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ARTICLE

Sealing system in culture vessels affects the *in vitro* development of mother-of-millions (*Kalanchoe delagoensis* Ecklon and Zeyher)

Sistema de vedação em frascos de cultura afeta o desenvolvimento *in vitro* de mãe-de-mil (*Kalanchoe delagoensis* Ecklon e Zeyher)

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Abstract: Sealing systems that provide greater permeability to gasses can favor plant development *in vitro*. This study aimed to evaluate the effectiveness of a low-cost sealing system that allows different CO_2-exchange rates [CO_2ER] to promote the *in vitro* growth of mother-of-millions (*Kalanchoe delagoensis*). Nodal segments of *K. delagoensis* were surface sterilized with sodium hypochlorite (2.5% active chlorine) and inoculated in culture vessels with Murashige and Skoog (MS) medium, sealed with rigid polypropylene lids with one (code: 1M; CO_2ER 21 µL L^{-1} s^{-1}) or two (code: 2M; CO_2ER 25 µL L^{-1} s^{-1}) gas-permeable membranes. In the control, the culture vessels were sealed with rigid polypropylene lids without membrane (code: 0M; CO_2ER 14 µL L^{-1} s^{-1}). After 45 days of culture, growth parameters were measured. Plants kept in culture vessels with 0M and 1M lids had a significant increase in the total plant length, main root, aerial part, and number of nodes when compared to the 2M sealing system. The number of leaves and plantlets along the leaf margin was 35.52% and 43.69% higher in plants grown in culture vessels with a 1M sealing system. In conclusion, sealing systems that allow gas exchange rates of up to 21 µL L^{-1} s^{-1} (0M and 1M) provided the greatest *in vitro* development of mother-of-millions.
Keywords: chandelier plant, culture vessel lid, gas-permeable membranes, *Kalanchoe*, micropropagation.

Resumo: Sistemas de vedação que proporcionam maior permeabilidade aos gases podem favorecer o desenvolvimento de plantas cultivadas *in vitro*. O objetivo deste estudo foi avaliar a eficiência de um sistema de vedação de baixo custo que permite diferentes taxas de trocas gasosas [CO_2ER] para promover o crescimento *in vitro* de mãe-de-mil (*Kalanchoe delagoensis*). Segmentos nodais de *K. delagoensis* foram desinfetados em hipoclorito de sódio (2.5%) e inoculados em fracos de cultura contendo meio MS, vedados com tampas rígidas de polipropileno com uma (código 1M; CO_2ER 21 µL L^{-1} s^{-1}) ou duas (código 2M; CO_2ER 25 µL L^{-1} s^{-1}) membranas permeáveis a gases. No controle, os frascos de cultura foram vedados com tampas rígidas de polipropileno sem membrana (código 0M; CO_2ER 14 µL L^{-1} s^{-1}). Após 45 dias de cultivo, parâmetros de crescimento foram avaliados. Plantas mantidas em frascos de cultura com tampas 0M e 1M tiveram aumento significativo no comprimento total da planta, raiz principal, parte aérea e número de nós quando comparadas ao sistema de vedação 2M. O número de folhas e plântulas ao longo da margem foliar foi 35,52% e 43,69% maior nas plantas cultivadas em frascos de cultura com sistema de vedação 1M. Concluindo, sistemas de vedação que permitem taxas de troca gasosa de até 21 **µL** L^{-1} s^{-1} (0M e 1M) proporcionaram o maior desenvolvimento *in vitro* de mãe-de-mil.
Palavras-chave: flor-da-fortuna, tampa do recipiente de cultura, membranas permeáveis a gases, *Kalanchoë*, micropropagação.

Introduction

In recent years, *Kalanchoe* has become a popular ornamental plant, especially as a potted plant (Kahraman et al., 2022). This genus belongs to the Crassulaceae family and includes approximately 140 species exhibiting semi-shrub, shrub, and rarely small tree habits (Smith et al., 2019). Among them, *Kalanchoe delagoensis* Ecklon and Zeyher (synonym *K. tubiflora* or *Bryophyllum tubiflorum*) is a succulent species originally from Madagascar (Akulova-Barlow, 2009), popularly known as mother-of-millons, chandelier plant, or devil's backbone (North Carolina, 2024). It is commonly used for ornamental purposes and has great pharmacological potential due to its secondary metabolites, for example, phenolic compounds and bufadienolides (Casanova et al., 2020; Katrucha et al., 2021). *K. delagoensis* is used in traditional medicine to treat allergies and wounds (Hsieh et al., 2013; García-Pérez et al., 2020). In addition to its anti-inflammatory and antioxidant properties, some studies have also shown that *K. delagoensis* has antimitotic properties, validating its great anti-cancer potential and reinforcing this species as a source of bioactive compounds of high medicinal value (Hsieh et al., 2013; García-Pérez et al., 2018).

In nature, *K. delagoensis* spreads through plantlets on the margin of the mother-plant leaves. However, the number of plantlets on the leaves can vary depending on environmental conditions (Akulova-Barlow, 2009). Micropropagation techniques have been proposed to propagate *Kalanchoe* species (Kertrung et al., 2018; Cui et al., 2019; Lozano-Milo et al., 2020) due to the recognized pivotal role of these applications in providing a large number of plantlets with high-quality standard. Moreover, these features could enhance the exploitation of phytochemical resources (Moraes et al., 2021).

The success of *in vitro* plant development largely relies on *in vitro* growth conditions created during the micropropagation process. The conventional *in vitro* cultivation systems are characterized by limited gas exchange, which determines high humidity, low CO_2 concentrations, and high ethylene levels in the culture vessel. Consequently, the photosynthetic capacity of *in vitro* shoots is also limited at high photosynthetic photon flux. They also present less ability to absorb nutrients from the medium and poor exchange of water vapor with the surroundings, which may affect the growth and differentiation of plant tissues and a lower survival rate during acclimatization (Xiao et al., 2011; Nguyen et al., 2020). In this conventional *in vitro* cultivation system, aluminum foil, polyvinyl chloride (PVC) film, and polypropylene or stainless-steel covers are usually used to seal the culture vessels (Saldanha et al., 2012; Oliveira Junior et al., 2022).

To minimize the negative effects caused by low gas exchange rates during the establishment of *in vitro* plants, sealing systems have been proposed that provide greater gas permeability. The use

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of gas-permeable membranes reduces the relative humidity and the concentration of ethylene inside the vessels, reduces the occurrence of hyperhydricity in plant tissues, and maintains the concentration of CO_2 in the microenvironment, favoring photosynthesis, which, in turn, promotes better vegetative growth of *in vitro* shoots (Saldanha et al., 2012; Batista et al., 2017). Porous membranes that promote higher gas exchange rates in *in vitro* culture have been commercialized, although expensive (Zobayed, 2005; Batista et al., 2017; Mamedes-Rodrigues et al., 2019). However, Saldanha et al. (2012) proposed an efficient and low-cost sealing system using polytetrafluoroethylene film and micropore, which have been demonstrated to be efficient for gas permeability, resulting in the optimization and viability of sealing systems in micropropagation. Several species grown in culture vessels with this low-cost sealing system had an increase in growth rate, and a higher survival rate when transferred to the field (Saldanha et al., 2012; Batista et al., 2017; Fortini et al., 2021; Ferreira et al., 2022; Jesus Santana et al., 2022).

In view of the ornamental and ethnopharmacological potential of *K. delagoensis* and the lack of information on its *in vitro* performance, the purpose of this study was to evaluate the influence of three types of sealing systems that allow different gas exchanges on the *in vitro* development of *K. delagoensis*. We hypothesize that the sealing system in culture vessels affects the *in vitro* development of mother-of-millions.

Materials and Methods

Plant material

Kalanchoe delagoensis individuals were collected in Jataí, GO (17º55'24" S 51º42'58" W) and immersed in water to prevent the plant from dehydrating. Thereafter, the leaves were removed from the stem. Under laminar flow, the stem was immersed in 70% (v v^{-1}) ethyl alcohol for 2 min, followed by a sodium hypochlorite commercial solution (commercial bleach, Super Global®, 2.5% active chlorine) with the addition of two drops of Tween 20® for 15 min. The explants were then washed four times in autoclaved deionized water.

Plant growth conditions

Nodal segments (2 cm long) were excised and inoculated into 250 mL glass vessels containing 35 mL of semi-solid culture medium consisting of MS salts (Murashige and Skoog, 1962), 3% sucrose (3% w v^{-1}), myo-inositol (0.001% w v^{-1}) and 0.8% agar (w v^{-1}) (Merck®, Darmstadt, Germany). The pH was adjusted to 5.7 ± 0.1 before autoclaving (20 min at 121°C and 1 × 105 Pa (1.1 kg cm^{-2} 121)). Five nodal segments were inoculated per vessel with three types of sealing, providing different levels of gas exchange: **(1)** rigid polypropylene caps (RPC) without a membrane (code 0 M; CO2 gas exchange rate [CO_2.ER] was 14 µL $L^{-1}s^{-1}$; **(2)** RPC with one orifice (10 mm) covered by a membrane (code 1M), with CO_2.ER 21 µL $L^{-1}s^{-1}$; **(3)** RPC covered by two membranes (code 2M) with CO_2.ER 25 µL $L^{-1}s^{-1}$ (Fig. 1). The levels of gas exchange [CO_2.ER] were determined by Batista et al. (2017). The membranes used in this study consisted of a layer of Amanco® polytetrafluoroethylene tape between two layers of Cremer® microporous tape (Saldanha et al., 2012). The plant material was maintained for 45 days in a growth room at 25 °C ± 1 with a 16-hour photoperiod and irradiance of 40 µmol $m^{-2}s^{-1}$.

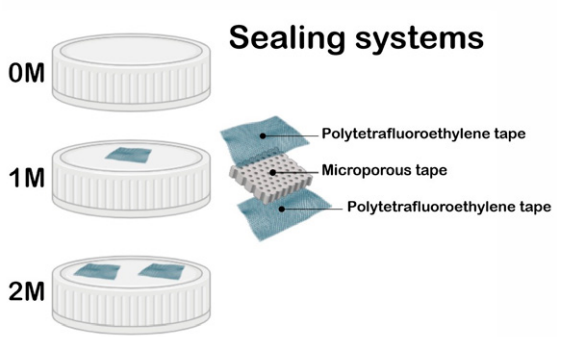

Fig 1. Detailed diagram of the sealing system with polypropylene caps without a membrane (0M - [CO_2.ER] 14 µL $L^{-1}s^{-1}$), with one membrane (1M - [CO_2.ER] 21 µL L^{-1} s^{-1}) and two membranes (2M - [CO_2.ER] 25 µL L^{-1} s^{-1}). The membranes were composed of polytetrafluoroethylene tape between two layers of microporous tape.

Morphometric analyses

At the end of 45 days, growth parameters such as total plant length (cm), primary root length (cm), shoot length (cm), leaf area per plant (cm^2), number of leaves, number of nodes, and plantlets formed on the margin of the mother-plant leaves were measured. The plant length, shoot length, and root were measured using a ruler. For leaf area analysis, the leaves were detached from the stem and photographed. The total leaf area was estimated using ImageJ v. 1.43u software (National Institutes of Health, USA).

Statistical analysis

The experiment was performed in a completely randomized design, and each treatment (0M, 1M, and 2M) consisted of 6 replicates, with 30 plants analyzed for each treatment. For all the results analyzed, the data were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test ($p \leq 0.05$), using R- Bio® version 171.

Results and Discussion

Gas exchange between the internal and external atmosphere of the *in vitro* culture vessels affected the development of *Kalanchoe delagoensis*. After 45 days of *in vitro* culture, new plants were formed from axillary shoot growth of the nodal segments in all treatments (Fig. 2). However, plants grown in 2M vessels (25 µL $L^{-1}s^{-1}$) were less vigorous when compared to plants grown in 0M (14 µL $L^{-1}s^{-1}$) and 1M (21 µL $L^{-1}s^{-1}$) vessels (Fig. 2). The number of gas-permeable membranes used during *in vitro* cultivation determines the gas flow rate between the microclimate inside and outside the containers (Batista et al., 2017).

The *K. delagoensis* plants grown under 0M and 1M conditions showed no difference in total length, reaching averages of 9.5 and 10 cm, respectively, and were considered suitable for acclimatization (Fig. 3a). However, plants grown in the 2M condition had lower total growth. The shoot length of plants grown in 2M was 35% and 40% shorter than in plants grown in 0M, and 1M, respectively (Fig. 3b). This parameter did not differ between the 0M and 1M treatments.

The number of nodes was higher in 0M and 1M compared to 2M (Fig. 3c). It is important to note that a lower number of nodes results in lower plantlet production in the next subcultivation. As observed for the aerial plant organs, root length was around 30% shorter in 2M than in the other treatments (Fig. 3d). The treatments with the lowest gas permeability (0M and 1M) provided the greatest growth in the shoot and the root, which was reflected in the total length of the plants grown under these conditions (Fig.3a, b, d).

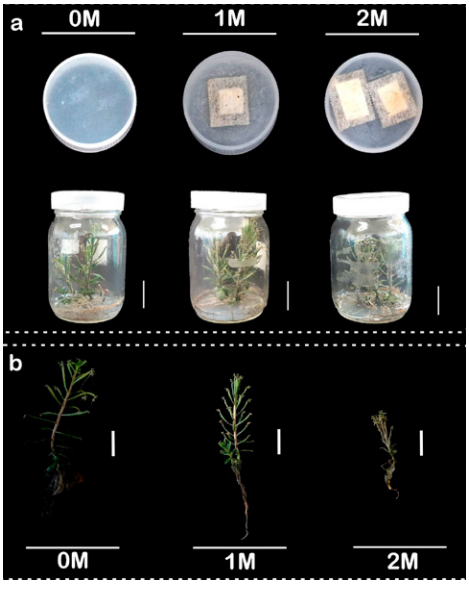

Fig. 2. *In vitro* development of *Kalanchoe delagoensis* under different sealing systems. (a) Culture vessels sealed with polypropylene caps without a membrane (code 0M - [CO_2.ER] of 14 µL L^{-1} s^{-1}), with one membrane (code 1M - [CO_2.ER] of 21 µL L^{-1} s^{-1}) and two membranes (code 2M - [CO_2.ER] 25 µL L^{-1} s^{-1}). (b) Plants were obtained at 0M, 1M, and 2M sealing conditions after 45 days. Bars: a = 3 cm; b = 2.5 cm.

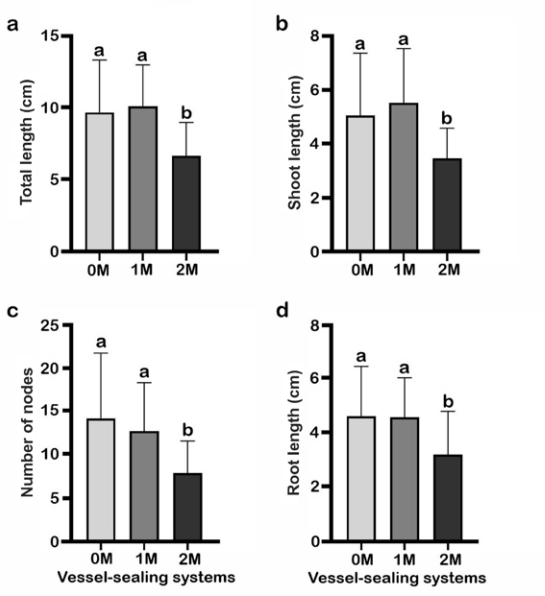

Fig. 3. Growth parameters of *Kalanchoe delagoensis* after 45 days in culture vessels sealed with polypropylene caps without a membrane (code 0M - [CO_2.ER] of 14 µL L^{-1} s^{-1}), with one membrane (code 1M - [CO_2.ER] of 21 µL L^{-1} s^{-1}) and two membranes (code 2M - [CO_2.ER] 25 µL L^{-1} s^{-1}). (a) Total length (shoot and root). (b) Shoot length (cm). (c) Number of nodes. (d) Root length. Means followed by the same letter do not differ by Tukey test ($P \geq 0.05$). (n = 6). The error bars describe the standard deviation.

It is important to consider that each species has a CO_2.ER optimum and this is related to its metabolic, physiological and genetic performance. In this study, we observed that *K. delagoensis* plants exhibited more vigor at CO_2.ER of up to 21 µL $L^{-1}s^{-1}$. In contrast, *Vernonia condensata* (Fortini et al., 2021) and *Pfaffia glomerata* (Saldanha et al., 2012; Batista et al., 2017) plants demonstrated greater vigor and biomass increase under two-membrane cultivation conditions (CO_2.ER of 25 µL $L^{-1}s^{-1}$). On the other hand, plantlets of *Mentha* species showed greater vigor and an increase in height, dry weight, and leaf area in sealing systems with four membranes (Oliveira et al., 2021). The differences in the species responses to the sealing system of culture vessels reinforce the importance of *in vitro* cultivation in different systems to obtain information and make it possible to modulate plant growth and development (Saldanha et al., 2012; Batista et al., 2017; Fortini et al., 2021; Oliveira et al., 2021).

There are also species, such as potatoes (*Solanum tuberosum*), in which growing conditions without membranes provide greater shoot length, fresh mass, and number of nodes (Mohamed and Alsadon, 2010). Increased gas flux in the *in vitro* environment results in reduced relative humidity and plant growth in these conditions has often been linked to the prevention of hyperhydricity and easier absorption of water and nutrients (Xiao et al., 2011). However, excessive water loss in these crops with greater gas exchange can change the nutritional characteristics, water, and osmotic potential of the medium, thus affecting plant development and growth (Gonçalves et al., 2008), as observed here. *K. delagoensis* is a succulent plant with Crassulacean Acid Metabolism (CAM), whose adaptation to arid climates allows it to survive but limits the speed of its growth. Environments with greater water availability can favor the growth of these species. In species with CAM metabolism, traditional *in vitro* cultivation, where gas exchange is low and relative humidity is high, promoted greater growth and photosynthetic activity than those cultivated in an *ex-vitro* environment (Malda et al., 1999).

In the present study, the number of leaves was higher in the 1M sealing system, in comparison to plants grown in 2M condition (Fig. 4a). No difference was observed between 0M and 1M treatments. The leaf area showed no significant difference among the treatments, although the 2M sealing system showed a smaller average (Fig. 4b). Increasing the number of leaves can improve light interception and, consequently, the capture of light energy to be used in the photosynthetic process. In parallel with the functions performed by leaves in primary metabolism, they are also responsible for the synthesis and storage of secondary compounds (Oliveira et al., 2021, García-Pérez et al., 2020). For the use of bioactives from *K. delagoensis*, it is interesting to use cultivation protocols in which the leaves are well formed but also numerous since one of the limiting factors for the extraction of chemical compounds in medicinal plants is the scarcity of biomass (Moraes et al., 2021).

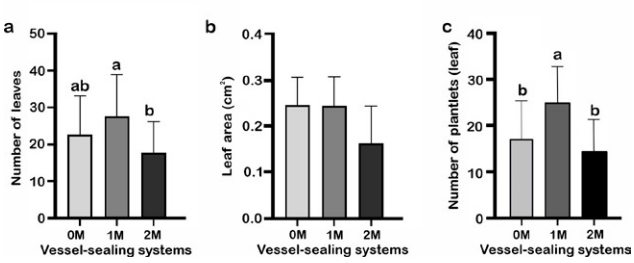

Fig. 4. Growth parameters of *Kalanchoe delagoensis* after 45 days in culture vessels sealed with polypropylene caps without a membrane (code 0M - [CO_2.ER] of 14 µL L^{-1} s^{-1}), with one membrane (code 1M - [CO_2.ER] of 21 µL L^{-1} s^{-1}) and two membranes (code 2M - [CO_2.ER] 25 µL L^{-1} s^{-1}). (a) Number of leaves. (b) Leaf area (cm^2). (c) Number of plantlets formed on the leaf margin. Means followed by the same letter do not differ by Tukey's test at 5% probability; values represent means (n = 6). The error bars describe the standard deviation.

The number of plantlets formed on the leaf margin, which will give rise to another plant, was significantly higher in the 1M sealing system (Fig. 4c), which may be directly associated with the more significant number of leaves observed in this sealing system (Fig. 4a). The more significant number of plantlets formed on the leaf margin may result in higher rates of plant multiplication during subcultivation, exponentially increasing the efficiency of the micropropagation system presented here.

In general, the sealing systems 0M and 1M provided *K. delagoensis* plants with more significant growth variables than the 2M sealing system. But, considering the leaf parameters analyzed here, the 1M condition may be even more advantageous since plants grown under conventional *in vitro* cultivation conditions (0M) generally present poorly differentiated and/or dysfunctional tissues, which limits their metabolic and physiological performance (Xiao et al., 2011; Nguyen et al., 2020, Ferreira et al., 2022). In species such as *Guazuma ulmifolia* and *Jacaranda cuspidifolia*, the cultivation in vessels with a porous membrane, similar to our 1M treatment, was sufficient to promote increased growth and leaf histodifferentiation (Ferreira et al., 2022; Jesus Santana et al., 2022). In this sense, further studies will be necessary to confirm whether the 1M sealing system promoted changes in the leaf histology of *K. delagoensis* in comparison to the conventional *in vitro* culture system (0M) to evaluate this hypothesis.

Conclusions

Sealing systems with gas exchange rates of up to 21 µL L^{-1} s^{-1} (0M and 1M) provided the greatest growth of *K. delagoensis*, under *in vitro* conditions. In addition, gas exchange rates higher than 25 µL L^{-1} s^{-1} (2M) impaired the growth of this species. We also concluded that the 1M sealing system seems more advantageous for micropropagation of *K. delagoensis*, since it provides more leaves and plantlets. The information obtained in this study will contribute to constructing efficient and viable micropropagation strategies and systems to obtain more vigorous plants with higher concentrations of bioactive compounds.

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Author Contribution

DIR: conceived and designed research; analyzed data; wrote the manuscript. **GMDO:** conducted experiments. **JRS**: contributed to statistical analysis; analyzed data; wrote the manuscript. **LASS:** conducted experiments; analyzed data; wrote the manuscript. **MLS**: analyzed data. **LLLD:** conducted experiments**;** wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability Statement

Data will be available on request.

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