sealed with caps without a PVC wrap or a cotton plug presented negligible leaf abscission due to the ease of gas exchange between the interior of the test tube and the external environment when using these types of closure. Zobayed et al. (2002) also obtained a decrease in leaf abscission by increasing aeration in *Annona squamosa* L. and *A. muricata* L.

It was observed that the presence of activated charcoal was a stimulus to the rooting of the explants, but a regression model adjusted for this variable was not found (Figure 2B). Studies on *Annona glabra* by Santana et al. (2008a), using culture medium with activated charcoal and the same type of closure used in this study, noted that aeration of the test tubes (caps without PVC lids or cotton plugs) provided the best results in root formation, independently of the presence or absence of sucrose in the culture medium.

The activated charcoal, although not a growth regulator, changes the composition of the medium and therefore, in some circumstances, improves or regulates plant growth *in vitro* (Figure 3A–C). Grattapaglia & Machado (1998) reported that coal simulated the dark conditions in which the roots normally do best, as well as having a dilution effect, retaining part of all the elements present in the environment, thus absorbing phenolic inhibitors of rooting. This fact explains its efficiency in the root induction of plants in *Annona glabra*, and also the reduction of leaf abscission. Similar results were found by Costa et al. (2006); by adding activated charcoal in the culture medium for the propagation of banana cv. 'Grand Naine' (AAA), they obtained better rooting of the shoots, but a smaller number of shoots per explant. Also Lemos & Blake (1996), studying early leaf abscission in explants of *Annona squamosa* cultured *in vitro* by using compounds of ethylene absorbent (activated charcoal and potassium permanganate - $KMnO_4$), achieved a significant reduction of leaf abscission.

Considering the variables analyzed in this study, it

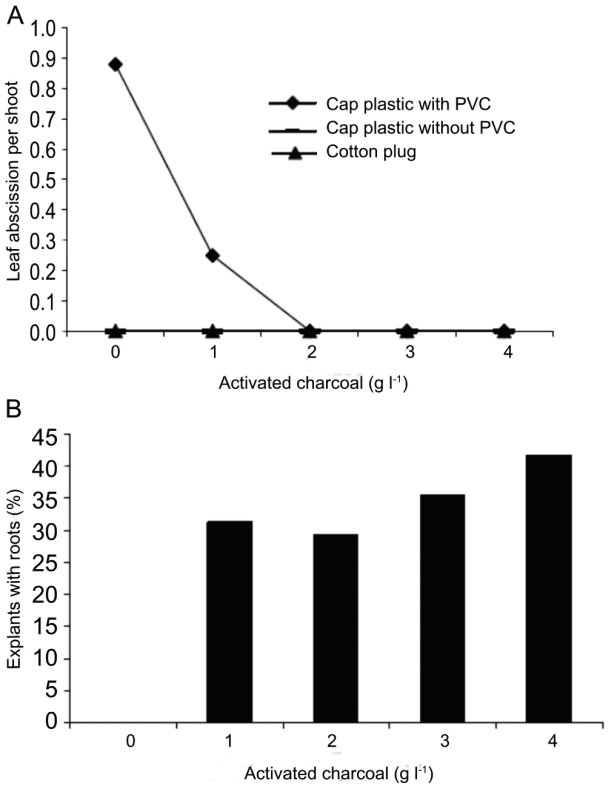

Figure 2. *In vitro* morphogenesis of *Annona glabra* depending on the concentration of activated charcoal: **A-** leaf abscission in tubes closed with plastic cap and PVC, cover with plastic cap without PVC and tubes sealed with cotton plug; **B-** percentage of explants with roots.

was found that the type of tube closure was the factor that most influenced the *in vitro* cultivation of *Annona glabra*. Closure of the tubes with a cotton plug and the plastic cap without PVC was the most appropriate, allowing gas exchange with the external environment by providing a better shoot growth.

The anatomical assessments found that the system of cultivation and the concentration of activated charcoal in the culture medium had a great influence on the variables studied. Tubes closed with a cotton plug and whose medium contained 3 g l^{-1} of activated charcoal had shoots with thicker leaves. Adaxial epidermis thickness was 34.7 µm, the abaxial epidermis 22.8 µm, palisade parenchyma 44 µm, the spongy parenchyma 81.4 µm and leaf thickness 182.9 µm. In test tubes with caps without PVC, the shoots had thicker leaves when the medium contained the highest concentrations of activated charcoal (3 and 4 g l^{-1}). Adaxial epidermis showed the greatest thickness (33.3 µm) at a concentration of 3 g l^{-1} of activated charcoal with no statistical difference using 4 g l^{-1} of activated charcoal. As for the thickness of the abaxial epidermis, palisade parenchyma, spongy parenchyma, and leaf, the best results were at a concentration of 4 g l^{-1} of activated charcoal (23,

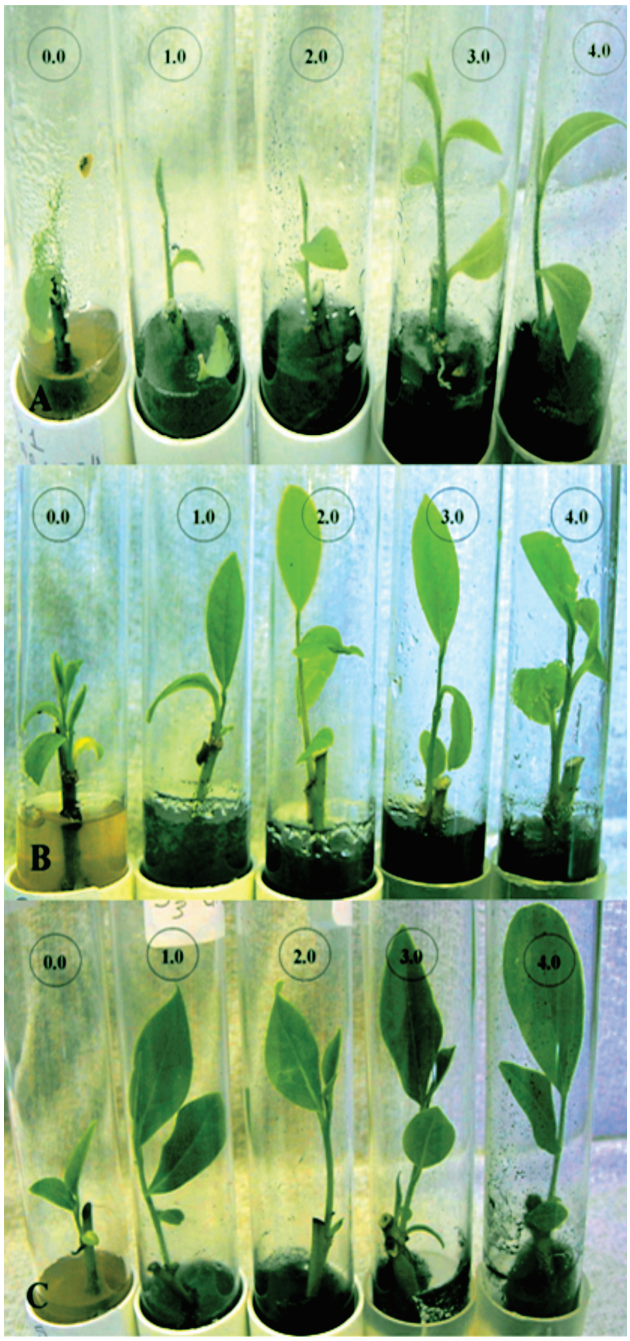

Figure 3. *In vitro* morphogenesis of *Annona glabra* depending on the concentration of activated charcoal (0, 1, 2, 3 and 4 g l^{-1}) and type of closure of culture tubes: **A-** test tubes closed with plastic caps wrapped in PVC; **B-** test tubes closed with plastic caps without PVC; **C-** test tubes closed with cotton plug.

49.4, 72.3 and 181.3 µm, respectively). However, for the thickness of the abaxial epidermis, there was no significant difference between the concentrations of 3 and 4 g l^{-1} of activated charcoal. In tubes closed with a cap plus PVC, the leaves were thicker in the culture medium without activated charcoal or when it was at a concentration of 1 g l^{-1}, except for the spongy parenchyma that showed no statistically significant difference for the concentration of 4 g l^{-1} of activated charcoal (Table 1).

It can be concluded, in general, that the higher concentrations of activated charcoal promoted increases in anatomic development of the *Annona glabra* leaves when the tubes were closed with a cotton plug or caps not wrapped in PVC. However, the highest averages were obtained when the tubes were closed with a cap plus PVC film and in the presence of 1 g l^{-1} of activated charcoal, which brought a greater blade thickness (Table 1). These data differ from studies previously reported for the same species performed by Santana et al. (2008b), who found that the greater thickness of leaf shoots was obtained when the tubes were closed with a plastic cap without PVC and grown in culture medium with activated charcoal and 4.9 µM of indolebutyric acid (IBA) in the absence of sucrose. These authors also suggested that it was necessary to improve the performance of plants grown *in vitro*, providing culture conditions that approximate those in which they grow *in vivo*, thus increasing survival rate. During *in vitro* cultivation of potatoes, when the containers were ventilated the leaves were anatomically thicker (Mohamed & Alsadon 2010), disagreeing with the findings of the present work.

Several authors have reported the advantages of encouraging anatomical development by increasing the aeration of the cultures (Leite et al. 2000; Calvete et al. 2002; Hazarika 2003; Fuentes et al. 2005; Skrebsky et al. 2006). Considering this aspect, when comparing anatomical characteristics, it appears that changes in these culture conditions are able to determine the adaptive plasticity of plants and, consequently, lead to plants anatomically and physiologically better adapted to the acclimatization process (Donnelly et al. 1985; Kozai & Kitaya 1995; Santana et al. 2008b).

For the *Annona glabra* shoots grown in tubes closed with a plastic cap and PVC combined, or not, with 1 g l^{-1} of activated charcoal, the culture medium in which favored the formation of thicker leaf, there was also greater leaf abscission, which is not favorable for the cultivation *in vitro*. On the other hand, when using the closure that allowed ventilation, there were increases in the thickness of the leaf tissues, though smaller when compared to those sealed with a plastic cap and PVC without activated charcoal or 1 g l^{-1} of activated charcoal, promoting greater number of shoots as well as higher percentage of responsive explants, increased shoot length and longer leaves.

The high leaf abscission observed in closed containers with a plastic cover and PVC, was probably caused by the buildup of volatile gases, particularly ethylene, which has a strong influence on the *in vitro* morphogenesis and induces the depletion of chlorophyll, leading to senescence and leaf abscission (Hazarika 2006). Nepomuceno et al. (2007) found that in *Anadenanthera colubrina* less leaf abscission occurred when ethylene antagonistic substances were used, making substantial

gains in the number of buds and number of shoots, showing that this gas is harmful to the *in vitro* cultivation of this species.

In cultures of *Scrophularia yoshimurae* T. Yamaz. (Scrophulariaceae), the aeration of the test tubes promoted a better shoot performance, although it reduced the number of shoots per explant (Lai et al. 2005). However, the type of test tube closure that allows gas exchange is critical during shoot multiplication phase of *Annona glabra*.

Conclusions

These results show that greater aeration of the growing containers allows for greater growth and lower leaf abscission in *Annona glabra* shoots grown *in vitro*; greater aeration reduces the thickness of the leaf tissues of shoots and the addition of activated charcoal to the culture medium reduces leaf abscission. Thus, control of morphogenesis *in vitro* can be achieved through proper management of the environmental conditions.

Table 1. Adaxial and abaxial epidermis, palisade, spongy parenchyma and leaf thickness in *Annona glabra* plants cultivated *in vitro*, depending upon the type of closure and concentrations of activated charcoal.

Type of closure	Concentrations of activated charcoal (g l^{-1})				
	0.0	1.0	2.0	3.0	4.0
Cotton plug	27.52C[y]c[z]	28.56Bbc	31.20 Ab	34.72 Aa	31.76 Ab
Cap without PVC	30.96Bab	26.24 Bc	29.60 Ab	33.28 Aa	31.04Aab
Cap with PVC	37.68 Aa	38.48 Aa	25.52Bbc	24.72 Bc	27.84 Bb
	Abaxial epidermis thickness (μm)				
Cotton plug	19.12 Bb	18.64 Bb	21.44 Aa	22.80 Aa	20.08 Bb
Cap without PVC	20.32 Bb	18.16 Bc	19.52Bbc	21.28Aab	22.96 Aa
Cap with PVC	27.20 Ab	29.44 Aa	17.52 Cc	16.73 Bc	18.96 Bc
	Palisade parenchyma thickness (μm)				
Cotton plug	36.80Bbc	34.32 Bc	39.52 Ab	44.00 Aa	38.16Bbc
Cap without PVC	35.68Bbc	31.84 Bc	38.32 Ab	38.88 Ab	49.36 Aa
Cap with PVC	42.46 Aa	41.76 Aa	31.52 Bb	34.16 Bb	34.24 Cb
	Spongy parenchyma thickness (μm)				
Cotton plug	59.20 Bb	64.16 Bb	76.96 Aa	81.36 Aa	62.08 Bb
Cap without PVC	72.96Bab	60.00 Bc	61.44Bbc	68.40Bbc	77.28 Aa
Cap with PVC	74.08Aab	82.08 Aa	55.12 Bc	65.52Bbc	77.84 Aa
	Leaf thickness (μm)				
Cotton plug	142.63Cc	145.67Bc	169.07Bb	182.87Aa	150.23Bc
Cap without PVC	159.90Bb	136.20Bc	148.90Bb	159.83Bb	181.33Aa
Cap with PVC	181.16Aa	191.78Aa	129.63Ac	141.10Cc	159.97Bb

[y] means followed by the same capital letter in each column do not differ at the probability level of 5%; [z] means followed by the same letter in each line do not differ at the probability 5% level, according to the Tukey test.

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