CYTOKININ AND FLASK SEALING AFFECT SHOOT PROLIFERATION AND *In Vitro* DEVELOPMENT OF *Jacaranda cuspidifolia* MART. MICROCUTTINGS

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ABSTRACT – Plant growth regulators (PGRs) and types of flasks sealing that allow gas exchange can favor the development of *in vitro* plant propagation systems. The objective of the present study was to evaluate the effects of cytokinin supplementation on the induction of shoot proliferation and the influence of gas exchange on the *in vitro* development of *Jacaranda cuspidifolia* Mart microcuttings. Nodal segments were cultured in medium supplemented with different concentrations (0.25; 0.5; 1.0 and 2.0 mg L^{-1}) of 6-benzyladenine (BA). In the control treatment, there was no addition of PGRs. After 30 days of culture, the microcuttings were isolated and transferred to a rooting medium supplemented with 1.0 mg L^{-1} indole-3-butyric acid. At this stage, the culture flasks were sealed with rigid polypropylene lids with a porous membrane (1 M; 21 µL L^{-1} s^{-1} CO_2) or with no membrane (0 M; 14 µL L^{-1} s^{-1} CO_2). Cytokinin supplementation induced the activation of axillary buds of *J. cuspidifolia*. The highest numbers of shoots were observed in explants cultivated in the presence of 0.5–1.0 mg L^{-1} BA. The microcuttings kept in flasks with a higher level of gas exchange (1M) had a higher percentage of rooting and greater root and shoot lengths. Microscopic analysis showed a greater differentiation of leaf tissues in plants kept in flasks with a higher level of gas exchange (1M). These plants also showed greater mesophyll thickness and, consequently, greater leaf blade thickness. The results provide new information for establishing an efficient *in vitro* propagation system for *J. cuspidifolia*.

Keywords: Cytokinin; Gas Exchange; Micropropagation.

CITOCININA E VEDAÇÃO DE FRASCOS AFETAM A PROLIFERAÇÃO DE GEMAS E O DESENVOLVIMENTO In Vitro DE MICROESTACAS DE Jacaranda cuspidifolia MART.

*RESUMO – Reguladores de crescimento (RCs) e tipos de vedação de frascos que permitem as trocas gasosas podem favorecer o desenvolvimento de sistemas de propagação **in vitro** de plantas. O objetivo do presente estudo foi avaliar os efeitos da suplementação de citocinina na indução de multibrotações e a influência das trocas gasosas no desenvolvimento **in vitro** de microestacas de **Jacaranda cuspidifolia** Mart. Segmentos nodais foram cultivados em meio suplementado com diferentes concentrações (0,25; 0,5; 1,0 e 2,0 mg L^{-1}) de 6-benziladenina (BA). No tratamento controle não houve adição de RCs. Após 30 dias de cultivo, as microestacas foram isoladas e transferidas para um meio de enraizamento suplementado com 1,0 mg L^{-1} de ácido indol-3-butírico. Nesta etapa, os frascos de cultura foram vedados com tampas rígidas de polipropileno com membrana porosa (1 M; 21 µL L^{-1} s^{-1} CO_2) ou sem membrana (0 M; 14 µL L^{-1} s^{-1} CO_2). A suplementação de citocinina induziu a ativação de gemas axilares de **J. cuspidifolia**. Os maiores números de gemas foram observados nos explantes cultivados na presença de 0,5 – 1,0 mg L^{-1} BA. As microestacas mantidas em frascos com maior nível de troca gasosa (1M) apresentaram maior porcentagem de enraizamento e maiores comprimentos de raiz e de parte aérea. A análise microscópica mostrou maior diferenciação dos tecidos foliares nas plantas mantidas em frascos*

com maior nível de troca gasosa (1M). Essas plantas também apresentaram maior espessura do mesofilo e consequentemente maior espessura da lâmina foliar. Os resultados obtidos fornecem novas informações para o estabelecimento de um sistema eficiente de propagação ***in vitro*** *para* ***J. cuspidifolia****.*

Palavras-Chave: Citocinina; Trocas gasosas; Micropropagação.

1. INTRODUCTION

Jacaranda cuspidifolia Mart (Bignoniaceae), popularly known as jacaranda de minas, caroba or caiá, is a species that occurs naturally in the south-central region of Brazil (Lorenzi, 2014). This species is used for urban landscaping and is commonly found in public areas due to the beauty of its violet flowers (Arruda, 2012; Moraes et al., 2013). The species contains chemical compounds that allow its use for the treatment of leishmaniasis (Fournet et al., 1994) and as an insecticide, depurative and antibacterial agent (Arruda et al., 2012). In addition, *J. cuspidifolia* has the potential to be exploited in the wood industry and for the recovery of degraded areas (Dutra et al., 2015).

J. cuspidifolia seedlings are produced from seeds (Lorenzi, 2014; Dutra et al., 2015). However, the variability in germination percentage (Parreira et al., 2011), the difficulty in acquiring large quatities of seeds (Debnath, 2004) and the long period of vegetative development (Gantait et al., 2011) have led to the search for alternatives propagation ways. In this context, vegetative propagation methods such as minicutting (Fauerharmel et al., 2020) and micropropagation systems (Malosso et al., 2012; Silva and Stefenon, 2014; Silveira et al., 2018) have been established for some *Jacaranda* spp due to their ornamental potential and phytochemical and ethnopharmacological relevance, contributing to their conservation and domestication (Silveira et al., 2013).

Cultivation of shoot tips and nodal segments is an important biotechnological strategy for the micropropagation and regeneration of plant tree species. The presence of pre-existing axillary buds accelerates the process of clonal multiplication and the production of *in vitro* plants on a large scale (Duclercq et al., 2011; Moura et al., 2012). The induction of shoot proliferation from shoot apex and nodal segments culture is the most common *in vitro* regeneration pathway for *Jacaranda* species (Malosso et al., 2012; Silveira et al., 2018), although somatic embryogenesis has also been induced from zygotic embryo culture of *Jacaranda acutifolia* (Bajaj, 1989). Cytokinin supplementation plays a crucial role in the induction of multiple shoots (Müller and Leyser, 2011) and therefore this plant growth regulator (PGR) is often added to the culture medium. This hormone stimulates cytokinesis and acts directly on the development of explants from the break in apical dominance, enabling the reprogramming and activation of axillary buds (Müller and Leyser, 2011) and consequently the induction of multiple shoots (Bielach et al., 2012). However, the responses of *Jacaranda* species to PGRs may vary. While *J. ulei* did not respond to the presence of cytokinin or auxin in the culture medium (Silveira et al., 2018), *in vitro* multiplication of *J. mimosifolia* was only observed in culture medium supplemented with high concentrations of cytokinin (Maruyama et al., 1993). As far as we know, no information has been obtained about the *in vitro* performance of *J. cuspidifolia*.

Conventional *in vitro* micropropagation is characterized by the addition of exogenous sources of carbohydrates to the culture medium and by the cultivation of plants in closed containers that avoid contamination and dehydration. Despite the efficiency of this technique for the large-scale production of plants with phytosanitary quality (Flôres et al., 2011; Silva et al., 2019, Silva et al., 2020), in this condition the plants are kept under high humidity and ethylene concentrations, which reduce the ability to perform gas exchange and photon flow, resulting in lower development and histodifferentiation of the plant organs (Xiao et al., 2011; Alvarez et al., 2012; Nguyen et al., 2020).

Cultivation under photomyxotrophic conditions, involving flask sealing systems that allow greater permeability of gases, has been carried out in order to minimize the changes caused by the *in vitro* environment. Thus, conditions that allow adequate rates of gas exchange can reduce the relative humidity inside the culture flasks and decrease the effects of hyperhydricity on plants. With the increase in gas exchange, the maintenance of CO_2 concentration is favored, photosynthesis is stimulated and the

ethylene concentration in the flasks is reduced (Kozai and Kubota, 2001). Currently, several models of membranes that promote higher rates of gas exchange in *in vitro* culture are commercialized (Zobayed, 2005). Microporous and polytetrafluoroethylene tapes are of low cost and have also been shown to be efficient for covering holes in culture flask lids, ensuring greater permeability of gases (Saldanha et al., 2012), which can help optimize and make the micropropagation systems viable for clonal propagation.

The objective of the present study was to determine the *in vitro* morphogenic potential of nodal segments of *J. cuspidifolia* to induce multiple shoots and to evaluate the *in vitro* development of microcuttings under different types of flasks sealing that allow gas exchange. We hypothesized that a gradual increase in cytokinin concentration in the culture medium would promote the activation and/or induction of axillary buds. Regarding the level of gas exchange, we believe that the use of an alternative system that allows greater permeability of gases will cause a general increase in the developmental parameters of *J. cuspidifolia*, associated with changes in leaf anatomy such as an increase in the thickness of photosynthetic tissues.

2. MATERIAL AND METHODS

2.1 Plant material

Seeds of *Jacaranda cuspidifolia* Mart (Bignoniaceae) were collected from adult individuals located in the municipality of Jataí, Goiás, Brazil (17º55'24'' S 51º42'58'' W). The seeds were washed in running water to remove surface residues and stored in a refrigerator.

2.2 Disinfestation and *in vitro* germination

For disinfestation, the collected seeds were immersed in 70° INPM ethyl alcohol (v/v) for 2 min, followed by a sodium hypochlorite solution (commercial bleach, Super Global®, 25% active chlorine) with the addition of two drops of Tween 20® for 15 min. The seeds were then subjected to laminar flow and washed 4 times in deionized and autoclaved water. Sixty seeds were inoculated into 250 ml flasks containing a culture medium consisting of MS salts (Murashige and Skoog, 1962), 3% sucrose, 100 mg L^{-1} inositol and 0.8% agar. The pH of the culture medium was adjusted to 5.7 ± 0.1 prior to autoclaving, which was performed for 20 min at 121°C and 1 × 105 Pa (1,1 kg cm^{-2}). Two seeds were inoculated per flask. After seed inoculation, the flasks were kept in a growth room at 25 °C ± 1 with a 16 hours photoperiod and 40 μmol m^{-2} s^{-1} irradiance.

2.3 Shoot proliferation induction

Sixty-day-old seedlings were sectioned into ± 2 cm long nodal segments which were vertically inoculated into a 250 ml flask containing MS medium, as described above, plus different concentrations (0.25; 0.5; 1.0; and 2.0 mg L^{-1}) of 6-benzyladenine (BA). In the control treatment, no PGRs were added (MS0). Each treatment consisted of 30 flasks with two nodal segments per flask. After inoculation, the flasks were kept in a growth room at 25 °C ± 1 with a 16 h photoperiod for 30 days. Subsequently, the number of shoots per explant and average length of axillary shoots per explant were evaluated.

2.4 Assessment of gas exchange levels in the growth and rooting of microcuttings

For rooting, microcuttings measuring more than 2.0 cm in length were isolated and inoculated into a 250 m flask containing MS medium, as described above, plus 1,0 mg L^{-1} of indole-3-butyric acid. Five microcuttings were inoculated per flask with two types of sealing: rigid polypropylene caps (RPC) (Vidrolabor®) without a membrane (0 M), with a CO_2 exchange rate (CO_2ER) of 14 μL L^{-1} s^{-1}, and RPC with a 10-mm orifice covered with a hydrophobic membrane (1 M) that allowed a CO_2ER of 21 μL L^{-1} s^{-1} (Batista et al., 2017). The lid hole was sealed with membranes consisting of a layer of Amanco® polytetrafluoroethylene tape between two layers of Cremer® micropore tape, as suggested by Saldanha et al., (2012). The microcuttings obtained from each cytokinin treatment were equally divided on both sealing conditions (0M and 1M). Plants grown under the 0M condition, which is the conventional *in vitro* propagation system, were used as control. Each flask sealing condition (0 M and 1M) was composed of 10 flasks. They were kept in a growth room for 60 days under the same conditions mentioned for germination. At the end of the experiment, the percentage of rooting, root length (cm), shoot length (cm), number of roots, leaves, nodes and shoots and the total plant length (cm) were evaluated.

2.5 Microscopic and micromorphometric analysis

Samples from the middle and internerval region of the third leaf of five plantlets developed under each of the flask sealing conditions (0M and 1M) were collected after 60 days of cultivation and fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18, v/v) (Johansen, 1940) for 72 hours. After this period, the samples were dehydrated through a graded ethanol series, and embedded into methacrylate resin (Historesin®, Leica Instruments, Germany). The embedded materials were transversely sectioned at 5 µm thickness with the aid of a hand-advancing rotating microtome (RM2235, 22 Leica Microsystems Inc., USA) and stained with toluidine blue, pH 4.8. Images were recorded using a Zeiss Axioskope microscope equipped with a U-Photo camera system (AxioCam HRc).

Micromorphometric analysis included three parameters: i) mesophyll thickness; ii) thickness of the epidermis on the abaxial and adaxial surfaces, and iii) thickness of the leaf blade. Each parameter was measured in triplicate using ImageJ® software (Rasband, 1997-2015).

2.6 Statistical analysis

The experiments were carried out in a completely randomized design. The data for the parameters evaluated in the experiment of shoot proliferation were submitted to analysis of variance (ANOVA) and the means were compared by Tukey's test ($P < 0.05$) when necessary. The morphometric and anatomical parameters obtained in the sealing flask experiment were submitted to the normality and homogeneity test (Bartlett), and evaluated by the F test to determine significant differences ($P < 0.05$) between the means of the two CO_2 permeability conditions. Statistical analysis was carried out using the Rbio software (Bhering 2017).

3. RESULTS

3.1 Cytokinins induce multiple shoots in *J. cuspidifolia* nodal explants

The concentration of cytokinin in the culture medium influenced the induction of *in vitro* responses in *J. cuspidifolia* microcuttings (Figure 1A-D). At 30 days of cultivation, only one axillary shoot took place in the absence of BA (MS0; Figure 1A). On the other hand, the treatments supplemented with BA induced the activation of axillary shoots of *J. cuspidifolia* in a dose-dependent manner (Figure 1B-D). The greatest number of shoots per explant was observed in the treatments supplemented with the highest concentrations of BA; concentrations of 1.0 and 2.0 mg L^{-1} BA had, on average, 3.0 and 4.2 shoots per explant, respectively (Figure 1E).

The length of the axillary shoots of microcuttings cultivated in the presence of cytokinin was reduced according to cytokinin concentration. The lowest BA concentration tested (0.25 mg L^{-1}) was the one that obtained shoots with the greatest length, although no significant differences were observed between treatments supplemented with 0.25, 0.5 and 1.0 mg L^{-1} BA (Figure 1F). The treatment supplemented with 2.0 mg L^{-1} BA induced shorter shoots (Figure 1F).

3.2 The flask sealing systems affect the growth and rooting of the microcuttings

The flasks sealing systems influenced the gas exchange between the internal and external environment and, consequently, the development of microcuttings of *J. cuspidifolia* (Fig. 2A, B). The microcuttings grown at 1M showed increments of 33.2% and 150% in the percentage of rooting (Figure 2C) and root length (Figure 2D), respectively, compared to plants grown under 0 M. The length of the aerial part of the plantlets was also 25% greater in this cultivation condition (Figure 2E). No differences were observed in the number of nodes (Figure 2F), leaves (Fig. 2G) or shoots (Figure 2H) between the both, 0M and 1M, flask sealing conditions.

The membrane sealing system contributed to a better development of the plantlets. The microcuttings grown under conditions of a higher CO_2 exchange rate (1M) showed greater total length compared to the conventional condition of *in vitro* cultivation (0M), with averages of 9.2 and 6.4 cm, respectively (Figure 2I). After 60 days of cultivation, 31 plantlets grown in the 1M condition presented roots and shoots with 6 cm in length, and were considered suitable for acclimatization. On the other hand, only 12 plantlets grown under 0M showed these characteristics.

3.3 The sealing of the flasks affects the differentiation of leaf tissues

The difference in gas exchange rates caused by the types of sealing of the culture flasks affected the

histological differentiation of *J. cuspidifolia* leaves. The leaf blade of this species consists of a uniseriate epidermis and dosiventral mesophyll composed of a palisade parenchyma layer and three to four layers of spongy parenchyma. However, plants grown in the system with a lower gas exchange (0M) rate showed a homogeneous mesophyll, poorly differentiated palisade parenchyma cells, and reduced intercellular spaces (Figure 3A) compared to plants grown at 1M condition, in which the mesophyll was already heterogeneous, dorsiventral and evident in the presence of intercellular spaces

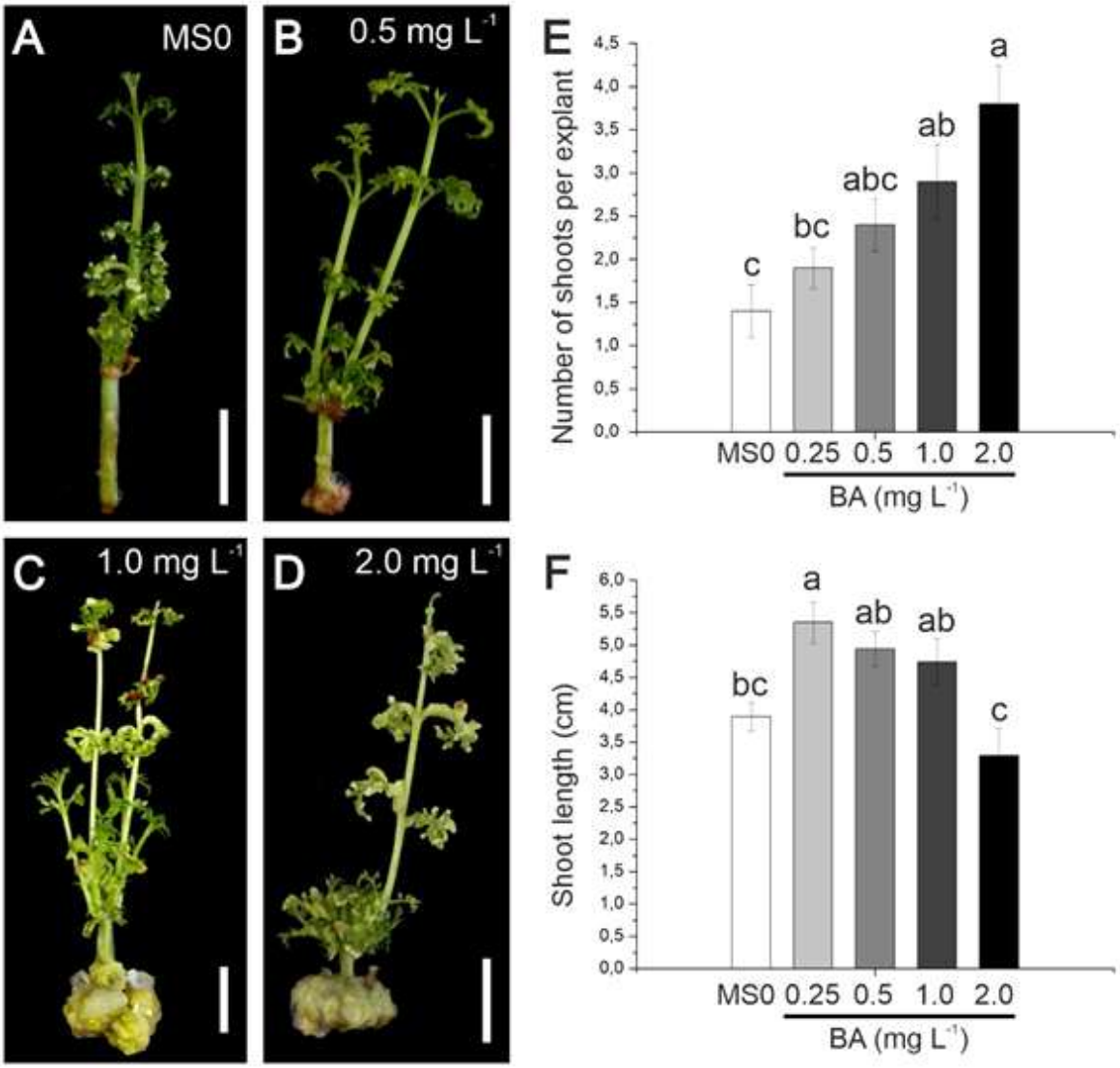

Figure 1 – Nodal segments of *Jacaranda cuspidifolia* grown in media supplemented with different concentrations of 6-benzyladenine (BA). (**a**) Nodal segment cultured in the absence of BA (MS0). (**b-d**) Nodal segments grown in media supplemented with 0.5 (**b**), 1.0 (**c**) and 2.0 mg L^{-1} BA (**d**). (**e**) Number of shoots per explant. (**f**) Length of shoot per explant. The means represented in each graph followed by the same letters did not differ from each other at the 5% probability level by Tukey's test. Error bars represent the standard deviation. Bars = 1 cm.

Figura 1 *– Segmentos nodais de* ***Jacaranda cuspidifolia*** *cultivados em meio suplementado com diferentes concentrações de 6-benziladenina (BA). (**a**) Segmento nodal cultivado na ausência de BA (MS0). (**b-d**) Segmentos nodais cultivados em meio suplementado com 0,5 (**b**), 1,0 (**c**) e 2,0 mg L^{-1} BA (d). (**e**) Número de gemas por explante. (**f**) Comprimento de um gemas por explante. As médias representadas em cada gráfico seguidas pelas mesmas letras não diferiram entre si pelo teste de Tukey ao nível de 5% de probabilidade. As barras de erro representam o desvio padrão. Barras de escala = 1 cm.*

(Figure 3B). There was no difference in epidermis thickness between the treatments evaluated (Figure 3C, D). However, the mesophyll thickness of plants grown under 1M was greater than that of plants grown at 0M (Figure 3E). The treatment with the highest CO_2 exchange rate (1M) also showed plants with greater leaf blade thickness compared to plants grown under 0M (Figure 3F).

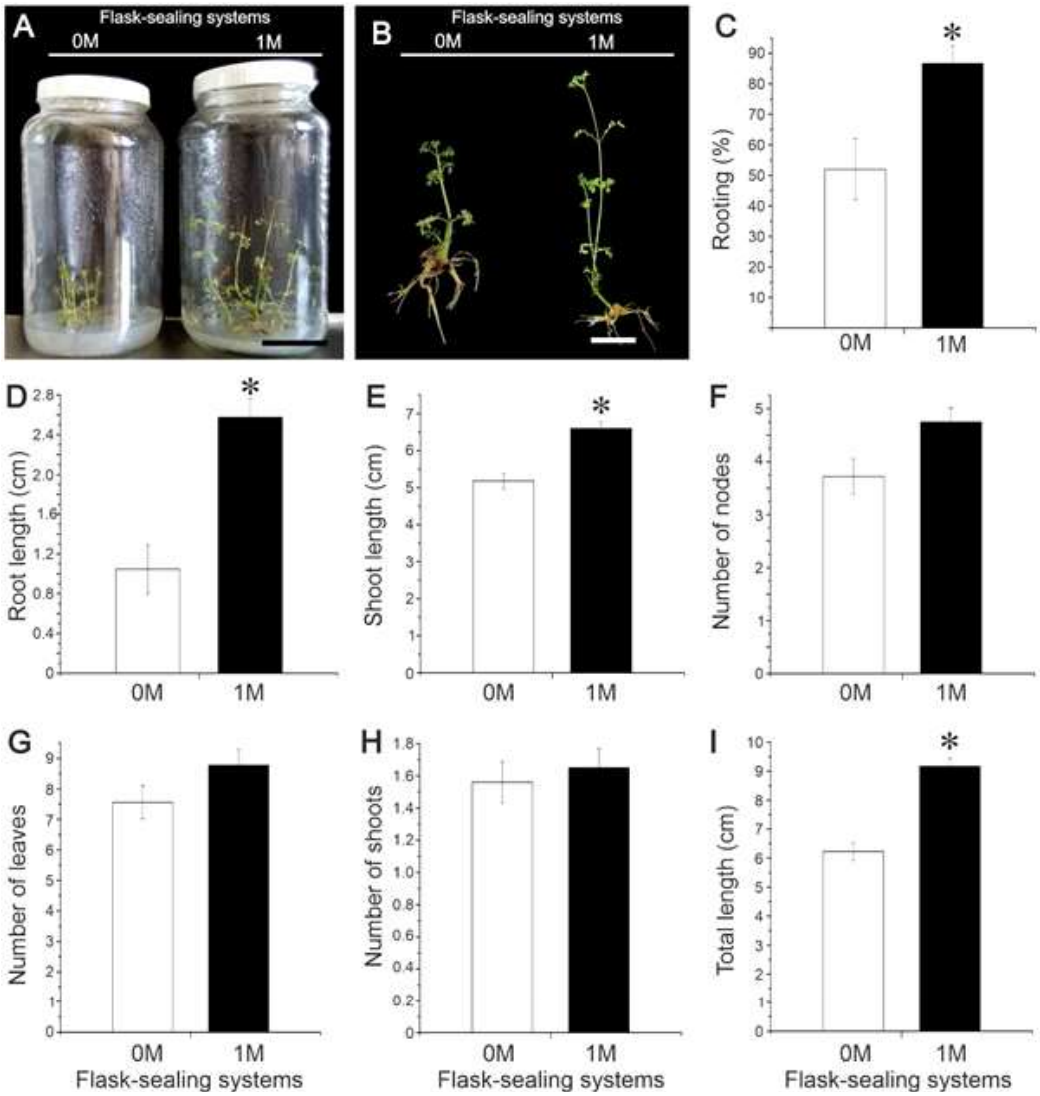

Figure 2 – Effect of gas exchange levels on growth and rooting of *Jacaranda cuspidifolia* microcuttings. (**a-b**) General view of explants at 60 days of cultivation in an environment sealed with polypropylene lids without a membrane (0 M) and with a membrane (1 M). (**c**) Percentage of rooting. (**d**) Root length (cm). (**e**) Length of shoot (cm). (**f**) Number of nodes per plant. (**g**) Number of leaves per plant. (**h**) Number of shoots per plant. (**i**) Total length (cm). Means with an asterisk (*****) were significantly different according to the F test ($P < 0.05$). Error bars represent the standard deviation. Bars: a = 5 cm; b= 2cm.

Figura 2 *– Efeito dos níveis de trocas gasosas no crescimento e enraizamento de microestacas de* ***Jacaranda cuspidifolia****. (**a-b**) Visão geral dos explantes aos 60 dias de cultivo em ambiente Vedado com tampas de polipropileno sem membrana (0 M) e com membrana (1 M). (**c**) Porcentagem de enraizamento. (**d**) Comprimento médio de raiz (cm). (**e**) Comprimento médio de gemas (cm). (**f**) Número de nós por planta. (**g**) Número de folhas por planta. (**h**) Número de gemas por planta. (**i**) Comprimento total (cm). As médias com asterisco (*****) foram significativamente diferentes de acordo com o teste F ($P < 0.05$). Barras de erro representam o desvio padrão. Barras de escala: a = 5 cm; b = 2cm.*

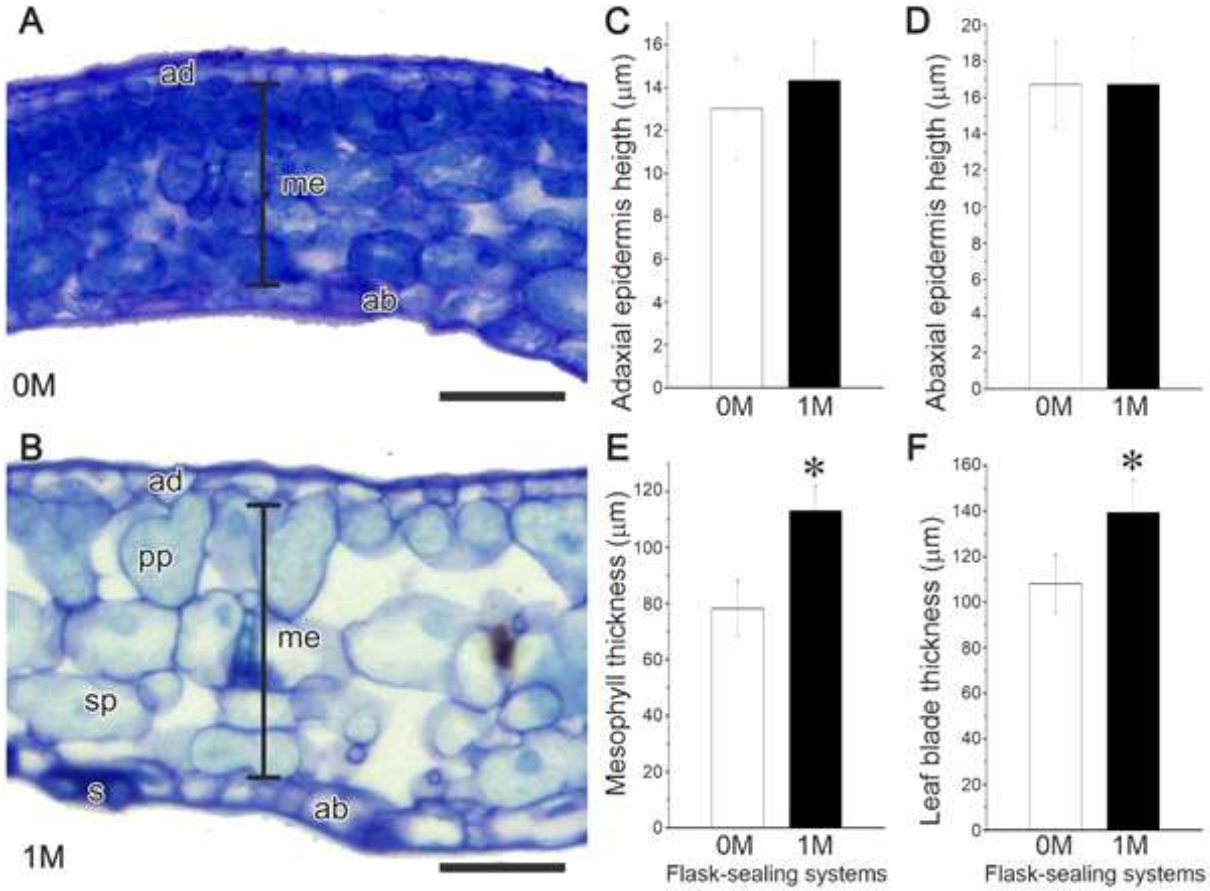

Figure 3 – Leaf anatomy of *Jacaranda cuspidifolia* microcuttings. (**a-b**) Leaf anatomy of plants at 60 days of cultivation in an environment sealed with polypropylene lids without a membrane (0 M) (**a**) and with a membrane (1 M) (**b**). (**c-d**) height of the epidermis cells of the adaxial (**c**) and abaxial (**d**) surfaces (µm). (**e**) mesophyll thickness (µm). (**f**) thickness of the leaf blade (µm). Means with an asterisk (*****) were significantly different according to the F test ($P < 0.05$). Error bars represent the mean standard deviation. Abbreviations: ab epidermis on the abaxial surface; ad epidermis on the adaxial surface; me mesophyll; pp palisade parenchyma; s stomata; sp spongy parenchyma. Bars: 50 µm.

***Figura 3** – Anatomia foliar de microestacas de **Jacaranda cuspidifolia**. (**a-b**) Anatomia foliar de plantas aos 60 dias de cultivo em ambiente vedado com tampas de polipropileno sem membrana (0 M) (**a**) e com membrana (1 M) (**b**). (**c-d**) altura das células das superfícies adaxial (**c**) e abaxial (**d**) da epiderme (µm). (**e**) espessura do mesofilo (µm). (**f**) espessura da lâmina foliar (µm). As médias com asterisco (*****) foram significativamente diferentes de acordo com o teste F ($P < 0.05$). As barras de erro representam o desvio padrão médio. Abreviaturas: ab face adaxial da epiderme; ad face adaxial da epiderme; me mesofilo; pp parênquima paliçádico; s estômato; sp parênquima lacunoso. Barras: 50 µm.*

4. DISCUSSION

The gradual increase in BA concentration led to a trend towards an increase in the number of active shoots, revealing a dose-dependent behavior of the nodal segments of *J. cuspidifolia*. This characteristic was also observed in the *in vitro* culture of *J. decurrens* nodal segments, in which the highest concentration of cytokinin evaluated also induced greater bud formation (Malosso et al., 2012). In the present study, 0.5 - 2.0 mg L^{-1} BA were the best concentrations for the induction of multiple shoots in *J. cuspidifolia* nodal segments. However, our results also showed a gradual reduction in bud length with increasing cytokinin concentrations, demonstrating the compensatory effect of cytokinins on plant development, since high concentrations induce greater formation/activation of axillary buds, but stimulate the formation of organs with reduced dimensions (Costa et al., 2020).

The alternative sealing system of culture flasks with low cost membranes influenced the *in vitro* development of *J. cuspidifolia* microcuttings. The higher gas permeability provided an increase in the rooting percentage and in the root length of the *J. cuspidifolia* microcuttings. Similar results were also observed during the *in vitro* rooting process of *Neoglaziovia variegata* with higher CO_2 exchange rates (Silveira et al., 2013). The length of the aerial part of *J. cuspidifolia* microcuttings was also greater in this culture condition, showing a result similar to that obtained by Saldanha et al. (2012) in the i*n vitro* cultivation of *Pfaffia glomerata* using low-cost vial sealing systems. The increase in gas exchange rates may favor carbon fixation in the photosynthetic process, in addition to having an acidifying effect on the cell environment, altering the action of the aminocyclopropanecarboxylate oxidase enzyme and reducing the amount of ethylene (Batista et al., 2017). In addition, the reduction in humidity increases transpiration and absorption of available nutrients, directly influencing the increase in the rate of plant growth and development (Arigita et al., 2010; Kozai, 2010; Iarema et al., 2012). Indeed, *in vitro* plants of *Guazuma ulmifolia* Lam. cultured in vessels sealed with a porous membrane showed a post-acclimatization survival rate 20.2% higher than plants previously grown under conventional *in vitro* propagation system (Jesus Santana et al., 2022). The better *in vitro* development of plants when grown in systems that allow greater gas permeability accelerates the hardening process and, consequently, acclimatization (Shin et al., 2014; Rodrigues et al., 2012). The transfer of plant materials from the *in vitro* to *ex vitro* environment is a critical phase of the micropropagation process. Therefore, technologies such as the use of sealing systems that allow high gas exchange rates, such as the one used here for *J. cuspidifolia*, are necessary in order to optimize and enable the use of micropropagation systems. However, it is important to mention that the increase in gas exchange rates in *in vitro* culture favors the development of explants as long as the external concentration of carbon dioxide does not change (Iarema et al., 2012; Fortini et al., 2021).

The *in vitro* plant growth parameters of *J. cuspidifolia* apparently reflected the anatomical differences observed in the leaf blade. While plants grown in a conventional sealing system (0M) had leaves with poorly differentiated photosynthetic tissues, dense and homogeneous mesophyll, plants grown in flasks with higher CO_2 exchange rate (1M) had leaves with differentiated palisade and spongy parenchyma, dorsiventral mesophyll and evident intercellular spaces. In this condition, the thickness of the mesophyll and leaf blade was greater than that of plants grown in the condition of lower CO_2 exchange rate. In the mesophyll, an increase in the palisade parenchyma can homogenize the light distribution (Vogelmann and Martin, 1993; Tholen et al., 2012), while an increase in the thickness of the spongy parenchyma can also promote light scattering, as well as lead to a greater diffusion of CO_2 in the tissue and consequently improve photosynthetic rates within a leaf (Niinemets et al., 2009; Tholen et al., 2012). In this sense, the increase in the mesophyll and in leaf blade thickness in the leaves of *J. cuspidifolia* cultivated in flaks with the membrane sealing system (1M) may allow a greater flow of gases, greater availability of cells to CO_2, optimization of the interception of light, and an increase in rubisco enzyme content per unit area, which can promote greater efficiency of both the carboxylation and the photosynthetic process (Mathan et al., 2021). The increase in photosynthetic rate in a gas exchange system was reported by Fortini et al. (2021) in *Vernonia condensata* leaves, with this system representing an efficient strategy for the production of larger and more vigorous plants. Anatomical analyses also showed that leaves of *Guazuma ulmifolia* cultured under conditions of higher gas exchange were significantly thicker and had more intercellular spaces than individuals cultured under conventional *in vitro* conditions (Jesus Santana et al., 2022). According to the authors, these anatomical features favored *ex vitro* acclimatization of those plants.

In summary, we describe here the potential of *J. cuspidifolia* nodal segments to induce multiple shoots when cultivated in a cytokinin-rich medium. We also demonstrated the beneficial effects of using sealing systems, which allow greater permeability of gases, on the rooting, histodifferentiation and *in vitro* development of microcuttings of this species. This information will contribute to the knowledge of the *in vitro* performance of *J. cuspidifolia*, as well as to the development of efficient and viable micropropagation systems for this important ornamental and medicinal woody tree species.

5. CONCLUSIONS

- The presence of BA in the culture medium was essential for the successful induction of multiple shoots;

- The highest number of shoots was observed in treatments supplemented with the highest concentrations of BA tested (0.5-2.0 mg L^{-1}). However, shoot length decreased with increasing BA concentration. Based on that, the concentrations 0.5 and 1.0 mg L^{-1} are recommended for multiple shoot induction.

- The use of a flask sealing system that allowed greater permeability of gases favored the rooting and the *in vitro* development of *J. cuspidifolia* microcuttings.

- The higher permeability of gases in the *in vitro* culture accelerated the photosynthetic tissue differentiation of *J. cuspidifolia* leaves.

6. AUTHOR CONTRIBUTIONS

DIR, and APCN designed the study; DKBF and LLLD established in vitro cultures; LASS performed statistical analyses; DKBF, LLLD, LASS and VCK assisted with histological and morphometrical evaluations; DKBF, LLLD, LASS and DIR wrote the manuscript; APCN and VCK revised the final version of the manuscript.

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