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Original Article

Brassavola tuberculata Hook.: *in vitro* growth and *ex vitro* establishment as a function of the micropropagation system and sucrose

Brassavola tuberculata Hook.: crescimento *in vitro* e estabelecimento *ex vitro* em função do sistema de micropropagação e da sacarose

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Abstract

This study examines the *in vitro* growth and *ex vitro* establishment of *Brassavola tuberculata* in relation to the micropropagation system and sucrose concentration employed in the *in vitro* culture. A completely randomized experimental design was utilized, employing a 2 x 5 factorial arrangement. The experimental period began with seedlings cultivated *in vitro* for 180 days, which were subsequently transferred to Murashige and Skoog culture media containing sucrose concentrations of 0, 15, 30, 45, or 60 g L^{-1}. The cultures were subjected to two micropropagation systems: conventional and gas exchange. After 90 days of *in vitro* cultivation, the plants were evaluated, transplanted into a substrate, and placed in a screened nursery for *ex vitro* cultivation. After 300 days of *ex vitro* cultivation, the survival and initial characteristics of the plants were assessed. The micropropagation system allowing gas exchange and sucrose concentrations up to 30 g L^{-1} enhanced the shoot and root growth of *in vitro* propagated plants. No noticeable anatomical differences were observed after 90 days of *in vitro* culture among the different sucrose concentrations and micropropagation systems used. In the *ex vitro* establishment, irrespective of sucrose concentration, the micropropagation system facilitating gas exchange positively influenced all evaluated characteristics.

Keywords: *in vitro* cultivation, photoautotrophic, photomixotrophic, acclimatization, ornamental horticulture, native species.

Resumo

Objetivou-se com este trabalho avaliar o crescimento *in vitro* e estabelecimento *ex vitro* de *Brassavola tuberculata* em função do sistema de micropropagação e da concentração de sacarose utilizados no cultivo *in vitro*. Foi utilizado o delineamento inteiramente casualizado e os tratamentos arranjados em esquema fatorial 2 x 5. Para o início do período experimental, foram utilizadas plântulas cultivadas *in vitro* por 180 dias, sendo transferidas para meios de cultivo Murashige e Skoog contendo 0, 15, 30, 45 ou 60 g L^{-1} de sacarose, e as culturas submetidas a dois sistemas de micropropagação: convencional ou com troca gasosa. Após 90 dias de cultivo *in vitro*, as plantas foram avaliadas e na sequência plantadas em substrato e acondicionadas em viveiro telado para o cultivo *ex vitro*. Após 300 dias de cultivo *ex vitro*, as plantas foram avaliadas quanto à sobrevivência e às mesmas características iniciais. A utilização do sistema de micropropagação que permite trocas gasosas, em conjunto com concentrações de sacarose de até 30 g L^{-1}, proporcionou aumento no crescimento de parte aérea e do sistema radicular das plantas propagadas *in vitro*. As diferentes concentrações de sacarose e os sistemas de micropropagação utilizados não apresentaram diferenças anatômicas perceptíveis aos 90 dias de cultivo *in vitro*. Já no estabelecimento *ex vitro*, independente da utilização de sacarose, o sistema de micropropagação que permite trocas gasosas influenciou positivamente todas as características avaliadas.

Palavras-chave: cultivo *in vitro*, fotoautotrófico, fotomixotrófico, aclimatização, horticultura ornamental, espécie nativa.

1. Introduction

The Orchidaceae family is recognized as one of the largest and most representative plant families among angiosperms, comprising species of high ornamental, nutritional, and pharmacological value. However, due to the extraction of native orchids driven by their captivating flowers, their populations have declined in various biomes. Consequently, many species have become vulnerable due to the systematic destruction of their habitats (Sasamori et al., 2020; Soares et al., 2020; Nongdam et al., 2023).

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The genus *Brassavola*, widely distributed throughout Brazil, possesses remarkable ornamental potential and resilience. Consequently, it is among the genera most threatened by unregulated collection and holds commercial interest for hybrid production (Xu et al., 2022; Vendrame et al., 2023). *Brassavola tuberculata* Hook. stands out as a prominent species within this genus.

Therefore, strategies for propagation and conservation must ensure the survival of these plants in their natural habitats while providing plant material for commercial production. In this regard, *in vitro* cultivation presents a viable alternative as it enables the conservation of pathogen-free material, production of elite plants, and maintenance of genetic diversity in the short, medium, and long term (Teixeira da Silva et al., 2017; Soares et al., 2020; Nongdam et al., 2023; Vendrame et al., 2023).

In vitro propagation offers several advantages over conventional propagation methods. However, it still faces technical and economic limitations, including high production costs, shortage of skilled labor, and the inability of plants with morphophysiological changes to survive in *ex vitro* environments. Consequently, scaling up production becomes challenging (Miranda et al., 2020; Nongdam et al., 2023).

Despite the widespread use of *in vitro* cultivation techniques for orchids, the *in vitro* propagation of *Brassavola* species still lacks established protocols, primarily due to the limited number of studies focused on these species (Pereira et al., 2022; Xu et al., 2022; Vendrame et al., 2023). Regarding *B. tuberculata*, only a few scientific articles have been published on its *in vitro* germination (Herrmann et al., 2011; Soares et al., 2012, 2020; Rosa et al., 2013), seed storage and viability (Macedo et al., 2014; Soares et al., 2014; Sousa et al., 2020), multiplication and *in vitro* rooting (Mengarda et al., 2017), and *ex vitro* acclimatization (Sousa et al., 2015).

Given the commercial, ornamental, and environmental demands associated with the Orchidaceae family, there is a current pursuit for sustainable production using advanced and cost-effective techniques (Teixeira da Silva et al., 2017; Nongdam et al., 2023). Consequently, there is a need for adaptations in *in vitro* cultivation protocols, particularly for native species. These adaptations should aim to enhance productivity and promote the development of plants with morphophysiological characteristics conducive to their *ex vitro* establishment. In this regard, modifications to micropropagation systems, commonly heterotrophic, and sucrose concentrations in culture media can lead to desirable traits in propagated plants (Ferreira et al., 2017; Ribeiro et al., 2019; Miranda et al., 2020; Santos et al., 2020; Nongdam et al., 2023).

Considering the above, numerous studies have explored the use of different micropropagation systems and sucrose concentrations in culture media for other cultivated species. However, this is the first study investigating the *in vitro* culture of *B. tuberculata* using photoheterotrophic, photoautotrophic, and photomixotrophic systems. Therefore, this study evaluates the *in vitro* growth and *ex vitro* establishment of *B. tuberculata* in relation to the micropropagation system and sucrose concentration employed in the culture.

2. Materials and Methods

2.1. In vitro germination

The experiment was conducted at the *In Vitro* Culture Laboratory of Flowers and Ornamental Plants and the orchidarium of the School of Agricultural Sciences at the Universidade Federal da Grande Dourados. Ripe fruits of *Brassavola tuberculata* Hook. were used as the study material. The fruits were derived from manual pollination and obtained from matrices over ten years old grown in a nursery covered by two 50% shade screens. The irradiance level was maintained at 235 µmol m-2 s-1, with a mean temperature of 22.6 ± 5 ºC and relative humidity of 73.9 ± 10%. The plants were irrigated with rotary micro-sprinklers positioned one meter above the plants, providing a daily water depth of 1 mm.

A sample of 0.005 g of seeds was weighed, and a tetrazolium test was conducted following the methodology described by Soares et al. (2014) to confirm viability. After confirming viability, another sample of 0.005 g of seeds was taken to an aseptic environment and disinfected according to Soares et al. (2020) to obtain the seed solution. For *in vitro* sowing, 1.0 mL of the suspension of disinfected seeds was inoculated in a culture flask with 60 mL of Murashige and Skoog culture medium (MS) with half the salt concentration (MS ½). The flasks had a capacity of 600 mL. The cultures were placed in a growth room with controlled temperature and photoperiod (25 ± 2 °C; 16 h) and an irradiance of 22 µmol m-2 s-1 provided by two white fluorescent lamps (6,500K).

2.2. In vitro growth

After 180 days of cultivation, the plants were standardized to a size of 4.0 ± 0.5 cm and subcultured in an aseptic environment to initiate the experimental period. Flasks with a capacity of 600 mL containing 60 mL of Murashige and Skoog culture medium (MS) solidified with 7.0 g L^{-1} of bacteriological agar (Himedia®, India) were used. The culture medium was supplemented with different sucrose concentrations (0, 15, 30, 45, and 60 g L^{-1}). The pH of the medium was adjusted to 5.8 using 0.1 M KOH before sterilization in an autoclave at 121 °C and 1 atm of pressure for 20 minutes. Each flask contained seven plants and was sealed with either a screw cap (conventional micropropagation system - CMS) or a screw cap with a hole and cotton filter (photoautotrophic or photomixotrophic micropropagation system with gas exchange - MSGE) to allow gas exchange.

After 90 days of subculture, the flasks were opened, and the plants were washed under running water to remove the culture medium. The plants were then evaluated using a digital caliper and precision scale to measure the number of leaves (NL), plant height (PLH) (mm), number of buds (NB), number of roots (NR), length of the largest root (LR) (mm), and fresh matter (FM) (g). The remaining culture medium in the flasks was heated until it returned to the liquid phase, and the pH was measured using a benchtop pH meter.

2.3. Visual anatomical analysis

To analyze the effects of the treatments on leaf tissues, samples from plants subjected to different micropropagation systems and sucrose concentrations were collected. Leaf fragments measuring 5 mm were cut from the central portion of the leaves. These fragments were fixed in F.A.A. (formaldehyde 35%, glacial acetic acid, and 50% ethanol) and stored in a refrigerator. The fragments were then progressively dehydrated in an alcohol series with tertiary butyl alcohol (Dankin & Hussey, 1985). After the dehydration process, the leaf fragments were infiltrated with paraffin and paraplast. Cross sections of 10-µm thickness were made using a manual rotary microtome. The sections were stained with safranin-orange G-fast Green FCF (Hagquist, 1974). The images were analyzed using the AxioVision version 3.1 computational application coupled to the micrometer eyepiece microscope.

2.4. Ex vitro growth

For the evaluation of *ex vitro* growth, the plants were transplanted into disposable transparent polypropylene containers with a capacity of 1,000 mL (20 x 10 x 5 cm). The containers were filled with a substrate consisting of one-third pink sphagnum moss (Agrolink, Holambra, Brazil) and two-thirds coconut fiber (Golden-Mix Chips, Amafibra) (1:1, v:v). After transplantation, the containers were placed in a protected nursery and maintained under the same conditions as the parent plants for 300 days.

Foliar fertilizations were conducted every 15 days using a solution of NPK 10:10:10 fertilizer at a concentration of 2.0 mL L^{-1}, along with micronutrients including magnesium (0.025%), boron (0.02%), copper (0.05%), iron (0.10%), manganese (0.05%), molybdenum (0.0005%), and zinc (0.05%), with a maximum chlorine content of 0.025%. At zero, 30, and 60 days after transplanting, plants were preventively disinfected with O-S-dimethyl-N-acetyl-phosphoram idothioate (4 mg L^{-1}) and mancozeb (4 mg L^{-1}). Foliar fertilization and disinfestation were carried out using a backpack sprayer with a capacity of 5 L.

After 300 days of *ex vitro* cultivation, the plants were removed from the containers and washed under running water to remove the substrate. The plants were then evaluated for the same initial characteristics (NL, PLH, PD, NB, NR, LR, LF, and FM). In order to investigate the hypothesis of increased plant growth during the *ex vitro* stage, and according to the treatments to which plants were exposed in the *in vitro* stage, their increases (I) regarding the initial values were calculated using the expression $I = (VF - VI)$. Where VI is the value of the variable before the plant was acclimatized, and VF is the value of the same variable after the *ex vitro* period, with its values expressed as a percentage and subjected to analysis of variance (Ribeiro et al., 2019).

2.5. Experimental design

A completely randomized experimental design was used with a 2 x 5 factorial design (two micropropagation systems and five sucrose concentrations) and five replicates of a culture flask with five plants each. The results were transformed using $\sqrt{(x + 1)}$ and subjected to analysis of variance. The Tukey test ($p < 0.05$) was used to compare the means of the quantitative factors. Regression curves were fitted to the significant factors. The statistical analysis was performed using the SISVAR software (Statistical Analysis Program v.5.3., Universidade Federal de Lavras, MG).

3. Results

3.1. In vitro growth

A significant effect was observed for the interaction between micropropagation systems and sucrose concentrations ($p < 0.05$) on the number of leaves (NL), buds (NB), length of the largest root (LR), fresh matter (FM), and pH of the culture media of *B. tuberculata* after 90 days of *in vitro* cultivation. Additionally, the micropropagation system had an isolated effect on plant height (PLH) and the number of roots (NR). The sucrose concentrations also had an isolated effect on NR.

Brassavola tuberculata plants exhibited higher NL, NB, FM, and pH when subjected to CMS. The calculated values included 26.95 leaves at a concentration of 17.12 g L^{-1} of sucrose, 9.28 shoots at a concentration of 17.59 g L^{-1} of sucrose, 0.47 g at a concentration of 17.16 g L^{-1} of sucrose, and a pH of 3.9 without the addition of sucrose. For LR, the highest calculated values were observed in MSGE when supplemented with 19.72 g L^{-1} of sucrose (32.21 mm) (Figure 1).

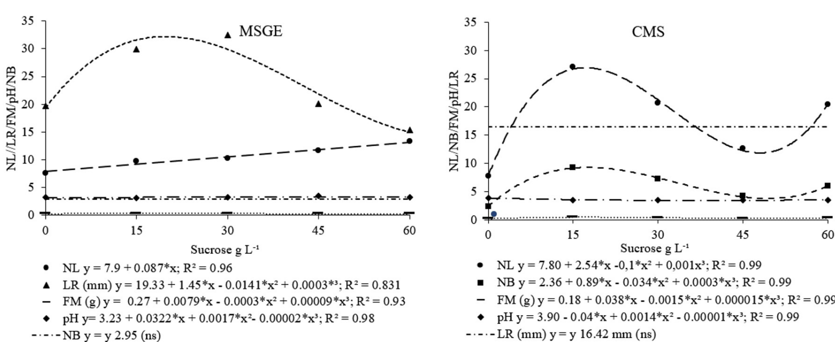

Figure 1. Number of leaves (NF), number of buds (NB), length of the largest root (LR), fresh matter (FM), and potential of hydrogen (pH) of *Brassavola tuberculata* Hook.. as a function of sucrose concentrations in the culture medium and micropropagation system. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE.

Regarding the isolated effect of the micropropagation system, it was observed that flasks under MSGE had larger plants (53.75 ± 19.40 mm) and a greater number of roots (4.97 ± 1.58 roots) (Figure 2).

Concerning the isolated effect of sucrose concentrations, plants grown in a culture medium supplemented with an average value of 34.37 g L^{-1} of sucrose showed a higher number of roots, with 5.02 roots (Figure 2C).

Figure 3 shows the variation in the morphological aspects of plants as a function of micropropagation systems and sucrose concentrations.

3.2. Histological slides

According to Figure 4, the cross-section images of leaves indicated that the material was in good conservation condition. The different sucrose concentrations and micropropagation systems used did not visually show anatomical differences in plants after 90 days of *in vitro* cultivation.

The characteristics common to *B. tuberculata* were observed in all cross-sections, including a uni-stratified epidermis with irregularly shaped and sized cells, thick cell walls, and a smooth, continuous cuticle surface throughout the leaf. The secondary wall cells showed no thickening, and the fiber bundles were arranged from the leaf adaxial to the abaxial face, displaying good organization. The presence of chloroplasts was noted, with homogeneous mesophilic organization, equifacial and slightly elongated isodiametric epidermal cells in the paradermic view, and straight and thick anticlinal walls (Figure 4).

Although *Brassavola* leaves are terete, the adaxial face corresponds to the grooved surface area of the leaf, while the abaxial face corresponds to the convex and rounded region. The chlorenchyma is homogeneous, with polyhedral cells of irregular size and thin walls, without a clear distinction between lacunous and palisade cells. In the central rib region, the upper mesophile cells appear more isodiametric, while in the lower mesophile region throughout the leaf, the cells tend to lengthen their anticlinal walls. Moreover, regarding conduction, the layers of vascular bundles tend to follow the conduplicate shape of the leaf (Figure 4).

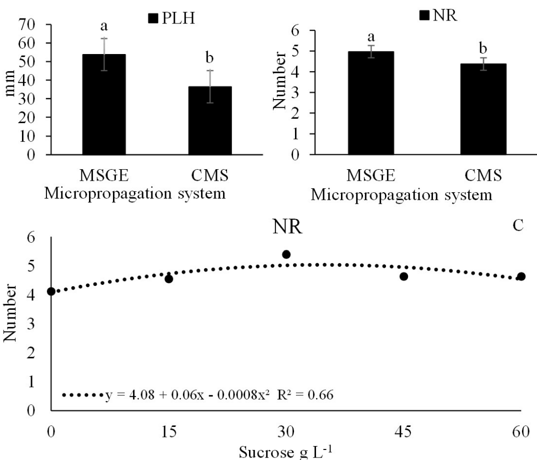

Figure 2. *In vitro* growth of *Brassavola tuberculata*Hook.: A) plant height (PLH) (mm); B) number of roots (NR) after 90 days of *in vitro* cultivation as a function of the micropropagation system, and (C) NR after 90 days of *in vitro* cultivation as a function of sucrose concentrations in the culture medium. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE.

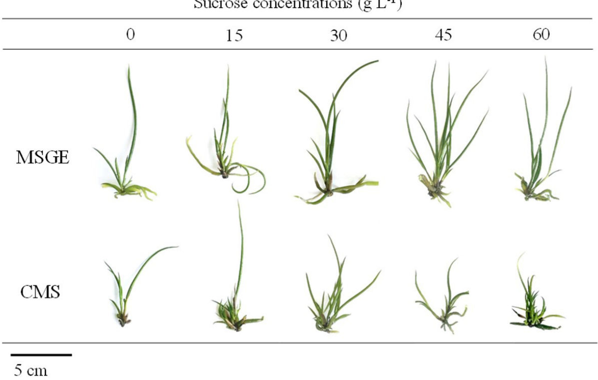

Figure 3. *Brassavola tuberculata* Hook. plants after 90 days of *in vitro* cultivation as a function of sucrose concentrations in the culture medium and micropropagation system. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE

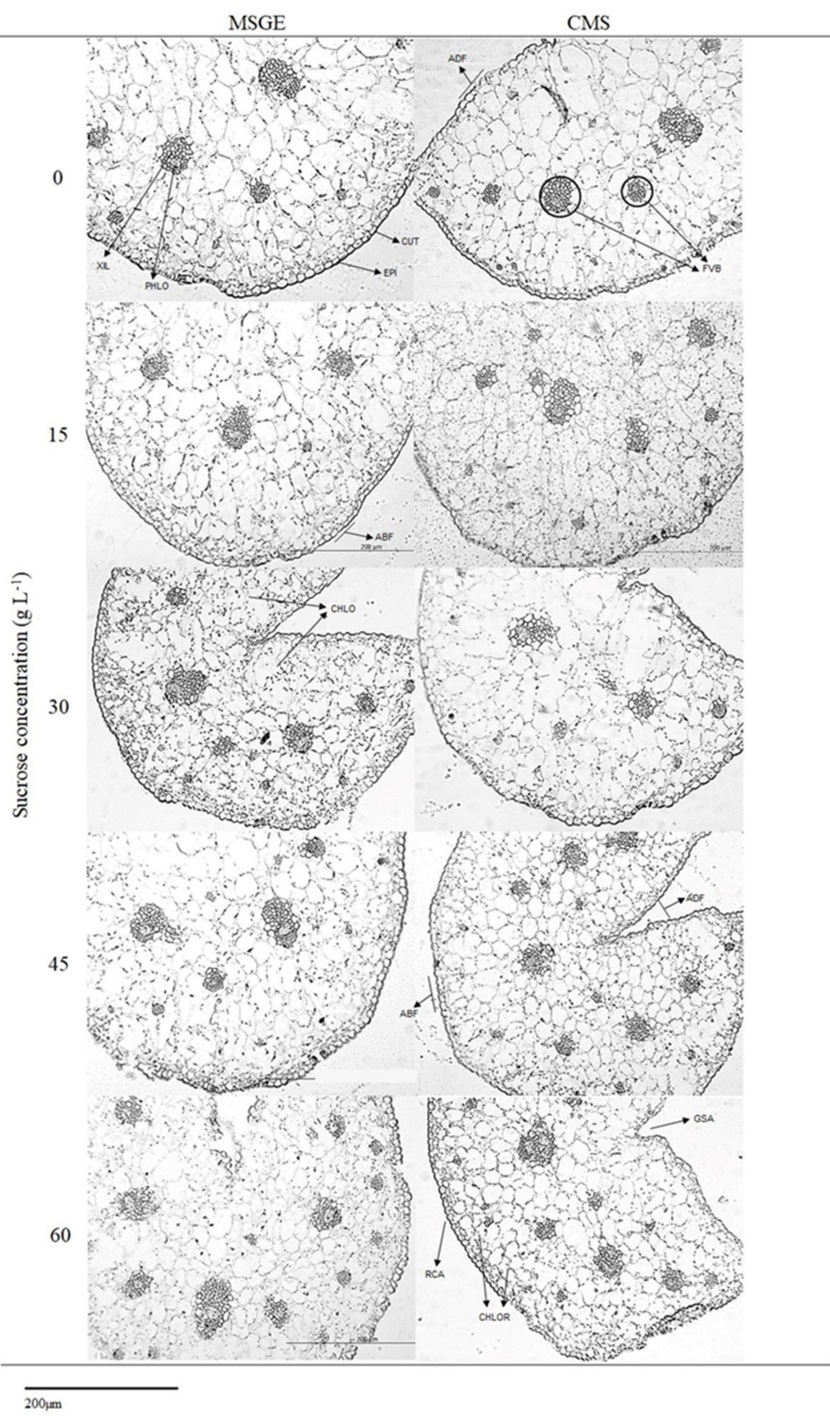

Figure 4. Cross-section of the median part of *Brassavola tuberculata* Hook. leaves after 90 days of *in vitro* cultivation as a function of sucrose concentrations in the culture medium and micropropagation system. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE. XIL - xylem; PHLO - phloem; CUT - cuticle; EPI - epidermis; ADF - adaxial face; ABF - abaxial face; FVB - fibrovascular bundle; CHLO - chloroplast; CHLOR - chlorenchyma; RCA - rounded convex region, and GSA - grooved surface area.

3.3. Ex vitro growth

After 300 days of *ex vitro* cultivation, the analysis of variance showed a significant effect of the interaction between micropropagation systems and sucrose concentrations ($p < 0.05$) on %SUR (survival), NL, NR, and LR. Furthermore, the micropropagation systems had an isolated effect on PLH and FM, while the effect of sucrose concentrations was observed only for PLH.

Regarding the interaction between micropropagation systems and sucrose concentrations, a higher %SUR was observed at the end of the experimental period when *B. tuberculata* plants were subjected to MSGE without sucrose in the culture medium, with %SUR = 82.32. MSGE without the addition of sucrose also provided the highest values for NL (46.73%).

For NR and LR, the highest calculated values were observed when using MSGE and a culture medium with 14.53 g L^{-1} of sucrose (169.85%) and 60.00 g L^{-1} of sucrose (234.40%), respectively (Figure 5).

Regarding the isolated effect of micropropagation systems, plants submitted to CMS showed the highest values for PLH (57.59 ± 34.86%). However, the highest FM values (259.91 ± 128.00%) were observed using MSGE (Figure 6).

Regarding the isolated effect of sucrose concentrations in the culture medium, the highest increases in PLH (89.06%) were observed using 44.90 g L^{-1} of this sugar (Figure 6C).

According to Figure 7, the conditions of *in vitro* cultivation did not limit plant growth during the *ex vitro* period. In general, MSGE showed the highest results for almost all parameters evaluated, suggesting that this micropropagation system may be suitable for cultivating this species.

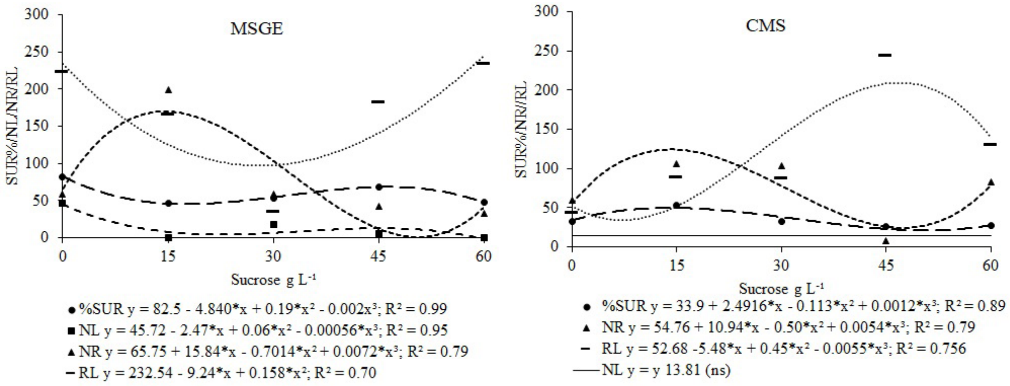

Figure 5. Survival (%SUR) and increases (%) in the number of leaves (NL), number of roots (NR), and length of the largest root (LR) of *Brassavola tuberculata* Hook.. as a function of sucrose concentrations and micropropagation system. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE.

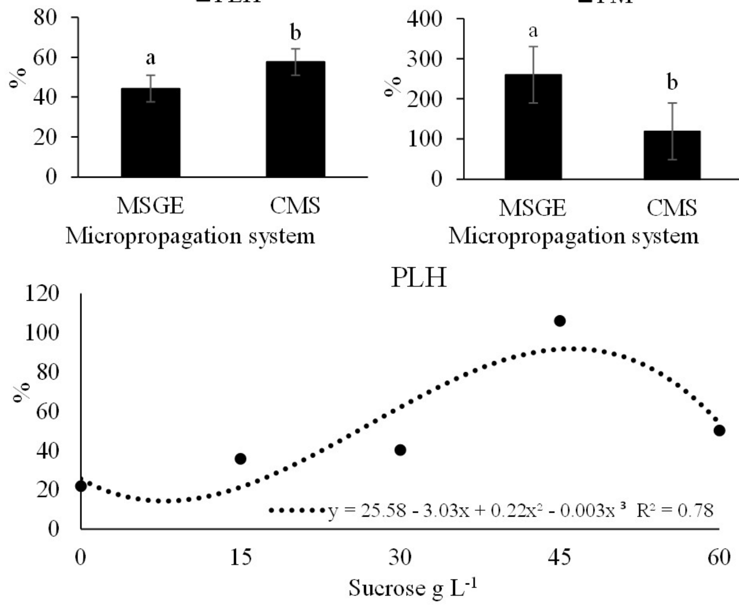

Figure 6. Increases in *Brassavola tuberculata* Hook..: A) plant height (PLH) (mm); B) fresh matter (FM) after 300 days of *in vitro* cultivation as a function of the micropropagation system, and (C) PLH after 300 days of *in vitro* cultivation as a function of sucrose concentrations in the culture medium. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE.

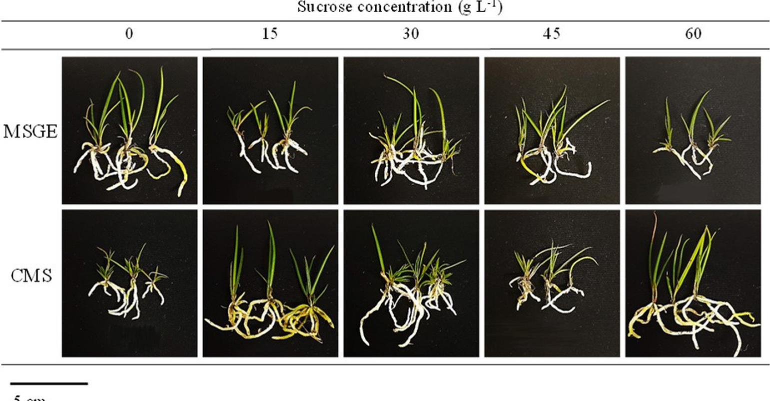

Figure 7. *Brassavola tuberculata* Hook. plants at 300 days of *in vitro* cultivation as a function of sucrose concentrations in the culture medium and micropropagation system. Conventional micropropagation system = CMS; micropropagation system with gas exchange = MSGE.

4. Discussion

4.1. In vitro growth

The observed results for NL in CMS suggest that its higher value may be related to the increased NB observed in the same treatment, leading to higher FM. This outcome could be attributed to the presence of ethylene gas and low CO_2 concentrations in the hermetic environment of CMS, which functions as a heterotrophic system. Consequently, it can reduce plant height and photosynthetic pigment content, thereby influencing the tillering of *in vitro*-grown plants (Teixeira da Silva et al., 2017; Miranda et al., 2020).

The results for LR, PLH, and NR in MSGE can be attributed to the utilization of this photomixotrophic propagation system, which enables gaseous exchange between the flask's interior and the atmospheric air, thus influencing the growth and development of *in vitro* plants (Silva et al., 2016; Fritsche et al., 2022)

These findings align with those of Ribeiro et al. (2019), where Denphal plants cultivated in flasks with ventilation caps displayed superior growth characteristics compared to those grown in a hermetic environment. Employing micropropagation under natural ventilation conditions can be an alternative to improving morphophysiological issues arising from the conventional *in vitro* culture system. MSGE, by allowing gas exchange, enhances photochemical efficiency and photosynthetic carbon assimilation, resulting in plants with more efficient metabolism and robustness (Fuentes et al., 2007; Kozai, 2010; Xiao et al., 2011; Fritsche et al., 2022).

Regarding sucrose, the optimal results for *in vitro* cultivation of *B. tuberculata* were achieved with concentrations around 15 and 30 g L^{-1}. Studies on different sucrose concentrations in the *in vitro* culture of native orchids, such as *Alatiglossum fuscopetalum* (Ferreira et al., 2017), *Oncidium flexuosum* (Caovila et al., 2016), *Cyrtopodium cachimboense* (Paulino et al., 2021), *Miltonia flavescens* (Lemes et al., 2014), and *Cattleya schilleriana* (Galdiano-Júnior et al., 2018), support our results. However, in contrast to our findings, Silva et al. (2014) observed that *Cattleya walkeriana* Gardner plants exhibited increased root system growth when cultured with the highest tested sucrose concentration (45 g L^{-1}) in flasks with ventilation caps. These observations suggest that the influence of sucrose may vary according to the orchid species studied, and the species examined here exhibits similar behavior to most reported in the scientific literature.

Regarding pH, the culture media were initially adjusted to 5.8 before the experimental period. However, a decrease in pH was observed for all treatments after 90 days of *in vitro* cultivation. The recommended pH range for adequate growth of most orchid species is between 5.0 and 6.5 (Faria et al., 2004). Additionally, plant tissue culture media often have low buffering capacity (Leifert et al., 1995). Therefore, some species can adjust the medium pH to values between 3.7 and 6.2 during the plant growth period, irrespective of the initial pH (Leifert et al., 1992).

In general, the pH decrease was more pronounced in MSGE. This decline may be attributed to water loss through gas exchange, occurring due to the different pressure and temperature gradients inside and outside the flask, concentration gradients of gases (CO_2 and H_2O, among others), and the speed and pattern of air movement around the flask (Xiao et al., 2011). Additionally, gas exchange and increased CO_2 levels may promote H_2CO_3 formation in the culture medium, leading to a decreased pH in this micropropagation system. However, Caovila et al. (2016) concluded in their research on sucrose concentrations and pH in the *in vitro* growth of *Oncidium flexuosum* that regardless of the pH value, the factor that most significantly influences the variables is the concentration of sucrose used.

This study demonstrates that photomixotrophic culture, with supplementation of up to 30 g L^{-1} of sucrose in the culture medium, provides favorable conditions for desirable characteristics in *B. tuberculata* plants during *ex vitro* cultivation, such as increased height and root system development.

4.2. Ex vitro growth

The results for %SUR and NL suggest that the use of photoautotrophic micropropagation systems, i.e., without adding carbohydrates to the culture medium and allowing gas exchange, promotes photosynthesis.

However, most orchids are cultivated using a conventional micropropagation system (heterotrophic) characterized by the absence of gas exchange and high humidity, leading to physiological and morphological disorders in plants, particularly stomatal malfunction, increased water loss through leaf tissue, and potential decrease in survival of orchid seedlings during the *ex vitro* acclimatization process (Majada et al., 2002; Cha-Um et al., 2010; Silva et al., 2016; Teixeira da Silva et al., 2017).

In the photomixotrophic system (with carbohydrate addition and gas exchange), the characteristics of NR, RL, and FM can be attributed to the increased aeration facilitated by MSGE. Furthermore, this system promotes photosynthesis by allowing gas exchange within the flask, leading to normalized transpiration and regulation of stomatal function. Under these conditions, explants can utilize endogenous and exogenous carbohydrates as an energy source (Kozai, 2010; Santos et al., 2020).

These results are consistent with Silva et al. (2014), who observed that *Cattleya walkeriana* Gardner plants grown in flasks with ventilation caps exhibited superior growth characteristics compared to those grown in a hermetic environment. Regarding sucrose concentrations, Galdiano-Júnior et al. (2013), in their study on the effect of sucrose on the number of roots in *Cattleya loddigesii* Lindley, observed the highest values with a concentration of 20.7 g L^{-1}, similar to the findings in our study (14.53 g L^{-1}). Additionally, the data obtained for LR align with those of Faria et al. (2004), who studied *Dendrobium nobil*e Lindl. plants and observed the longest root length with a concentration of 60 g L^{-1} sucrose.

Regarding the isolated effect of sucrose on PLH, our results are similar to those reported by Besson et al. (2010), who observed a significant difference in shoot length of *Miltonia flaviscens* Lindl. with medium supplementation of 30 and 45 g L^{-1} of sucrose. However, Galdiano-Júnior et al. (2013) observed higher shoot length values for *Cattleya loddigesii* Lindl. with lower sucrose concentrations (21.5 g L^{-1}). These observations indicate that the concentration of sucrose used in the culture media can influence different plant materials in distinct ways.

The higher PLH observed when using CMS alone may be attributed to the transfer to the *ex vitro* environment. These plants need to complete their autotrophism by increasing their metabolic rates (Teixeira da Silva et al., 2017), thus utilizing their photoassimilates for height growth. Therefore, plants grown *in vitro* using a conventional system exhibit an increased growth rate during the *ex vitro* phase. Meanwhile, plants from MSGE, which had already initiated rusting during *in vitro* cultivation, only maintained this growth rate (Ribeiro et al., 2019).

In orchid species propagation protocols, it is crucial to consider not only the number of micropropagated plants but also the morphophysiological quality of the resulting plants. Thus, the findings of this study, combined with those previously reported in the scientific literature for the Orchidaceae family, support the inference that photoautotrophic and photomixotrophic micropropagation systems promote the *ex vitro* establishment of plants when cultivated *in vitro*, making them recommended for the *in vitro* cultivation of *B. tuberculata*.

5. Conclusion

The use of a micropropagation system that allows gas exchange, along with sucrose concentrations of up to 30 g L^{-1}, enhances the shoot and root growth of *B. tuberculata* plants propagated *in vitro*. For *ex vitro* establishment, the micropropagation system that allows gas exchange is recommended regardless of the sucrose concentration used.

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References

BESSON, J.C.F., OLIVEIRA, L.K., BONETT, L.P. and STEFANELLO, S., 2010. Fontes e concentrações de carboidratos no crescimento vegetativo e no enraizamento *in vitro* de *Miltonia flavescens* Lindl. *Revista Brasileira de Biociências*, vol. 8, no. 1, pp. 9-13.

CAOVILA, L.E., GIANINI, P.F. and PEDROSO-DE-MORAES, C., 2016. Concentração de sacarose e índices de Ph no crescimento *in vitro* de *Oncidium flexuosum* SIMS. (Orchidaceae). *Revista em Agronegócio e Meio Ambiente*, vol. 9, no. 3, pp. 531-545. http://dx.doi.org/10.17765/2176-9168.2016v9n3p531-545.

CHA-UM, S., ULZIILBAT, B. and KIRDMANEE, C., 2010. Effects of temperature and relative humidity during *in vitro* acclimatization, on physiological changes and growth characters of Phalaenopsis adapted to in vivo. *Australian Journal of Crop Science*, vol. 4, pp. 750-756.

DANKIN, M.E. and HUSSEY, R.S. 1985. Staining and histopathological techniques in nematology. In: K. R. BARKER, C. C. CARTER and J. N. SASSER, ed. *An advanced treatise on Meloidogyne*. Raleigh: North Carolina State University, pp. 39-48.

FARIA, R.T., RODRIGUES, F.N., OLIVEIRA, L.V.R. and MÜLLER, C., 2004. *In vitro Dendrobium nobile* plant growth and rooting in different sucrose concentrations. *Horticultura Brasileira*, vol. 22, no. 1, pp. 780-783. http://dx.doi.org/10.1590/S0102-05362004000400023.

FERREIRA, W.M., VASCONCELOS, M.C., SILVA, C.C.N., OLIVEIRA, H.R. and SUZUKI, R.M., 2017. Asymbiotic germination, multiplication and development of *Alatiglossum fuscopetalum* (Orchidaceae) as affected by culture medium, sucrose and growth regulators. *Iheringia. Série Botânica*, vol. 72, no. 1, pp. 57-65. http://dx.doi.org/10.21826/2446-8231201772106.

FRITSCHE, Y., DEOLA, F., SILVA, D.A., HOLDERBAUM, D.F. and GUERRA, M.P., 2022. *Cattleya tigrina* (Orchidaceae) *in vitro* regeneration: main factors for optimal protocorm-like body induction and multiplication, plantlet regeneration, and cytogenetic stability. *South African Journal of Botany*, vol. 149, pp. 96-108. http://dx.doi.org/10.1016/j.sajb.2022.05.059.

FUENTES, G., TALAVERA, C., DESJARDINS, Y. and SANTAMARÍA, J.M., 2007. Low exogenous sucrose improves *ex vitro* growth and photosynthesis in coconut *in vitro* plantlets if grown *in vitro* under high light. *Acta Horticulturae*, no. 748, pp. 151-155. http://dx.doi.org/10.17660/ActaHortic.2007.748.18.

GALDIANO-JÚNIOR, R.F., MANTOVANI, C. and LEMOS, E.G.M., 2018. Carbohydrates on the growth of *Cattleya schilleriana* Reichb.f. seedlings (Orchidaceae). *Ciência Rural*, vol. 48, no. 7, pp. e20160977. http://dx.doi.org/10.1590/0103-8478cr20160977.

GALDIANO-JÚNIOR, R.F., MANTOVANI, C., FARIA, R.T. and LEMOS, E.G.M., 2013. Concentrações de sacarose no desenvolvimento *in vitro* e na aclimatização de *Cattleya loddigesii* Lindley. *Semina: Ciências Agrárias*, vol. 34, no. 2, pp. 583-592. http://dx.doi.org/10.5433/1679-0359.2013v34n2p583.

HAGQUIST, C.W., 1974. Preparation and care of microscope slides. *The American Biology Teacher*, vol. 36, no. 1, pp. 414-417. http://dx.doi.org/10.2307/4444888.

HERRMANN, M.H., FREITAS, E.M. and PÉRICO, E., 2011. Cultivo *in vitro* de plântulas de orquídea em meio de cultura alternativo. *Current Agricultural Science and Technology*, vol. 17, no. 1-4, pp. 162-166.

KOZAI, T., 2010. Photoautotrophic micropropagation-environmental control for promoting photosynthesis. *Propagation of Ornamental Plants*, vol. 10, no. 4, pp. 188-204.

LEIFERT, C., MURPHY, K.P. and LUMSDEN, P.J., 1995. Mineral and carbohydrate nutrition of plant cell and tissue cultures. *Critical Reviews in Plant Sciences*, vol. 14, no. 2, pp. 83-109. http://dx.doi.org/10.1080/07352689509701923.

LEIFERT, C., PRYCE, S., LUMSDEN, P.J. and WAITES, W.M., 1992. Effect of medium acidity on growth and rooting of different plants growing *in vitro*. *Plant Cell, Tissue and Organ Culture*, vol. 30, no. 1, pp. 171-179. http://dx.doi.org/10.1007/BF00040019.

LEMES, C.S.R., SORGATO, J.C., SOARES, J.S. and ROSA, Y.B.C.J., 2014. Culture media and sucrose on initial *in vitro* growth of *Miltonia flavescens*. *Ciência Rural*, vol. 46, no. 3, pp. 499. http://dx.doi.org/10.1590/0103-8478cr20150368.

MACEDO, M.C., ROSA, D.B.C.J., SOARES, J.S., TATARA, M.B., HOFFMANN, N.T.K. and ROSA, Y.B.C.J., 2014. Armazenamento de sementes e aclimatização de *Brassavola tuberculata* Hook. *Semina: Ciências Agrárias*, vol. 35, no. 6, pp. 2883-2894. http://dx.doi.org/10.5433/1679-0359.2014v35n6p2883.

MAJADA, J.P., FAL, M.A., TADEO, F. and SÁNCHEZ-TAMÉS, R., 2002. Effects of natural ventilation on leaf ultrastructure of *Dianthus caryophyllus* L. cultured *in vitro*. *In Vitro Cellular & Developmental Biology. Plant*, vol. 38, no. 3, pp. 272-278. http://dx.doi.org/10.1079/IVP2001271.

MENGARDA, L.H.G., COLA, G.P.A., OLIVEIRA, S.C. and FREITAS, A.R., 2017. Multiplication, rooting *in vitro*, and acclimatization of *Brassavola tuberculata* Hook. (Orchidaceae), an orchid endemic to the brazilian Atlantic Rainforest. *Bioscience Journal*, vol. 33, no. 3, pp. 730-738. http://dx.doi.org/10.14393/BJ-v33n3-32987.

MIRANDA, N.A., XAVIER, A., OTONI, W.C., GALLO, R., GATTI, K.C., MOURA, L.C., SOUZA, D.M.S.C., MAGGIONI, J.H. and SANTOS, S.S.O., 2020. Quality and intensity of light in the *In Vitro* development of microstumps of *Eucalyptus urophylla* in a photoautotrophic system. *Forest Science*, vol. 66, no. 6, pp. 754-760. http://dx.doi.org/10.1093/forsci/fxaa027.

NONGDAM, P., BELESKI, D.G., TIKENDRA, L., DEY, A., VARTE, V., EL MERZOUGUI, S., PEREIRA, V.M., BARROS, P.R. and VENDRAME, W.A., 2023. Orchid micropropagation using conventional semi-solid and temporary immersion systems: a review. *Plants*, vol. 12, no. 5, pp. 1136. http://dx.doi.org/10.3390/plants12051136. PMid:36904000.

PAULINO, M.A.P., MARTINS, V., SILVA, A.P.R., KARSBURG, I.V., SILVA, J.C., CORBELLINI, M. and RONDON, M.J.P., 2021. Desenvolvimento *in vitro* de *Cyrtopodium Cachimboense* l. C. Menezes em diferentes níveis de sacarose. *Brazilian Journal of Development*, vol. 7, no. 2, pp. 18844-18860. http://dx.doi.org/10.34117/bjdv7n2-500.

PEREIRA, S.T.S., SORGATO, J.C., VENDRAME, W.A., FARIA, R.T. and PIVETTA, K.F.L., 2022. Light and culture medium formulations for *in vitro* germination and development of *Brassavola perrinii*. *Revista Ciência Agronômica*, vol. 53, no. e20207362, pp. 1-7. http://dx.doi.org/10.5935/1806-6690.20220037.

RIBEIRO, L.M., SORGATO, J.C., SCALON, S.P.Q., SOARES, J.S. and RIBEIRO, I.S., 2019. Influência da luz, ventilação natural e tamanho do frasco no crescimento e desenvolvimento de denphal (Orchidaceae). *Agrária*, vol. 14, no. 3, pp. e5957. http://dx.doi.org/10.5039/agraria.v14i3a5957.

ROSA, Y.B.C.J., MARQUES JÚNIOR, G.A., SOARES, J.S., ROSA, D.B.C.J., MACEDO, M.C. and CEZAR, A.M.A., 2013. Estudo da viabilidade de sementes de *Brassavola tuberculata* Hook. em função do período de armazenamento, tempo de cultivo e tratamento pré-germinativo. *Ornamental Horticulture (Campinas)*, vol. 19, no. 2, pp. 155-160. http://dx.doi.org/10.14295/rbho.v19i2.655.

SANTOS, G.C., CARDOSO, F.P., MARTINS, A.D., PASQUAL, M., OSSANI, P.C., QUEIROZ, J.M., REZENDE, R.A.L.S. and DÓRIA, J., 2020. Effect of light and sucrose on photoautotrophic and photomixotrophic micropropagation of *Physalis angulate*. *Bioscience Journal*, vol. 36, no. 4, pp. 1353-1367. http://dx.doi.org/10.14393/BJ-v36n4a2020-47738.

SASAMORI, M.H., ENDRES-JÚNIOR, D. and DROSTE, A., 2020. Conservation of *Vriesea flammea* LB Sm., an endemic Brazilian bromeliad: effects of nutrients and carbon source on plant development. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 80, no. 2, pp. 437-448. http://dx.doi.org/10.1590/1519-6984.215276. PMid:31291407.

SILVA, A.B., LIMA, P.P., OLIVEIRA, L.E.S. and MOREIRA, A.L., 2014. Crescimento *in vitro* e anatomia foliar de *Cattleya walkeriana* (Gardner, 1839) cultivada em sistema de ventilação natural. *Revista Ceres*, vol. 61, no. 6, pp. 883-890. http://dx.doi.org/10.1590/0034-737X201461060001.

SILVA, A.B., REIS, C.O., CAZETTA, J.O., CARLIN, S.D., LANDGRAF, P.R.C. and REIS, M.C., 2016. Effects of exogenous proline and a natural ventilation system on the *in vitro* growth of orchids. *Bioscience Journal*, vol. 32, no. 3, pp. 619-626. http://dx.doi.org/10.14393/BJ-v32n3a2016-31368.

SOARES, J.S., ROSA, Y.B.C.J., MACEDO, M.C., SORGATO, J.C., ROSA, D.B.C.J. and ROSA, C., 2012. Cultivo *in vitro* de *Brassavola tuberculata* (Orchidaceae) em meio de cultura alternativo suplementado com diferentes concentrações de açúcar e carvão ativado. *Magistra*, vol. 24, no. 3, pp. 226-233.

SOARES, J.S., ROSA, Y.B.C.J., TATARA, M.B., SORGATO, J.C. and LEMES, C.S.R., 2014. Identificação da viabilidade de sementes de orquídeas pelo teste de tetrazólio. *Semina: Ciências Agrárias*, vol. 35, no. 5, pp. 275-284. http://dx.doi.org/10.5433/1679-0359.2014v35n5p2275.

SOARES, J.S., SORGATO, J.C. and RIBEIRO, L.M., 2020. Protocolo para germinação assimbiótica e desenvolvimento inicial de protocormos de orquídeas nativas do Cerrado brasileiro. *Rodriguésia*, vol. 71, pp. e01332018. http://dx.doi.org/10.1590/2175-7860202071095.

SOUSA, G.G., OTUBO, B.M.R., SORGATO, J.C., SOARES, J.S. and RIBEIRO, L.M., 2020. Armazenamento de sementes e concentrações de ágar no cultivo *in vitro* de *Brassavola tuberculata* Hook. (Orchidaceae). *Iheringia. Série Botânica*, vol. 75, pp. E2020017. http://dx.doi.org/10.21826/2446-82312020v75e2020017.

SOUSA, G.G., ROSA, Y.B.C.J., MACEDO, M.C. and SOARES, J.S., 2015. Aclimatização de *Brassavola tuberculata* com a utilização de ANA em diferentes substratos. *Horticultura Brasileira*, vol. 33, no. 02, pp. 208-215. http://dx.doi.org/10.1590/S0102-053620150000200012.

TEIXEIRA DA SILVA, J.A., HOSSAIN, M.M., SHARMA, M., DOBRÁNSZKI, J., CARDOSO, J.C. and ZENG, S., 2017. Acclimatization of *in vitro*-derived *Dendrobium*. *Horticultural Plant Journal*, vol. 3, no. 3, pp. 110-124. http://dx.doi.org/10.1016/j.hpj.2017.07.009.

VENDRAME, W.A., XU, J.J. and BELESKI, D.G., 2023. Micropropagation of *Brassavola nodosa* (L.) Lindl. using SETIS™ bioreactor. *Plant Cell, Tissue and Organ Culture*, vol. 153, no. 1, pp. 67-76. http://dx.doi.org/10.1007/s11240-022-02441-y.

XIAO, Y., NIU, G. and KOZAI, T., 2011. Development and application of photoautotrophic micropropagation plant system. *Plant Cell, Tissue and Organ Culture*, vol. 105, no. 2, pp. 149-158. http://dx.doi.org/10.1007/s11240-010-9863-9.

XU, J.J., BELESKI, D.G. and VENDRAME, W.A., 2022. Effects of culture media and plant growth regulators on *in vitro* propagation of *Brassavola nodosa. In Vitro Cellular & Developmental Biology. Plant*, vol. 58, no. 6, pp. 931-941. http://dx.doi.org/10.1007/s11627-022-10276-7.