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In vitro conservation of *Notylia barkeri* Lindl.

Conservación *in vitro* de *Notylia barkeri* Lindl.

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ABSTRACT

The ornamental significance of orchid species lies in their morphological characteristics. *Notylia barkeri* Lindl. is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Therefore, it is imperative to institute preservation methods to safeguard its long-term viability. The objective was to establish an *in vitro* conservation protocol for *N. barkeri*. Different concentrations of abscisic acid (0.1 and 2 mg•L^{-1}) and ancymidol (0.1 and 2 mg•L^{-1}) were evaluated in the Murashige and Skoog medium. After 180 days, the survival percentage, number and length of shoots, number of leaves, and number and length of roots were evaluated. The highest survival percentage (85.71 %) was observed in control treatment. While in 1 mg•L^{-1} of abscisic acid, 57.14 % was observed. The lowest percentages were observed in ancymidol. It was observed that at 1 mg•L^{-1} of abscisic acid, the shoot length was reduced without affecting survival, as opposed to when ancymidol was used. Additionally, 1 mg•L^{-1} of abscisic acid reduced the number and length of roots. Obtained data may contribute to the conservation of this species of ornamental interest.

KEY WORDS: Germplasm preservation, orchid, ornamental, abscisic acid, ancymidol.

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Revista Bio ciencias
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RESUMEN

La relevancia ornamental de las especies de orquídeas radica en sus características morfológicas. *Notylia barkeri* Lindl. está catalogada en el Apéndice II de la Convención sobre el Comercio Internacional de Especies Amenazadas de Fauna y Flora Silvestres. Por tanto, resulta imperativo instituir métodos de preservación para salvaguardar su viabilidad a largo plazo. El objetivo fue establecer un protocolo de conservación *in vitro* de *N. barkeri*. Se evaluaron diferentes concentraciones de ácido abscísico (0.1 y 2 mg•L^{-1}) y ancimidol (0.1 y 2 mg•L^{-1}) en medio Murashige y Skoog. Después de 180 días se evaluó el porcentaje de supervivencia, número y longitud de brotes, número de hojas, número y longitud de raíces. El mayor porcentaje de supervivencia (85.71 %) se observó en tratamiento testigo. Mientras que en 1 mg•L^{-1} de ácido abscísico se observó 57.14 %. Los menores porcentajes se observaron en ancimidol. Se observó que en 1 mg•L^{-1} de ácido abscísico se reduce la longitud de brotes sin afectar su supervivencia a diferencia de cuando se utilizó ancimidol. Además, en 1 mg•L^{-1} de ácido abscísico se redujo el número y longitud de raíces. Nuestros resultados pueden contribuir a la conservación de esta especie con interés ornamental.

PALABRAS CLAVE: Preservación del germoplasma, orquídea, ornamental, ácido abscísico, ancimidol.

Introduction

Habitat fragmentation, a consequence of anthropogenic-induced land-use change, has a substantial impact on wild orchid populations (Mafakheri *et al*., 2022). The direct loss of natural habitats used by wild orchid populations for their survival and reproduction has generated threats of extinction (Baider & Florens, 2021), as well as, illegal extraction and sometimes the traditional use of these species (Castillo-Pérez *et al*., 2022). These phenomena trigger negative effects on the ecology and gene pool of members of the Orchidaceae family (Boucher *et al*., 2017). All this contributes to the fact that several orchids are on national and international lists of endangered and/or threatened species.

Notylia barkeri Lindl. is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This appendix includes species that are not necessarily at risk of extinction but whose potential use needs to be regulated to prevent practices that are incompatible with their survival mechanisms. This orchid is a small dimension epiphyte, with yellow flowers in inflorescences that have 50-100 flowers each (Damon, 2008; Hernández-Orta, 2019). This species has a fragrance composed of 20 chemical compounds, with

Revista Bio ciencias
ISSN 2007-3380

β-bisabolene at 40.61% (with anticancer properties) and 1,8-cineole at 29.35% (used in perfumes) being the most predominant (Cancino & Damon, 2007). Coupled with this *N. barkeri* has the potential to be a participant in the ornamental orchid trade. Orchid trade has been increasing exponentially in the last decade (Tiwari *et al*., 2024).

This species is found in various tropical and subtropical regions of the Americas, particularly in tropical forests (Valen, 2016). In Mexico, it has a distribution from Jalisco and Tamaulipas to the south, including Chiapas, Tabasco, and Quintana Roo (Hernández-Orta *et al.,* 2019). However, it is believed that due to deforestation resulting from land-use changes, this species may face a reduction in its wild population and be categorized as threatened.

Effective orchid conservation requires both *in-situ* and *ex-situ* strategies, with the latter including artificial propagation through plant tissue culture (PTC) (Wraith *et al.,* 2020). *In vitro* conservation safeguards germplasm of great interest through the use of an artificial medium and controlled incubation conditions (Chauhan *et al.,* 2019). Existing literature on *in vitro* conservation of orchids reveals a growing interest in the application of plant growth inhibitors to improve the efficiency of conservation protocols (Zhou *et al.,* 2021; Targu *et al.,* 2023). Previous reports have explored various inhibitors with promising results in terms of suppressing tissue overgrowth and preserving long-term viability; however, significant variability in the response of different orchid species to these inhibitors is observed. This technique has allowed for the *in vitro* preservation of different orchid species such as Laelia (*Laelia anceps*) (Ramírez-Mosqueda *et al.,* 2019), *Stanhopea tigrina* (Cruz-Cruz *et al.,* 2022), and vanilla (*Vanilla planifolia*) (Bautista-Aguilar *et al.,* 2021).

In vitro preservation through minimal growth involves specific strategies focused on reducing nutrients in the culture medium and applying growth inhibitors (Chauhan *et al.,* 2019). These inhibitors include substances such as abscisic acid (ABA), ancymidol (ANC), paclobutrazol (PBZ), polyethylene glycol (PEG), and other osmoregulators such as sorbitol and mannitol, as modified by (Mancilla-Alvarez *et al.,* 2019; Pujasatria *et al.,* 2020).

The *N. barkeri* conservation is crucial for biodiversity preservation and endangered species protection. This orchid, in particular, possesses unique characteristics that require a specialized approach for long-term conservation. Establishing an *in vitro* conservation protocol using plant growth inhibitors represents an innovative and effective means of ensuring its survival. Despite advances in this field, some limitations remain to be addressed, such as the diversity of inhibitors, the amount of inhibitor application, and preservation time. However, the scope of this research encompasses generating practical and applicable guidelines. Therefore, the methodological approach focuses on rigorously evaluating different inhibitors, with the aim of developing an effective and reproducible protocol that can be implemented in the conservation of *N. barkeri* and potentially extrapolated to other threatened plant species.

Revista Bio ciencias
ISSN 2007-3380

Material and Methods

Vegetal material

Plants of *N. barkeri* previously established *in vitro* were used; these originated from the asymbiotic germination of seeds of this orchid. The orchid seed pods were collected from coffee plantations in the locality of La Palma, Córdoba, Veracruz, Mexico. For germination, MS medium (Murasnige & Skoog, 1962) supplemented with 30 g•L^{-1} sucrose and without plant growth regulators (PGRs) was employed. The pH of the culture medium was adjusted to 5.8 ± 0.2, 2.5 g•L^{-1} of Phytagel® was added as a gelling agent, and 20 mL of medium was dispensed into "G" type flasks. The flasks containing the culture medium were autoclaved at 1.5 kg•cm^{-2} and 121 °C for 15 minutes. Cultures were incubated at 24 ± 2 °C, under artificial irradiance with LED lights at 50 µmol•m^2•s^{-1} in a photoperiod of 16 hours of light and 8 hours of darkness. All reagents used were of the Sigma Aldrich brand.

In vitro preservation of *Notylia barkeri* Lindl.

Individual shoots measuring 0.5-1.0 cm in length were transferred to test tubes (22 × 220 mm) containing 15 mL of MS culture medium supplemented with 30 g•L^{-1} sucrose. The effect of the growth inhibitors abscisic acid (ABA) and ancymidol (ANC) at different concentrations (0, 1, and 2 mg•L^{-1}) was analyzed. The pH of the culture medium was adjusted to 5.8 ± 0.2, and 2.5 g•L^{-1} of Phytagel® was added as a gelling agent. The test tubes were autoclaved at 1.5 kg•cm^{-2} and 121 °C for 15 minutes. Cultures were incubated at 25 ± 2 °C under an irradiance of 50 µmol•m^{-2}•s^{-1} provided by LEDs in a photoperiod of 16 hours light and 8 hours dark. After 180 days, the survival rate, number of shoots, shoot length, number of leaves, and number of roots per explant were recorded.

In vitro regeneration and acclimatization

After 180 days of preservation, individual shoots measuring 1.0-2.0 cm in length obtained from the 1 mg•L^{-1} ABA treatment were transferred to the MS medium described above, supplemented with 2 mg•L^{-1} 6-benzylaminopurine (BAP). The pH, sterilization of the culture media and incubation were carried out as described above for *in vitro* preservation. After 30 days of culture, the number of shoots per explant was assessed.

Subsequently, for acclimatization, shoots with a height between 3 and 5 cm and optimal root development were selected and rinsed with running water. Then, the shoots were planted in sterile peat + agrolite (1:1 v/v) using 60-cavity trays (5 × 5 × 8 cm). The plants were kept in closed trays for 14 days and subsequently maintained under greenhouse conditions (with natural light irradiance of 130 µmol•m^{-2}• s^{-1}, at 30 ± 2 °C and 60 ± 5 % RH). Gro-Green® (20-30-10 NPK) foliar fertilizer (1 mg•L^{-1}) was applied once every 7 days. One irrigation per day was established, and after 60 days of growth, the survival percentage was determined.

Revista Bio ciencias
ISSN 2007-3380

Statistical analysis

A completely randomized design was utilized in all experiments. The data obtained were statistically processed using IBM SPSS Statistics software (version 21). An ANOVA followed by a Tukey test ($p \leq 0.05$) was conducted to determine if there were significant differences between treatments. Percentage data were transformed to the arcsine of the square root of the percentage to fulfill the assumptions of normality and equality of variance.

Results and Discussion

In vitro preservation of *Notylia barkeri* Lindl.

After 180 days of *in vitro* preservation, significant differences were observed between the evaluated treatments (Table 1). The control treatment exhibited the highest survival percentage (85.71 %), followed by the treatment with 1 mg•L^{-1} ABA (67.14%) and the treatment with 2 mg•L^{-1} ABA with 28.57 % (Figure 1A, B, and C, respectively). The treatments containing ANC drastically affected the survival of *N. barkeri* shoots (Figures 1D and E). The lowest survival percentage (14.28 %) was observed in the treatment with 1 mg•L^{-1} of ANC. For the number of shoots, there were no significant differences (Table 1). Shoots with a length of 2.64 cm were observed in the control treatment, followed by 1.11 cm in 1 mg•L^{-1} ABA and 1.00 cm in 2 mg•L^{-1} ABA. There was a drastic decrease in shoot length in the treatments containing ABA. The highest number of leaves per explant (5.42) was obtained in the control treatment, while the addition of ABA to the culture medium significantly decreased leaf formation. On the other hand, the addition of ANC, at any of the concentrations evaluated, resulted in a drastic reduction in leaf formation. Regarding the number and length of roots, the control treatment generated a greater number and length of roots compared to the treatments involving the addition of ABA. The addition of ANC did not generate root formation in *N. barkeri* shoots preserved *in vitro* for 180 days.

Revista Bio ciencias
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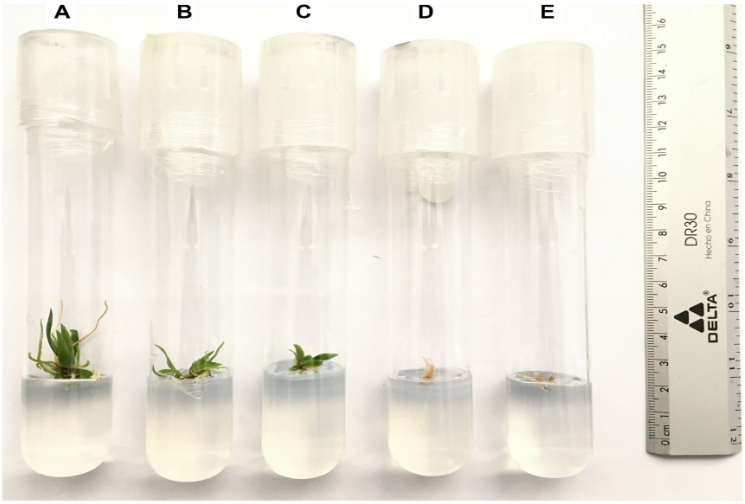

Figure 1. *In vitro* preservation of *Notylia barkeri* Lindl after 180 days of culture. A) Control, B) 1 mg•L^{-1} of ABA, C) 2 mg•L^{-1} of ABA, D) 1 mg•L^{-1} of ANC and E) 2 mg•L^{-1} of ANC.

Source: Own elaboration based on results.

Table 1. Effect of different concentrations of ABA and ANC on the *in vitro* preservation of *Notylia barkeri* Lindl.

Treatment (ABA/ANC)	Survival (%)	Number of shoots	Shoot length	Number of leaves	Number of roots	Root length
0 mg•L $^{-1}$	85.71 ± 0.41^{a}	1.00 ± 0.00^{a}	2.64 ± 0.12^{a}	5.42 ± 1.50^{a}	6.42 ± 0.84^{a}	1.69 ± 0.11^{a}
1 mg•L^{-1} ABA	67.14 ± 0.29^{b}	1.14 ± 0.14^{a}	1.11 ± 0.07^{b}	3.28 ± 0.42^{ab}	3.14 ± 0.59^{b}	1.39 ± 0.16^{a}
2 mg•L^{-1} ABA	28.57 ± 0.48^{c}	1.14 ± 0.14^{a}	1.00 ± 0.09^{b}	3.42 ± 0.42^{ab}	2.42 ± 0.36^{b}	0.85 ± 0.05^{b}
1 mg•L^{-1} ANC	14.28 ± 0.26^{d}	0.85 ± 0.12^{a}	0.44 ± 0.08^{c}	1.85 ± 0.34^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
2 mg•L^{-1} ANC	28.57 ± 0.26^{c}	0.71 ± 0.16^{a}	0.31 ± 0.08^{c}	1.71 ± 0.47^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

The mean ± standard error is represented. Different letters express significant differences (Tukey, $p \leq 0.05$).

Source: Own elaboration based on results.

Revista Bio ciencias
ISSN 2007-3380

In this study, *N. barkeri* germplasm was conserved *in vitro* for 180 days under minimal growth, and the plant material from the conservation program was successfully regenerated and adapted. Plant germplasm conservation aims to preserve both intraspecific and interspecific genetic variability harbored by individuals belonging to the same species (Priyanka *et al.,* 2021). However, plant tissue culture techniques offer an effective alternative by enabling the seedling preservation through *in vitro* germplasm banks, guaranteeing pathogen-free plants, conserved in reduced spaces, with low costs, and providing controlled environments for efficient handling, both in the short and medium-term, of plant material (Shahzad *et al.,* 2017).

Abscisic acid (ABA) is a key plant growth regulator (PGR), with one of its main functions being growth inhibition (Chen *et al.,* 2020). In this study, it was observed that the addition of ABA to the culture medium generated a reduction in shoot length, number of leaves, as well as, number and length of roots. However, it affected the survival percentage. The efficiency of ABA in the *in vitro* preservation of plant germplasm has been reported in several orchid species. Ramírez-Mosqueda *et al.* (2019) observed that it was necessary to use 2 mg•L^{-1} of ABA in the *in vitro* conservation of *Laelia anceps*, while Cruz-Cruz *et al. (*2022) added 2 mg•L^{-1} of ABA in the *in vitro* preservation of *Stanhopea tigrina*. However, in this study, it was observed that 2 mg•L^{-1} of ABA reduced the survival of *N. barkeri* shoots.

The use of ancymidol caused a drastic reduction in the survival of *N. barkeri* shoots preserved *in vitro*. This contrasts with Mancilla-Álvarez *et al.* (2019), who reported that 2 mg•L^{-1} of ANC is effective in the *in vitro* conservation of malanga, drastically reducing growth variables such as shoot length, number of shoots, leaves, and roots, without affecting shoot survival. This compound modulates plant physiology, mainly the gibberellins inhibition, reducing the growth and development of *in vitro* preserved plants (Al-Ajlouni *et al.,* 2023).

Recently, ABA as well as ANC are two growth inhibitors frequently used for *in vitro* plant germplasm preservation protocols (Ramírez-Mosqueda *et al.,* 2019; Mancilla-Álvarez *et al.,* 2019; Cruz-Cruz *et al.,* 2022). However, the susceptibility of different plant species to these compounds needs to be well-thought-out when applying them to conservation strategies such as minimal growth. This finding may have important implications in the planning of conservation strategies since the ANC presence could represent a potential threat to the development and growth of this species.

In vitro regeneration and acclimatization

After 30 days of culture, regeneration of 3.4 shoots per explant was obtained in a culture medium supplemented with 2 mg•L^{-1} of BAP (Figure 2A). After sixty days in the acclimatization process, 82 % survival was observed in *N. barkeri* plants (Figure 2B).

Revista Bio ciencias
ISSN 2007-3380

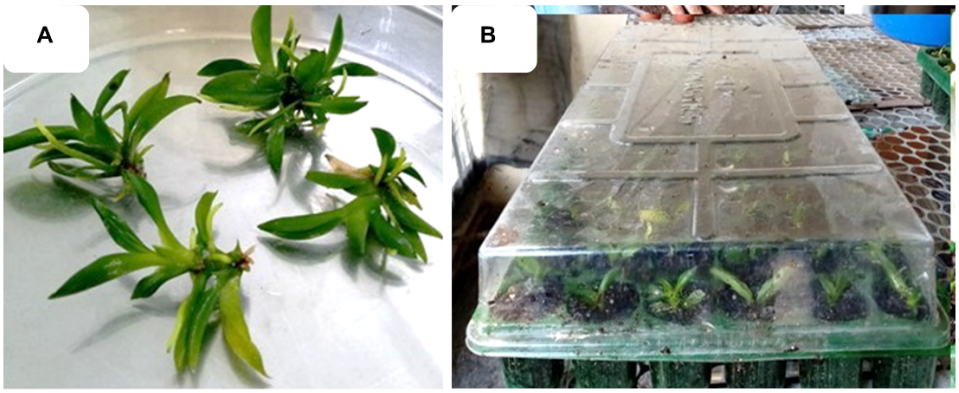

Figure 2. *In vitro* regeneration and acclimatization of conserved *N. barkeri* germplasm. A) Shoot proliferation on MS medium with 2.0 mg•L^{-1} of BAP, B) Acclimatized plants after eight weeks of culture.

Source: Own elaboration based on results.

Finally, the success of an *in vitro* preservation protocol relies on the ability to regenerate and acclimatize plants from the preserved germplasm (Murthy *et al.,* 2018; Mancilla-Alvarez *et al.,* 2019). BAP is a synthetic plant growth regulator that is frequently used during the *in vitro* shoot proliferation phase in several orchids such as *Catasetum integerrimum* (Castillo-Pérez *et al.,* 2022), *Dryadella zebrina* (dos Santos Anjos *et al.,* 2021), and *Phalaenopsis philippinensis* (Khorshidi, 2023). This is in agreement with the obtained data, allowing shoot regeneration from the preserved material of *N. barkeri* in ABA for 180 days. However, PTC innovations such as temporary immersion systems could be utilized if large numbers of plants are required from the conserved germplasm (Vendrame *et al.,* 2023). In this study, the high acclimatization percentages obtained guarantee the success of the described protocol.

Conclusions

An effective method for *in vitro* conservation and regeneration of *N. barkeri* using ABA was established. Additionally, it was demonstrated that the use of ANC is not viable due to its negative impact on growth and survival, and therefore, it is not recommended for the *in vitro* preservation of this species. Obtained data could be applied in future conservation programs for this orchid species, including the establishment of *in vitro* germplasm banks.

Revista Bio ciencias
ISSN 2007-3380

Authors' contribution

García-Merino: Research, Methodology, Writing the Original Draft. Ramírez-Mosqueda: Work conceptualization, Writing-Revision and Editing, Visualization. Mata-Alejandro: Conceptualization, Revision and Editing, Visualization. López-Larios: Conceptualization, Methodology, Revision and Editing, Visualization. López-Aguilar: Conceptualization and Revision.

All authors of this manuscript have read and accepted the published version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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