Colegio de Postgraduados

Clonal micropropagation and *ex-situ* conservation of *Rhyncholaelia digbyana* (Lindley) Schltr

Chi-Ramírez Maura, R.[1]; Herrera-Cool, Gilbert J.[2]; Sánchez-Contreras, A.[1]; López-Puc, Guadalupe[1*]

[1] Centro de Investigación y Asistencia en Tecnología y Diseño del estado de Jalisco AC. Subsede Sureste. Parque Científico y Tecnológico de Yucatán. Tablaje catastral 31264 km 5.5 Carr. Sierra papacal- Chuburna puerto, C.P. 97302. Mérida, México.

[2] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Chetumal, km 25 Carretera Chetumal-Bacalar, Xul-Ha, Quintana Roo, México. C.P. 77963.

* Correspondence: glopez@ciatej.mx

ABSTRACT

Objective: To obtain protocols for clonal micropropagation, crop planning, and *in vitro* conservation of *Rhyncholaelia digbyana* (Lindley) Schltr.

Methodology: The effects of the Kundson C basal medium and benzylaminopurine concentration were evaluated for clonal micropropagation. The treatment with the greatest number of shoots formed per apex was selected for crop planning. Experiments were conducted to determine the effect of basal medium Murashige and Skoog concentration at 2.2 gL^{-1} and 4.4 gL^{-1}; sorbitol, mannitol, and sucrose at 1, 2, and 3% on slow growth.

Results: The best treatment for clonal micropropagation and crop planning was identified as 21.60 gL^{-1} Knudson C with 8.80 μM benzylaminopurine. This treatment resulted in uniform-sized shoots produced. The multiplication process can yield 10,240 seedlings in 12 months. Slow growth was achieved using Murashige and Skoog basal media at 2.2 gL^{-1} with 1% mannitol.

Implications: More experiments must be conducted to determine the best shoot induction conditions and improve resource efficiency.

Conclusions: These findings represent the first report on micropropagation and *ex-situ* conservation to preserve germplasm for this species as an important resource for the floriculture industry.

Keywords: mannitol, minimum growth, orchids conservation, plant tissue culture, sorbitol.

Citation: Chi-Ramírez, M. R., Herrera-Cool, G. J., Sánchez-Contreras, A., &López-Puc, G. (2024). Clonal micropropagation and *ex-situ* conservation of *Rhycncholaelia digbyana* (Lindley) Schltr. *Agro Productividad*. https://doi.org/10.32854/agrop.v17i6.2832

Academic Editor: Jorge Cadena Iñiguez
Guest Editor: Daniel Alejandro Cadena Zamudio

Received: February 19, 2024.
Accepted: May 17, 2024.
Published on-line: July 04, 2024.

Agro Productividad, 17(6). June. 2024. pp: 141-150.

INTRODUCTION

Orchids are highlighted in floriculture, with a global revenue of more than 400 million US dollars (Kanlayavattanakul *et al.*, 2018). Further, orchids have ethnopharmacological activities (Kaur *et al.*, 2022). Conventionally, orchid propagation is 2 to 3 years. The availability of prized commercial orchids is limited due to the paucity of plant materials. Plant tissue culture is crucial in ensuring a supply of raw materials for the floriculture industry (Bhattacharyya *et al.*, 2023; Cazar *et al.*, 2023). Plant tissue culture of orchids has been achieved with different focus. For example, propagation from asymbiotic seed germination of orchids such as *Dracula felix* (Luer), *Thrichocentrum stramineum*, *Thunia marshalliana*, and *Dendrobium densiflorum* (Quijia-Lamiña *et al.*, 2023; Ramos-Ortiz *et al.*, 2020; Pongener & Ranjan Deb, 2019). Clonal micropropagation from shoot tips culture of *Brassavola nodosa* and *Doritis pulcherrima* (Xu *et al.*, 2022; Mondal *et al.*, 2013). On the other hand, clonal micropropagation from Transverse thin cell layer (t-TCL) segments of *Malaxis acuminata* (Bhattacharyya *et al.*, 2022). Clonal micropropagation from apices has been evaluated in species such as *Delonix regia*, *Origanum scabrum*,

Abelmoschus esculentus, and *Trichilia pallida*, using basal media such as Murashige and Skoog basal media (MS) (Alexopoulos *et al.*, 2023; de Souza *et al.*, 2021; de Oliveira Costa *et al.*, 2020). In orchids, MS is the most used basal media for micropropagation (Ahmadi *et al.*, 2023; Xu *et al.*, 2022; Bhattacharyya *et al.*, 2022;); as well as Knudson C (Pathak *et al.*, 2022; Ramos-Ortiz *et al.*, 2020), and Vacin and Went (Quijia-Lamiña *et al.*, 2023; Sunitibala & Neelashree, 2018). For clonal micropropagation, cytokinins such as benzilamkinetinynopurine (BAP), kinetin, and zeatin are plant growth regulators (PGRs) that induce cell division, generation of shoots, initiation, and elongation of roots. Orchid micropropagation requires a crop planning if the purpose is to market them. This planning involves organizing time, materials, labor, and space to produce the number of plants needed according to the market demand in a stipulated time. On the other hand, the Orchidaceae family faces threats due to the disturbed integrity of the ecosystems (Fonge *et al.*, 2019; Debnath & Kumaria, 2023). Although *in situ* conservation is essential for preserving plant species and maintaining genetic variability, it poses risks, such as invasion by pests, diseases, and natural disasters. Thus, *ex-situ* conservation is indicated for the conservation (Generoso *et al.*, 2023). *Ex-situ* conservation is achieved through seed banks, a relatively low-cost method for preserving the genetic diversity of many individuals. An alternative to reduce labor and time spent is to induce slow plant growth to reduce the turnover frequency (Chappell *et al.*, 2020; Generoso *et al.*, 2023). Slow or minimum growth is a biotechnological tool for medium-term plant germplasm conservation under *in vitro* conditions, reduces the metabolic activity, *i.e.*, the growth rate of *in vitro* cultures, by maintaining them on a modified growth medium or in altered culture conditions (Chauhan *et al.*, 2019). Physical factors chemical substances for supplementation of culture media can be applied to conserve plants by the slow-growth method (Mayo-Mosqueda *et al.*, 2022; El-Hawaz *et al.*, 2019). These factors are evaluated individually or combined. In tissue culture media, slow growth using chemical substances is induced using sorbitol, sucrose, or mannitol. Mannitol is a sugar alcohol that causes osmotic stress by reducing plant water uptake, resulting in slower plant growth in tissue culture media (Chappell *et al.*, 2020). Mannitol has been evaluated for slow growth in *Morus alba*, *Vanilla planifolia*, *Saccharum* spp., and *Xanthosoma* spp. (Espinosa-Reyes *et al.*, 2021; Bello-Bello *et al.*, 2015; Bello-Bello *et al.*, 2014; Rayas *et al.*, 2013). Sorbitol is also an osmoregulatory agent used to slow the growth of bananas, potatoes, and Asparagus (Singh *et al.*, 2021; Muñoz *et al.*, 2019; Thakur *et al.*, 2015;). Sucrose, another osmolyte, has also been evaluated for in vitro conservation through minimal growth in species such as *Morus alba*, *Dioscorea alata*, and *Dioscorea rotundata* (Espinosa-Reyes *et al.*, 2021; Díaz *et al.*, 2015). *Rhyncholaelia digbyana* (Lindl.) Schltr is distributed in southern Mexico, Guatemala, Honduras, and Costa Rica. It is an epiphytic plant that produces white or greenish, aromatic, long-lasting flowers. This species has the largest flower of the orchids found in the Yucatan Peninsula and has economic importance in hybridization processes (Wrigth *et al.*, 2017; Sánchez-Martínez *et al.*, 2002). Hence, this research aims to obtain clonal micropropagation, crop planning, and the conditions for conserving *in vitro* plants of *Rhyncholaelia digbyana*.

MATERIALS AND METHODS

Plant material

Fully closed *Rhycncholaelia digbyana* from five capsules fully closed were collected from experimental Field El Tormento, Campeche, Mexico, at the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP). The methods for disinfection of capsules and asymbiotic germination of plants are described by (López Puc & Herrera Cool, 2022).

Clonal micropropagation from apices

For *in vitro* establishment, the apices were extracted from *Rhyncholaelia digbyana in vitro* plants of Bank germplasm of orchids of Research Center of Jalisco (CIATEJ). The apex size of 1 cm^2 was taken and placed separately according to treatment. The factors evaluated were the basal medium's concentration Knudson C (KC) and the BAP concentration, with two levels of each factor and one central. Point. The central point was the intermediate level of each factor. The dependent variables were the number of shoots per apex, shoot length, number of leaves, and leaf length. Five repetitions per treatment were performed, and the experimental unit was one explant per flask. All treatments were supplemented with 15% coconut water and gel rite at 0.23% w/v. The apices were sown in clonal propagation treatments for 90 days. Conditions of the photoperiod room were 23±2 °C, 16/8 (light/darkness). The pictures of shoot development from the apex were captured using a Nikon stereoscopic microscope (zoom 4, observation of 640 LP/mm).

Crop planning

A plan was devised for the large-scale propagation of *Rhyncholaelia digbyana* plants through micropropagation. The most effective treatment for inducing the highest number of shoots per apex was selected. To produce around 10,000 plants, the number of propagation cycles required was calculated. The initial explants used were apices from *in vitro* seedlings (cycle 1), then at 3, 6, 9, and 12 months of culture, plants obtained were multiplied (cycles 2-4) subcultures in the same multiplication medium. During each cycle, the number of shoots developed was counted to estimate the multiplication rate of shoots per cycle.

Slow growth for *in vitro* conservation

Slow growth experiments were conducted using 2 cm plantlets from asymbiotic germination and established in a factorial experiment of 2×2^3 for conservation. The three factors were the concentration of basal medium (2.2 gL^{-1} and 4.4 gL^{-1}), type of carbon source (sorbitol, mannitol, and sucrose), and carbon source concentration (1, 2, and 3% w/v). Two controls evaluated were MS at 2.2 gL^{-1} and 4.4 gL^{-1}) without a carbon source. All treatments were supplemented with Gelrite® SIGMA. Treatments with 2.2 gL^{-1} of MS basal medium were solidified with 3.1 gL^{-1} of Gelrite®, and treatments with 4.4 gL^{-1} MS were solidified with 2.2 gL^{-1} of Gelrite®. The media pH was adjusted to 5.7 before sterilization by autoclave at 121 °C for 15 minutes. Five plantlets per treatment

were cultured in 150×25 mm tubes. After being cultured in a conservation medium for six months, variables such as plantlet growth and root growth in millimeters and new shoots and leaves were evaluated.

The independent contribution of each variable on the induction of shoots and slow growth was analyzed by analyzing each response variable. Significant differences were calculated according to the LSD test at the 5% significance level using Stat graphics® Centurion XVI statistical software. Data were presented as means ± standard error.

RESULTS AND DISCUSSION

Clonal micropropagation from apices

This study presents the clonal propagation protocol by inducing *Rhyncholaelia digbyana* shoots from apices as explants. The induction of shoots from apices as an explant is possible because apical dominance is inhibited by the action of cytokinins, promoting the activation of axillary buds (de Souza *et al.*, 2021). An advantage of clonal propagation is that it provides greater genetic stability since meristematic activation is less favorable to somaclonal variation events (de Oliveira Costa *et al.*, 2020). In this study, clonal micropropagation was induced using Knudson C. This basal media is among the most used for micropropagation of orchids. It has been used in propagation from leaf explants of *Vanda cristata*. (Pathak *et al.*, 2022), as well as shoot tip culture by *Doritis pulcherrima* Lindl. (Mondal *et al.*, 2013).

The induction of shoots was significantly greater in the treatments with the highest concentrations of BAP (T3, T4, and T5). The highest average number of shoots was obtained using 21.60 gL^{-1} of KC with 8.80 μM BAP (T5) that showed a maximum multiplication rate (4.83±1.16, Table 1) like different authors (de Souza *et al.*, 2021) obtained between 5 and 7 shoots per explant by adding 2.0 μM BA to the MS medium. A similar response was obtained by Ram *et al.* (2014), where the maximum average shoot multiplication was 4.92 shoots per explant by adding 2.2 μM of BAP to the MS medium. It is considered an acceptable multiplication rate in an intermediate range. The Pareto chart standardized showed that BAP concentration had a significant effect on the variables of the number of shoots and leaves (Figure 1A and Figure 2A, respectively). This agrees with what was obtained by De Souza (2020), where the addition of BAP to the culture medium promoted the formation of axillary shoots from apices in *Trichilia pallida*. However, BAP concentration did not significantly affect shoot and leaf length (Figure 1B and Figure 2B, respectively). Regarding shoot length, lower concentrations of KC produced longer shoots. A significant effect of KC concentration was found for the variable of shoot length (Figure 1B) and number of new leaves (Figure 2A) at lower concentrations; these results can be related to the growth habit characteristics of epiphytic orchids, presenting nutritional minimal requirements (Mamani Sánchez *et al.*, 2022). While for shoot production and leaf length, there was no significant effect (Figure 1A and Figure 2B).

On the other hand, the number and length of leaves, T1 (17.80 gL^{-1} KC, 4.40 μM BAP), was the treatment that showed the highest number of leaves compared to the other treatments. (Table 1). T5 presented the highest number of new shoots and shoot length

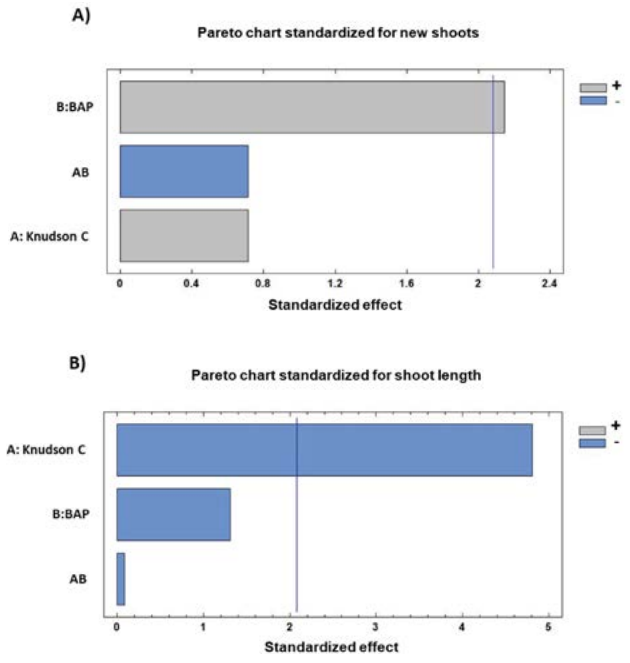

Figure 1. Main effects of benzylaminopurine (BAP) and Knudson C basal medium on the number of new shoots, shoot length during clonal micropropagation of *Rhyncholaelia digbyana* (Lindl.) Schltr.

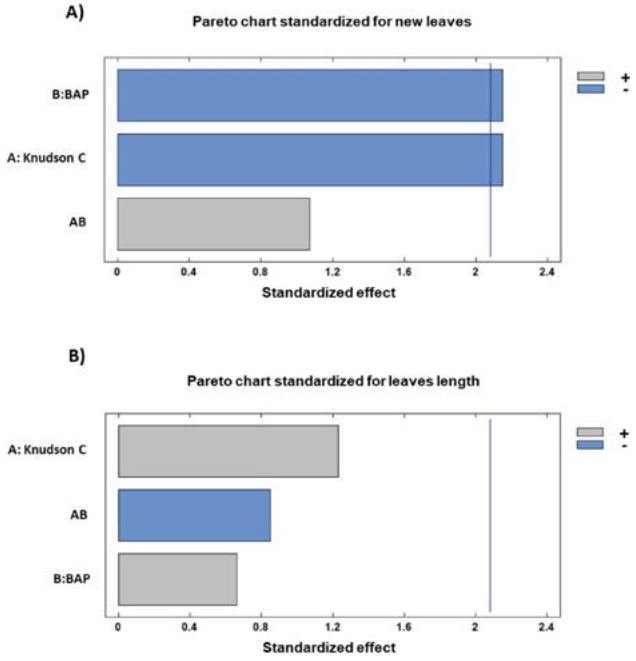

Figure 2. Main effects of BAP and the Knudson C basal medium on the number and length of leaves during clonal micropropagation of *Rhyncholaelia digbyana* (Lindl.) Schltr.

and was the third treatment with the largest shoots. Regarding the number of leaves, it was the second treatment with the highest number of leaves, so it was selected as the best treatment due to the morphology of the shoots with uniform size and the highest number of shoots produced.

Table 1. Clonal propagation from the apices of *Rhyncholaelia digbyana* (Lindley) Schlter.

Treatments	KC (gL^{-1})	BAP (μM)	Number of Shoots	Shoot length of the new shoots (mm)	Number of leaves	Leaves length (mm)
T1	17.80	4.40	3.33 ± 0.51^{a}	24.83 ± 4.30^{b}	3.83 ± 0.40^{b}	13.0 ± 1.89^{c}
T2	28.40	4.40	3.33 ± 1.75^{a}	14.83 ± 7.35^{b}	2.66 ± 1.36^{a}	9.33 ± 1.96^{a}
T3	17.80	12.0	4.33 ± 1.03^{ab}	19.83 ± 5.63^{ab}	3.00 ± 0.63^{ab}	11.0 ± 4.09^{ab}
T4	28.40	12.0	4.66 ± 0.81^{ab}	17.50 ± 3.50^{a}	3.33 ± 0.81^{ab}	8.50 ± 0.83^{a}
T5	21.60	8.80	4.83 ± 1.16^{b}	19.00 ± 3.68^{ab}	3.50 ± 0.54^{ab}	9.83 ± 1.47^{a}

Different letters indicate significant statistical differences according to the Tukey test ($p\leq0.05$). KC: Knudson C Basal Medium, BAP: 6 benzylaminopurine.

Crop planning

The plan to produce approximately 10,000 seedlings of *Rhyncholaelia digbyana* was carried out, considering that T5 produces an average of 4.8 shoots. For planning, 40 apex explants from 40 mother plants were used, each apex being planted individually in a jar with its respective culture medium for the development of shoots. At the end of the cycle, 160 average seedlings were obtained; in turn, the apices were obtained from these seedlings. Subsequently, the formation of shoots was induced again. The multiplication process is repeated until 10,240 seedlings are obtained from 2,560 induction flasks (Table 2).

Slow growth for *in vitro* conservation

The slow growth of *Rhyncholaelia digbyana* was achieved using mannitol at 1% like potatoes (Chappell *et al.*, 2020); However, on *Morus alba*, mannitol reduced growth but did not improve survival and physical condition of the explants during their *in vitro* conservation (Espinosa-Reyes *et al.*, 2021). There is evidence that depending on the response of the specific genotypes to media containing carbon sources, in *Catasetum integerrimum*, *Castanea* spp., slow growth can be obtained using sucrose (López Puc & Herrera Cool, 2022; Gomes *et al.*, 2021) the sorbitol was the best option for *Epidendrum chlorocorymbos* and *Vanilla planifolia* conservation (Lopez-Puc, 2013; Divakaran *et al.*, 2006). The sorbitol allowed more development of all variables' growth evaluated in *Rhyncholaelia digbyana*. Hence, sorbitol can be an adequate way to allow development after a slow growth period. MS medium at 2.2 and 4.4 gL^{-1} allows the slow growth of *Rhyncholaelia digbyana* plantlets, while in *Vanilla planifolia*, MS concentration was 1.5 gL^{-1} (Divakaran *et al.*, 2006). MS

Table 2. Crop plan for the production plants from apices of *Rhyncholaelia digbyana* (Lindley) Schlter with 1×4 rate multiplication of seedling.

Cycle number	Vegetal material source	Glass containers**	Plants obtained	Time (months)
1	40*	40	160	3
2	160	160	640	6
3	640	2560	2560	9
4	2560	2560	10240	12

* Seedlings (Initial source of apex explant). ** apex per glass container with 30 mL of 21.60 gL^{-1} Knudson C and 8.80 μM BAP.

at 2.2 gL^{-1} was used to conserve *Arnica montana* and Epindendroideae orchids (Petrova *et al*., 2021; Menezes-Sá *et al*., 2019). These results are relevant because there is scarce information about the *in vitro* propagation of *Rhyncholaelia digbyana*, so developing a clonal propagation protocol can be used for large-scale plant production. On the other hand, the slow growth protocol established will allow germplasm preservation, saving resources and replanting time.

The treatment with the lowest plant growth was SL5 (2.2 gL^{-1} of MS, 2% Sucrose); T5 had no significant difference with SL1-T4 and SL6-T12 treatments. Despite T5 being the best treatment for slow growth, the plant morphology was disproportionate since it had greater root growth in a proportion of 1 to 6 (2.75 mm of plant growth and 16.75 mm of root growth). In SL6 (2.2 gL^{-1} MS, 3% Sucrose), an average plant growth of 3.75 was observed, with 11 new leaves. However, in SL5 and SL6, the sucrose darkens the growing medium culture. Whereas SL10 (2.2 of gL^{-1} MS, 1% mannitol), an average of 5.25 mm of plant and root growth was obtained, 0.25 and 0.75 shoots and roots, respectively (Table 3), so this treatment was selected for the conservation of *Rhyncholaelia digbyana*. During a slow growth period, the carbon source had a significant effect on the variable for new shoots formed (Figure 3A), plant growth (Figure 3B), and new leaves formed (Figure 3C). The effect of the carbon source and MS strength had a significant effect on root growth variable (Figure 3D).

Table 3. Seedlings development of *Rhyncholaelia digbyana* (Lindley) Schlter in slow growth conditions.

Treatments	MS (gL^{-1})	Carbon source (%)		Plant growth (mm)	Root growth (mm)	New shoots	New leaves
SL1	4.4	Sucrose	10	5.50 ± 3.3^{abc}	1.00 ± 0.8^{a}	1.00 ± 0.1^{ab}	9.00 ± 2.1^{abcd}
SL2	4.4		20	3.75 ± 3.8^{ab}	0.50 ± 1.0^{a}	0.50 ± 0.1^{a}	5.25 ± 1.7^{abc}
SL3	4.4		30	3.5 ± 2.3^{ab}	0.75 ± 0.9^{a}	0.75 ± 0.1^{ab}	6.5 ± 2.4^{abc}
SL4	2.2		10	4.0 ± 0.8^{ab}	14.75 ± 3.5^{ab}	0.50 ± 0.1^{a}	2.00 ± 0.7^{ab}
SL5	2.2		20	2.7 ± 0.9^{a}	16.75 ± 5.0^{ab}	0.25 ± 0.1^{a}	0.75 ± 0.2^{a}
SL6	2.2		30	3.7 ± 2.7^{ab}	11.00 ± 4.9^{ab}	1.25 ± 0.2^{abc}	0.75 ± 0.2^{a}
SL7	4.4	Mannitol	10	7.0 ± 2.8^{abcd}	14.75 ± 7.0^{ab}	0.25 ± 0.1^{a}	3.00 ± 1.1^{ab}
SL8	4.4		20	5.00 ± 6.2^{ab}	11.25 ± 3.9^{ab}	0.00 ± 0.0^{a}	1.50 ± 0.6^{ab}
SL9	4.4		30	6.50 ± 3.6^{abcd}	10.75 ± 8.3^{ab}	0.25 ± 0.1^{a}	2.00 ± 0.4^{ab}
SL10	2.2		10	5.25 ± 1.3^{ab}	5.25 ± 5.1^{ab}	0.25 ± 0.1^{a}	0.75 ± 0.1^{a}
SL11	2.2		20	5.25 ± 1.5^{ab}	15.75 ± 4.8^{ab}	0.25 ± 0.0^{a}	1.25 ± 0.2^{ab}
SL12	2.2		30	5.25 ± 1.0^{ab}	11.75 ± 2.3^{ab}	0.00 ± 0.0^{a}	1.50 ± 0.2^{ab}
SL13	4.4	sorbitol	10	10.75 ± 2.4^{cde}	15.25 ± 3.7^{ab}	4.00 ± 0.2^{bcd}	18.25 ± 5.4^{cde}
SL14	4.4		20	8.75 ± 2.9^{bcde}	12.00 ± 2.4^{ab}	5.25 ± 0.5^{d}	23.50 ± 5.5^{e}
SL15	4.4		30	8.25 ± 2.5^{bcde}	9.25 ± 2.0^{ab}	4.50 ± 0.3^{cd}	23.25 ± 6.9^{e}
SL16	2.2		10	12.75 ± 3.6^{e}	43.25 ± 11.0^{c}	5.75 ± 0.2^{d}	25.25 ± 3.3^{e}
SL17	2.2		20	11.5 ± 3.8^{de}	28.75 ± 9.1^{bc}	5.00 ± 0.2^{d}	21.25 ± 4.3^{de}
SL18	2.2		30	8.25 ± 2.9^{bcde}	21.25 ± 7.1^{abc}	3.00 ± 0.3^{abcd}	14.25 ± 2.7^{bcde}

Different letters indicate significant statistical differences according to the Tukey test ($P\leq0.05$). MS: Murashige and Skoog basal medium.

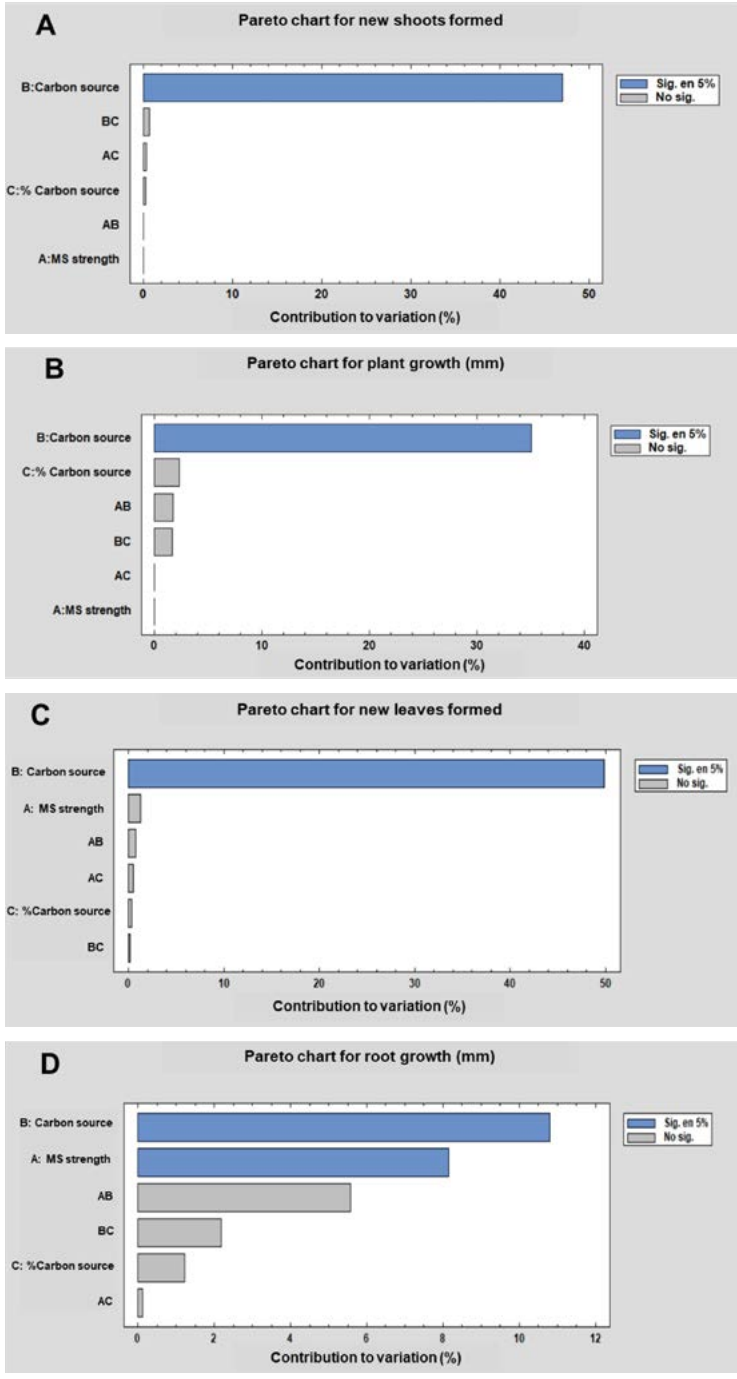

Figure 3. The Pareto charts for plant and root growth, and the number of shoots and leaves formed during the slow growth period of *Rhyncholaelia digbyana* (Lindley) Schlter.

CONCLUSION

This work represents the first report on micropropagation and *ex-situ* conservation to preserve germplasm for this species as an important resource for the floriculture industry.

ACKNOWLEDGMENTS

This study was financed with the resources of Project Fomix Campeche-CONACyT, "Establishment of an in vitro germplasm bank of native orchids from the state of Campeche for their sustainability- 96713.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Alexopoulos AA, Kartsonas E, Karras S, Mavrommati E, Petropoulos SA, Papafotiou M. (2023). In vitro Propagation of Origanum scabrum (Boiss. & Heldr.): An Endemic Medicinal Plant of Greece. *Plants, 12*(11):1-14. https://doi.org/10.3390/plants12112118

Bello-Bello J, García-García G, Iglesias-Andreu L. (2015). Conservación de vainilla (*Vanilla planifolia* Jacks.) bajo condiciones de lento crecimiento *in vitro*. *Revista Fitotecnia Mexicana, 38*(2): 165-171.

Bello-Bello J, Poot-Poot W, Iglesias-Andreu L, Caamal-Velázquez H, Díaz-Sánchez C. (2014). Comparison of the effect of osmoregulatory and growth inhibitors on *in vitro* conservation of sugarcane. *Agrociencia*, 48: 439–446.

Bhattacharyya P, Kumar S, Lalthafamkimi L, Sharma R, Kumar D, Singh D, Kumar S. (2023). Molecular and phytomedicinal stability of long term micropropagated *Malaxis acuminata*: An endangered terrestrial orchid of biopharmaceutical importance. *South African Journal of Botany*, 155: 372-382. https://doi.org/10.1016/j.sajb.2023.01.051

Bhattacharyya P, Lalthafamkimi L, Sharma T, Kumar D, Kumar A., Kumar D, Kumar S. (2022). Metabolic and tissue-specific expression profiling in micropropagated plants of *Malaxis acuminata*: an endangered medicinal orchid. *Plant Cell, Tissue and Organ Culture, 151*(3): 535-549. https://doi.org/10.1007/s11240-022-02369-3

Cazar ME, Abad DH, Idrovo AM, Barrera DA. (2023). Assessment of antioxidant activities of *Epidendrum secundum* J, a terrestrial orchid from southern Ecuadorian highlands. *South African Journal of Botany*, 154:380-386.https://doi.org/10.1016/j.sajb.2023.01.050

Chappell AL, Koym JW, Scheuring DC, Miller JC, Vales MI. (2020). Incorporation of mannitol in tissue culture media for long-term Storage of potatoes at moderately low temperature and effect on subsequent micropropagation. *American Journal of Potato Research, 97*(4): 439-446. https://doi.org/10.1007/s12230-020-09780-6

Chauhan R, Singh V, Quraishi A. (2019). *In vitro* conservation through slow-growth storage. In synthetic seeds: germplasm regeneration, preservation and prospects. Ed. Springer. United Kingdom. https://doi.org/10.1007/978-3-030-24631-0_19

de Oliveira Costa A, Silva LA, Duarte IM, Machado M, da Silva GZ, da Silva DF, da Costa Netto AP, Rocha DI. (2020). Shoot proliferation and *in vitro* organogenesis from shoot apex and cotyledonary explants of royal poinciana (*Delonix regia*), an ornamental leguminous tree. *Trees-Structure and Function, 34*(1): 189-197. https://doi.org/10.1007/s00468-019-01910-w

de Souza PP, de Oliveira LF, Ribas LL. (2021). An efficient protocol for *in vitro* propagation of *Trichilia pallida* Swartz (Meliaceae): a potential tree for natural insecticide production. *Plant Cell, Tissue and Organ Culture, 144*(2):469-476. https://doi.org/10.1007/s11240-020-01958-4

Debnath S, Kumaria S. (2023). Insights into the phytochemical accumulation, antioxidant potential, and genetic stability in the *in vitro* regenerants of *Pholidota articulata* Lindl., an endangered orchid of medicinal importance. *South African Journal of Botany*, 152: 313-320. https://doi.org/10.1016/j.sajb.2022.11.033

Divakaran M, Babu KN, Peter KV. (2006). Conservation of Vanilla species, *in vitro*. *Scientia Horticulturae, 110*(2): 175-180. https://doi.org/10.1016/j.scienta.2006.07.003

El-Hawaz RF, Adelberg J, Naylor-Adelberg J, Eisenreich R, Van der Meij J. (2019). The effect of slow-growth strategy on the production of *Petunia×hybrida* Vilm. microcuttings. *In Vitro Cellular and Developmental Biology-Plant, 55*(4): 433-441. https://doi.org/10.1007/s11627-019-09996-0

Espinosa-Reyes A, Silva-Pupo J, Pérez-Pérez L, Joâo-Zambela A. (2021). Effect of mineral salts and the use of mannitol on the *in vitro* conservation of *Morus alba* L. *Pastos y Forrajes*, 44: 1-6. https://orcid.org/0000-0002-9918-641X

Flores-Hernández A, Robledo-Paz A, Jimarez-Montiel MJ. (2017). Culture medium and agar substitutes for *in vitro* growth of orchids. *Revista Mexicana de Ciencias Agrícolas, 8*(6): 1315-1328.

Fonge BA, Essomo SE, Bechem TE, Tabot PT, Arrey BD, Afanga Y, Assoua EM. (2019). Market trends and ethnobotany of orchids of Mount Cameroon. *Journal of Ethnobiology and Ethnomedicine, 15*(29). https://doi.org/10.1186/s13002-019-0308-1

Generoso A, Carvalho V, Sales R, Brito NL, Viana A, Pereira TN. (2023). New evidence for *in vitro* conservation of nodal segments of the passion fruit Rio Dourado (*Passiflora edulis*). *Acta Scientiarum Agronomy*, 45. https://doi.org/10.4025/actasciagron.v45i1.59498

Gomes F, Clemente M, Figueiredo P, Costa RL. (2021). Medium-term *in vitro* conservation of *Castanea* spp. hybrid clones. *Vegetos, 34*(1): 127-137. https://doi.org/10.1007/s42535-020-00184-9

Kanlayavattanakul M, Lourith N, Chaikul P. (2018). Biological activity and phytochemical profiles of Dendrobium: A new source for specialty cosmetic materials. *Industrial Crops and Products*, 120: 61-70. https://doi.org/10.1016/j.indcrop.2018.04.059

Kaur H, Sena S, Jha P, Lekhak MM, Singh SK, Goutam U, Arencibia AD, Kumar V. (2022). *Arundina graminifolia* (D.Don) Hochr. (Orchidaceae): A review of its medicinal importance, phytochemistry and pharmacology activities. *South African Journal of Botany*, 150: 956-964. https://doi.org/10.1016/j.sajb.2022.08.048

López Puc G, Herrera Cool GJ. (2022). Germinación asimbiótica, conservación *ex situ* e *in vitro* regeneración de plantas de *Catasetum integerrimum* Hook. *Polibotánica, 53*(27). https://doi.org/10.18387/polibotanica.53.9

Lopez-Puc G. (2013). An effective *in vitro* slow growth protocol for conservation of the orchid *Epidendrum chlorocorymbos* Shltr. *Tropical and Subtropical Agroecosystems*, 16: 61-68. http://www.redalyc.org/articulo.oa?id=93927469007

Mamani Sánchez B, Eva A, Mamani M, Noelia A, Pardo M. Pinedo MN. (2022). Germinación *in vitro* de *Epidendrum secundum* con diferentes agentes gelificantes y concentraciones de agua de coco. *Acta nova, 10*(3):345-359.

Mayo-Mosqueda A, García-Hernández E, Noguera-Savelli E, Cetzal-Ix W, Alatorre-Cobos F. (2022). Advances in breeding, bioprospecting, and *in Vitro* Culture of *Laelia* Orchid Species. *Horticulturae, 8*(103):1-19. https://doi.org/10.3390/horticulturae8020103

Mondal T, Aditya S. Banerjee N. (2013). *In vitro* Axillary Shoot Regeneration and Direct Protocorm-like Body Induction from Axenic Shoot Tips of *Doritis pulcherrima* Lindl. *Plant Tissue Culture & Biotech, 23*(2):251-261.

Pathak P, Kumari A, Thakur B. (2022). Regeneration competence of an endangered orchid, *Vanda cristata* Wall. ex Lindl. using leaf explants: A study *in vitro*. *South African Journal of Botany*, 151: 1018-1024. https://doi.org/10.1016/j.sajb.2022.10.054

Pongener A, Ranjan DC. (2019). *In vitro* seed germination and propagation of *Thunia marshalliana* Rchb. f. on substrata of low-cost options. *Plant Cell Biotechnology and Molecular Biology, 20*(6): 204-211. https://www.researchgate.net/publication/333612255

Quijia-Lamiña PH, Baquero LE, Kane ME, Zettler LW. (2023). *In vitro* Seed Germination and Seedling Development of *Dracula felix* (Luer) Luer —An Orchid Native to Ecuador. *Diversity, 15*(732):1-20. https://doi.org/10.3390/d15060732

Ram B, Rathore, TS, Derajira B. (2014). An efficient protocol for micropropagation and genetic stability analysis of *Melia dubia* Cav.-an important multipurpose tree. *Int. J. Curr. Microbiol. App. Sci*, (3) 7. http://www.ijcmas.com

Ramos-Ortiz S, Rangel-Guerrero LM., Pedraza-Santos ME, Chávez-Ávila VM, Martínez-Ávalos JG., Sánchez-Vargas NM, Martínez-Palacios A. (2020). Clonal propagation of *trichocentrum stramineum* (Orchidaceae), a threatened species endemic to Mexico. *Botanical Sciences, 98*(2): 355-365. https://doi.org/10.17129/BOTSCI.2468

Rayas A, Cabrera M, Santos A, Basail M, López Jorge Medero V, Beovides Y. (2013). Efecto del manitol y el nitrato de plata en la conservación *in vitro* de la malanga (*Xanthosoma* spp.). *Revista Colombiana Biotecnología, 15*(1): 167–171.

Sánchez-Martínez A, Sarmiento M, Andrews J. (2002). *Rhyncholaelia digbyana* Schltr. In Orquídeas de Campeche. Ed. Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). México.

Sarmah D, Kolukunde S, Sutradhar M, Singh BK, Mandal T, Mandal N. (2017). A Review on: *In vitro* Cloning of Orchids. *International Journal of Current Microbiology and Applied Sciences, 6*(8): 1909-1927. https://doi.org/10.20546/ijcmas.2017.609.235

Wrigth J, Ferris B, Mann S. (2017). Species and orchids of the Nigth *Rhyncholaelia digbyana*. *Bulletin of the Orchid Society of Camberra*, 1-11.

Xu JJ, Beleski DG, Vendrame WA. (2022). Effects of culture methods and plant growth regulators on *in vitro* propagation of *Brassavola nodosa* (L.) Lindl. hybrid. *In Vitro* Cellular and Developmental Biology – *Plant, 58*(6): 931-941. https://doi.org/10.1007/s11627-022-10276-7