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Portada



El proceso comunitario de aprendizaje y aprovechamiento sustentable de hongos comestibles a partir de residuos agrícolas, como rastrojo de maíz y frijol, representa una alternativa agroecológica que fortalece la soberanía alimentaria, promueve el uso responsable de recursos locales y contribuye a la revitalización del conocimiento etnomicológico tradicional. La participación comunitaria, el intercambio gastronómico y la transmisión intergeneracional de saberes reflejan la estrecha relación entre patrimonio biocultural, innovación tecnológica y resiliencia social en contextos rurales indígenas.

The community-based process of learning and sustainable use of edible mushrooms through agricultural residues, such as maize and bean straw, represents an agroecological alternative that strengthens food sovereignty, promotes the responsible use of local resources, and contributes to the revitalization of traditional ethnomycological knowledge. Community participation, gastronomic exchange, and the intergenerational transmission of knowledge reflect the close relationship between biocultural heritage, technological innovation, and social resilience in indigenous rural contexts.

por/by
Rafael Fernández Nava

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**INFLUENCE OF THE CULTURE
MEDIUM COMPOSITION ON THE
ASYMBIOTIC GERMINATION OF
Cyrtopodium macrobulbon (LEX.) G.A.
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ABSTRACT: *Cyrtopodium macrobulbon* (Lex.) G.A. Romero & Carnevali is a terrestrial orchid native to the Yucatán Peninsula, valued for its ornamental and ethnobotanical importance. Like other Orchidaceae species, it faces reproductive challenges due to specific pollinator requirements, long capsule maturation periods, and its dependence on mycorrhizal fungi for germination. Asymbiotic germination offers an effective alternative, allowing seeds to develop under sterile conditions in nutrient-rich media without fungal associations. Enhancing germination percentages requires a comprehensive assessment of the culture medium. This study assessed the effects of basal medium type [Murashige & Skoog (MS) with or without vitamins], its concentration (100% or 50%), and carbon source (sucrose or sorbitol) on the asymbiotic germination of *Cyrtopodium macrobulbon*. Seeds were cultured under controlled conditions for 24 weeks, and germination percentages were recorded. Results showed that the carbon source had the most significant influence, with sucrose promoting higher germination percentages. The highest germination percentage (57.37%) was obtained with full-strength MS medium without vitamins, supplemented with 3% sucrose. This protocol provides a solid foundation for *in vitro* germination of *Cyrtopodium macrobulbon* and represents a valuable tool for its propagation, conservation, and sustainable use.

Key words: *Cyrtopodium macrobulbon*, *in vitro*, orchid conservation, sorbitol

RESUMEN: *Cyrtopodium macrobulbon* (Lex.) G.A. Romero & Carnevali es una orquídea terrestre nativa de la Península de Yucatán, valorada por su importancia ornamental y etnobotánica. Al igual que otras especies de Orchidaceae, enfrenta desafíos reproductivos debido a los requisitos específicos de polinización, a los largos períodos

de maduración de la cápsula y a su dependencia de hongos micorrízicos para la germinación. La germinación asimbiótica ofrece una alternativa efectiva, permitiendo que las semillas se desarrollen en condiciones estériles, en medios ricos en nutrientes, sin la presencia de asociaciones fúngicas. Mejorar las tasas de germinación requiere una evaluación integral del medio de cultivo. Este estudio evaluó los efectos del tipo de medio basal [Murashige y Skoog (MS) con o sin vitaminas], de su concentración (100% o 50%) y de la fuente de carbono (sacarosa o sorbitol) en la germinación asimbiótica de *Cyrtopodium macrobulbon*. Las semillas se cultivaron en condiciones controladas durante 24 semanas y se registraron los porcentajes de germinación. Los resultados mostraron que la fuente de carbono tuvo la influencia más significativa, ya que la sacarosa promovió porcentajes más altos de germinación. El mayor porcentaje de germinación (57.37%) se obtuvo con el medio MS puro, sin vitaminas, suplementado con 3% de sacarosa. Este protocolo proporciona una base sólida para la germinación *in vitro* de *Cyrtopodium macrobulbon* y constituye una herramienta valiosa para su propagación, conservación y uso sostenible.

Palabras clave: *Cyrtopodium macrobulbon*, *in vitro*, conservación de orquídeas, sorbitol.

INTRODUCTION

The Orchidaceae family comprises approximately 35,000 species distributed worldwide, except in polar and desert regions, and is diverse mainly in tropical areas. Approximately 70% of orchids are epiphytic or lithophytic, while around 30% are terrestrial species (De, 2020; Deb *et al.*, 2022; Kumar *et al.*, 2024; Kunakhonnuruk *et al.*, 2018; Paramanik *et al.*, 2024).

Orchids are monocotyledonous, herbaceous, angiosperm, and perennial plants. They display two distinct growth patterns: monopodial and sympodial. In monopodial growth, the stem develops from a single bud and produces leaves at the apex. In contrast, sympodial growth occurs when the stem or rhizome extends horizontally along the ground, generating multiple buds that develop into pseudobulbs, from which the leaves emerge (De, 2020).

These plants are cultivated for their ornamental beauty, but they also possess nutraceutical, pharmacological, and ethnobiological properties. They produce bioactive compounds, including alkaloids, phenanthrenes, flavonoids, and terpenes. This diversity of compounds has led indigenous communities worldwide to use these plants in traditional medicine (Castillo-Pérez *et al.*, 2024; Choudhary & Kumar, 2024; Paramanik *et al.*, 2024).

Orchids encounter several threats, including habitat destruction, climate change, and illegal trade. In addition, their reproductive success is constrained by intrinsic seed traits: orchid seeds are dust-like, lack endosperm, and contain a highly reduced, undifferentiated embryo surrounded by a thin testa, often with a large internal air space (Prasongsom *et al.*, 2022). These morphological features are associated with physiological dormancy and extremely limited internal nutrient reserves, making germination dependent on specific mycorrhizal associations. Consequently, natural germination percentages are typically low, ranging from 1% to 5% (Salazar-Mercado & Botello-Delgado, 2020). As a result, all species in the Orchidaceae family are included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), meaning that their trade must be regulated to prevent endangerment (Kumar *et al.*, 2024).

Orchids reproduce sexually or asexually. Sexual reproduction depends on birds and insects attracted by visual or olfactory cues and nectar. However, it faces limitations such as specific pollinator requirements and long seed maturation (Salazar-Mercado & Botello-Delgado, 2020; Tiwari *et al.*, 2024).

Asymbiotic germination mimics the nutritional support provided by symbiotic fungi by using sterile, nutrient-enriched media supplemented with added sugars, enabling controlled conditions, reducing contamination risk, and enabling efficient seedling production (Jeldes-Cajas *et al.*, 2024). This process involves six stages, from embryo enlargement to shoot formation from the protocorm (Yamazaki & Miyoshi, 2006). Protocols must be species-specific because of the varying nutritional and environmental needs across species, making culture medium evaluation

crucial for *in vitro* propagation and conservation. Mexico has about 1,300 orchid species, with 41 in the Yucatán Peninsula (Mayo-Mosqueda *et al.*, 2020), including the notable *Cyrtopodium macrobulbon*, valued for its ornamental and ethnobiological significance.

Cyrtopodium macrobulbon is a terrestrial orchid distributed across Mexico and Central America, from Sinaloa in Northern Mexico to Panama. It inhabits diverse ecosystems, ranging from xerophytic scrublands to sub-deciduous forests, typically growing on rocks covered with organic matter or in well-drained soils. This sympodial species exhibits annual growth, producing lateral inflorescences from developing stems that bloom in spring. Pseudobulbs mature in summer and shed their leaves after the rainy season. The sap from its pseudobulbs is used as an adhesive in amate paper making, a tradition of the Otomi people (León-Peralta & Ojascastro, 2024). In traditional medicine, it is employed to treat wounds, burns, inflammation, and urinary issues (Castillo-Pérez *et al.*, 2024). Although these applications are based on empirical use in humans, (Castillo-Pérez *et al.*, 2024) reported *in vivo* pharmacological tests in mice, where an ethanolic extract from roots demonstrated strong analgesic activity, achieving 75% pain inhibition and suggesting potential anti-inflammatory properties.

Given its ornamental and cultural relevance and the threats it faces, propagation strategies are vital to its conservation. As asymbiotic germination must be species-specific, this study evaluates the effects of various media and carbon sources on the germination of *Cyrtopodium macrobulbon* seeds, aiming to develop an *in vitro* germination protocol.

MATERIALS AND METHODS

Plant material

The Ethnobiological Garden of Campeche, Mexico, donated a seed capsule of *Cyrtopodium macrobulbon*. The capsule was placed in a manila envelope and stored in a desiccator containing 1 Kg of silica gel. The sample was allowed to mature for 15 days at a controlled temperature of 23 ± 2 °C.

Disinfection of Plant Material

After the capsule was fully matured, as indicated by a color change from green to yellowish-green or yellow, it was cleaned with a detergent solution and then rinsed with distilled water. While working in a laminar flow hood, the capsule was placed in a 1% commercial sodium hypochlorite solution containing 10 µL of detergent and left to soak for 10 minutes. After this, the capsule underwent three rinses with sterile distilled water. Finally, briefly immersed in 96% ethanol and flamed using a Bunsen burner.

Seed Extraction and Counting

The sterilised capsule was set in a clean Petri dish and sliced lengthwise into two halves with a scalpel. Using a spatula, 0.5 g of seeds was removed and measured on an analytical balance. The extracted seeds were placed in flasks containing 50 mL of sterile distilled water and shaken gently to ensure thorough homogenization of the suspension. The average number of seeds in a 0.5 mL sample of the solution was determined by placing that volume into a Petri dish, staining with safranin, and manually counting the seeds under a stereomicroscope. The average number of seeds recorded was 480.

Asymbiotic Germination

The study utilized a 2^3 factorial experimental design to assess how three different factors influence asymbiotic germination. The factors were (A) two types of basal media: MS with vitamins (Sigma MS-5519; Technical sheet: <https://tinyurl.com/MS-5519-components>) and without vitamins (Sigma MS-5524; Technical sheet: <https://tinyurl.com/MS-5524-components>); (B) two salt strengths: full strength ($4.4 \text{ g} \cdot \text{L}^{-1}$) and half strength ($2.2 \text{ g} \cdot \text{L}^{-1}$); and (C) two carbon sources: sucrose and sorbitol at 3% WV⁻¹ (Both SIGMA S9378 and S3889, respectively). This

design resulted in eight treatment combinations, each with six replicates, yielding 48 experimental units. Furthermore, four control conditions were used: MS with vitamins and MS without vitamins, each at full and half strength, all lacking a carbon source. The experiment began by adding 0.5 mL of the seed solution to the culture media (35 mL per culture vessel) and incubating the culture in a growth chamber at 25°C in complete darkness for 2 weeks. It was then transferred to a photoperiod of 16 hours of light and 8 hours of darkness for 22 weeks. At the conclusion of the experiment, the number of germinated seeds was counted, and the germination percentages were calculated.

Statistical Analysis

The data were analyzed using Statgraphics Centurion® version 16.1.03. An analysis of variance (ANOVA) was conducted, followed by mean comparisons using Fisher's Least Significant Difference (LSD) test at a 5% significance level.

RESULTS

Cyrtopodium macrobulbon seeds began to germinate three months into the experiment. The experiment lasted six months, after which the number of germinated seeds was recorded. Germination percentages were calculated, and the data were subsequently analyzed.

Analysis revealed that the carbon source exerted the most significant influence on the asymbiotic germination of *Cyrtopodium macrobulbon* seeds, with the combined effects of MS medium type and salt concentration also playing notable roles (Fig. 1A). Germination percentages tended to increase in media supplemented with MS with vitamins at full strength (100%) (Fig. 1B). However, these increases were not statistically significant. ANOVA results indicated that media containing 100% MS without vitamins produced the highest germination percentages, highlighting the significant interaction between MS medium type and concentration (Fig. 1A and 1C). Furthermore, sucrose was more effective than sorbitol in enhancing asymbiotic seed germination (Fig. 1B).

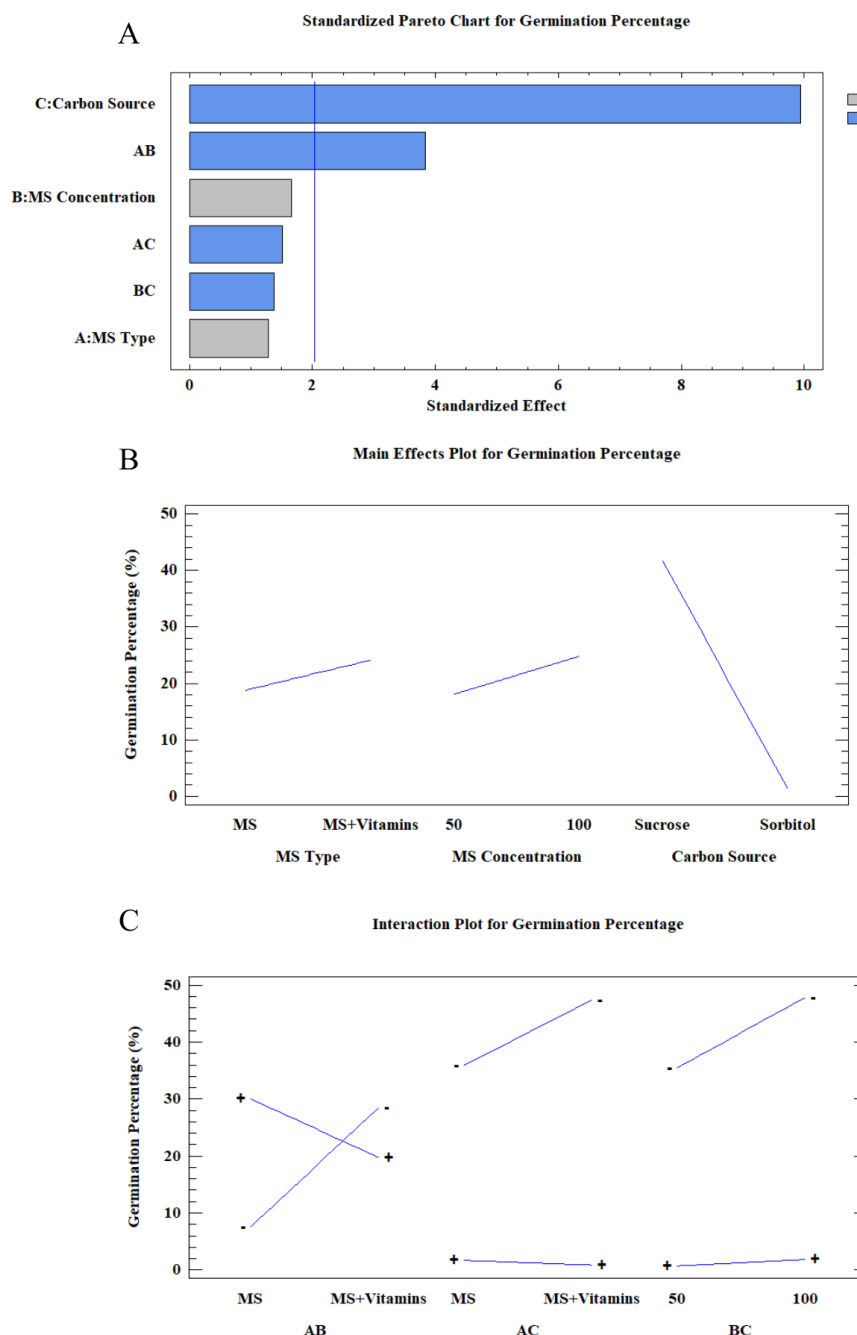


Figure 1. Effect of MS type, salt concentration, and carbon source on the germination percentage of *Cyrtopodium macrobulbon* seeds. (A) presents a standardized Pareto chart, (B) shows the main effects plot, and (C) displays the interaction plot. Symbols “-” and “+” indicate the lower and higher levels of each factor evaluated: sucrose (-) and sorbitol (+) for carbon source; MS without vitamins (-) and MS with vitamins (+) for MS medium type; and 50% MS (-) and 100% MS (+) for MS salt concentration.

Figura 1. Efecto del tipo MS, la concentración de sal y la fuente de carbono en el porcentaje de germinación de semillas de *Cyrtopodium macrobulbon*. (A) presenta un diagrama de Pareto estandarizado, (B) muestra el gráfico de efectos principales y (C) muestra el gráfico de interacción. Los símbolos “-” y “+” indican los niveles inferior y superior de cada factor evaluado: sacarosa (-) y sorbitol (+) para la fuente de carbono; MS sin vitaminas (-) y MS con vitaminas (+) para el tipo de medio MS; y 50 % MS (-) y 100 % MS (+) para la concentración de sal MS.

Table 1 presents the effects of MS basal medium type, salt concentration, and carbon source on the germination percentage of *Cyrtopodium macrobulbon* seeds. The results reveal significant differences among treatments, as indicated by Fisher's LSD test ($p \leq 0.05$). The media containing sucrose produced the highest germination percentages. The greatest percentage, $57.37 \pm 18.55\%$, was found in full-strength MS medium without vitamins (MS-Suc). This result was not significantly different from that of half-strength MS medium with vitamins and sucrose ($\frac{1}{2}$ MS+Suc). In contrast, media supplemented with sorbitol showed the germination percentage of only $3.47 \pm 14.66\%$ in MS medium without vitamins at full strength (MS-Sor). This indicates that sorbitol is not a suitable carbon source for the germination of this species.

Table 1. Effect of MS basal medium, its salt concentration, and carbon source on the germination percentage of *Cyrtopodium macrobulbon* seeds.

Tabla 1. Efecto del medio basal MS, su concentración de sal y la fuente de carbono sobre el porcentaje de germinación de semillas de *Cyrtopodium macrobulbon*.

Treatment	Germination percentage (%)
MS-Suc	57.37 ± 18.55^a
$\frac{1}{2}$ MS-Suc	13.87 ± 14.66^c
MS-Sor	3.47 ± 3.10^{cd}
$\frac{1}{2}$ MS-Sor	0.96 ± 0.73^{cd}
MS+Suc	39.23 ± 16.63^b
$\frac{1}{2}$ MS+Suc	55.59 ± 11.24^a
MS+Sor	0.20 ± 0.32^d
$\frac{1}{2}$ MS+Sor	1.52 ± 3.33^{cd}

Means $\pm$ standard deviation followed by different letters indicate significant differences according to Fisher's LSD multiple comparison test ($p \leq 0.05$). MS⁻ = MS without vitamins, MS⁺ = MS with vitamins.

The carbon source is the most critical factor influencing germination percentage, with sucrose significantly outperforming sorbitol. Efficient germination is achieved using full-strength MS with vitamins and sucrose as the carbon source. These findings highlight the importance of both individual factors and their interactions in optimizing germination protocols.

The seeds of *Cyrtopodium macrobulbon* at different stages of asymbiotic germination are illustrated in Fig. 2. Media supplemented with sucrose not only produced the highest germination percentages but also resulted in the formation of large protocorm-like bodies (PLBs) in various shades of green (Fig. 2H). In contrast, many seeds cultured in MS sorbitol-supplemented media (Fig. 2G) failed to germinate; among those that did, growth was limited, and color exhibited slight variation. It is important to highlight that, despite achieving successful germination and six months having passed since the start of the experiment, only two seeds developed fully formed shoots with roots (Fig. 2I)

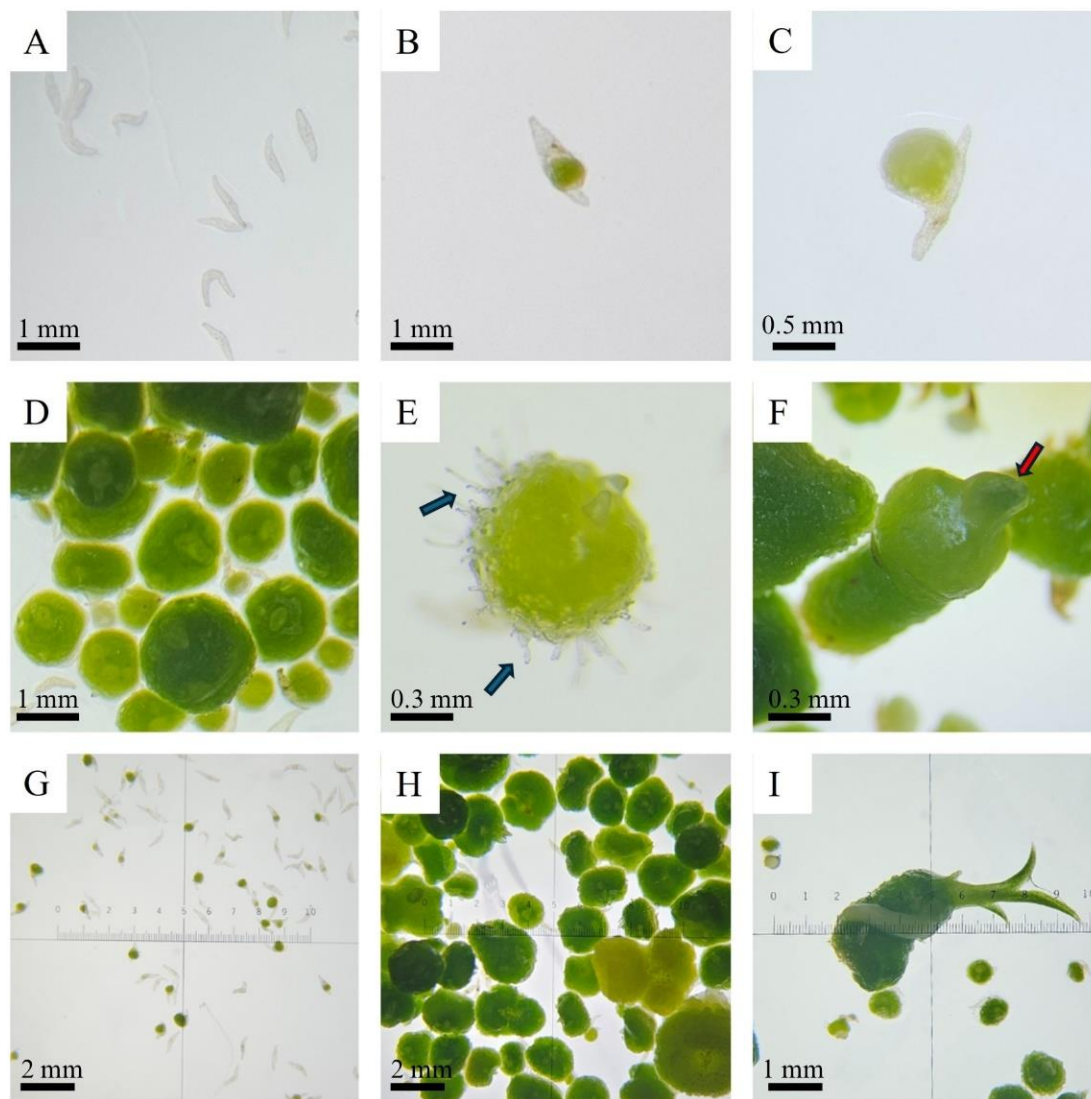


Figure 2. *Cyrtopodium macrobulbon* seeds at different stages of asymbiotic germination, as described by Yamazaki & Miyoshi (2006), in media with varying sources of carbon. (A) Seeds without embryo growth. (B) Embryo growth and green coloring inside the seed coat. (C) Embryo breaks through and emerges from the seed coat. (D) Protocorm-like bodies (PLBs) of various sizes. (E) Rhizoid development (blue arrows) on the PLB surface. (F) Shoot differentiation and apical shoot formation (red arrow) on the PLB. (G) Seeds germinated in media with sorbitol as the carbon source. (H) PLBs formed in media with sucrose as the carbon source. (I) Shoot and root of *Cyrtopodium macrobulbon* growing from a PLB.

Figura 2. Semillas de *Cyrtopodium macrobulbon* en diferentes etapas de germinación asimbiótica, según lo descrito por Yamazaki y Miyoshi (2006), en medios con diferentes fuentes de carbono. (A) Semillas sin crecimiento embrionario. (B) Crecimiento embrionario y coloración verde dentro de la cubierta de la semilla. (C) El embrión rompe y emerge de la cubierta de la semilla. (D) Cuerpos similares a protocormos (PLB) de varios tamaños. (E) Desarrollo de rizoides (flechas azules) en la superficie del PLB. (F) Diferenciación del brote y formación del brote apical (flecha roja) en el PLB. (G) Semillas germinadas en medios con sorbitol como fuente de carbono. (H) PLB formados en medios con sacarosa como fuente de carbono. (I) Brote y raíz de *Cyrtopodium macrobulbon* creciendo a partir de PLB.

DISCUSSION

Asymbiotic germination of orchids has been documented across a wide variety of species, with germination percentages varying by species and culture medium. For instance, (Kaur, 2023) reported a germination of 65.05% for *Cypripedium cordigerum* D. Don seeds cultured on a modified terrestrial orchid medium (BM-1). (Kim *et al.*, 2021) achieved 87.5% germination of *Pelatantheria scolopendrifolia* (Makino) seeds cultured on half-strength MS medium. (Paramanik *et al.*, 2024) reported a germination percentage of 83.5% for *Vanda tesellata* (Roxb.) seeds cultured on full-strength MS medium. Additionally, (Samala & Thipwong, 2023) obtained 94.69% seed germination for *Dendrobium cruentum* Rehb. f. using the Vacin and Went (VW) medium supplemented with banana and potato homogenates.

Germination responses to different culture media treatments can vary with medium composition. In the present study, asymbiotic germination of *Cyrtopodium macrobulbon* reached 57.37%. Although the MS medium type factor was not statistically significant, the highest germination percentage was observed in MS medium without vitamins at full salt concentration. However, no significant differences were detected between this treatment and the medium containing vitamins at 50% MS concentration, which produced the second-highest germination values. These results suggest that both culture conditions were suitable for promoting seed germination in this species. The role of vitamins in plant tissue culture has been widely documented, as they participate in metabolic pathways that support cell growth, embryogenesis, and organ development across several species, including as maize, beans, peas, and tobacco (Tomar, 2018).

Research has demonstrated that exogenous vitamins can significantly stimulate and enhance seed germination. These vitamins function as enzyme cofactors, facilitating hormone biosynthesis and bolstering antioxidant systems, thereby safeguarding plant cells from oxidative stress (Chen *et al.*, 2021; Xin Chi *et al.*, 2021; Sahito *et al.*, 2024). Incorporation of vitamins has been shown to confer benefits for germination; however, it is important to note that the effectiveness of these vitamins may vary based on the distinct physiological requirements of each orchid species (Pierik *et al.*, 1988).

In the case of the epiphytic orchid *Catasetum integerrimum*, (López Puc & Herrera Cool, 2022) reported that asymbiotic seed germination occurred only on MS medium without added vitamins, whereas no germination was observed on vitamin-supplemented MS medium. In contrast, in the present study, germination was also recorded in media containing vitamins, although the highest values were obtained in MS medium without vitamins. Conversely, (Castillo-Pérez *et al.*, 2022) achieved germination percentages of up to 88% for the same species after 90 days of culture on MS medium enriched with vitamins, activated charcoal (AC), and sucrose as the carbon source. These contrasting results suggest that orchid seeds response to vitamin supplementation may depend on factors such as medium composition, carbon source, and species-specific physiological requirements.

For *Cyrtopodium macrobulbon* (Carranza-Álvarez *et al.*, 2015) reported that seed germination occurred on MS medium, but did not specify whether vitamins were included. In their study, they achieved 87.4% germination on MS medium supplemented with activated charcoal (AC), compared to only 15.9% on medium without this additive. Germination responses were observed after 30 days of culture. In contrast, in the present study, germination began after three months. These differences may be attributed to factors such as seed immaturity or inbreeding depression, as demonstrated by (Oikonomidis *et al.*, 2024), who found that these factors significantly increased the percentage of empty and non-viable seeds in three species of the *Ophrys* genus.

On the other hand, (Carranza-Álvarez *et al.*, 2015) reported higher germination percentages in *Cyrtopodium macrobulbon* in media supplemented with activated charcoal (AC), which may contribute to medium darkening and conditions favorable for terrestrial orchid species. In addition, the beneficial effects of AC in plant tissue culture have been its ability to adsorb inhibitory compounds such as phenolics that may accumulate in the culture medium and negatively affect tissue development. Although activated charcoal was not evaluated in the present study, these findings suggest that future research could explore its potential role in improving asymbiotic germination and early seedling development in this species.

The carbon source significantly influenced seed germination. Treatments supplemented with sucrose resulted in substantially higher germination percentages, whereas germination in media containing sorbitol was very low. The superior performance of sucrose may be attributed to its rapid hydrolysis into monosaccharides, which provide readily available energy and help maintain a favorable osmotic balance for seed development. This impact has also been studied in orchids during different growth stages. For example, (Chi-Ramírez *et al.*, 2024) investigated the effects of sucrose and sorbitol in minimal growth protocols for *Rhyncholaelia digbyana*. They found that this species thrived better in media supplemented with sorbitol, while growth was restricted in media containing sucrose or mannitol. In contrast, (Lopez-Puc, 2013) found that for *Epidendrum chlorocorymbos* Schltr., the best way to conserve the plant is to culture it in media containing 1% sorbitol, as this concentration effectively restricts seedling growth. Similarly, (López Puc & Herrera Cool, 2022) demonstrated that for *Catasetum integerrimum*, media supplemented with sucrose promotes proper growth for propagation, while a concentration of 2% sorbitol limits development.

Research on the role of sorbitol in seed germination is relatively limited, and results vary among species (Jain *et al.*, 2010) reported that sorbitol induced osmotic stress in maize (*Zea mays* L. cv. Ganga Safed-2), inhibiting germination and reducing leaf growth. Similarly, (Johnson & Kane, 2013) observed that sorbitol did not improve germination of *Bletia purpurea* seeds and hindered later developmental stages, particularly rhizoid formation, suggesting that this species may not effectively absorb or metabolize sorbitol. These findings are consistent with the present study, where sorbitol resulted in very low germination percentages compared with sucrose. Likewise, (Vegas-García *et al.*, 2019) reported germination percentages of 50–63% in African palm (*Elaeis guineensis* Jacq.) embryos cultured *in vitro* on MS medium supplemented with either sucrose or sorbitol as the carbon source, indicating that the response to sorbitol can vary by species.

In contrast, other studies have reported beneficial effects of sorbitol in different plant species. (Perez-Jimenez *et al.*, 2021) found that peach (*Prunus persica* L. Batsch) seeds treated with sorbitol achieved germination percentages above 80%, which was attributed to differences in osmotic potential and stress responses associated with carbohydrate sources. Likewise (Çalık Koç *et al.*, 2024) reported that sorbitol priming improved seedling emergence and early growth in lentil plants. These contrasting findings indicate that the effects of sorbitol on germination and early development are highly species-dependent and may be influenced by differences in the metabolic capacity to utilize polyols as carbon sources.

CONCLUSIONS

The findings of this study indicate that the carbon source plays a critical role in the asymbiotic germination of *Cyrtopodium macrobulbon* seeds. Sucrose proved to be the most effective carbon source, achieving germination percentages of up to 57.37%, whereas sorbitol treatments resulted in little to no germination and appeared to inhibit seed development. The combination of full-strength MS medium without vitamins and 3% sucrose proved to be the most effective treatment for promoting *in vitro* germination. These results provide valuable information for developing a standardized germination protocol to support the long-term conservation of this native, ethnobotanically important species. Beyond its conservation value, this protocol provides a reproducible and scalable platform for *ex situ* cultivation, enabling orchid growers, conservation programs, and germplasm repositories to implement evidence-based strategies to safeguard *Cyrtopodium macrobulbon* and potentially other terrestrial orchids facing similar ecological pressures.

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