ARTÍCULO CORTO

An easy hardening off method for micropropagated *Guarianthe skinneri* (Orchidaceae) plantlets

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Abstract

This study reports an easy protocol for hardening off plantlets of the tropical orchid *Guarianthe skinneri*, which is a culturally relevant plant species in several countries of the Central American region. In order to increase the *post vitro* survival rate of micropropagated plants, we included a "preparation step" before transferring the plants from the laboratory to the greenhouse. This preparation step (two weeks of duration in Dalla Rosa and Laneri or KO7 medium) consisted in the ventilation of the culture vessel. Results showed that, in comparison with the control treatment, ventilation of the culture vessel (aperture of 88 mm^2) yielded the greatest survival (90%) and best appearance (3.4 on a hedonic scale of 0.0 to 4.0) of plants. Results are explained with base in the hydric relations of the plants with their microenvironment.

Keywords:
Acclimatization
in vitro propagation
Micropropagation
Orchids
Water balance

Un método simple para endurecer plántulas micropropagadas de *Guarianthe skinneri* (Orchidaceae)

Resumen

Este estudio reporta un protocolo simple para el endurecimiento de plántulas de la orquídea tropical *Guarianthe skinneri*, la cual es una especie vegetal de importancia cultural en varios países de la región centroamericana. Con el fin de incrementar la tasa de supervivencia *post vitro* de las plantas micropropagadas, incluimos un "paso de preparación" antes de transferir las plantas del laboratorio al invernadero. Este paso de preparación (de dos semanas de duración en medio Dalla Rosa y Laneri o KO7) consistió en la ventilación del recipiente de cultivo. Los resultados mostraron que, en comparación con el tratamiento control, la ventilación del recipiente de cultivo (apertura de 88 mm^2) produjo mayor supervivencia (90%) y mejor apariencia (3.4 en una escala hedónica de 0.0 a 4.0) de las plantas. Los resultados se explican con base en las relaciones hídricas de las plantas con su microambiente.

Palabras clave:
Aclimatación
Propagación *in vitro*
Micropropagación
Orquídeas
Balance hídrico

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1. Introduction

Orchids have been one of the most appreciated and admired groups of ornamental plants for many centuries by different civilizations, due to the great beauty of their flowers (Ticktin et al., 2023). They are mainly used as potted ornamentals and for cut flowers. *Guarianthe (Cattleya)* is one of the most popular genera of orchids in the international market, such as *Cattleya skinneri* (Pant et al., 2020), which has been reclassified as *Guarianthe skinneri* (Dressler and Higgins, 2003). This species is culturally important in several countries of the Central American region; for example, in Costa Rica was declared the national flower in 1939 (Abarca, 2020), while in Southern Mexico, it is commonly known as "Candelaria" and is the symbol of a religious festival (Ovando et al., 2023).

In order to increase the rate of propagation of this species, many laboratories have undertaken processes for its micropropagation with successful results (Hernández-Domínguez et al., 2024; Leyva-Ovalle et al., 2020). However, a frequent problem with orchid in vitro propagation is the lack of ability of *ex vitro* plants to adequately acclimatizing in field conditions (Hartmann et al., 1997; Ovando et al., 2005), particularly in hostile, warm, humid, and propitious areas for the development of pathogens as in the humid tropics. *Ex vitro* orchids have been reported to survive from 30% to 50%, due to the morphological and physiological changes of the plant in the *in vitro* stage (Hew and Yong, 1997; Poniewozik et al., 2021). Therefore, it is necessary to develop an effective protocol to "harden off" orchid plants before the acclimatization phase to prepare morpho-physiologically the plants.

This study proposed the establishment of a "preparation phase" that includes the modification of culture conditions in the last *in vitro* stage, such as the micro-ventilation of the container in order to determine their effects on the quantity and quality of *G. skinneri* vitroplants.

2. Materials and Methods

2.1. Plant material

Six-month-old plantlets from an *in vitro* propagation system were used, which were in the multiplication stage. Plants had a size ranging between 1.5 cm and 2.0 cm in height. Before the experiment, plants were sub-cultivated three times in media with 1.0 mg L^{-1} of the cytokinin 6-benciladenine. Plants had four to five leaves and six to eight roots.

2.2. Culture medium

The culture medium used was KO7 (Dalla Rosa and Laneri, 1977), whose composition is: $Ca(NO_3)_2.4H_2O$ (1 g L^{-1}), $MgSO_4.7H_2O$ (0.25 g L^{-1}), KH_2PO_4 (0.25 g L^{-1}), $(NH_4)_2SO_4$ (0.5 g L^{-1}), $MnSO_4.4H_2O$ (7.5 mg L^{-1}), $FeSO_4.7H_2O$ (27.85 mg L^{-1}), NaEDTA.$2H_2O$ (37.25 mg L^{-1}). The medium was supplemented with coconut water (150 mL L^{-1}), sucrose (20 g L^{-1}), agar (8 g L^{-1}) and 0.5 g L^{-1} of activated charcoal; the last one to avoid oxidation of the culture medium and roots of the plants. The pH of the culture medium was adjusted to 5.5, deposited in 100 mL flasks (baby food jars) with screw plastic cap in 20 mL volumes and autoclaved at 121 °C for 15 minutes.

2.3. Effect of the ventilation of the culture container

A complete random experiment was designed to evaluate the effect of the ventilation of the culture vessel, with four treatments and three replicates, 12 experimental units (one plant per culture container). The bottles used were "baby food jars" with capacity of 110 mL and with a mouth area of 1590 mm^2. Ventilation was done by circular perforation of the bottle caps and prior to autoclaving the windows were covered with Whatman No. 40 filter paper. Treatments were Control (without ventilation), T1 (44 mm^2), T2 (88 mm^2) and T3 (132 mm^2).

The "preparation" period had a duration of two weeks, under a luminous intensity of 660 lux with a photoperiod of 12 hours and room temperature of 28 °C, after which the plantlets went to the greenhouse for their acclimatization for 30 days, where the conditions were average temperature of 30 °C and relative humidity of 70%.

2.4. Variables and data analysis

After the preparation period (two weeks), water content of the plant and of humidity of the remainder culture medium was determined. After the greenhouse period (30 days), the plant survival, appearance of plantlets (on a hedonic scale of 0 to 4), leaf and cuticle thickness, and number and size of stomata were evaluated. Data were processed by analysis of variance and means separation by the Tukey method (α 0.05).

3. Results and Discussion

The influence of the ventilation of the culture container for two weeks, followed by 30 days of acclimatization, on the survival of the tropical orchid *Guarianthe skinneri* is showed in Figure 1. It is remarkable that while the water content in the culture medium diminished (Figure 1A), the plants accumulated most water in their tissues (Figure 1B).

Plants response to water stress by dramatically complex mechanisms, including drought avoidance via enhancing capacity of getting water and drought tolerance mainly via improving osmotic adjustment ability and increasing cell wall elasticity to maintain tissue turgidity (Taticharoen et al., 2023; Xu et al., 2010). In this study, plants grown with ventilation may have experienced rapid adjustments in their water relations.

Several authors have pointed out that plants grown *in vitro* exhibit physiological and anatomical modifications depending on culture conditions, especially, the leaves undergo temporary modifications that prevent them from adequate adaptation (Buyun et al., 2021; George and Sherrington, 1984; Hazarika, 2006). In our study, the treatment of 44 mm^2 of ventilation area promoted the greatest thickening of the leaves and of cuticle thickness (Table 1,

ANOVA P <0.05), being the last variable one of the most important, since the cuticle is a waxy layer that protects the top of the leaves from solar radiation. Similar results were found by Ramos et al. (2001), who proved that ventilation of the culture vessel and the addition of the phytohormone abscisic acid (ABA) to the culture medium favors leaf development (greater thickness, fewer stomas, and more chloroplasts), allowing a better survival of vitroplants of *Tagetes erecta* when these are transferred to the field. Figure 2 shows a comparison between the thickness of the leaves in the treatment of 44 mm^2 and the control without ventilation.

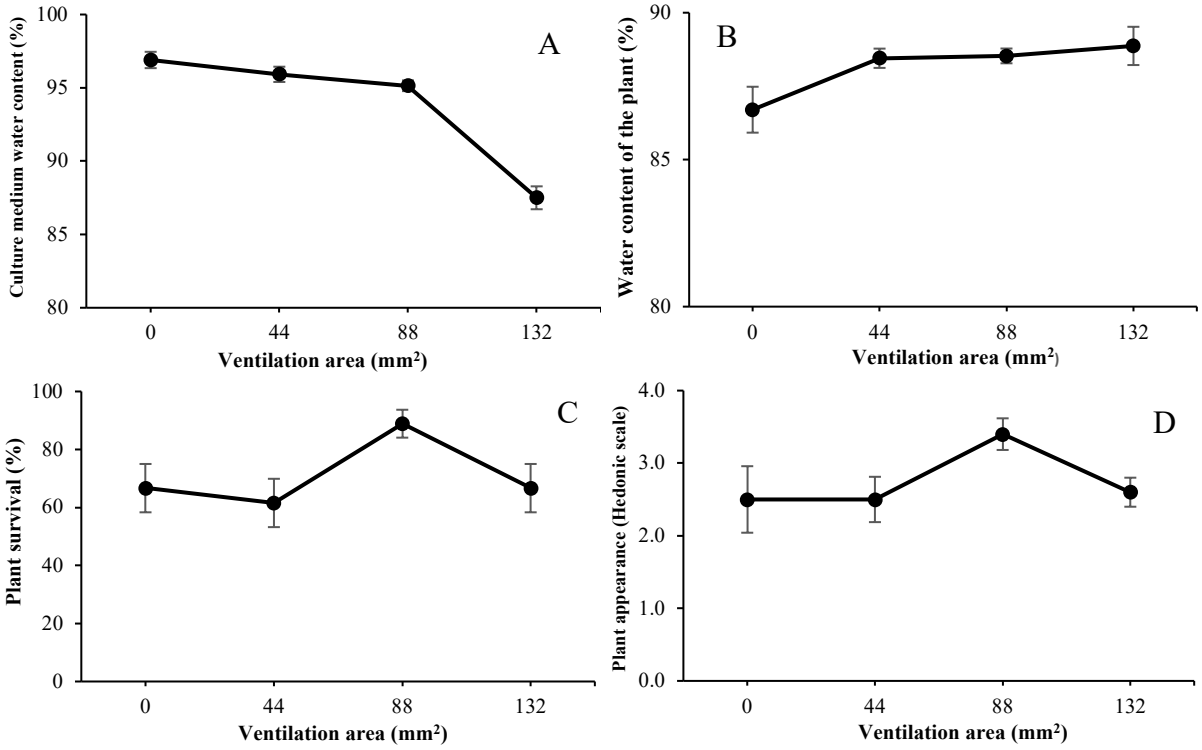

Figure 1. Influence of the ventilation of the culture container on the micropropagation of the tropical orchid *Guarianthe skinneri*. After two weeks of laboratory incubation: A) Effect on the water content of the remainder culture medium; B) Effect on the plant water content. After 30 days in greenhouse: C) Effect on the survival of the plants; D) Effect on the appearance of the plants.

Table 1. Foliar anatomical variables of *ex vitro* plantlets of the tropical orchid *Guarianthe skinneri* grown in culture containers with ventilation.

Ventilation area (mm^2)	Leaf thickness (µm)	Cuticle thickness (µm)	Leaf stomata/mm^2	
			Adaxial	Abaxial
0	446.4 ± 70.0ab	4.6 ± 0.4c	14.6 ± 2.1a	60.4 ± 11.1b
44	509.0 ± 64.6a	12.2 ± 1.5a	3.9 ± 1.1b	89.3 ± 15.2a
88	410.4 ± 26.3b	5.1 ± 1.8bc	3.2 ± 1.4b	119.5 ± 17.3a
132	383.2 ± 15.9bc	7.4 ± 2.0b	3.9 ± 1.8b	108.3 ± 9.1a

The data with different letters indicate Tukey statistical difference (α 0.05).

The stomata number per square millimeter showed that the control plants (microenvironment with high relative humidity) have more stomata on the adaxial surface of the leaf in comparison to the rest of treatments, which, in nursery conditions, leads to a faster dehydration of the plantlet. It is possible that ventilation of the containers reduces the relative humidity in them, allowing better stomatal functioning, increases the production of epicuticular waxes, and allows the increase of CO_2 (Paz-Silva et al., 2004).

However, the treatment with better results was that of 88 mm^2 of ventilation had a survival rate of almost 90% and an appearance value of 3.4 (Figures 1C, 1D). These results contrast with that mentioned by several authors, who cite 30% to 50% of *ex vitro* survival rates for orchids in hostile, hot, humid climates, and favorable for the development of phytopathogenic microorganisms (Ovando et al., 2005). Although the present study was carried out in the dry season, the conditions were of excessive heat and with relatively high ambient humidity. Figure 3 shows plants grown in containers with 88 mm^2 of ventilation and plants grown in closed containers.

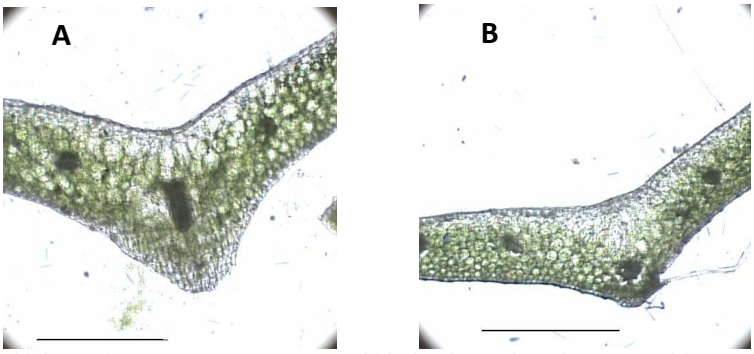

Figure 2. Comparison of leaf thickness in *Guarianthe skinneri* orchid plantlets cultured *in vitro* with vessel ventilation (44 mm^2, A) and the control without ventilation (B). The bar represents 500 µm.

Figure 3. *Guarianthe skinneri* orchid plants grown in closed containers and with 88 mm^2 of ventilation.

Based on the results obtained, the micropropagation process of the tropical epiphytic orchid *G. skinneri* is improved when a "preparation phase" of the plantlets is used for *ex vitro* conditions; this stage lasts for two weeks and consists in the decrease of the relative humidity of the container (through ventilation of 88 mm^2). This finding is similar to that of Fuentes et al. (2001), who found that the ventilation of the culture container and reduction of saccharose together with increased light intensity minimized the problems of low survival and slow growth of vitroplants of several species. Ventilation helped plants to have better stomatal control and reduced leaf water loss, while reducing saccharose to half the normal concentration produced higher rates of photosynthesis.

4. Conclusion

The hardening off method designed in this work consisted in the culture of six-month-old plants in KO7 medium supplemented with coconut water, sucrose, and activated charcoal, for two weeks. In the best treatment, the culture containers had ventilation of 88 mm^2 and was incubated under a luminous intensity of 660 lux with a photoperiod of 12 hours and room temperature of 28 °C. After the transference of the plants to greenhouse conditions (average temperature of 30 °C and relative humidity of 70%) the plant survival was about 90%.

Conflict of interest

The authors declare that they have no conflict of interest

References

Abarca SO. 2020. Rendidos ante la Guaria morada. Revista Herencia, 33(1), 135-179.

Buyun L, Ivannikov R, Batsmanova L, Taran N, Kovalska L, Gurnenko I, Maryniuk M. 2021. Structural adaptation and antioxidant response of *Guarianthe bowringiana* (O'Brien) Dressler & WE Higgins (Orchidaceae Juss.) seedlings during *ex vitro* acclimatization. Acta Agrobotanica, 74(1), 7422.

Dalla Rosa M, Laneri U. 1977. Modification of nutrient solutions for germination and growth "in vitro" of some cultivated orchids and for the vegetative propagation of *Cymbidium* cultivars. American Orchid Society Bulletin, 46, 813-820.

Dressler RL, Higgins WE. 2003. *Guarianthe*, a generic name for the "Cattleya" skinneri complex. Lankesteriana, **7**, 37-38.

Fuentes G, Aguilar M, Talavera C, Espadas F, Coello J, Quiroz A, Maust B, Santamaría J. 2001. ¿La ventilación del contenedor y la disminución de sacarosa del medio, mejoran la sobrevivencia y crecimiento de plantas cultivadas *in vitro* al ser transferidas al campo? En: Memorias del IX Congreso Nal. de Biotecnología y Bioingeniería. SMBB. Veracruz, Ver., México. Septiembre de 2001.

George EF, Sherrington PD. 1984. Plant propagation by tissue culture. Exegetics Limited. New York.

Hartmann TH, Kester DE, Davies FT, Geneve RL. 1997. Plant propagation. Principles and practices. 6th Edn. Prentice Hall. New Jersey.

Hazarika BN. 2006. Morpho-physiological disorders in in vitro culture of plants. Scientia Horticulturae, 108(2), 105-120.

Hernández-Domínguez E, López-Aguilar DR, Orduño-Cruz A, Zetina-Córdoba P, Ramírez-Mosqueda, MA 2024. Micropropagation of *Guarianthe skinneri* (Bateman) Dressler & WE Higging in temporary immersion bioreactors. Methods in Molecular Biology, 2759, 149-156.

Hew CS, Yong JW. 1997. The physiology of tropical orchids in relation to the industry. World Scientific.

Leyva-Ovalle OR, Bello-Bello JJ, Murguía-González J, Núñez-Pastrana R, Ramírez-Mosqueda MA. 2020. Micropropagation of *Guarianthe skinneri* (Bateman) Dressler et WE Higging in temporary immersion systems. 3 Biotech, 10, 26.

Ovando I, Damon A, Bello R, Ambrosio D, Albores V, Adriano L, Salvador M. 2005. Isolation of endophytic fungi and their mycorrhizal potencial for the tropical epiphytic orchids *Cattleya skinneri, C. aurantiaca* and *Brassavola nodosa*. Asian Journal of Plant Sciences, 4(3), 309-315.

Ovando I, Ruiz-González S, Adriano-Anaya L, Salvador-Figueroa M. 2023. Flor de la Candelaria: (*Guarianthe skinneri*; Orchidaceae). La biotecnología a su rescate. KDP-Amazon, United States of America.

Pant M, Negi A, Singh A, Gautam A, Rawat M. 2020. Cattleya orchids: A mini review. Journal of Critical Reviews, 7(12), 2394-5125.

Paz-Silva R, Villegas-Monter A, Trejo-López C, Terrazas-Salgado T, Cervantes-Martínez C. 2004. Niveles de sacarosa y pérdida de agua del medio de cultivo durante el enraizamiento *in vitro* de dos portainjertos de vid. Revista Chapingo serie Horticultura, 10(2), 119-125.

Poniewozik M, Parzymies M, Szot P, Rubinowska K. 2021. *Paphiopedilum insigne* morphological and physiological features during *in vitro* rooting and *ex vitro* acclimatization depending on the types of auxin and substrate. Plants, 10(3), 582.

Ramos AN, Aguilar M, Quiroz A, Santamaría J. 2001. Efecto del ABA exógeno en la anatomía de hojas de plantas de *Tapetes erecta* cultivadas *in vitro*. En: Memorias del IX Congreso Nal. de Biotecnología y Bioingeniería. SMBB. Veracruz, Ver., México. Septiembre de 2001.

Taticharoen T, Matsumoto S, Chutteang C, Srion K, Malumpong C, Abdullakasim S. 2023. Response and acclimatization of a CAM orchid, *Dendrobium* Sonia 'Earsakul' to drought, heat, and combined drought and heat stress. Scientia Horticulturae, 309, 111661.

Ticktin T, Charitonidou M, Douglas J, Halley JM, Hernández-Apolinar M, Liu H, Phelps J. 2023. Wild orchids: A framework for identifying and improving sustainable harvest. Biological Conservation, 277, 109816.

Xu Z, Zhou G, Shimizu H. 2010. Plant responses to drought and rewatering. Plant Signaling & Behavior, 5(6), 649-654.